MAURITIUS RESEARCH COUNCIL

MOLECULAR MECHANISMS OF ANTIOXIDANT PROTECTION IN HEALTH AND DISEASE

Final Report

September 2006

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MAURITIUS RESEARCH COUNCIL, UNSOLICITED RESEARCH GRANT SCHEME

MOLECULAR MECHANISMS OF ANTIOXIDANT PROTECTION IN HEALTH AND DISEASE: POTENTIAL OF APPLICATION AND CHARACTERIZATION OF ANTIOXIDANT ACTIONS OF ENDEMIC PLANT EXTRACTS

SEPTEMBER 2006

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MOLECULAR MECHANISMS OF ANTIOXIDANT PROTECTION IN HEALTH AND DISEASE: POTENTIAL OF APPLICATION AND CHARACTERIZATION OF ANTIOXIDANT ACTIONS OF ENDEMIC PLANT EXTRACTS

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OBJECTIVES OF PROJECT

The broad objectives of the study were primarily to

- Determine the antioxidant capacity of polyphenolic rich endemic Mauritian plant extracts.
- Establish prophylactic strategies based on bioactive components of Mauritian endemic plants.
- Study the molecular mechanisms of antioxidant protection in the congenital malformations in diabetic and non-diabetic pregnancies.

The specific objectives allied to the above centered on

- Assessing the effects of endemic Mauritian plant extracts on the levels of isoprostanes in oxidised low density lipoproteins and in plasma and urines of animal models.
- Finding out if endemic plant extracts decreases oxidative damage to molecular components, proteins, lipids and DNA animal models of Parkinson Disease and Alzheimer disease *in vitro.*
- Establishing neuroprotective properties of Mauritian endemic plants
- Correlating dietary status with the levels of oxidative damage.

This project has been extremely prolific in the amount and quality of data generated and the extent of the scientific research and review papers, as listed below, that were published and/or are line for publication.

Although our original objectives had to be revisited in the light of initial findings and the unavailability of specific facilities, the set goals pertaining to the setting the grounds for the assessment of the molecular mechanisms of antioxidant protection in health and disease were achieved beyond expectations.

We were extremely fortunate to establish other contacts through Prof Okezie Aruoma, notably at the University of Pisa, Pisa, Italy and Seoul National University, Seoul, Korea.

The two PhD students Miss Vidushi Neergheen and Mr Muhammad Soobrattee conducted tissue culture work using a number of established cell lines to investigate the molecular mode of actions of endemic plant extracts and their chemopreventive potential. This was a major training initiative for the students in the context of transfer of competency to future Mauritian scientists.

Whilst the prevailing logistics did not permit the evaluation of the efficacy of the endemic plant extracts in animal models, the data acquired in the cellular and in vitro studies will form the basis for future investigations, one that will involve translation research to scientifically investigate the health benefits of the Mauritian edible endemic plants.

PREVIOUS REPORTS

A first progress report compiling research activities from July 2002-May 2003 was submitted to the Mauritius Research council in May 2003. The report indicated that the selection of plant species for this study was based either on their importance in folk medicine or their high phenolic content as revealed by previous work carried in the department of Biosciences, at the University of Mauritius. A summary of a extensive literature review made on antioxidant assay protocols was highlighted. The report also emphasized on the colorimetric determination of total phenols, proanthocyanidins and flavonoids of endemic plant extracts and the optimisation of HPLC and antioxidant assays.

A second progress report based on research activities during the period May 2003-April 2004 was focused on the detailed analysis of polyphenolic classes and individual components of 26 endemic plant extracts. A multi system analysis (independent complementary assaying methods: Trolox equivalent Antioxidant capacity (TEAC), ferric reducing antioxidant power (FRAP), hypochlorite scavenging, hydroxyl radical scavenging, copper–phenanthroline DNA oxidation assay was employed.

The third progress report submitted in April 2005 covered research work undertaken at London South Bank University (UK) and University of Pisa (Italy) during the period June 2004 to August 2004 and data generated at the University of Mauritius between August 2004 and February 2005. The activities were geared towards the establishment of microsome peroxidation assay, the setting of animal cell culture lines and development of the comet assay. The techniques acquired at the above mentioned institutions were used for further characterisation of in vitro antioxidant activities of Mauritian endemic plant extracts.

SCIENTIFIC RESEARCH AND REVIEW PAPERS EMANATING FROM THE PROJECT FUNDED BY THE MAURITIUS RESEARCH COUNCIL (in annex)

1. V.S Neergheen, M.A Soobrattee, T. Bahorun and O.I. Aruoma (2005). Phenol contents and antioxidant activities of some Mauritian endemic plants from the Ebenaceae and Myrtaceae families, In Proceedings of Bioresources towards drug development. Edited by A. Gurib-Fakim, P. Ramasami. H. Li Kam Wah, S. Jhaumeer Laulloo and A. Subratty. University of Mauritius Press, Réduit, Mauritius.

2. V.S. Neergheen, T. Bahorun, M.A Soobrattee, and O.I. Aruoma (2006). Characterisation of phenolic constituents in Mauritian endemic plants as determinants of their antioxidant activities *in vitro*. *Journal of Plant Physiology* **163** 787-799.

3. V. Mersch-Sundermann, T. Bahorun, T. Stahl, V.S. Neergheen, M.A. Soobrattee, O.I. Aruoma, R. Wohlfarth, R. Sobel, H.E.Brunn and T. Schmeiser (2006). *Monimiastrum globusum*, an endemic Mauritian plant, exhibited both DNA-damaging potency and chemopreventive effects towards BaP-induced genotoxicity in human derived cells *Toxicology in Vitro* (in press).

4. V.S. Neergheen, T. Bahorun and O.I. Aruoma (2007). Inhibition of lipid peroxidation and free radical scavenging potential of Mauritian endemic plants used in ethnomedicine *Pharmaceutical Biology* (in press).

5. M. A. Soobrattee, V. S. Neergheen, A. Luximon-Ramma, O. I. Aruoma and T. Bahorun (2006). Phenolics content and antioxidant actions of the Rubiaceae, Ebanaceae, Celastraceae, Erythroxylaceae and Sterculaceae families of Mauritian endemic plants. *Toxicology in Vitro* (Revision Submitted).

6. D. Ramful, T. Bahorun, E. Aumjaud, V.S. Neergheen, M. Soobrattee, S. Naidu and O.I. Aruoma (2006). Antioxidative propensity of *Eugenia pollicina* leaf extract in multi-system antioxidant and free radical scavenging analysis

models. *Nutrition Research* (submitted for publication).

7. O I. Aruoma, T. Bahorun and L-J Jen. (2003). Neuroprotection by bioactive components in medicinal and food plant extracts. *Reviews in Mutation Research* **544** 203-215.

8. M.Soobrattee, T. Bahorun and O.I. Aruoma (2005). Antioxidant and neuroprotective potentials. In: *Oxidative Stress, Inflammation and Health.* Edited by Y-J Surh and L Packer, Taylor and Francis: Boca Raton: London, New York, Singapore, pp 501-524.

9. M.A. Soobrattee, V.S. Neergheen, A.Luximon-Ramma, O.I. Aruoma and T. Bahorun (2005). Phenolics as potential antioxidant therapeutic agents. Mechanisms and actions. *Mutation Research* **579** 200-213.

10. V.S. Neergheen, M.A. Soobrattee, T. Bahorun and O.I. Aruoma (2005). A survey of the Mauritian endemic flora for potential prophylaxis, *In Proceedings of the 7th Annual meeting of Agricultural Scientists*, Réduit, Mauritius 2005, 1-11.

11. T. Bahorun, M.A. Soobrattee, V. Luximon-Ramma and O.I. Aruoma (2006). Free radicals and Antioxidants in Cardiovascular health and disease. *Internet Journal of Medical Update* Volume **1**, (2) Number 2 and July-December 2006 http://www.geocities.com/agnihotrimed/vol1_no2_jul-dec2006.htm.

12. M.A Soobrattee, T. Bahorun and O.I. Aruoma (2006). Chemopreventive actions of phenolic antioxidants in cancer *Biofactors*, **27**,19-35

This report also includes details of unpublished data as follows:

- Research work undertaken at the University of Mauritius during the period March 2005 and February 2006 on the iron (II) ions chelating activities of Mauritian endemic plant extracts.
- Research work undertaken at the College Veterinary Medicine, Seoul National University, Seoul, South Korea (in collaboration with Professor Kyang-Sun Kang).

EVALUATION OF CHEMOPREVENTIVE POTENTIAL OF MAURITIAN ENDEMIC PLANT EXTRACTS ON A NUMBER OF CANCER CELL LINES

INTRODUCTION

Cancer is a multifactorial and multimechanism disease characterised by three distinct stages: initiation, promotion and progression (Surh, 1999). Cancer is one of the most common causes of morbidity and mortality, with more than 7 million deaths each year worldwide (WHO). In Mauritius, the death rate from cancer for the year 2004 is about 11.3% and the incidence of cancer in particular of the lung, breast, colon and rectum, stomach is expanding at a fast rate (Annual Digest, 2004). Conventional therapies like radiotherapy, chemotherapy, immunotherapy, surgery have not been completely successful as portrayed by the unexpectedly high morbidity and mortality from cancer. This has led to a surge of interest in finding alternative strategies that can control this deadly disease and research has been directed towards developing effective anticancer therapies which can complement the existing therapies.

There is renewed interest in the use of plant-derived phytochemicals and herbal extracts to prevent cancer from the standpoint of their ability to enhance the elimination of carcinogens and their reactive metabolites or by halting/reversing the tumour promotion/progression stages (Aruoma et al, 2005; Kundu and Surh, 2005). Natural antioxidants with free radical scavenging and metal chelating effects have also been reported to reduce oxidative stress and metal induced carcinogenicity. Disrupted cellular signalling cascades are centre to cancer biology (Kundu and Surh, 2005) and therefore targeting the intracellular signalling network can represent a rational approach for chemoprevention. A wide range of signals including tumour promoters, ROS, UV, cytokines and other inflammatory mediators have the ability to turn on and off the relaying events in the cellular signalling network thereby transmitting improper signalling to diverse downstream target molecules in cellular dysfunction. The aberrant activation of MAPKs (JNK, p38 MAPK, ERK 1/2) and protein kinase C transmit inflammatory and mitogenic signals to downstream transcription factors (NF-κB, AP1). Thus induction of the latter leads to tumour promotion by transactivating several target genes including pro-inflammatory mediators like COX 2 and *i*NOS, several anti-apoptotic proteins, cell cycle regulatory proteins and kinases that disrupt gap junction intercellular communication (Kundu and Surh, 2005). Several dietary phytochemicals in particular

curcumin, (-)-epigallocatechin gallate, gingerol, capsaicin, resveratrol, genistein have been reported to inhibit upstream kinases activation and to silence the downstream components of cellular signalling network, thus they offer a unique strategy for preventing cellular proliferation and ultimately carcinogenesis (Aruoma et al, 2005; Kundu et al, 2003; Kundu and Surh, 2005; Primanio et al, 2001).

GJIC has been hypothesized to regulate growth, differentiation and apoptosis (Loewenstein, 1990; Lucke et al, 1999; Wilson et al, 2000; Yamasaki and Naus, 1996). Tumour cells are characterised by dysfunctional GJIC resulting in a lack of growth control by the inability to differentiate and by resistance to apoptosis (Hix et al, 2004). Thus up-regulation of GJIC represents an interesting approach for chemoprevention. The gap junctions structure is transcribed and translated from a highly conserved family of genes coding for connexins which assembled into hexamers forming connexons which aligned with connexons of opposing contiguous cell membrane forming a channel for passive cytoplasmic diffusion of ions and other polarized and non-polarized molecules up to a molecular mass of 1 kDa (Bruzzone et al, 1996; Kumar and Gilula, 1996). Various tumour promoters and oncogenes down-regulate GJIC (Matesic et al, 1994) while several dietary factors have been suggested to modulate GJIC by induction of various signal transducing systems which can prevent inhibition of GJIC and therefore suppress tumorigenesis (Sigler and Ruch, 1993; Nielsen et al, 2000).

Moreover the intrinsic link between apoptosis and GJIC suggest that agents that can increase GJIC should assist apoptosis-inducing chemotherapeutic agents. Apoptosis has been proposed as an important target for cancer chemoprevention as it prevents the proliferation of neoplastic transformed cells and enhances the elimination of genetically unstable and damaged cells (D'Agostini et al, 2005). Two major pathways trigger the apoptotic process, which is characterized by cell shrinkage, chromatin condensation, internucleosomal DNA fragmentation and formation of apoptotic bodies. The extrinsic pathway is induced by activation of death receptors on the cell surface, whereas the intrinsic pathway involves an increase of mitochondrial permeability and cytochrome *c* release (Hu and Kavanagh, 2004; Okada and Mak, 2003). Several chemopreventive agents can stimulate apoptosis via JNK and p38 MAPK activation, which subsequently activate the caspases (Stadheim et al, 2000).

The cytotoxicity of the plant extracts was evaluated against the WB-F344 rat liver

epithelial cells using the MTT assay. In addition, the ability of the extracts to prevent GJIC inhibition in the WB cells was also investigated. The anti-proliferative effects of the plant extracts on human breast cancer cell lines MCF-7 (estrogen-receptor positive) and MDA-MB-231 (estrogen-receptor negative) were also tested and the apoptosis inducing effect of the plant extracts on the cancer cell lines was determined.

MATERIAL AND METHODS

- **Plant extracts**

50g of the leaves were extracted first with acetone/water (70/30 v/v) (2 x 400 ml) and finally with pure methanol (3 x 400 ml). Filtrates were concentrated *in vacuo* and the resulting aqueous extract was washed with dichloromethane to remove lipids before being freeze-dried. Part of the lyophilized material was dissolved in 50% ethanol and used for the cell culture treatment while the other part was dissolved in water at a final 1:5 fresh weight: volume ratio for the determination of the chelating effect.

- **Iron (II) chelating activity**

The method of Dorman et al (2003) was adapted to study the chelating activity of the plant extracts on iron (II) ions. The reaction mixture contained 200 µl of plant extract (varied concentrations) and 50 µl of $FeCl_2.4H_2O$ (0.5 mM). The reaction volume was made up to 1 ml with distilled deionised water and incubated for 5 minutes at room temperature. After incubation, 50 µl of ferrozine (2.5 mM) was added and the purple coloration formed was read at 562 nm. The absorbance of the reaction mixture was read both before and after the addition of ferrozine as to cater for possible interferences caused by the plant extract. The controls contained all the reaction reagents except the extract or the positive control substance. EDTA was used as a positive control. The chelating activity was calculated according to the following equation:

$$\text{Chelating activity [\%]} = \left(\frac{(Abs_{control} - Abs_{sample})}{Abs_{control}} \right) \times 100$$

The analyses were made in triplicates and the results were expressed as IC_{50} mg fresh weight/ml.

- **Cell culture**

WB-F344 (WB) rat liver epithelial cells

Normal WB-F344 (WB) rat liver epithelial cells were cultured in D-media (Formula No. 78-5470EF, Gibco BRL, Grand Island, NY, USA) containing 3 ml/L PSN (penicillin/streptomycin/ neomycin) mixture (Gibco BRL, Grand Island, NY, USA) in the presence of 5% fetal bovine serum (Gibco BRL, Grand Island, NY, USA). Cells were incubated in a 37 °C humidified incubator containing 5% CO_2 and 95% air. Cells were grown in 75 mm tissue culture plates and the culture medium was changed every other day. Cells were treated with the different plant extracts dissolved in 50% ethanol at a final concentration of 0.1, 0.25, 0.5, 0.75, 1, 1.25, 1.5, 2 mg fresh weight/ml and incubated for 24 h.

MCF-7 and MDA-MB 231 human breast cancer cells

MCF-7 cells (ATCC) and MDA-MB 231 cells (ATCC) were cultured in D-media (EMEM containing 50% increase of all essential amino acids except glutamine, 50% increase of all vitamins and 100 % increase of all non-essential amino acids) supplemented with 5% FBS (fetal bovine serum; Gibco Laboratories, USA) and 3 ml/l PSN antibiotic mixture (Gibco Laboratories, USA). Exponentially growing cultures were maintained in humidified atmosphere of 5% CO_2 at 37 °C in 75 mm tissue culture plates and the culture medium was changed every other day. Cells were treated with the plant extracts at a final concentration of 0.1, 0.5, 0.75, 1, 1.25, 1.5, 3 mg fresh weight/ml and incubated at 37°C for 48 hours.

Cytotoxicity test/ Cell proliferation assay

The cytotoxic effects of the plant extracts were measured by the MTT assay, based on the ability of live cells to convert tetrazolium salt into purple formazan. The cells were seeded in 24-well microplates and incubated overnight. Then the cells were treated with different concentrations of the test compounds or their vehicles for 24 h for the WB F344 rat liver epithelial cells and for 48 h for the human breast cancer cells. At the end of the treatment period, 50 µl of MTT stock solution (5 mg/ml, Sigma, St Louis, MO, USA) was added to each well and the plates were further incubated for 4 h at 37°C. The supernatant was removed and 500 µl of DMSO were added to each well to solubilize the

water insoluble purple formazan crystals, and then transferred into 96-well microplate for reading. The absorbence of the resulting solution was measured at 540 nm using a Multiscan MCC 340 microplate reader (Titertek, USA). All the measurements were performed in triplicate. Results are expressed as the percentages cell viability with respect to control or vehicle-treated cells.

Bioassay of GJIC

The scrape loading/dye transfer (SL/DT) technique was adapted from El-Fouly et al (1987). Cells were pretreated with the plant extracts for 24 h prior to the addition of hydrogen peroxide (750 µM) for 1 h. The GJIC assay was conducted at non-cytotoxic dose levels of the samples, as determined by the MTT assay. Following incubation, the cells were washed twice with 2 ml of PBS. Lucifer yellow was added to the washed cells and three scrapes were made with a surgical steel-bladed scalpel at low light intensities. The three scrapes were performed to ensure that the scrape traversed a large group of confluent cells. After 3 minutes incubation period with Lucifer yellow, the cells were washed thrice with PBS and then fixed with 2 ml of a 4 % formalin solution. The distance travelled by the dye in a perpendicular direction to the scrape was observed under an inverted fluorescent microscope (Olympus, Melville, NY, USA).

Apoptosis assay

Apoptosis of MCF-7 and MDA-MB 231 breast cancer cell lines were analyzed morphologically by nuclear DNA staining using Hoechst 33258 (Sigma, St Louis, MO, USA). The cells were plated in 35 mm culture dishes overnight and the latter were treated with the plant extracts at varied concentrations and incubated at 37°C for 48 h. Following this incubation period, control and plant extracts-treated cells were washed with PBS and fixed in 4% paraformaldehyde in PBS for 20 min, stained with Hoechst 33258 at 1 µg/ml in PBS for 30 min. Stained cells were washed thrice with PBS and fixed with 1 ml of 4% paraformaldehyde in PBS. The nuclear morphology were observed under a fluorescent microscope (Olympus, Melville, NY, USA) through UV-filter.

Statistical analysis

All spectrophotometric measurements were made on a Unicam spectrophotometer

interfaced with Unicam UV Series Vision 32 software (Version 1.22, Unicam Instruments, Cambridge, UK). Results are expressed as mean value ± standard deviation (n = 3).

RESULTS

The ferrous ion chelating activity of the plant extracts were measured using ferrozine, a powerful chelating reagent, to indicate the presence of a chelator in the reaction system. Ferrozine forms a complex with free Fe^{2+} but not with Fe^{2+} bound by extracts. In the presence of chelating agents, the complex formation of ferrous ion and ferrozine is altered and this can be monitored by the decrease in the absorbance at 562 nm. All the Mauritian endemic plant extracts demonstrated the ability to chelate iron (II) ions in a concentration-dependent manner. *Syzygium commersonii*, *Eugenia tinifolia* and *Syzygium glomeratum* were the most potent Fe^{2+} ions chelators as shown in Figure 3. The IC_{50} values for all the extracts were estimated from the linear response curve and is depicted in Figure 3. EDTA (10-100 µM) was used as a positive control and the calculated IC_{50} value is 0.021 mg/ml.

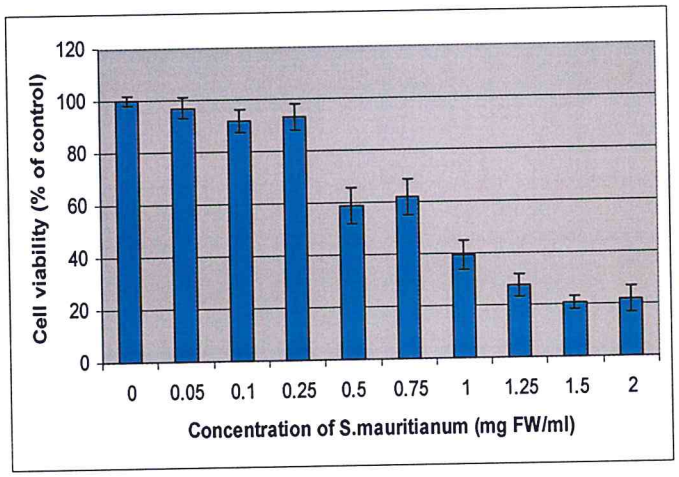
Cytotoxicity against WB F344 rat liver epithelial cells

The cytotoxicity was determined by the MTT assay and various concentrations of the plant extracts were tested. Results showed that *E. orbiculata, M.obovata, S.commersonii, S. mauritianum*, *S.venosum*, inhibited cell growth in a dose-dependent manner (Figure 1) while *C. orientalis*, *C. lanceolata*, *C. macrocarpa*, *E. sideroxyloides*, *E.elliptica*, *E.pollicina*, *E.tinifolia*, *M.acutisepalum* and *M.globosum* were not cytotoxic at a concentration range of 0.05-1.5 mg FW/ml. In the same vein, a standard preparation of EMX and papaya extract showed very low cytotoxicity between 10 and 100 µl/ml and 0.1 to 0.7 µg/ml respectively.

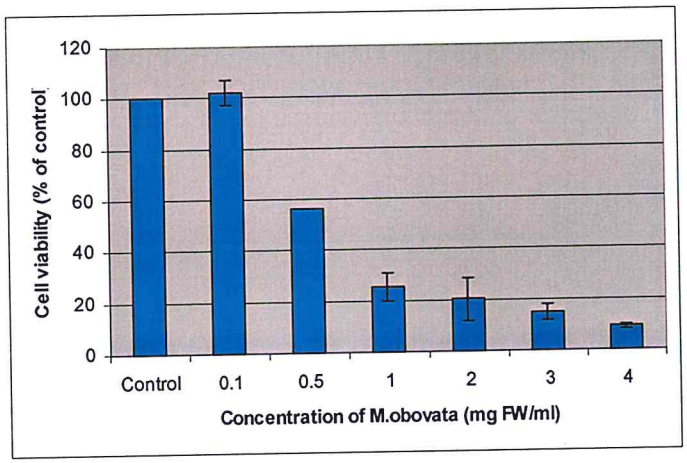
Table 1. Concentration of plant material that caused 50% iron (II) chelating activity. The IC_{50} values were determined from a calibration curve for each plant extract.

Plant species	Fe^{2+} chelating activity (IC_{50} mg FW/ml)
Cassine orientalis	23 ± 0.68
Chassalia grandifolia	42 ± 1.16
Chassalia coriacea var. johnstonii	27 ± 5.32
Chassalia lanceoloata subsps latifolia	32 ± 1.15
Coffea mauritiana	52 ± 1.23
Coffea macrocarpa	67 ± 2.1
Diospyros neraudii	3.2 ± 0.12
Diospyros revaughanii	2.1 ± 0.41
Diospyros tesselleria	1.15 ± 0.21
Diospyros mellanida	2.5 ± 0.08
Erythroxylum sideroxyloides	19 ± 2.36
Eugenia elliptica	5
Eugenia orbiculata	4.35 ± 0.21
Eugenia pollicina	4.6 ± 0.01
Eugenia tinifolia	1.05 ± 0.07
Fernelia buxifolia	13.85 ± 0.07
Maytenus pyria	16 ± 3.4
Monimiastrum acutisepalum	8.9 ± 0.14
Monimiastrum globosum	4.75 ± 0.78
Myonima nitens	22.05 ± 0.07
Myonima obovata	18.6 ± 1.13
Syzygium commersonii	0.85 ± 0.21
Syzygium glomeratum	1.5 ± 0.14
Syzygium mauritianum	6.2 ± 0.1
Syzygium venosum	4.65 ± 0.07
Trochetia boutoniana	1.6 ± 0.09

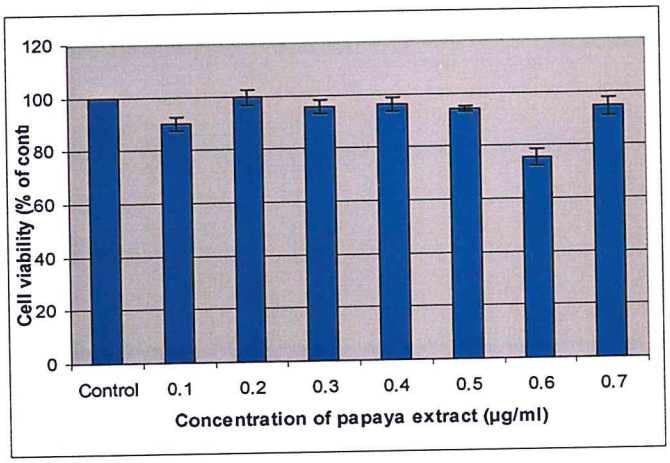
Figure 1: Cytotoxicity in the WB cells after treatment with various concentrations of plant extracts. The cells were incubated with (A) *S.mauritianum*; (B) *M.obovata* ; (C) papaya extract and (D) EMX for 24 h. Cell viability was assayed by the MTT assay. Results are expressed as percentages of cell survival compared to the untreated control (mean ± SD, n = 3).



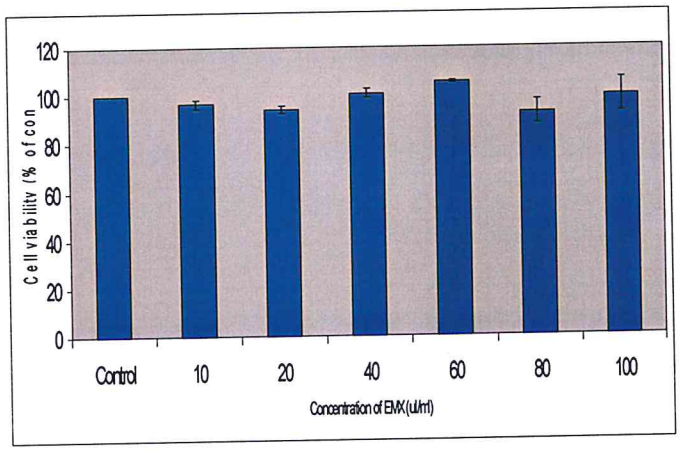
A



B



C



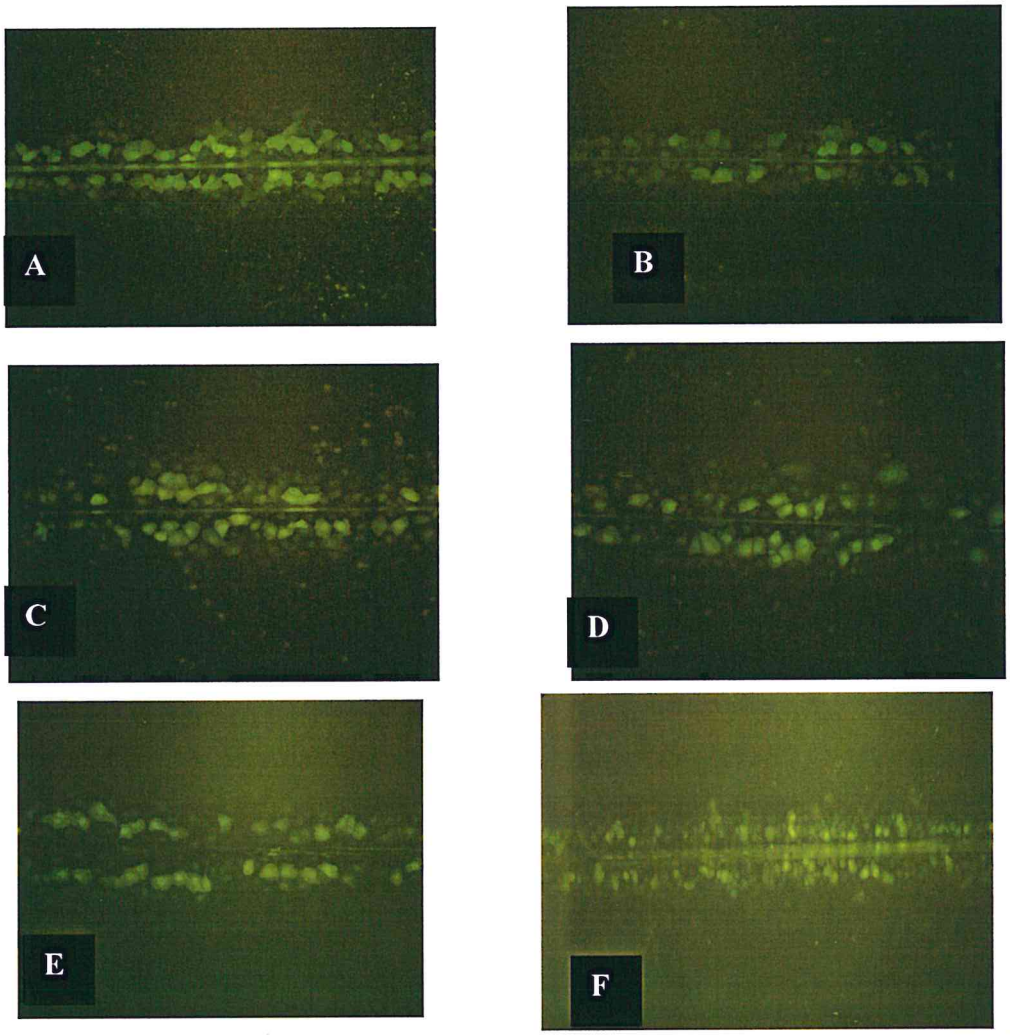
D

Effect of Mauritian endemic plant extracts on GJIC

The GJIC of WB F344 cells were assessed using SL/DT assay following 24 h treatment with the plant extracts and further 1 h incubation with hydrogen peroxide (H_2O_2). H_2O_2 is a well-known cancer promoter that inhibits GJIC in WB-F344 rat liver epithelial cells in a dose- and time-dependent manner. The GJIC of the control cells did not decrease during the experimental incubation period (Figure 2A). Treating the cells with 750 µM H_2O_2 for 1 h showed a subsequent inhibition of GJIC as detected by the distance travelled by the fluorescent dye Lucifer yellow (Figure 2B). Cells pre-treated with the

plant extracts in particular C. orientalis (0.5 mg FW/ml), EMX (200 µl/ml), *E.pollicina* (0.5 and 0.75 mg FW/ml); *S.commersonii* (0.25 mg FW/ml) showed preventive effects on the inhibition of GJIC induced by 750 µM H_2O_2 (Figure 2 C-F). *E. tinifolia* at a concentration of 0.5 and 0.75 mg FW/ml also showed partial restoration of the GJIC while all the other plant extracts tested did not show any significant recovery of GJIC.

Figure 2: Effect of Mauritian endemic plant extracts on GJIC by the SL/DT assay. A: Control; B: H_2O_2 (750 µM); C: H_2O_2 + *E.pollicina* extract (0.5 mg FW/ml); D: H_2O_2 + *E.pollicina* extract (0.75 mg FW/ml); E: H_2O_2 + *S. commersonii* extract (0.25 mg FW/ml); F H_2O_2 + EMX (200 µl/ml)



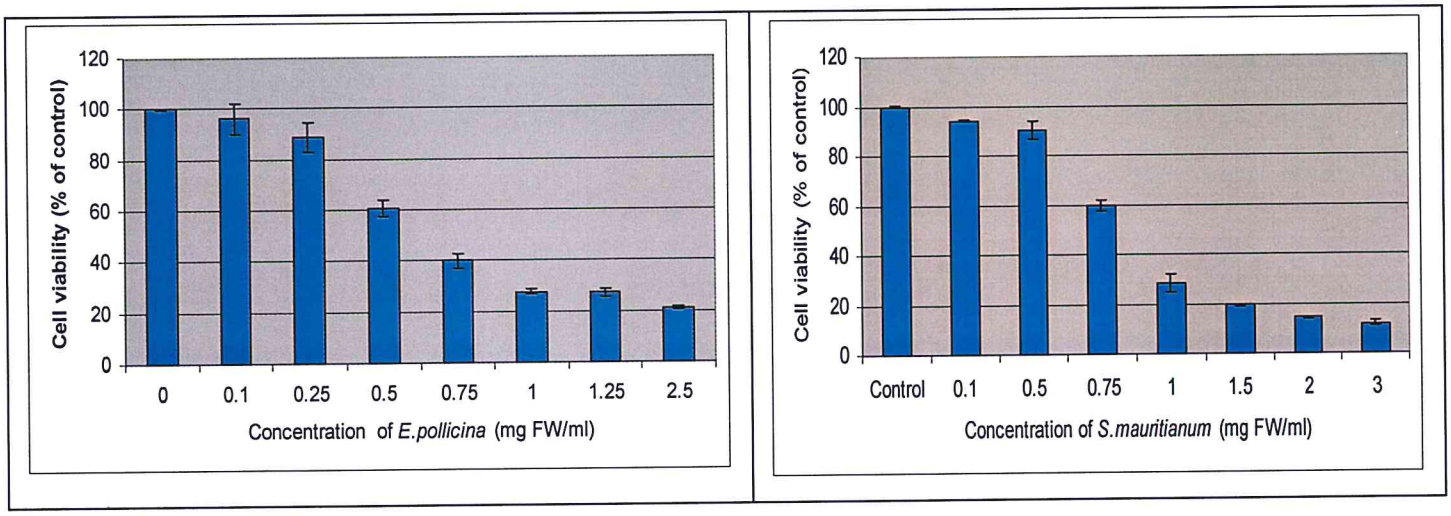
Anti-proliferative activity of some Mauritian endemic plant extracts against MCF-7 and MDAMB 231 breast cancer cells

The effect of the Mauritian endemic plant extracts on the growth of two human breast cancer lines: MDAMB 231 and MCF-7 cells were evaluated by treating the cells with a series of concentrations (0.05-4 mg FW/ml) of the plant extracts for 48 hrs. Cell growth

was determined by the MTT assay and only extracts of *E.pollicina*, *E.orbiculata*, *E. tinifolia*, *M.obovata*, *S.commersonii*, *S.mauritianum*, *S.venosum* inhibited MDAMB 231 cell proliferation in a dose- dependent manner (Figure 3A). *E.pollicina*, *S.commersonii*, *S.mauritianum* exhibited cytotoxicity activity with a calculated IC_{50} value of 0.65, 0.6 and 0.81 mg FW/ml respectively. Five of the extracts, *E.pollicina*, *M.obovata*, *S.commersonii*, *S.mauritianum* and *S.venosum* dose-dependently inhibited MCF-7 cells proliferation. *E.tinifolia*, *E.orbiculata*, *E.elliptica*, *M. acutisepalum* and *M.globosum* were weak inhibitors of cell proliferation. The calculated IC_{50} values are shown in Table 2

Figure 3: Inhibition of (A) MDAMB 231 human breast cancer cell proliferation (B) MCF-7 human breast cancer cells by *E.pollicina* and *S.mauritianum*. Cells were treated with various concentrations of the test compounds for 48 h and cell viability was determined by the MTT assay. Results are expressed as percentages of proliferation compared to the untreated control (mean ± SD, n = 3).

A



B

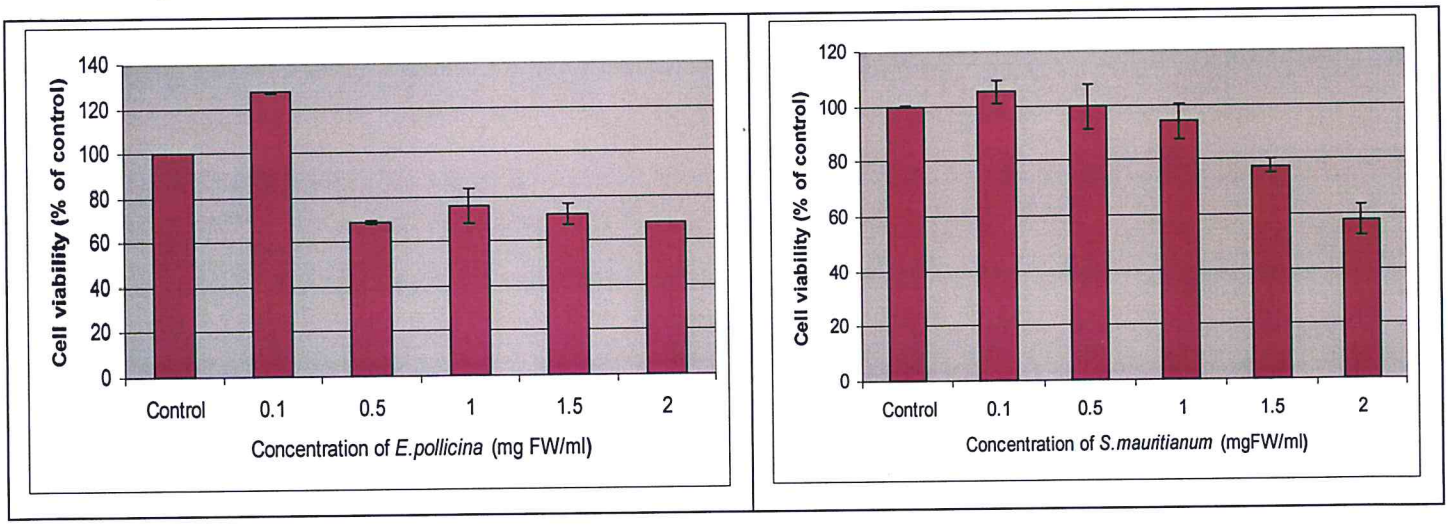
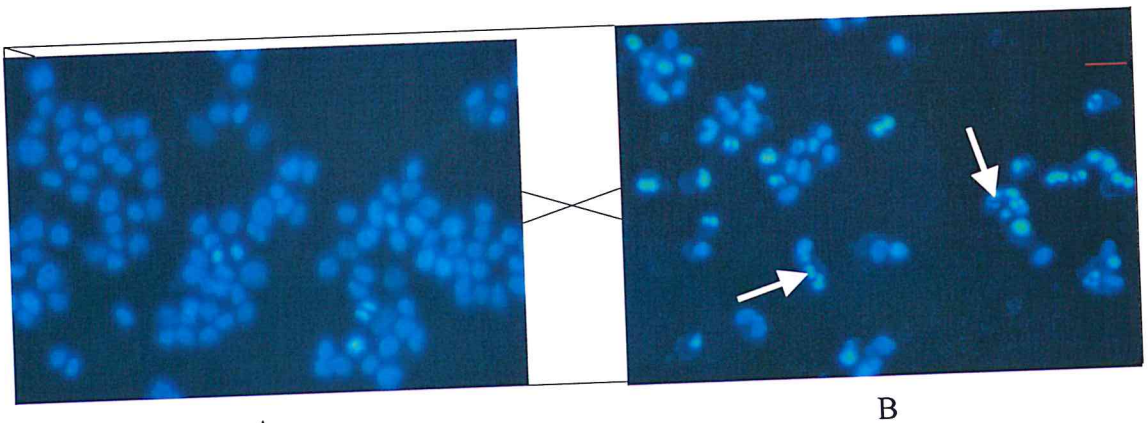


Table 2: *In vitro* cytotoxic activity of the Mauritian endemic plant extracts tested against human breast cancer cell lines (MDA MB 231 and MCF-7). The calculated IC_{50} values are shown and results are expressed as mg fresh weight/ml and are mean of three replicates. NA: IC_{50} not obtained, NC: not cytotoxic within the concentration range tested.

Plant species	MDA MB 231 cells	MCF-7 cells
E. elliptica	> 3	NC
E. orbiculata	1.06	> 3
E. pollicina	0.65	NA
E. tinifolia	1.3	NC
M. acutisepalum	2.0	NA
M. globosum	2.5	NA
M. obovata	1.0	0.1
S.commersonii	0.6	2.35
S. mauritianum	0.65	> 2
S.venosum	1.15	> 3
C. orientalis	0.3	2.3
C. macrocarpa	NC	1.6
C. lanceolata	NA	1.2
E. sideroxyloides	NC	NC
D. tesselaria	NC	NC

Anti-apoptotic effect of Mauritian endemic plant extracts

The ability of the plant extracts to induce apoptosis in the cancer cell lines was determined by nuclear staining using the Hoechst dye and the nuclear morphology was subsequently visualized under a fluorescent microscope. Out of the ten plant extracts studied only *E. pollicina* induced apoptosis in the MCF-7 cells as shown in Figure 4B. Cells treated with 0.5 mg FW/ml *E. pollicina* extract was characterised by DNA fragmentation and formation of apoptotic bodies. The other plant extracts although they exhibited strong cytotoxic activities against the cancer lines were not potential inducers of apoptosis under the experimental condition used.



A B

Figure 4: MCF-7 human breast cancer cells treated with (A) vehicle and (B) *E. pollicina* extract. Figure 4B shows the presence of fragmented DNA and apoptotic bodies as indicated by the arrows

DISCUSSION

The likelihood of transition metal ions to catalyse the generation of free radicals in particular hydroxyl radicals and their possible implication in several pathological conditions have been widely described (Kehrer et al, 2000). Thus iron chelators are of potential interest as prophylactics. The Mauritian endemic plant extracts showed concentration dependent iron (II) chelation activity. This chelating activity of the plant extracts cannot be ascribed to one particular class of phenolic compound but may result from the synergistic influence of the different classes of compounds present in the extract. Literature data has reported that the metal chelating potential can be ascribed to specific functional groups and flavones with the 4-oxo function and a 3 or 5' OH group are important metal chelators (Mira et al, 2002).

This study also sought to establish the possible potential chemopreventive effects of Mauritian endemic plant extracts by virtue of their ability to inhibit cell proliferation and induce apoptosis in MCF-7 and MDA MB231 human breast cancer cells. The genus Eugenia, Syzygium and Myonima showed promising anti-proliferative activities against

both human breast cancer cells. These plant extracts are very rich sources of phenolic antioxidants including phenolic acids, flavan-3-ols, flavonol derivatives (Neergheen et al, 2006) and these phytochemicals may in part contribute to the anti-proliferative effects of the plant extracts.

Considerable attention has been focussed on the ability of phenolic rich plant extracts to induce apoptosis in cancer cell lines (Herman-Antosiewicz and Singh, 2004; D'Agostini et al, 2005). Apoptosis has been proposed as a novel target for cancer prevention (Sun et al, 2004; Steele and Kelloff, 2005) and the rationale is the elimination of neoplastic cells that fail to respond to the cell regulatory signals. *E.pollicina* extract inhibited cell proliferation significantly and induced apoptosis in MCF-7 cells at a concentration of 0.5 mg FW/ml. Several experimental studies have suggested the importance of plant extracts and naturally occurring phytochemicals as inducers of apoptosis and their potential application as chemopreventive agents (Chiang et al, 2005; Jo et al, 2005). For instance the extract of *Ganoderma lucidum*, an oriental fungus, at a concentration of 500 µg/ml significantly inhibited the proliferation of MCF-7 cells and the antiproliferative effect was attributed to cell cycle arrest at G1 phase and also to the induction of apoptosis (Hu et al, 2002). The ethanolic extract of *Solagrum nigrum* ripe fruits has been shown to inhibit the proliferation of human MCF-7 breast cancer cells and to induce cell death by apoptosis (Son et al, 2003). The mechanism underlying apoptotic induction by *E.pollicina* may result from the phenolic components which have been reported to induce apoptosis via multiple mechanisms in particular by triggering the CD95-CD95L signalling pathway; activating the caspases, decreasing the expression of Bcl-2 and cyclin D1, up-regulating p21 and proapoptotic protein Bax and by the MAPK activation that results in p53 up-regulation (Clement et al, 1998; Hu et al, 2002; She et al, 2001; She et al, 2002).

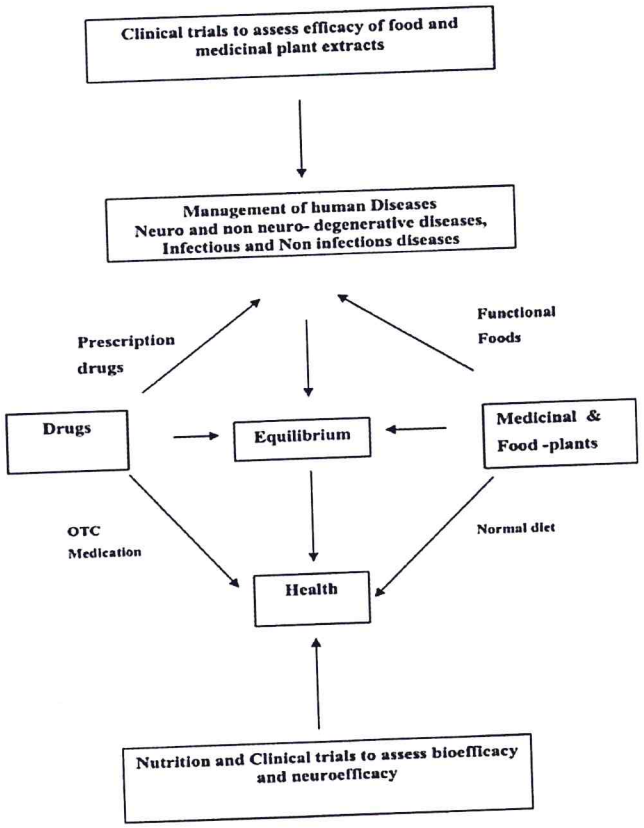
In addition substantial body of evidence from experimental studies has identified GJIC as a key target for chemoprevention (Passani et al, 1998; Trosko et al, 2005) and several naturally-occurring compounds like green tea, resveratrol, caffeic acid phenyl ester, epicatechin, ginsenoside Rb_2 have been shown to either upregulate GJIC in stem cells or prevent the down regulation of GJIC by tumour promoters (Kang et al, 2000; Na et al, 2000; Nielsen et al, 2000; Sigler and Ruch, 1993). Mechanistic studies have shown that tumour promoters have the ability to down-regulate GJIC via the activation of protein kinase C, MAPK, hyperphosphorylation of the major gap junction protein called connexin 43, the disappearance of gap-junctional plaques in plasma membrane and also

through an increase in intracellular Ca^{2+} levels which can trigger closure or degradation of the gap junctions (Matesic et al, 1994; Madhukar et al, 1983; Warn-Cramer et al, 1998). Extracts of *C. orientalis*, *E.pollicina* and *S.commersonii* significantly restored GJIC at a concentration of 0.5, 0.5 and 0.25 mg FW/ml respectively. The molecular mechanism of the preventive effect of the plant extracts on the inhibition of GJIC by H_2O_2 has to be further studied to provide further details on the chemopreventive nature of the endemic plant extracts.

RECOMMENDATION

Strategies for the intervention and prevention of cancers, diabetes, cardiovascular disease, HIV/AIDS and neurodegenerative diseases requires an understanding of the basic molecular mechanism(s) by which prophylactic agents may potentially prevent or reverse the promotion or progression of the diseases.

Future work should embrace the search for neuroprotectants, anti-inflammatory therapeutics and cognitive enhancers (hence defining the role for flavonoids, and phenolic compounds in food plants and medicinal plants that are endemic to Mauritius) with their translation to public health concerns.



A strategy to facilitate definition of the prophylactic potentials of diet, nutritional/food supplements and medicinal plants From Aruoma *et al* 2005 Mutation Research **579**: 1-5)

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ANNEX

Phenol contents and antioxidant activities of some Mauritian endemic plants from the Ebenaceae and Myrtaceae family

V S Neergheen, M A Soobrattee, T Bahorun* and O I Aruoma

Abstract

There is an emerging interest for the evaluation of natural antioxidants from food and plant sources as possible prophylactic agents in the management of several clinical disorders. This prompted the current investigation where 10 Mauritian endemic plant extracts were analyzed for their total phenolics, proanthocyanidins, flavonoids and antioxidant capacity. The antioxidant propensities were assessed by five highly reproducible assays: the trolox equivalent antioxidant capacity (TEAC), ferric reducing antioxidant potential (FRAP), an assay involving hypochlorite, deoxyribose method and the copper-phenanthroline (Cu-phen) assay. The amount of total phenolics varied widely and ranged from 7 to 103 mg gallic acid equivalent/g fresh weight. Flavonoids were relatively low, ranging from 4 to 17 mg quercetin equivalent/g fresh weight while proanthocyanidin was estimated to range between 1 and 40 mg cyanidin chloride equivalent/g fresh weight. HPLC screening of the total extracts showed the presence of several flavan-3-ols derivatives namely (+)-catechin, (-)-epicatechin, (-)-epicatechin gallate, (-)-epigallocatechin gallate, (-)-epigallocatechin, procyanidin B1 and B2. Greatest antioxidant activities assessed by TEAC, FRAP and hypochlorite scavenging were observed in *Syzygium* and *Diospyros* species whilst both *Coffea macrocarpa* and *Coffea mauritiana* had weak antioxidant activities in these assays. The *Coffea* species however were potent inhibitors of deoxyribose degradation in the deoxyribose assay. The Cu-phen assay revealed the pro-oxidant nature of the *Syzygium* species and *Diospyros mellanida* at a concentration of 5-10 g FW/L but the prooxidant index did not approach that of the reference ascorbic acid. A clearcut correlation was established between antioxidant activities of the extracts and total phenol (TEAC: r=0.96, FRAP: r=0.81, HOCl: r=0.92) while weaker correlation was observed with total proanthocyanidin and total flavonoid contents. The data suggest that the plant extracts rich in polyphenols can be promising sources of prophylactic antioxidants.

Introduction

With the growing recognition of oxidative stress mediated pathologies[1-3], interest has been rekindled towards the evaluation of naturally-derived antioxidants. There is also an urge for the development of natural antioxidants in the food industry driven in part by toxicity concerns on the use of synthetic antioxidants, butylated hydroxyanisole (BHA)

and butylated hydroxytoluene (BHT)[4,5]. Many health-related properties including anticancer, antimicrobial, anti-inflammatory activities, effects on capillary fragility, and an ability to inhibit human platelet aggregation have been ascribed to phenolics[6,7]. Plant phenolics have also been reported to act as free radical scavengers, complexers of pro-oxidant metals, reducing agents and quenchers of singlet oxygen[8]. Thus plants rich in these compounds can therefore be potential sources of natural antioxidants. In this view some Mauritian endemic plants were examined for their phenolic contents and antioxidant propensities.

Mauritius is endowed by a unique flora, out of which 45% are strict endemics. A number of these endemics have been found to be useful in traditional medicine for prophylaxis and cure of diseases. From the list of Mauritian endemic plants studied, *Diospyros* species are well known for their antibacterial, antifungal, antiviral, antihelminthic, antiprotozoan, and antimalarial properties whilst *Syzygium glomeratum* are used in the treatment of migraine[9]. Thus the current investigation aimed at screening the Mauritian endemic flora mainly species from the Myrtaceae and Ebenaceae family for their phenolic, proanthocyanidin, flavonoid contents in relation to their antioxidant activities. It seems clear that the *Diospyros* and *Syzygium* species could be potential sources of dietary polyphenolic antioxidant compounds that can be further evaluated *in vivo* by clinical trials.

Experimental

Chemicals

2,2'-Azino-bis(3-ethylbenzthioziozline-6-sulphonic acid) (ABTS) and (2,4,6-tri(2-pyridyl)) s-triazine (TPTZ) were purchased from Sigma Co. (St. Louis, MO, USA). Trolox C (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), a water-soluble analogue of vitamin E, HPLC grade of (+)-catechin ((+)-C), (-)-epicatechin ((-)-EC), (-)-epigallocatechin ((-)-EGC), (-)-epicatechin-3-gallate ((-)-ECG), (-)-epigallocatechin-3-gallate ((-)-EGCG), procyanidin dimers B1 and B2 and gallic acid (GA) were

purchased from Sigma-Aldrich (Germany). HPLC grade of quercetin and cyanidin chloride were obtained from Extrasynthèse (Genay, France). All other reagents used were of analytical grade.

Plant material

Mature leaves of *Syzygium commersonii*, *Syzygium glomeratum* and *Syzygium mauritianum* were obtained from Brise Fer forest (centre of Mauritius). *Syzygium venosum* was collected from Le Pétrin conservation management area while the *Diospyros* and *Coffea* species were obtained from the Magenta nature reserve. The leaf specimens were harvested in 2002, immediately weighed and kept at –20 °C prior to extraction.

Extraction

Leaves (50 g) were extracted first with acetone/water (70/30 v/v) (2 × 400 mL) and finally with pure methanol (3 × 400 mL). Filtrates were concentrated *in vacuo* and the resulting aqueous extract was washed with dichloromethane to remove lipids before being freeze-dried. Part of the freeze-dried extract (corresponding to 1 g FW) was dissolved in water for antioxidant assays, this solution being adjusted at a final 1/2 plant fresh weight/volume ratio. An aliquot of the freeze-dried extract (corresponding to 1 g FW) was dissolved in absolute methanol for storage at –20 °C for subsequent analyses, this solution being adjusted at a final 1/5 leaves fresh weight/volume ratio.

Determination of total phenol content

Total phenol content was determined by the method adapted from Singleton *et al*[10] using the Folin-Ciocalteu reagent. To each tube, 0.25 mL of the extract was added followed by 3.75 mL of distilled water and 0.25 mL of Folin-Ciocalteu reagent. After 3 minutes, 20% sodium carbonate was added. The tubes were capped, mixed thoroughly and heated at 40 °C for 40 minutes. The blue coloration was read at 685 nm against a

blank standard. Results were expressed in mg of gallic acid/g fresh weight of plant material.

Determination of total proanthocyanidin content

The HCl/butan-1-ol assay adapted from Porter *et al*[11] was used to quantify the total amount of proanthocyanidins. To each tube, 0.25 mL of the methanolic extract was added, followed by 3 mL of butan-1-ol-HCl solution and 0.1 mL of $NH_4Fe(SO_4)_2.12H_2O$ in 2M HCl. The tubes were capped and incubated for 40 minutes at 95 °C. After cooling in the dark, the red coloration was then read at 550 nm against a blank standard. The amount of proanthocyanidins was expressed in mg of cyanidin chloride/g fresh weight.

Determination of total flavonoid content

The $AlCl_3$ method[12] was used for the quantification of the total flavonoid content of methanolic plant extracts. 1.5 mL of 2% $AlCl_3.6H_2O$ was added to equal volumes of the extract. The mixture was shaken and the absorbance was read at 440 nm. Flavonoid contents were expressed in mg quercetin equivalent/g fresh weight.

High performance liquid chromatography (HPLC)

HPLC analyses of total plant extracts were carried out using a HP 1100 series liquid chromatography system comprising vacuum degasser, quaternary pump, autosampler, thermostatted column compartment and diode array detector. Analyses are carried out at 25 °C after filtration of total extracts on Millipore (0.22 μm) and injection (30 μL) on a Waters Spherisorb ODS-2 column (5 μm particle size, 80 Å pore size) by an acidified acetonitrile-water gradient. Elution with a flow rate of 0.7 mL/min was as follows: 0-30 minutes, 0-15% B in A; 30-50 minutes, 15% B in A; 50-60 minutes, 15-25% B in A; 60-90 minutes, 15-100% B in A; 90-100 minutes, 100-0% B in A (Solvent A: acetonitrile/water, 1/9 v/v, pH 2.6; Solvent B: acetonitrile/water, 1/1 v/v, pH 2.6). The absorption wavelength was selected at 280 nm for proanthocyanidins and phenolic acids

and 360 nm for the detection of flavonoids. The identification of the compounds was carried out based on the retention times and the peak area from the photodiode array detection in comparison with authentic standards.

Qualitative analysis of (+)-catechin, (-)-epicatechin, (-)-epicatechin gallate, (-)-epigallocatechin, (-)-epigallocatechin gallate, procyanidin B1 and B2 and gallic acid were carried out by comparison with authentic samples. Absorption wavelength was selected at 280 nm.

Antioxidant activity

TEAC assay

The ABTS/MnO_2 method[13] was used to evaluate the ability of the plant extracts to scavenge the preformed radical cation $ABTS^{\cdot+}$. The $ABTS^{\cdot+}$ radical was generated by a reaction between ABTS (0.5 mM) and activated MnO_2 (1mM) in phosphate buffer (0.1 M). To 3 mL of the $ABTS^{\cdot+}$ solution, 0.5 mL of plant extracts was added and the decay in absorbance was followed for 15 minutes at 734 nm. TEAC values were expressed in μmol trolox equivalent/g fresh weight for the plant extracts.

FRAP assay

The ferric reducing antioxidant power (FRAP) assay gives an indication of the reducing ability of the plant extract. This method was adapted from Benzie and Strain[14]. FRAP reagent was freshly prepared by mixing together 10 mM 2,4,6-tripyridyl triazine (TPTZ) and 20 mM ferric chloride in 0.25 M acetate buffer, pH 3.6. Plant sample (100 μL) was added to 300 μL of water followed by 3 mL of FRAP reagent. The absorbance was read at 593 nm after 4 minutes incubation at ambient temperature against a blank of distilled water. A calibration curve of ferrous sulphate (100-1000 μmol/L) was used, and results were expressed in μmol Fe(II)/g fresh weight of plant material.

Hypochlorite scavenging activity

This assay is adapted from Weiss *et al*[15]. The reaction mixture contained taurine (150 mM), sodium hypochlorite (600 mM), plant extract (variable concentrations), phosphate saline buffer (pH 7.4) in a final volume of 1 mL. The solution was mixed thoroughly and potassium iodide (2 M) was added. A yellow coloration was developed and the absorbance was read at 350 nm. The results were expressed as IC_{50} values.

Deoxyribose assay

The hydroxyl radical scavenging potential of the extracts was determined using the deoxyribose assay[16,17]. The reacting mixture contained in a final volume of 1 mL the following reagents: 200 µL KH_2PO_4-KOH (100 mM), 200 µL deoxyribose (15 mM), 200 µL $FeCl_3$ (500 µM), 100 µL EDTA (1 mM), 100 µL ascorbic acid (1 mM), 100 µL H_2O_2 (10 mM) and 100 µL sample. Reaction mixtures were incubated at 37 °C for 1 h. At the end of the incubation period, 1 mL 1% (w/v) thiobarbituric acid (TBA) was added to each mixture followed by the addition of 1 mL 2.8% (w/v) trichloroacetic acid (TCA). The solutions were heated in a water bath at 80 °C for 20 min to develop the pink coloured MDA-$(TBA)_2$ adduct. As turbidity was encountered, the MDA-$(TBA)_2$ chromogen was extracted into 2 mL butan-1-ol and its absorbance measured at 532 nm. Results were expressed as the percentage inhibition of deoxyribose oxidation/ g fresh weight of plant material.

Copper-phenanthroline (Cu-phen) assay

The Cu-phen assay[18] was carried out essentially as described by Aruoma[19]. Reaction mixture contained in a final volume of 1.2 mL, the following reagents in order of addition indicated: 100 µL 1,10-phenanthroline (1.8 mM stock solution made up in water having initially dissolved the crystals in 50 µL ethanol), 480 µL copper(II) chloride (250 µM), 300 µL DNA (1.68 mg/mL) and 120 µL KH_2PO_4-KOH buffer at pH 7.4 (100 mM). Ascorbate (stock solution: 2.88 mM) or extracts (100 µL) were added to

initiate the reaction. The tubes were incubated at 37 °C for 1 hour. At the end of the incubation period, 100 µL 0.1 M EDTA was added to stop the reaction. DNA damage was assessed by adding 1 mL 1% (w/v) TBA and 1 mL 25% (v/v) HCl followed by 15 minutes incubation at 80 °C. The pink chromogen formed was extracted into butan-1-ol and the absorbance measured at 532 nm. Results were expressed as the absorbance at 532 nm.

Statistical analysis

Results are expressed as mean value ± standard deviation (n = 3). Simple regression analysis was performed to calculate the dose-response relationship of standard solutions used for calibration as well as for the test samples. Linear regression analysis was performed, quoting the correlation coefficient r_{xy}.

Results and Discussion

The implication of free radicals in the pathogenesis of a wide range of degenerative disorders led to the suggestion that antioxidants could be used as prophylactic and chemopreventive agents. This accounts for an upsurge of interest in the therapeutic potential of medicinal plants as antioxidants. The present study thus elaborates on the polyphenolic composition as well as on the antioxidant activities of the leafy total extracts. The data suggest the richness of the Mauritian endemic plants with high levels of phenol compounds measured in the *Syzygium* and *Diospyros* species. The total phenolic content of the plant extracts varied from 7 to 103 mg/g FW with a maximum detected in *Syzygium commersonii* (103 ± 1.2 mg/g FW) followed by *Syzygium glomeratum* (84 ± 1.3 mg/g FW). Extracts of the *Diospyros* species also showed high levels of phenolics whilst both *Coffea macrocarpa* and *Coffea mauritiana* contained lower amounts with 24 ± 0.31 and 7 ± 0.23 mg/g FW respectively (Figure 1). Using the same methodology, Santos *et al*[20] reported high phenolic content in leaves of *Stryphnodendron adstringens*, *Stryphnodendron polyphyllum* and *Dimorphandra mollis*, 3 medicinal plants used in Brazil.

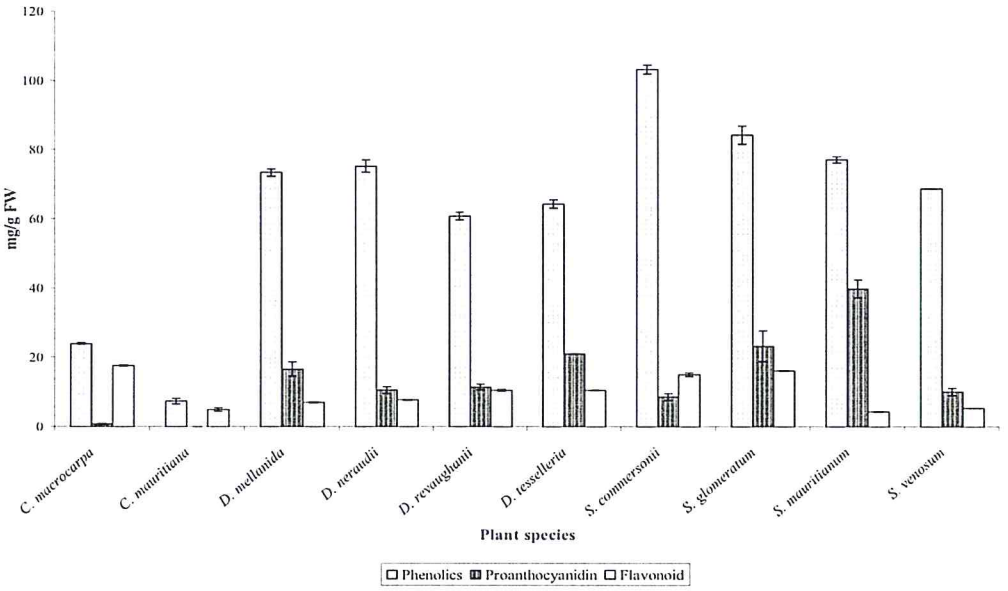


Figure 1: Total phenol, total proanthocyanidin and total flavonoid content of the plant extracts of *Coffea*, *Diospyros* and *Syzygium* species. Results are expressed as mg gallic acid equivalent/g FW for total phenol, as mg cyanidin chloride equivalent/g FW for total proanthocyanidin and as mg quercetin equivalent/g FW for total flavonoid.

Highest flavonoids content was measured in *Coffea macrocarpa* (18 ± 0.7 mg/g FW), *Syzygium glomeratum* (16 ± 0.5 mg/g FW) and *Syzygium commersonii* (15 ± 0.1 mg/g FW). Proanthocyanidin content was relatively high ranging between 0.9 and 40 mg/g FW with maximum level detected in *Syzygium mauritianum*. HPLC analyses showed the presence of proanthocyanidins including B1 and B2 dimers, and catechins comprising mainly (+)-catechin, (-)-epicatechin, (-)-epicatechin gallate, (-)-epigallocatechin gallate and (-)-epigallocatechin (Table 1).

These substances are widely discussed with regards to their ability to scavenge free radicals, superoxide and hydroxyl radicals[21,22]. Literature data have shown the presence of procyanidin B2 in plant extracts to be associated with (-)-EC[23,24]. This is the case for most of the studied plant extracts, however no (-)-EC has been detected in *Diospyros*

mellanida and Diospyros tessellaria under the experimental condition used despite the presence of procyanidin B2 (Table 1).

Table 1: Distribution of flavan-3-ol derivatives in *Coffea, Diospyros* and *Syzygium* Mauritian endemic species

(+++) = very prominent; (++) = prominent; (+) = present; tr = trace; (-) = not detected. Estimated from the peak area of the HPLC profiles.

	Catechins					Procyanidin dimers	
Plant species	**(+)-C**	**(-)-EC**	**(-)-ECG**	**(-)-EGCG**	**(-)-EGC**	**B1**	**B2**
Coffea macrocarpa	(+)	(++)	(++)	(+)	(+)	(+)	(+)
Coffea mauritiana	(+)	(+)	_	(++)	(+)	Tr	(+)
Diospyros mellanida	(+++)	_	(+)	(++)	(+++)	-	(+)
Diospyros neraudii	Tr	(+)	(+)	(+)	-	-	-
Diospyros revaughanii	(+)	(+)	(++)	(+)	-	-	(+)
Diospyros tesselleria	(+++)	-	-	(++)	(++)	(+++)	(++)
Syzygium commersonii	(+++)	-	-	(+++)	(+++)	(+++)	-
Syzygium glomeratum	(+)	(+++)	(+++)	(++)	(+)	(+)	(+)
Syzygium mauritianum	(+++)	(+++)	-	(+++)	(+++)	-	(++)
Syzygium venosum	(+++)	-	(+++)	-	(+)	(+++)	(++)

The mechanism of the reactions involving antioxidants is complex and depends on the physico-chemical parameters of the test reagents and substrates suggesting a multimethod approach for the assessment of antioxidant activity[25,26]. Although several screening approaches have been proposed, an estimation of the antioxidant potential by using the TEAC, FRAP, oxygen radical absorption assay (ORAC), ferric reducing antioxidant power (FRAP), total oxyradical scavenging capacity (TOSC), the deoxyribose assay, assays involving oxidative DNA damage, assays involving reactive nitrogen intermediates (e.g. $ONOO^-$), Trolox equivalent antioxidant capacity (TEAC) and the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay can provide preliminary information, giving a basis for further characterization procedures[26]. Mauritian endemic plant extracts exhibit a wide range of antioxidant potentials. The scavenging potential of the plant extracts as assayed by the TEAC method varied between 72 and 1485 µmol/g FW (Table 2) with an antioxidative potency in the following order: *S. commersonii* > *S.*

glomeratum > *S. mauritianum* > *S. venosum* > *D. tessellaria*> *D. revaughanii* > *D. neraudii* > *D. mellanida* > *C. macrocarpa* > *C. mauritiana*. Comparing data obtained on Mauritian endemic plants with other plant species obtained in the same assaying system revealed that the total antioxidant efficacy of Mauritian plants is relatively high compared with the results of similar studies of wild medicinal plants used in New Mexico and in West Africa[27]. In addition, Pietta *et al*[28] reported the total antioxidant activity of some selected medicinal plants in the range of 2.57-5.12 mM trolox. A systematic survey of the relative levels of antioxidant activities in selected British medicinal plant species showed that leaves of *Geranium pretense*, *Geum urbanum* and *Viola tricolor* were potent scavengers of $ABTS^{\cdot+}$ radical cation with an activity of 2.22 ± 0.34, 1.67 ± 0.21 and 1.46 ± 0.32 mmol Trolox equivalent/g dry weight respectively[29].

Table 2: Phenolic content and antioxidant activities of the Mauritian endemic plant extracts

Species	TEAC[a]	FRAP[b]	HOCl[c]	Deoxyribose[d]
Coffea macrocarpa	447 ± 7.07	175 ± 1.95	0.976	26.6 ± 0.98
Coffea mauritiana	73 ± 3.67	34 ± 1.8	6.737	23.9 ± 2
Diospyros mellanida	818 ± 6.29	696 ± 23.52	0.255	17.5 ± 0.5
Diospyros neraudii	911	396 ± 8.81	0.266	16.8 ± 1.2
Diospyros revaughanii	945 ± 15.71	607 ± 25.6	0.275	20.2 ± 1.5
Diospyros tessellaria	989 ± 9.43	517 ± 13.98	0.289	Prooxidant
Syzygium commersonii	1485 ± 40.71	1392 ± 64.58	0.256	Prooxidant
Syzygium glomeratum	1351± 170.6	460 ± 3.33	0.262	5 ± 0.58
Syzygium mauritianum	1088 ± 23.35	568 ± 3.67	0.255	Prooxidant
Syzygium venosum	1028 ± 71.38	770 ± 25.71	0.248	Prooxidant

All analyses were mean of triplicate measurements ± standard deviations.
[a]Results expressed in units µmol trolox equivalent/g fresh weight
[b]Values are expressed in units of µmol Fe(II)/g fresh weight
[c]IC_{50} is defined as the concentration sufficient to obtain 50 % of a maximum scavenging activity
[d]Results are expressed as % inhibition of deoxyribose oxidation by 1 g FW/L plant extract.

In the FRAP assay the order of potency was *S. commersonii* > *S. venosum* > *D. mellanida* > *D. revaughanii* > *S. mauritianum* > *D. tessellaria* > *S. glomeratum* > *D. neraudii* > *C. macrocarpa* > *C. mauritiana* whereas in the hypochlorite scavenging

system the antioxidant activities were as follows: *S. venosum* ≈ *S. mauritianum* ≈ *D. mellanida* ≈ *S. commersonii* ≈ *S. glomeratum* ≈ *D. neraudii* ≈ *D. revaughanii* ≈ *D. tessellaria* > *C. macrocarpa* > *C. mauritiana* (Table 2). In a previous work on *Cassia fistula* vegetative and reproductive organs, the TEAC and FRAP data varied respectively from 93 to 992 µmol Trolox equivalent/g dry weight and 51 to 811 µmol Fe(II)/g dry weight with maximum activity in pod extracts[30]. High FRAP values have also been measured for a series of medicinal plants including *Melissa officinalis*, *Mentha piperita*, *Ocimum basilicum*, *Salvia officinalis*, *Satureja hortensis* and *Majoranna hortensis* from the Labiatae family[31]. Studies conducted by Bahorun *et al*[32] on the HOCl scavenging potential of *Crataegus monogyna*, showed IC_{50} value of the order of 8.49 mg dry weight/L for floral buds. However in a similar system, *Cynara cardunculus* L. exhibited weak protective activity against damage by HOCl[33].

The ability of the plant extracts to inhibit the hydroxyl radical-dependent deoxyribose degradation showed that *Coffea macrocarpa* extract exhibited the highest inhibitory activity (27%) followed by *Coffea mauritiana* (24%) (Table 2). *Syzygium commersonii*, *Syzygium mauritianum*, *Syzygium venosum* and *Diospyros tessellaria* were found to increase oxidative damage of the deoxyribose sugar and were therefore suggested to exhibit prooxidant actions in the deoxyribose assay. This prooxidant activity may result from the ability of the extracts to redox cycle the metal ion required for $OH^{\cdot}$ generation and thus increase the radical production[34,35]. Screening the antioxidant activities of 20 traditional Mediterranean and Chinese medicinal herbs, Schinella *et al*[35] observed that most of the extracts showed an inhibitory activity of 30% $OH^{\cdot}$ in the deoxyribose system which is comparable to the antioxidant action of the Mauritian endemic plants. Similar studies conducted on *Cassia fistula* showed that the extract provided 30% protection against deoxyribose oxidative damage at a concentration 125 µg/mL[36]. The ability of the plant extracts to stimulate DNA oxidative damage was evaluated using the copper-phenanthroline assay. *Syzygium commersonii*, *Syzygium mauritianum*, *Syzygium venosum* and *Diospyros mellanida* extracts at concentrations 5-10 g FW/L were able to

stimulate DNA damage. Although the apparent prooxidant nature of plant extracts can be attributed to the phenolic compounds, the extent of the prooxidant index is nowhere near that of the reference ascorbic acid.

In rationalizing the antioxidant potential of the plant extracts in terms of phenolic classes, it is clear from the regression correlation coefficient data that the antioxidant capacity based on TEAC, FRAP and hypochlorite scavenging was associated more strongly with total phenols ($r = 0.957$, $r = 0.814$, $r = 0.925$ respectively) and to a lower extent with total flavonoid ($r \leq 0.28$) and total proanthocyanidin ($r \leq 0.63$) content. The overall antioxidant potency of the plant extracts may result from a concerted action of the cocktail of antioxidants present in the extract. Similar linear relationships have been observed between phenolic content and the antioxidant capacity of *Cassia fistula* plant extracts[30,37]. The strong antioxidative activities of *Eucommia ulmoides* (Du Zhong) leaf extracts in different lipid peroxidation models were also related to the high polyphenolic content mainly flavonoids and phenolic acids[38]. Similar observations were made with the free radical scavenging activities of *Xanthosoma violaceum*[39] leaf extracts and *Capparis spinosa*[40] buds. Such positive relationship between phenolics content and antioxidant activity has also been reported in fruits[41,42], beverages[43,44], vegetables[45], juices[46], wines[47] and cell cultures[48].

Conclusions

In the light of the results obtained it is clear that *Syzygium* and *Diospyros* species are potential sources of phenolics and antioxidants. This suggests that antioxidants from Mauritian endemic plant extracts could have great relevance in the prevention and therapeutics of free radical mediated diseases. These preliminary findings thus provide ground for further detailed investigations of the bioactive phytochemicals in these plant extracts and benefit a rationale for clinical trials using the endemic plants for such conditions as diabetes, neurodegenerative and cardiovascular diseases where oxidative and nitrosative stress are involved.

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Characterization of the phenolic constituents in Mauritian endemic plants as determinants of their antioxidant activities in vitro

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Mauritian endemic plants;
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Myrtaceae genus;
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Summary

The phenolic constituents of Mauritian endemic plants from the Rubiaceae and Myrtaceae family were assessed and correlated with their potential antioxidant activities in vitro. The antioxidant activities of the plant extracts ranged from 0.27 to 1.49 mmol Trolox equivalent/g FW and from 0.20 to 1.39 mmol Fe(II) equivalent/g FW in the TEAC and FAP assays, respectively, with *Syzygium commersonii* showing the highest activity in these two systems. *Eugenia orbiculata* and all the *Syzygium* species were effective scavengers of hypochlorous acid while *Monimiastrum acutisepalum* was the most potent inhibitor of deoxyribose degradation. The plant extracts inhibited microsomal lipid peroxidation with low IC_{50} s ranging from 0.02 to 1.75 mg FW/mL when reaction was initiated with Fe^{3+}/ascorbate and from 0.093 to 1.55 mg FW/mL in the AAPH-dependent lipid peroxidation. The potential prooxidant nature of the plant extracts was compared with ascorbate (250 μM) using copper–phenanthroline assay. The plant extracts at concentrations up to 5 g FW/L were not prooxidant. However, *Myonima nitens*, *Syzygium commersonii*, *Syzygium glomeratum* and *Syzygium mauritianum* at concentrations of 10 g FW/L had potency approaching 50% of the prooxidant activity of ascorbic acid in vitro, suggesting relative safeties. The total phenolics influenced the antioxidant activities in the TEAC, FRAP and HOCl scavenging assays whereas a negative correlation was observed with the deoxyribose assay. The high levels of polyphenolic compounds and the

Abbreviations: AAPH, 2, 2′-azobis(2-amidinopropane) hydrochloride; FRAP, ferric reducing antioxidant power; TEAC, Trolox equivalent antioxidant capacity

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significant antioxidant activities of these Rubiaceae and Myrtaceae plant family make them suitable candidates as prophylactic agent.
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Introduction

There is a growing body of evidence suggesting that free radicals play an important role in the development of tissue damage and pathological events in living organisms (Aruoma, 1998; Lefer and Granger, 2000; Smith et al., 2000; Bhatia et al., 2003; Olinski et al., 2003; Peuchant et al., 2004). Lipid peroxidation of fats and fatty acids in foods results not only in their spoilage but is also a source of peroxyl and hydroxyl radicals that are associated with carcinogenesis, mutagenesis and aging (Yagi, 1987; Finkel and Holbrook, 2000). Therefore, antioxidants that scavenge reactive oxygen species (ROS) and reactive nitrogen species (RNS) may be of major importance in preventing the onset and/or the progression of oxidative pathologies and provide protection to foods.

Many health-related properties including anticancer, antiviral, antimicrobial, anti-inflammatory activities, antioxidant properties, effects on capillary fragility, and an ability to inhibit human platelet aggregation have been ascribed to phenolics (McGregor et al., 1999; Spignoli, 2000). The physiological benefits of the plant phenolics have been attributed to their potential role in inhibiting lipid peroxidation, modulating cell signal transduction pathways and inducing apoptosis (Lin et al., 1999; Hou et al., 2004; Wiswedel et al., 2004).

The tropical island of Mauritius is endowed with a unique flora, resulting from colonization and adaptive radiation of plant species from different sources. Tropical plants are generally tolerant of high levels of environmental stress induced by ultraviolet radiation thereby explaining the high levels of phenolic compounds in endemic plant species previously studied (Toyokuni et al., 2003). Several of these endemics have already been reported in the traditional pharmacopoeia against a wide range of disorders including bronchitis, diabetes, asthma and inflammatory diseases (Gurib-Fakim, 1996). The antioxidant activity of some Mauritian endemic plants of the Rubiaceae and Myrtaceae family comprising the *Myonima*, *Fernelia*, *Eugenia*, *Monimiastrum* and *Syzygium* species (Table 1) were examined using the antioxidant assays Trolox equivalent antioxidant capacity (TEAC), ferric reducing antioxidant power (FRAP), hypochlorous acid (HOCl) scavenging, deoxyribose, copper–phenanthroline and lipid peroxidation. The antioxidant potency was then related to their total phenolic, proanthocyanidin and flavonoid content. This study is the first of its kind to report the antioxidant effectiveness of some selected Mauritian endemic plants. Thus the endemic plants from the Rubiaceae and Myrtaceae family could be potential sources of dietary polyphenolic antioxidant compounds that may have potential benefits in health and disease management.

Materials and methods

Plant material

Mature leaves of *Syzygium commersonii*, *Syzygium glomeratum*, *Syzygium mauritianum*, *Eugenia pollicina* were obtained from Brise Fer forest (centre of Mauritius). *Fernelia buxifolia*, *Syzygium*

Table 1. Endemic plants used in this study

Family	Plant species	Common name	Traditional use
Rubiaceae	*Fernelia buxifolia*	Bois Chauve souris, Bois de buis	
Rubiaceae	*Myonima nitens*	Petit bois de pintade	
Rubiaceae	*Myonima obovata*	–	
Myrtaceae	*Eugenia elliptica*	–	
Myrtaceae	*Eugenia orbiculata*	–	
Myrtaceae	*Eugenia tinifolia*	Bois de nefles	Purgative
Myrtaceae	*Monimiastrum acutisepalum*	Bois de Clous	
Myrtaceae	*Syzygium commersonii*	Bois de Pomme	
Myrtaceae	*Syzygium glomeratum*	Bois de Pomme	Use in the treatment of migraine
Myrtaceae	*Syzygium mauritianum*	–	
Myrtaceae	*Syzygium venosum*	–	

venosum, *Myonima nitens*, *Myonima obovata* were collected from Le Pétrin conservation management area while those of *Monimiastrum acutisepalum* and *Eugenia orbiculata* were obtained from Le Pétrin broadwalk. *Eugenia tinifolia* and *Eugenia elliptica* were from Le Mondrain natural reserve. The leaf specimens were harvested in 2002, immediately weighed and kept at −20 °C prior to extraction. Voucher specimens have been deposited at the Department of Biosciences, Faculty of Science, University of Mauritius.

Extraction

50 g of the leaves were extracted first with acetone/water (70/30 v/v) (2 × 400 ml) and finally with pure methanol (3 × 400 ml). The plant material was macerated overnight at 4 °C in the organic solvents. The filtrates were concentrated *in vacuo* and pooled together. The resulting aqueous extract was washed with dichloromethane to remove lipids before being freeze-dried. Part of the freeze dried extract (corresponding to 1 g FW) was dissolved in water for antioxidant assays, this solution being adjusted at a final 1/2 plant fresh weight/volume ratio. An aliquot of the freeze dried extract (corresponding to 1 g FW) was dissolved in absolute methanol for storage at −20 °C for subsequent analyses, this solution being adjusted at a final 1/5 leaves fresh weight/volume ratio.

Determination of total phenol content

Total phenol content was determined by the method adapted from Singleton and Rossi (1965) using the Folin–Ciocalteu reagent. To each tube, 0.25 mL of the extract was added followed by 3.75 mL of distilled water and 0.25 mL of Folin–Ciocalteu reagent. After 3 min, 20% sodium carbonate was added. The tubes were capped, mixed thoroughly and heated at 40 °C for 40 min. The blue coloration was read at 685 nm against a blank standard. Results were expressed in milligrams of gallic acid/g fresh weight of plant material.

Determination of total proanthocyanidin content

The HCl/Butan-1-ol assay adapted from Porter et al. (1986) was used to quantify the total amount of proanthocyanidins. To each tube, 0.25 mL of the methanolic extract was added, followed by 3 mL of 1 BuOH–HCl solution and 0.1 mL of $NH_4Fe(SO_4)_2 \cdot 12H_2O$ in 2 M HCl. The tubes were capped and incubated for 40 min at 95 °C. After cooling in the dark, the red coloration was then read at 550 nm against a blank standard. The amount of proanthocyanidins was expressed in miiligrams of cyanidin chloride/g fresh weight of plant material.

Determination of total flavonoid content

The $AlCl_3$ method (Lamaison and Carnet, 1990) was used for the quantification of the total flavonoid content of methanolic plant extracts. A total of 1.5 mL of 2% $AlCl_3 \cdot 6H_2O$ was added to equal volumes of the extract. The mixture was shaken and the absorbance was read at 440 nm. Flavonoid contents were expressed in milligrams quercetin equivalent/g fresh weight of plant material.

High-performance liquid chromatography (HPLC)

HPLC analyses of total plant extracts were carried out using a HP 1100 series liquid chromatography system comprising vacuum degasser, quaternary pump, autosampler, thermostatted column compartment and diode array detector. Analyses are carried out at 25 °C after filtration of total extracts on Millipore filter paper (0.22 μm) and injection (30 μl) on a Waters Spherisorb ODS-2 column (5 μm particle size, 80 Å pore size) by an acidified acetonitrile-water gradient. Elution with a flow rate of 0.7 mL/min was as follows: 0–30 min, 0–15% B in A; 30–50 min, 15% B in A; 50–60 min, 15–25% B in A; 60–90 min, 15–100% B in A; 90–100 min, 100–0% B in A (Solvent A: acetonitrile/water, 1/9 v/v, pH 2.6; Solvent B: acetonitrile/water, 1/1 v/v, pH 2.6). The absorption wavelength was selected at 280 nm for proanthocyanidins and phenolic acids and 360 nm for the detection of flavonoids. The identification and quantification of the compounds were carried out based on the retention times and the peak area from the photodiode array detection in comparison with authentic standards. Quantitative analysis of (+)-catechin, (−)-epicatechin, (−)-epicatechin gallate, (−)-epigallocatechin, (−)-epigallocatechin gallate, procyanidin B1 and B2 and gallic acid were carried out by comparison with authentic samples.

Hydrolysis of flavonoid conjugates and quantitative analysis of aglycones released

Hydrolysis of flavonoid conjugates was carried out essentially as described by Crozier et al. (1997). An aliquot of 1 g fresh weight equivalent

freeze-dried extract was dissolved in 20 mL 60% methanol containing 100 μg morin as internal standard. Recovery of the compounds from this extract was monitored by the use of morin. Five milliliters of 6 M HCl was added to each extract, which was refluxed at 90 °C for 2 h. After cooling the flavonoid aglycones were exhaustively extracted using ethyl acetate. The latter phase was evaporated to dryness and taken in absolute methanol prior to analysis by HPLC. The methanolic hydrolysed extract was subjected to HPLC analysis and quercetin, myricetin, kaempferol, apigenin and luteolin were identified and quantified within the plant extracts.

Antioxidant activity

Trolox equivalent antioxidant capacity (TEAC assay)

The ABTS/MnO_2 method (Campos and Lissi, 1997) was used to evaluate the ability of the plant extracts to scavenge the preformed radical cation $ABTS^{+}$. The $ABTS^{+}$ radical was generated by a reaction between ABTS (0.5 mM) and activated MnO_2 (1 mM) in phosphate buffer (0.1 M). To 3 mL of the $ABTS^{+}$ solution, 0.5 mL of plant extracts was added and the decay in absorbance was followed for 15 min at 734 nm. TEAC values were expressed in μmol trolox equivalent/g fresh weight for the plant extracts as previously described. The absorption wavelength was selected at 360 nm for the detection of the flavonoid aglycones.

Ferric reducing antioxidant power (FRAP assay)

The FRAP assay gives an indication of the reducing ability of the plant extract. This method was adapted from Benzie and Strain (1996). FRAP reagent was freshly prepared by mixing together 10 mM 2,4,6-tripyridyl triazine (TPTZ) and 20 mM ferric chloride in 0.25 M acetate buffer, pH 3.6. Plant sample (100 μL) was added to 300 μL of water followed by 3 mL of FRAP reagent. The absorbance was read at 593 nm after 4 min incubation at ambient temperature against a blank of distilled water. Results were expressed in μmol Fe (II)/g fresh weight of plant material.

Potential to scavenge preformed hypochlorous acid (HOCl assay)

HOCl was prepared by adjusting the pH of a 1% (v/v) solution of NaOCl to 6.2 with dilute sulphuric acid. The working concentration of the stock solution was determined spectrophotometrically by measuring its absorbance at 235 nm and applying a molar extinction coefficient of $100\,M^{-1}\,cm^{-1}$ (Weiss et al., 1982; Aruoma, 1994a). The reaction mixture contained taurine (10 mM), HOCl (1 mM), plant extract (variable concentrations), phosphate saline buffer (pH 7.4) in a final volume of 1 mL. The solution was mixed thoroughly and incubated for 10 min at ambient temperature followed by the addition of 10 μL of potassium iodide. A yellow coloration was developed and the absorbance was read at 350 nm. The results were expressed as IC_{50} values (mg FW/mL).

Potential to inhibit deoxyribose degradation (deoxyribose assay)

The hydroxyl radical scavenging potential of the extracts was determined using the deoxyribose assay (Halliwell et al. 1987; Aruoma, 1994a, b). The reacting mixture contained in a final volume of 1 mL the following reagents: 200 μL KH_2PO_4–KOH (100 mM), 200 μL deoxyribose (15 mM), 200 μL $FeCl_3$ (500 μM), 100 μL EDTA (1 mM), 100 μL sample, 100 μL H_2O_2 (10 mM) and 100 μL ascorbic acid (1 mM). Reaction mixtures were incubated at 37 °C for 1 h. At the end of the incubation period, 1 mL 1% (w/v) thiobarbituric acid (TBA) was added to each mixture followed by the addition of 1 mL 2.8% (w/v) trichloroacetic acid (TCA). The solutions were heated in a water bath at 80 °C for 20 min to develop the pink coloured MDA-$(TBA)_2$ adduct. As turbidity was encountered, the MDA-$(TBA)_2$ chromogen was extracted into 2 mL butan-1-ol and its absorbance measured at 532 nm. Results were expressed as the percentage inhibition of deoxyribose degradation/g fresh weight of plant material.

Inhibition of microsomal lipid peroxidation induced by Fe^{3+}/ascorbate

Beef liver microsomes were prepared by tissue homogenization with ice-cold 0.25 M sucrose-10 mM Tris buffer, pH 7.4 with 1 mM EDTA. Microsomal fractions were isolated by removal of the nuclear fractions at 1500 rpm for 5 min and removal of mitochondrial fraction at 5000 rpm for 15 min. The supernatant was then centrifuged in a Beckman Optima™ ultracentrifuge at 100 000*g* for 60 min at 4 °C. The pellet was then washed in 0.25 M NaCl, pH 7.5. The membranes were suspended in saline (0.25 M, pH 7.5) and stored in aliquots at −20 °C. Microsomal protein was determined by the Lowry–Folin method (Lowry et al., 1951). The reacting

mixture contained in a final volume of 1 mL the following reagents: 200 µL phosphate-buffered saline [3.4 mM Na_2HPO_4/NaH_2PO_4 (pH 7.4), 0.15 M NaCl], 200 µl microsome (0.5 mg/mL microsomal protein), 400 µL of plant extract, 100 µL iron (III) chloride (1 mM) and 100 µL ascorbate (1 mM). The mixture was incubated for 1 h at 37 °C. At the end of the incubation period, 100 µL 2% BHT was added followed by 1 mL 1% (w/v) TBA and 2.8% (w/v) TCA. Addition of BHT to the reaction mixture minimizes erroneous increases in color due to iron ion-dependent hydroperoxide decomposition during the acid heating stage. The solutions were heated in a water bath at 80 °C for 20 min to develop the pink coloured MDA-$(TBA)_2$ adduct. As turbidity was encountered, the MDA-$(TBA)_2$ chromogen was extracted into 2 mL butan-1-ol and its absorbance measured at 532 nm. Results are expressed as IC_{50} (mg FW/mL).

2, 2′-azobis(2-amidinopropane) hydrochloride (AAPH)-induced lipid peroxidation

The reaction mixture contained in a final volume of 0.8 mL the following reagents: 200 µL of beef liver microsomes (0.5 mg/mL microsomal protein final concentration) suspended in 0.1 M potassium phosphate buffer, pH 7.5, 400 µL sample and 200 µL AAPH (20 mM) to initiate peroxidation. The mixture was incubated at 37 °C for 1 h and the solution gently shaken at 10 min interval. After incubation, 1.6 mL TCA–TBA–HCl stock solution (15% w/v TCA, 0.375% TBA, 0.25 N HCl) was added. The solution was heated in a boiling water bath for 15 min. After cooling, the precipitate was removed by centrifugation and the absorbance of the resulting supernatant measured at 532 nm. Results are expressed as IC_{50} (mg FW/mL).

Inhibition/stimulation of the copper–phenanthroline-dependent DNA oxidation (Cu-phenanthroline assay)

The Cu-phenanthroline assay was conducted essentially as described by Aruoma (1994a). Reaction mixture contained in a final volume of 1.2 mL, the following reagents in order of addition indicated: 100 µL 1.10-phenanthroline (1.8 mM stock solution made up in water having initially dissolved the crystals in 50 µL ethanol), 480 µL copper (II) chloride (250 µM), 300 µL DNA (1.68 mg/mL) and 120 µL KH_2PO_4–KOH buffer at pH 7.4 (100 mM). 100 µL of ascorbate (2.88 mM) or extracts (100 µL) were added to initiate the reaction. The tubes were incubated at 37 °C for 1 h. At the end of the incubation period 100 µL 0.1 M EDTA was added to stop the reaction. DNA damage was assessed by adding 1 mL of 1% (w/v) TBA in 0.05 M NaOH and 1 mL 25% (v/v) HCl followed by 15 min incubation at 80 °C. The pink chromogen formed was extracted into 2 mL of butan-1-ol and the absorbance measured at 532 nm. Results were expressed as the absorbance at 532 nm.

Statistical analysis

Results are expressed as mean value ± standard deviation ($n = 3$). Simple regression analysis was performed to calculate the dose–response relationship of standard solutions used for calibration as well as for the test samples. Linear regression analysis was performed, quoting the correlation coefficient r_{xy}.

Results

Polyphenolic content of the plant extracts

The phenolic composition of the Mauritian endemic plant extracts is presented in Table 2. The amount of total phenolics varied widely and ranged from 10 to 103 mg/g fresh weight. Leaves of *Syzygium commersonii* showed the highest levels (103 ± 1.2 mg/g FW) followed by *Syzygium glomeratum* (84 ± 1.3 mg/g FW) and *Syzygium mauritianum*, which contained similar levels (77 ± 2.6 mg/g FW), and *Syzygium venosum* (69 ± 0.91 mg/g FW). Moderate but comparable values varying between 23 and 31 mg/g FW were observed in *Eugenia tinifolia*, *Eugenia orbiculata*, *Myonima nitens* and *Myonima obovata* while *Eugenia elliptica*, *Fernelia buxifolia*, *Monimiastrum acutisepalum* contained lower amounts with 16 ± 0.4, 15 ± 0.6, 13 ± 0.9 and 10 ± 0.4 mg/g FW, respectively.

Proanthocyanidin contents ranged from 3 to 40 mg/g FW with maximum level in *Syzygium mauritianum*. Lower amounts were recorded in the other *Syzygium* species (*Syzygium glomeratum*: 23 ± 4.5 mg/g FW, *Syzygium venosum*, 10 ± 1 mg/g FW and *Syzygium commersonii*: 9 ± 1 mg/g FW). *Eugenia* species contained between 7 and 19 mg/g FW. High levels were also observed in both *Myonima* species with 21 ± 1.1 mg/g FW in *M. nitens* and 26 ± 3.1 mg/g FW in *M. obovata*. *Monimiastrum* species and *Fernelia buxifolia* were poor proanthocyanidin sources. HPLC data show the prominent presence of procyanidins B1 and B2 in most of the studied species. Procyanidin B1 ranged from 63 and

Table 2. Phenolic content and antioxidant activities of the Mauritian endemic plant extracts

Species	Total[a] phenolics	Total[b] proanthocyanidins	Total[c] flavonoid	FRAP[d]	TEAC[e]	HOCl[f]	Deoxyribose[g]
Fernelia buxifolia	13±0.86	3±0.33	4±0.05	224±2.71	309±7.07	2.07	20±0.67
Myonima nitens	25±0.39	21±1.13	6±0.21	335±29.96	409±1.05	0.69	16±0.75
Myonima obovata	31±1.47	26±3.06	6±0.15	374±2.8	445±5.24	0.65	7±1.67
Eugenia elliptica	16±0.37	13±0.54	6±0.02	290±4.06	373±3.14	0.58	13±1.59
Eugenia orbiculata	28±1.0	19±0.24	10±0.27	308±49.06	1463±35.32	0.15	16±0.23
Eugenia tinifolia	23±1.36	7±1.36	10±0.02	200±11.81	590±10.48	0.48	26±1.73
Monimiastrum acutisepalum	10±0.35	5±0.95	4±0.05	245±7.62	273±3.143	1.40	32±2.02
Syzygium commersonii	103±1.20	9±0.97	15±0.08	1392±64.58	1485±40.71	0.17	Prooxidant
Syzygium glomeratum	84±1.33	23±4.45	16±0.54	460±3.33	1351±170.6	0.15	5±0.58
Syzygium mauritianum	77±2.63	40±2.55	4±0.1	568±3.67	1088±23.35	0.23	Prooxidant
Syzygium venosum	69±0.91	10±1.04	5±0.1	770±25.71	1028±71.38	0.16	Prooxidant

All analyses were mean of triplicates measurements ± standard deviations.
[a]Results expressed in mg gallic acid equivalent/g fresh weight.
[b]Results expressed in mg cyanidin chloride equivalent/g fresh weight.
[c]Results expressed in mg quercetin equivalent/g fresh weight.
[d]Values are expressed in units of μmol Fe(II)/g fresh weight.
[e]Results expressed in units μmol trolox equivalent/g fresh weight.
[f]Values expressed as IC_{50} (mg FW/ml).
[g]Results are expressed as % inhibition of deoxyribose oxidation/g FW plant extract.

6484 μg/g FW while procyanidin B2 varied between 157 and 1221 μg/g FW. *Syzygium commersonii* and *Syzygium venosum* contained the highest level of B1 (6484±8.6 and 5873±7.8 μg/g FW, respectively). Procyanidin B1 was not detected in *Fernelia buxifolia*, *Myonima nitens* and *Syzygium mauritianum*. The highest B2 content was measured in *Syzygium mauritianum* and *Syzygium venosum* while the other plant species were marked by lower levels with values ranging from 157 to 871 μg/g FW (Table 3).

The total flavonoid content ranged from 4 to 16 mg/g FW with optimum amounts assayed in *Syzygium glomeratum* (16±0.5 mg/g FW) and *Syzygium commersonii* (15±0.1 mg/g FW). HPLC screening of the hydrolysed extract showed that quercetin and kaempferol were the main flavonol aglycones present while myricetin was detected only in *Myonima nitens* and *Syzygium commersonii* (Table 4), whilst the flavone aglycone. Apigenin and luteolin were not detected. Quercetin was the predominant aglycone ranging between 34 and 7975 μg/g FW, the maximum being present in *Monimiastrum acutisepalum*. High levels of kaempferol aglycones were measured in *Eugenia elliptica* (3531±72 μg/g FW), followed by *Syzygium mauritianum* (2149±44 μg/g FW), *Myonima nitens* (1441±29 μg/g FW) and *Myonima obovata* (871±18 μg/g FW).

Table 3 shows the distribution of flavan-3-ol derivatives ((+)-C, (−)-EC, (−)-EGC, (−)-ECG and (−)-EGCG and gallic acid in Mauritian endemic plant extracts. (+)-C was distributed in all species studied ranging from 113 to 6801 μg/g FW with highest level in *Eugenia orbiculata* (Fig. 1). The amount of (−)-EC varied from 119 and 7636 μg/g FW. *Eugenia tinifolia*, *Syzygium mauritianum*, *Eugenia orbiculata* and *Syzygium glomeratum* were characterized by high levels while the lowest amount was detected in *Eugenia elliptica*. (−)-EGC was common in the extracts with values ranging between 101 and 3649 μg/g FW except in *Monimiastrum globosum*. Significant and comparable levels of (−)-EGCG and (−)-ECG was recorded in *Syzygium commersonii* (8128±4.0 μg/g FW) and in *Syzygium venosum* (8512±51.9 μg/g FW), respectively. The concentrations of flavan-3-ols and gallic acid varied widely between different plant species but high values has been obtained mainly in plant extracts of the Myrtaceae family.

Free radical scavenging activity and reducing power of the plant extracts

The scavenging potential of the plant extracts as assayed by the TEAC method varied between 273 and 1485 μmol/g FW. *Syzygium commersonii* exhibited the highest activity (1485±40.7 μmol/g FW) followed by *Eugenia orbiculata* (1463±35.2 μmol/g FW), *Syzygium glomeratum* (1351±17.1 μmol/g FW), *Syzygium mauritianum* (1088±23.5 μmol/g FW) and *Syzygium venosum* (1028±71.4 μmol/g FW). *Fernelia buxifolia*, *Myonima*

Table 3. HPLC data of (+)-catechin, (−)-epicatechin, (−)-epicatechin gallate, (−)-epigallocatechin gallate, (−)-epigallocatechin, procyanidin B1, procyanidin B2 and gallic acid from the endemic plant extracts. Values are expressed in μg/g fresh weight

Plant species	(+)-C	(−)-EC	(−)-ECG	(−)-EGCG	(−)-EGC	B 1	B 2	GA
Fernelia buxifolia	118 ± 0.51	–	–	–	333 ± 5.1	–	–	29 ± 0.63
Myonima obovata	932 ± 4.04	–	399 ± 3.24	–	101 ± 1.55	194 ± 0.26	157 ± 3.05	–
Myonima nitens	482 ± 2.09	–	1085 ± 7.36	–	104 ± 1.59	–	–	48 ± 0.38
Eugenia tinifolia	3855 ± 16.72	7636 ± 25.59	–	2447 ± 1.93	1057 ± 1.62	2327 ± 3.09	731 ± 1.47	478 ± 6.3
Eugenia orbiculata	6801 ± 29.51	–	–	1583 ± 2.47	3649 ± 5.9	3652 ± 4.86	–	494 ± 6.52
Eugenia elliptica	182 ± 0.78	119 ± 0.4	–	–	113 ± 1.73	146 ± 0.19	–	96 ± 1.27
Monimiastrum acutisepalum	573 ± 2.49	786 ± 4.21	223 ± 1.81	–	111 ± 1.7	63 ± 0.08	–	43 ± 0.57
Syzygium commersonii	2927 ± 12.70	–	–	8128 ± 4.02	2104 ± 3.22	6485 ± 8.62	–	376 ± 4.95
Syzygium glomeratum	762 ± 3.31	3635 ± 31.67	8513 ± 51.92	2035 ± 5.03	711 ± 1.89	1128 ± 1.50	871 ± 1.69	144 ± 1.89
Syzygium venosum	2035 ± 8.83	–	3444 ± 28.04	–	674 ± 1.32	5873 ± 7.81	1120 ± 2.17	439 ± 5.78
Syzygium mauritianum	3850 ± 16.71	5780 ± 50.37	–	4866 ± 8.33	2484 ± 8.05	–	1221 ± 2.37	782 ± 1.32

Table 4. Concentration of plant material that caused 50% inhibition of microsome lipid peroxidation

Plant species	IC_{50} (mg/ml)Fe^{3+}/ascorbate	IC_{50} (mg/ml) AAPH
Eugenia orbiculata	0.02	0.093
Syzygium venosum	0.077	0.1
Syzygium mauritianum	0.085	0.33
Syzygium glomeratum	0.09	0.105
Eugenia elliptica	0.12	0.63
Myonima obovata	0.14	0.43
Syzygium commersonii	0.162	0.26
Eugenia tinifolia	0.25	0.19
Myonima nitens	0.285	0.22
Monimiastrum acutisepalum	0.4	0.68
Monimiastrum globosum	0.64	0.75
Fernelia buxifolia	1.75	1.55

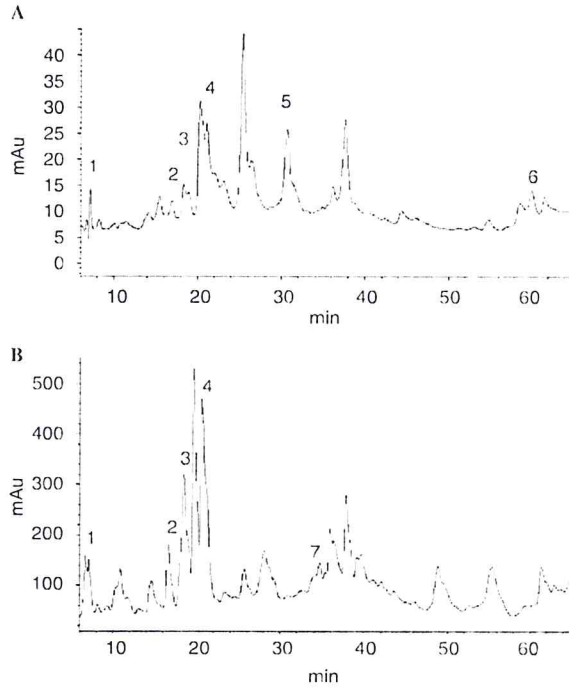


Figure 1. HPLC data of aglycones namely kaempferol, myricetin and quercetin from the hydrolysed plant extracts. Values are expressed in μg/g fresh weight.

obovata and *Myonima nitens* which are members of the Rubiaceae family had low TEAC values (Table 2). The FRAP values showed a very similar pattern as the TEAC values with *Syzygium commersonii* exhibiting the highest ferric reducing capacity (1392 ± 61.6 μmol/g

FW). However compared to *Syzygium commersonii* all the other plant extracts had lower FRAP values with data varying between 200 and 770 μmol/g FW (Table 2).

In the assessment of the abilities of the plant extracts to scavenge hypochlorous acid, *Eugenia orbiculata*, *Syzygium glomeratum*, *Syzygium venosum*, *Syzygium commersonii* and *Syzygium mauritianum* were found to be effective scavengers with very low IC_{50} values ranging between 0.15 and 0.16 mg FW/mL. *Fernelia buxifolia* and *Monimiastrum acutisepalum* were poor scavengers of HOCl as indicated by their high IC_{50} values (Table 2). The ability of the plant extracts to inhibit the reaction in the hydroxyl radicals-dependent deoxyribose assay was evaluated. The extracts were generally weak inhibitors of deoxyribose degradation. However *Monimiastrum acutisepalum* extract exhibited the highest inhibitory activity (32%) followed by *Eugenia tinifolia* (26%). Addition of *Syzygium commersonii*, *Syzygium venosum* and *Syzygium mauritianum* in the reaction mixtures seemed to stimulate deoxyribose degradation. A further examination of the potential prooxidant characteristics was carried out using the copper-phenanthroline-dependent DNA damage assay.

The lipid peroxidation induced by Fe^{3+}/ascorbate and the water-soluble azo-initiator AAPH were evaluated. Of the endemic plants studied the most potent inhibitor of microsomal lipid peroxidation in both assay system was *Eugenia orbiculata* (Table 4) and the inhibition occurred in a dose-dependent manner. The *Syzygium* species were also very effective at protecting the microsomes against Fe^{3+}/ascorbate-dependent lipid peroxidation with IC_{50} values ranging between 0.08 and 0.16 mg FW/mL. Higher calculated IC_{50} values ranging between 0.1 and 0.33 mg FW/mL were observed for the Syzygium extracts in the lipid peroxidation system stimulated by AAPH. Relatively lower activities were measured for *Monimiastrum* and *Myonima* species under these particular experimental conditions (Table 4). The lowest activity was observed with *Fernelia buxifolia*.

The potential prooxidant activity of the extracts was evaluated by measuring levels of oxidative damage to the sugar moiety of DNA chelated to the copper–phenanthroline complex. The extracts were not prooxidant at concentrations below 5 g FW/L. However, *Syzygium commersonii*, *Syzygium glomeratum*, *Syzygium venosum*, *Syzygium mauritianum*, *Myonima nitens* and *Myonima obovata* at 10 g FW/L had prooxidant activity only approaching 50% that of 240 μM ascorbate (Table 6). The prooxidant activity of ascorbic acid (240 μM) was used as 100% for the basis of estimating the potential prooxidant activities (Table 5).

Discussion

There is continuing interest on the screening of medicinal plants with a view to determine new sources of natural antioxidants (Kähkönen et al., 1999; Mensor et al., 2001). Of the endemic plant extracts examined, only *Eugenia tinifolia* and *Syzygium glomeratum* have been reported to have medicinal uses (Table 1). The former species is known to have purgative properties while *S. glomeratum* has been used in the treatment of migraine (Gurib-Fakim, 1996). Data related to the nature of phytochemicals and indications of the majority of extracts investigated are lacking and need to be substantiated. The present study elaborates on the polyphenolic composition, as well as on the antioxidant activity of leafy total extracts. High levels of phenolic compounds were measured in species like *S. commersonii*,

Table 5. Concentration of plant material that caused 50% inhibition of microsome lipid peroxidation, initiated by [a]Fe^{3+}/Ascorbate and [b]AAPH. Results are the mean of triplicate experiments ± standard deviation and are expressed as mg fresh weight/ml

Plant species	IC_{50}^{a}	IC_{50}^{b}
Eugenia orbiculata	0.02 ± 0.002	0.093 ± 0.003
Syzygium venosum	0.077 ± 0.006	0.1 ± 0.002
Syzygium mauritianum	0.085 ± 0.009	0.33 ± 0.007
Syzygium glomeratum	0.09 ± 0.01	0.105 ± 0.004
Eugenia elliptica	0.12 ± 0.009	0.63 ± 0.01
Myonima obovata	0.14 ± 0.01	0.43 ± 0.009
Syzygium commersonii	0.162 ± 0.004	0.26 ± 0.003
Eugenia tinifolia	0.25 ± 0.053	0.19 ± 0.001
Myonima nitens	0.285 ± 0.020	0.22 ± 0.062
Monimiastrum acutisepalum	0.4 ± 0.044	0.68 ± 0.015
Fernelia buxifolia	1.75 ± 0.09	1.55 ± 0.020

Table 6. Effect of plant extracts and ascorbic acid (240 μm) on copper 1–10 phenanthroline dependent DNA damage

Plant extract (g FW/L)	Extent of DNA damage (A_{532} nm)	Plant extract (g FW/L)	Extent of DNA damage (A_{532} nm)	Plant extract (g FW/L)	Extent of DNA damage (A_{532} nm)
		Eugenia orbiculata		*Syzygium commersonii*	
None	0.000	0.1	0.004	0.1	0.000
Ascorbic acid (240 μM)	0.179	0.5	0.015	0.5	0.005
		1.0	0.026	1.0	0.013
		5.0	0.057	5.0	0.049
		10	0.075	10	0.105
Monimiastrum acutisepalum		*Myonima obovata*		*Syzygium glomeratum*	
0.1	0.000	0.1	0.003	0.1	0.007
0.5	0.003	0.5	0.011	0.5	0.019
1.0	0.009	1.0	0.023	1.0	0.031
5.0	0.017	5.0	0.048	5.0	0.054
10	0.026	10	0.083	10	0.101
Syzygium mauritianum		*Syzygium venosum*		*Myonima nitens*	
0.1	0.000	0.1	0.010		
0.5	0.003	0.5	0.034	0.1	0.005
1.0	0.017	1.0	0.047	0.5	0.008
5.0	0.057	5.0	0.064	1.0	0.015
10	0.139	10	0.097	5.0	0.063
		10	0.109		
Fernelia buxifolia		Eugenia elliptica			
0.1	0.006	0.1	0.000		
0.5	0.011	0.5	0.000		
1.0	0.021	1.0	0.008		
5.0	0.039	5.0	0.021		
10	0.056	10	0.034		

S. glomeratum and *S. mauritianum*. Phenolic constituents identified were gallic acid, proanthocyanidins including B1 and B2 dimers, conjugated flavonoids containing mainly quercetin and kaempferol and catechins comprising mainly of (+)-catechin, (−)-epicatechin, (−)-epicatechin gallate, (−)-epigallocatechin gallate and (−)-epigallocatechin. These substances are widely discussed with regards to their ability to scavenge free radicals, peroxyl, superoxide and hydroxyl radicals (Hatano et al., 1989; Silva et al., 2002; Strlič et al., 2002; Vaya et al., 2003).

Given that the mechanisms of action of naturally occurring antioxidants can be diverse in vivo, a comprehensive prediction of the antioxidant efficacy initially in vitro requires a multiplicity of assessing methods with various implications for molecular targets (Aruoma 1994a, 2003). In this study, a number of well-established in vitro assays were used to characterize the direct antioxidant activities of the plant extracts. Whilst the TEAC system involved the potential scavenging of an artificially generated $ABTS^{\cdot}$ radical (Campos and Lissi, 1997), the reaction mechanisms invloved in the deoxyribose, HOCl, copper-phenthroline and lipid peroxidation assays, are such that the ability to react with the biologically relevant reactive oxygen species can be tested (Weiss et al., 1982; Aruoma, 1994a, 1996). Mauritian endemic plant extracts exhibited a wide range of antioxidant potentials. For instance the antioxidant potency of the extracts in TEAC system was in the following order: *S. commersonii* > *E. orbiculata* > *S. glomeratum* > *S. mauritianum* > *S. venosum* > *E. tinifolia* > *M. obovata* > *M. nitens* > *E. elliptica* > *F. buxifolia* > *M. acutisepalum*. In the FRAP assay the order of potency was *S. commersonii* > *S. venosum* > *S. mauritianum* > *S. glomeratum* > *M. obovata* > *M. nitens* > *E. orbiculata* > *E. elliptica* > *M. acutisepalum* > *F. buxifolia* > *E. tinifolia* whereas for the HOCl assay the order was *E. orbiculata* ~ *S. venosum* ~ *S. mauritianum* ~ *S. commersonii* ~ *S. glomeratum* > *E. tinifolia* > *M. obovata* > *M. nitens* > *F. buxifolia* > *E. elliptica* > *M. acutisepalum*. It is clear that due to the complex nature of the different phytochemical classes, the antioxidant capacities of plant extracts cannot be evaluated using a single method. This justifies the use of a multiple-method approach in antioxidant activity assessment as recommended in literature (Frankel

and Meyer, 2000; Aruoma, 1996, 2003; Aruoma et al. 1996). Lipid peroxidation of microsomes assayed with TBA was also used to evaluate the efficacy of the Mauritian plant extracts to protect membrane mimetics. However, the inhibition of lipid peroxidation produced different results when different initiators of lipid peroxidation were used. The order of activity in the Fe^{3+}/ascorbate-dependent peroxidation was as follows *E. orbiculata* > *S. venosum* > *S. mauritianum* > *S. glomeratum* > *E. elliptica* > *M. obovata* > *S. commersonii* > *E. tinifolia* > *M. nitens* > *M. acutisepalum* > *F. buxifolia*. In the system where lipid peroxidation was stimulated by AAPH the hierarchy of activity based on the IC_{50} values was in the following decreasing order: *E. orbiculata* > *S. venosum* > *S. glomeratum* > *E. tinifolia* > *M. nitens* > *S. commersonii* > *S. mauritianum* > *M. obovata* > *E. elliptica* > *M. acutisepalum* > *F. buxifolia*, suggesting that the inhibitory activity was intrinsically linked to peroxyl radical scavenging. Thus plants from the Myrtaceae family mainly *Eugenia* and *Syzygium* species had high measurable antioxidant activity under this experimental condition with IC_{50s} ranging from 0.012–0.25 mg FW/mL. The ability of the Mauritian endemic plant extracts in inhibiting lipid peroxidation suggests a potential prophylactic importance uses in food matrix as preservatives.

It is interesting to compare data obtained on Mauritian endemic plants with other plant species obtained in the same assaying systems. This study has revealed that the total antioxidant content of Mauritian plants is relatively high compared with the results of similar studies of wild medicinal plants used in New Mexico and in West-African plants (VanderJagt et al., 2002). The range of antioxidant values measured in those studies were 27–972 and 3.9–26.3 μmol Trolox equivalent/g dry weight, respectively, compared to a range of 412–2861 μmol Trolox equivalent/g dry weight observed in the Mauritian endemic plants. In addition, Chen et al. (2005) reported the total antioxidant activity of some selected medicinal plants in particular *Ampelopsis sinica*, *Ampelopsis humiforlia*, *Potentilla Freyniana*, *Selaginella labordei* and *Chrysanthemum multiflorum* in the range of 26.1–61.2 mM Trolox at a concentration of 10 mg/mL. In a previous work on *Cassia fistula* vegetative and reproductive organs we reported TEAC and FRAP data varying respectively from 93 to 992 μmol Trolox equivalent/g dry weight and 51–811 μmol Fe(II)/g dry weight with maximum activity in pod extracts (Luximon-Ramma et al., 2002). Mantle et al. (2000) reported high $ABTS^{\bullet+}$ and the superoxide radical ($O_2^{\bullet-}$) scavenging for British medicinal plants and suggested that those showing appreciable antioxidant activity be considered for use in the treatment of the neurodegenerative condition Alzheimer's disease.

Studies conducted by Bahorun et al. (1996) on the HOCl scavenging potential of *Crataegus monogyna*, showed IC_{50} value of the order of 8.49 mg dry weight/L for floral buds. However in a similar system, *Cynara carduncula* L. exhibited weak protective activity against damage by HOCl (Valentão et al., 2002). *Monimiastrum acutisepalum* provided 32% inhibition of deoxyribose degradation although it showed weaker scavenging activities towards $ABTS^{\bullet}$ radical and hypochlorous acid. This could be attributed to the fact that the assays used to determine the antioxidant activity are based on different mechanisms with different expected results, thereby indicating that extracts showing poor antioxidant properties in one assay system should not be categorized as poor sources of antioxidants. This is an important issue for studies to assess the antioxidant potentials of bioactive components in both food plants and medicinal plants (Aruoma 2003). *Syzygium commersonii*, *Syzygium mauritianum* and *Syzygium venosum* were found to increase oxidative damage of the deoxyribose sugar and were therefore suggested to exhibit prooxidant actions in the deoxyribose assay. In this particular assay, compounds may act as prooxidants because of their ability to redox cycle the metal ion required for $^{\bullet}OH$ generation and thus increase the radical production (Aruoma, 1994a, b, 1996; Li and Xie, 2000; Schinella et al., 2002). Similar studies conducted on *Cassia fistula* showed that the extract provided 30% protection against deoxyribose oxidative damage at a concentration 125 μg/mL (Munasinghe et al., 2001) while *Poria cocos* and *Coptis chinensis*, both powerful Chinese and Mediterranean folkloric medicine, showed an inhibitory activity higher than 40% (Schinella et al., 2002). The ability of the plant extracts to inhibit lipid peroxidation was comparable to the protective effect of *Evolvulus alsinoides* (0.089 mg/mL), *Sida cordifolia* (0.1 mg/mL) and *Cynodon dactylon* (0.61 mg/mL), three medicinal plants traditionally used in the management of neurodegenerative diseases (Auddy et al., 2003). In addition, the inhibitory activity of 10 Korean medicinal plants against assessed by Kang et al. (2003) against lipid peroxidation was evident at concentrations of 0.2 mg/mL.

As some of the plant extracts were able to stimulate oxidative damage to deoxyribose by possibly interacting with the iron ions in the reacting mixture, the prooxidant activity of all the plant extracts were evaluated using the copper–phenanthroline assay. *S. commersonii*, *S.*

mauritianum, *S. venosum* and *M. nitens* stimulated DNA damage at the highest concentrations used (Table 5). Although the apparent prooxidant nature of plant extracts can be attributed to the phenolic compounds, the extent of the prooxidant index is nowhere near that of the reference ascorbic acid. Studies have revealed that phenolic antioxidants can induce damage to carbohydrates, proteins and DNA in in vitro assays (Aruoma, 1994a), but the reactions can be inhibited if proteins such as albumin are included in the reaction mixture (Smith et al., 1992). Hollman et al. (1996) and Manach et al. (1998) have argued that the effective concentrations of the bioactive compounds mainly quercetin following the consumption of a flavonoid rich food is in the range 300–650 nM. Thus the prooxidant actions seen for the Rubiaceae and Myrtaceae family of endemic plants in in vitro context are unlikely to pose problems in vivo.

In rationalizing the antioxidant potential of the plant extracts in terms of phenolic classes, it is clear from the regression correlation coefficient data that the antioxidant capacity based on TEAC, FRAP and HOCl was associated more strongly with total phenols ($r = 0.778$, 0.759, and 0.714, respectively) and to a lower extent with total flavonoid and total proanthocyanidin content. Correlation with individual compounds quantified by HPLC in the plant extracts were significantly high (TEAC/(−)-ECG, $r = 0.908$; FRAP/(−)-EGCG, $r = 0.922$; HOCl/(−)-ECG, $r = 0.798$) indicating the important contribution of these compounds in the antioxidant scavenging and reducing potential of the extracts. Despite the striking linear correlation between antioxidant activities and phenolic contents in the plant extracts, it is, however, difficult to ascribe the antioxidant potency of the plant extracts to a specific polyphenolic class or to individual substances since the overall antioxidant effect of the extracts may be due to the synergistic influence of the different phenolic classes.

Similar linear relationships have been observed between phenolic content and the antioxidant capacity of *Cassia fistula* plant extracts (Luximon-Ramma et al., 2002; Siddhuraju et al., 2002) whilst the antioxidant activity of *Eucommia ulmoides* (Du Zhong) leaf extracts were strongly related to the high flavonoid and phenolic acid content (Yen and Hsieh, 1998). The phenolic/antioxidant activity correlation does not seem to be restricted to the endemic plants, as similar observations have been made for fruits (Luximon-Ramma et al., 2003), beverages (McPhail et al., 1999; Richelle et al., 2001), vegetables (Velioglu et al., 1998), juices (Gil et al., 2000), wines (Burns et al., 2000) and callus cell cultures (Bahorun et al., 2003).

Knowledge regarding the chemical nature and mechanisms of action of antioxidants, and their important role in disease prevention and treatment is rapidly evolving. Data pertaining to the composition of traditional plants will allow for rational recommendations of their use. In the light of the results obtained it is clear that Mauritian endemic plant extracts in particular the genus Eugenia and Syzygium could be a potential source of prophylactic antioxidants against reactive oxygen species and reactive nitrogen species. This would benefit a rational for clinical trials using the endemic plants for such conditions as Alzheimer, Parkinson, diabetes, cancer, viral and cardiovascular diseases where free radical mechanisms are implicated. Plants have played a dominant therapeutic role in the treatment of human ailments since time immemorial and it has been reported that 50% of all pharmaceuticals are derived from plant sources. Mauritian endemic plants thus represent an interesting source of untapped bioactive compounds and considerable effort is warranted towards the conservation of the Mauritian flora mainly because of their prospective therapeutic effects. Thus the management of endemic plant resources is of utmost significance for the identification of novel prophylactic compounds and for the sustainable future use of these plants.

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Assessment of the DNA damaging potency and chemopreventive effects towards Bap-induced genotoxicity in human derived cells by Monimiastrum globosum, an endemic Mauritian plant

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ABSTRACT

Naturally occurring compounds have protective effects towards mutagens and carcinogens. The leaf extract of *Monimiastrum globosum* (Bois de Clous), a Mauritian endemic plant from the *Myrtaceae* family, was studied for its potency to induce DNA damage in human HepG2 hepatoma cells using DNA migration as a biological endpoint in the alkaline single cell gel electrophoresis (SCGE) assay. This was contrasted with the ability to modulate the benzo[a]pyrene (BaP)-dependent DNA damage in human hepatoma cells. *M. globosum* caused genotoxicity in HepG2 cells at concentrations exceeding 3 mg fresh weight (FW) per ml cell culture in the absence of cytotoxicity. Pre-treatment of the cells with 12.2 µg FW/ml to 1.56 mg FW/ml led to a pronounced antigenotoxic effect towards BaP-induced DNA damage. DNA migration (OTM) was reduced by 66%, 81.5% and 74% for 49, 98 and 195 µg FW/ml, respectively. A U-shaped dose-response curve was derived for *M. globosum* indicating genotoxic effects in high doses and antigenotoxic effects in low doses. *M. globosum* extract had total phenolics (15 mg/g FW) with flavonoids (aglycones and conjugates: 8 mg/g FW) and proanthocyanidins (3 mg/g FW) as major phenolic subclasses. The hydrolysis of conjugated flavonoids yielded the aglycones quercetin (606 µg/g FW) and kaempferol (117.8 µg/g FW) while HPLC-MS/MS analysis of the total extract revealed free flavonoids such as quercetin (19.2 µg/g FW) and myricetin (2.5 µg/g FW). The antioxidant activity of the extract of *M. globosum*, assessed by the FRAP and TEAC assays yielded values of 275±3.82 µmol/g FW and 346±4.2 µmol/g FW, respectively.

Keywords: *Monimiastrum globosum*, antigenotoxicity, polyphenols, flavonoids, Antioxidant assays, Single cell gel electrophoresis, Comet assay, BaP-induced DNA damage

Abbreviations: BaP, benzo(a)pyrene; FRAP assay, Ferric reducing/antioxidant power (FRAP) assay; FW, fresh weight; *M. globosum, Monimiastrum globosum;* TEAC assay, Trolox equivalent antioxidant capacity (TEAC) assay, ROS, radical oxygen species; SCGE assay, single cell gel electrophoresis (comet) assay

INTRODUCTION

Accumulating data from *in vitro* and short-term *in vivo* studies as well as long-term carcinogenicity studies with chemically-treated animals continue to show that phytochemicals could possess antimutagenic and anticarcinogenic effects (Mitscher et al., 1992; Ferguson, 1994; Gupta et al., 2001; Waters et al., 1996; Starvic, 1996). Additionally, epidemiological studies support that chemopreventive effects are associated with the intake of plant materials (Cline and Hughes, 1998; Dreosti, 1998). However, under some experimental conditions, these phytochemicals exhibit genotoxic and/or mutagenic effects by themselves or potentiate the effect of other xenobiotics (Stich, 1991; Anderson et al., 1998; Yoshino et al., 1999; Mersch-Sundermann et al., 2004). Thus it is of paramount importance to investigate the circumstances under which phytochemicals, used in traditional medicine and/or potential prophylactic agents exhibit beneficial and harmful effects.

Monimiastrum globosum (M. globosum) (Bois de Clous) (Figure 1) is a small, erect and slender tree, which can reach 5 m high, found in the Mauritian indigenous highland forests. It is an endemic species of Mauritius from the *Myrtaceae* family. A chemosystematic survey had previously shown that the plant contained relatively large amounts of phenolics, most probably due to the fact that tropical plants are tolerant of high levels of environmental stress induced by ultraviolet radiation. The effects of the high phenolic contents of *M. globosum* and other Mauritian endemic plants have been studied on the promoter activities of antioxidant enzymes and it has been shown that phenolics were proportionally associated with Cu, Zn-SOD promoter activity and were inversely correlated with catalase promoter activity

(Toyokuni et al., 2003). The chemopreventive potentials of the extract might reside in their abilities to modulate the expression of antioxidant enzyme genes thereby warranting further studies of the extract in other systems. The aim here was to evaluate the genotoxic and antigenotoxic, potency of the *M. globosum* extract on human cells and to assess its contents of antioxidant phenolic compounds.

In the antigenotoxicity experiments we used benzo(a)pyrene, a polycyclic aromatic hydrocarbon (PAH), which is a strong mutagen and suspected human carcinogen (IARC, 1987) at a concentration of 50 µM. PAH is ubiquitously distributed in the environment, although the principle exposure route for humans is through the diet (Phillips et al, 1999). BaP has been shown to induce gene mutations, chromosomal aberrations and other types of genotoxic effects in both *in vitro* and *in vivo* studies (IARC, 1973, 1983). For exerting its genotoxic and/or carcinogenic effect, BaP has to be activated by CYP-450 isoenzymes to a variety of mutagenic and carcinogenic electrophiles which can covalently bind to the DNA, RNA or proteins (Zytkovicz, et al 1981). Besides that, the generation of BaP-metabolites, especially through the aryl hydrocarbon receptor (AhR)-dependent upregulation of CYP1A1, may directly form radical oxygen species (ROS) through redox cycling or may disrupt mitochondrial electron transport, leading to more ROS formation and depletion of ATP (Elbekai et al., 2004).

MATERIALS AND METHODS

Chemicals

DMSO and benzo[a]pyrene (BaP) (CAS: 50-32-8, purity > 99%) were purchased from Sigma Aldrich (Steinheim, Germany). Normal melting point agarose (NMA) was from Merck (Darmstadt, Germany) and low melting point agarose (LMA) was obtained from Serva (Heidelberg, Germany). Dulbeccos Minimal Essential Medium (DMEM), fetal calf serum (FCS) and antibiotics (penicillin, streptomycin) were from Invitrogen, Karlsruhe, Germany.

BaP was dissolved in sterile DMSO (purity >99%). Quercetin, kaempferol and morin were from Extrasynthèse (Genay, France). Myricetin was purchased from Sigma-Aldrich (Steinheim, Germany). Fisetin was purchased from Roth (Karlsruhe, Germany). All other chemicals and reagents were from Sigma, Germany.

Plant material (*M. globosum *extract)

Leaves of *M. globusum* (Figure 1) were harvested at Le Mondrain Natural reserve; Mauritius, in 1999 and frozen at -30°C until use. Voucher specimen has been deposited at the Mauritius Herbarium, Mauritius Sugar Industry Research Institute, Réduit and at the Department of Biosciences, Faculty of Science, University of Mauritius. Frozen mature leaves (25 g) were crushed using a Waring blender, in 500 ml methanol/water (70/30 v/v) and left to macerate for 24 h at 4 °C. After filtration the residue was homogenized in 500 ml acetone/water and left again to macerate for 24 h at 4 °C before finally being filtered and extracted with 1000 ml absolute methanol. The combined filtrates were reduced to the aqueous phase *in vacuo* at 37 °C before being washed with dichloromethane (3 x 150 ml) to remove fat-soluble substances and chlorophylls. The aqueous extract was concentrated and divided into two equal aliquots. The first part was freeze-dried and re-dissolved in methanol at a final 1:5 fresh weight: volume ratio for storage at -30°C. This was used for the analysis of phenolic compounds. The second aliquot was used to determine antioxidant activity.

Human HepG2 hepatoma cells

HepG2 cells were kindly provided by Dr. Firouz Darroudi, Leiden University Medical Center "LUMC", The Netherlands (Natarajan and Darroudi, 1991). The cells were cultured in DMEM supplemented with 15 % fetal calf serum and 100 U/ml penicillin and 0.1 mg/ml streptomycin in a 5% CO_2 atmosphere at 37°C. Only cells of passage 5-17 were used in the experiments.

Alkaline single cell gel electrophoresis (SCGE)

The SCGE assay (Comet assay) was carried out as described by Singh et al. (1991) according to the guidelines developed by Tice et al. (1990, 2000) and Klaude et al. (1996) with slight modifications. Briefly, HepG2 cells were plated onto multiwell systems at a density of 1 x 10^5 cells /ml culture medium. After 24 h of growth the cells were exposed to dilutions of *M. globosum* extract corresponding to 2-6250 µg FW/ml culture medium for 24 h (genotoxicity assay). Cytotoxicity of the plant extract towards HepG2 cells was determined by the trypan blue method. A viability of ≥ 80 % was considered as no relevant cytotoxic effect. Then, a suspension of 1 x 10^4 cells was mixed with 100 µl of 0.5 % LMA in PBS and transferred to normal melting agarose-coated (1.5%) slides. Two slides were prepared for each concentration of the compound tested. The slides were covered with a coverslip and the agarose was allowed to solidify in a refrigerator at 4°C. Thereafter, the coverslips were removed and the slides kept in a lysing solution (pH 10) containing 2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris, 1% Triton-X 100 and 10% DMSO over 1 h at 4°C. After alkali unwinding (1 mM Na_2EDTA and 0.3 mM NaOH), pH 13.5, for 20 min., the slides were electrophoresed at 25 V and 300 mA for another 20 min. Subsequently, the slides were rinsed two times with 400 mM Tris buffer (pH 7.5), stained with 80 µl ethidium bromide solution (0.02% in water) and analyzed with a Leica fluorescence microscope (Leica DMLS; excitation filter: BP 546/10 nm; barrier filter: 590 nm) connected to a computerized image analysis system (Kinetic Imaging 3.1, Optilas, München, Germany). The Olive tail moment (OTM), calculated according to Olive et al. (1992), was used as indicator of DNA damage. For antigenotoxicity testing, after 24h cell growth the cell cultures were pre-treated with dilutions of *M. globosum* extract (2-6250 µg FW/ml) for another 24h. Then, the medium was removed, the cell culture washed twice with PBS buffer and after that exposed to 50 µM Bap for another 24h. In the SCGE assay, 102 systematically screened cells per concentration were

evaluated and the test was carried out three times. The reported OTM are the mean values ± standard deviation (SD) of three independent experiments. For *M. globosum* genotoxicity and antigenotoxicity experiments the significance was calculated in comparison to the negative control (Aqua dest.) and BaP reference, respectively, using the Student's t-test (* $p \leq 0.05$, ** $p \leq 0.01$).

Total phenolic content analysis

Total phenolics were determined by the method of Singleton and Rossi (1965) using the Folin-Ciocalteu reagent. 0.25 ml of diluted samples was added to 3.5 ml of distilled water in screw-capped test tubes followed by 0.5 ml Folin-Ciocalteu solution. After 3 mins, 1 ml of sodium carbonate (1 %) was added and the test tubes were properly shaked before incubating in boiling water bath for 1 min. The tubes were then allowed to cool in darkness. The absorbance of the blue colour that developed was read at 685 nm using gallic acid as standard. Results were expressed in mg of gallic acid/g fresh weight.

Total proanthocyanidin content analysis:

The HCl/butan-1-ol assay of Porter et al. (1986) was used to quantify the total proanthocyanidins. 0.25 ml of extract was added to 3 ml of a 95 % solution of n-Butanol/HCl (95:5 v/v) in stoppered test tubes followed by 0.1 ml of a solution of $NH_4Fe(SO_4)_2.12H_2O$ in 2 M HCl. The tubes were incubated for 40 mins at 95°C. The absorbance of the red coloration that developed was read at 550 nm. Data obtained was expressed in mg of cyanidin chloride/g fresh weight.

Total Flavonoid content analysis

The $AlCl_3$ method adapted from Lamaison and Carnat (1990) was used for the determination of the total flavonoid content of the methanolic extracts. 1.5 ml of extracts was added to equal

volumes of a solution of 2 % $AlCl_3.6H_2O$ (2 g in 100 ml methanol). The mixture was vigorously shaken and absorbance was read at 440 nm after 10 mins incubation. Data were expressed in mg quercetin equivalents/g fresh weight.

High performance liquid chromatography

HPLC analyses of hydrolysed total extract of *M. globusum* were carried out using a Hewlett Packard 1100 series liquid chromatography system equipped with a vacuum degasser, quaternary pump, auto-sampler, thermostated column compartment and diode array detector. After filtration on Millipore (0.22 µm) 30µl of 25% (v/v) aqueous methanolic extracts was injected onto a Spherisorb ODS 2 RP 18 column (5 µm pore size, 4.6 mm id x 150 mm) eluted by an acidified acetonitrile-water gradient. Elution with a flow rate of 0.7 ml/min at 25 °C was as follows: 0-30 minutes, 0-15% B in A; 30-50 minutes, 15% B in A; 50-60 minutes, 15-25% B in A; 60-90 minutes, 15-100% B in A; 90-100 minutes, 100-0% B in A (Solvent A: acetonitrile/water, 1/9 v/v, pH 2.6; Solvent B: acetonitrile/water, 1/1 v/v, pH 2.6). Quercetin and kaempferol were identified and quantified in the extracts after acid hydrolysis of flavonoid conjugates essentially as described in Crozier et al. (1997) with morin as an internal standard. An aliquot corresponding to 3 g fresh mass of plant extract was extracted with 20 ml of 60 % aqueous methanol containing 50µg morin as an internal standard. 5 ml of 6 M HCl was added to the extract, which was then refluxed at 90 °C for 2 hrs. After cooling the flavonoid aglycones were extracted using 2 x 25ml of ethyl acetate. The organic phase was evaporated to dryness and taken in absolute methanol prior to analysis by HPLC. Absorption wavelengths were selected at 280 and 360 nm.

HPLC-MS/MS analysis of flavonoid content

Mass spectrometry following HPLC separation was applied in order to authenticate the flavonoid components of *M. globosum*. Quercetin, myricetin and kaempferol were used as

internal standards. Plant extract solution 10mg of the freeze dried *M. globosum* sample was dissolved in 0.5 ml distilled water. An HPLC Agilent 1100 Series System (Waldbronn, Germany) interfaced with a Micromass Quattro Micro (Manchester, UK) MS/MS detector (triple quadrupole) was used for the chemical composition analysis. Luna 3µ C 18 (2) column (150 x 3.0mm) from Phenomenex (Aschaffenburg, Germany) was used for the chromatographic separation of the compounds prior to ms analysis. The column temperature was 60°C, the injection volume of each sample was 20 µl with an constant flow of 0.2 ml/min. The mobile phase for the flavonols was acetonitrile / water 50 /50 (V/V). Quantification was carried out with external standard solutions (20 µg/L), which were prepared by dissolving 2 µg of each flavonol compound in 100 mL distilled water. The limit of detection (LOD) was 2 µg/L for each compound.

Trolox equivalent antioxidant capacity (TEAC) assay

To determine the radical-scavenging ability of the plant extract, the TEAC assay was carried out according to the method of Campos and Lissi (1996). This assay measures the electron-donating ability of the plant extract to the artificially generated $ABTS^{+\cdot}$ radical which was monitored by the decay in absorbance at 734 nm. Results were expressed as µmol Trolox equivalent per gram fresh weight.

Ferric reducing/antioxidant power (FRAP) assay

The FRAP assay was carried out as recommended by Benzie and Strain (1996). It measures the change in absorbance at 593 nm owing to the formation of a blue colored Fe^{II}-tripyridyltriazine (Fe^{2+}-TPTZ) compound from the colorless oxidized Fe^{III} form (Fe^{3+}-TPTZ) by the action of electron donating antioxidants. Results were expressed as µmol Fe (II) per gram fresh weight.

RESULTS

The outcome of the SCGE analysis with *M. globosum* extract is shown in Figure 2. Significant DNA damage was caused only at a concentration higher than 1.56 mg FW/ml culture medium. *M. globosum* extract corresponding to 3.12 mg FW/ml exhibited a moderate and significant increase of DNA migration (OTM=3.0±0.27) in comparison to the *Aqua dest.* negative control (OTM=1.33±0.3); whereas the application of 6.25 mg FW led to a pronounced DNA-damaging effect in HepG2 cells with an OTM of 5.87±1.2 which exceeded the DNA migration of the Bap (50 µM) positive control (OTM=3.68±0.98) by more than 50%. Concentrations >6 mg of *M.globosum* led to cytotoxicity not acceptable for evaluation.

The studies of the antigenotoxic properties of *M. globosum* over a wide dose range resulted in a U-shaped dose-response curve (Figure 3). Pre-treatment of the cultures with both relatively low (<24.4 µg FW/ml) and extremely high (>3.12mg FW/ml) concentrations of *M. globosum* did not result in any significant antigenotoxic effect towards BaP-induced DNA migration in HepG2 cells. In comparison to the negative control (OTM=1.37±0.55) 50µM BaP caused a significant DNA damage with an OTM of 3.85±0.82. *M. globosum* treatment with concentrations of 12.2-1.562 µg FW/ml prior to BaP exposure resulted in a significant DNA-protective effects with regard to BaP-induced genotoxicity. The strongest antigenotoxic effect of *M. globosum* in HepG2 cells was found for 98 and 195 µg FW/ml with an 81.5 und 74% decrease of BaP-induced DNA damage, respectively.

No cytotoxicity could be observed in the genotoxicity or antigenotoxicity experiments with the plant extract. Exposure of HepG2 cells to the *M. globosum* extract at concentrations between 6.1 and 6250 µg FW/ ml culture medium resulted in a viability of ≥ 80 % (data not shown).

Phenolic analyses of total extract of *M. globosum* showed that the total phenolic content was 15 mg/g FW with total flavonoids and proanthocyanidins amounting respectively to 8 mg/g and 3 mg/g FW. The qualitative and quantitative determination of individual flavonoid

glycosides in plants was difficult, as most of the reference compounds are not commercially available. The hydrolysis of flavonoid glycosides to aglycones is therefore a practical approach for the quantitative determination of flavonoids in plant extracts. HPLC analysis of hydrolysed flavonoid conjugates in *M.globosum* showed that quercetin (606 µg/g fresh weight) and kaempferol (117.8 µg/g FW) were the main flavonol aglycones present. The HPLC-MS/MS analysis of *M.globosum* extract revealed the presence of free flavonoids at the concentration of 19.2 and 2.5 µg/g FW for quercetin and myricetin, respectively, whereas fisetin and kaempferol were not detectable, i.e. under detection limit of 2 µg/L. The antioxidant propensities of the *M. globosum* extract were determined using the TEAC and FRAP assays. Values of 346 ± 4.2 µmol/g FW and 275 ± 3.8 µmol/g FW were obtained respectively.

DISCUSSION

The phytochemical composition of *M. globosum,* makes it a potential source of chemopreventive compounds. Former investigations provided evidence that *M .globosum* contains antioxidative phenolic components such as (-)epicatechin gallate, quercetin-3-O-galactoside (hyperoside), kaempferol-3-glucoside (astragalin), quercetin-3-O-glucoside (isoquercitrin) and polymeric proanthocyanidins (Bahorun et al., 2001; Toyokuni et al., 2003) which may be responsible for protective effects towards chemically-induced DNA damage (Dauer et al., 2003; Knasmueller et al., 2000).

We assessed the geno- and antigenotoxic potency of the leaf extract of *M. globosum* towards BaP-induced DNA damage in human cells . The HEPADNA study (QLK1-CT-1999-0810) supports the assumption that human-derived, metabolically competent HepG2 hepatoma cells are a suitable tool for the detection of genotoxicity and by extension, cytoprotective, antigenotoxic and cogenotoxic effects, especially when using the alkaline single cell electrophoresis (SCGE) assay (Uhl et al., 2000; Knausmueller et al., 2004; Kassie et al., 2003;

Laky et al., 2002; Mersch-Sundermann et al., 2004; van Borstel 1998). The HepG2/SCGE assay was utilised in the experiments described here.

The results of the study demonstrated that extracts of *M. globosum* have modulatory, i.e. antigenotoxic effects on BaP-induced DNA migration in HepG2 cells. The exposure to relatively low concentrations of *M. globosum* (24.4 µg FW per ml culture medium) significantly inhibited the Bap-induced genotoxicity in HepG2 cells ($p \leq 0.05$) (Figure 3). The no observable effect level was 6.1 µg FW/ml. However, the exposure of the cell cultures to *M. globosum* extract alone resulted in a significant increase in DNA migration ($p<0.01$) when concentrations of >3 mg FW/ml were used in the cell cultures (Fig.2) without detection of any relevant cytotocicity. These results indicate that constituents of the complex plant mixture act as genotoxicants. Hence, our studies clearly demonstrated that *M. globosum* possess the character of a "Janus"-(anti)genotoxicant, a term used to designate compounds behaving as genotoxin or antigenotoxin, depending on the experimental conditions used (De Flora and Ramel, 1988). In this case high concentrations of the plant extract led to genotoxicity in the SCGE assay while low concentrations showed antigenotoxic effects towards BaP-induced genotoxicity in HepG2 cells.

The possible genotoxic effects of the plant extract may be attributed to the presence of phytophenolic components including tannins, catechins, flavones, isoflavones (Austin et al 1992; Kashiwada et al, 1993, Finlay et al, 1994; Duarte et al 1999; Ray et al, 2005). It has been reported that the genotoxicity might be related to hydrogen peroxide formation arising from the auto-oxidation of phenolic molecules (Rueff et al, 1989). In addition, flavonoids inhibit topoisomerase I and/or II enzymes i. e. interfering with the replication and transcription processes inhibiting the relegation of DNA double strand breaks and enhancing the formation of cleavable DNA-enzyme complexes (Ferguson, 2001; Ray et al, 2005). These effects could lead to accumulated DNA breaks and mutations thus contributing significantly to genotoxicity of phenolic rich extracts.

The bioactivity of *M. globusum* extract can be attributed to its phytochemical constituents, amongst which flavan-3-ol and flavonol derivatives are the main subclasses. Use of HPLC-MS/MS analysis enabled the estimation of the levels of the flavonols quercetin, myricetin, fisetin and kaempferol. The total free flavonol content found was 21.7 µg/g FW (19.2 µg/g FW quercetin and 2.5 µg/g FW myricetin). The antigenotoxic effect of *M. globosum* extract may be caused by enzyme induction (e. g. phase II enzymes such as glutathione-S-transferases which are responsible for the detoxification of BPDE, the ultimate genotoxic metabolite of BaP), or inhibition (especially of CYP1A1, an enzyme required for the metabolic activation of BaP), antioxidant and scavenging properties of the polyphenolics contained in it. . Additionally, scavenging of bioactive molecules represents one of the most important mechanisms in antimutagenesis and anticarcinogenesis (Rice-Evans et al., 1996).

Using independent antioxidant assays, *M. globosum* leaf extract has been shown to exhibit potent antioxidant and free radical scavenging propensities ascribed to its polyphenolic richness, more particularly to its flavonoid content. It is however suggested that the overall activity of the plant extract may be due to the synergistic effects of all phenolic sub-classes. Literature data widely suggest the use of plant phenolics as dietary supplements and therapeutic agents. This has been attributed to the multidimensional effect of the phenolics including free radical scavenging activity, metal chelation, inhibition of oxidases and alteration of gene expression notably the antioxidant response elements (ARE) which involves the phase II detoxifying enzymes (Yu et al., 1997; Vanderjagt et al., 2002). The radical scavenging TEAC and FRAP values obtained with the *M. globosum* extract suggests that this plant species can be a promising source of natural antioxidants. It could be ascribed to the presence of the flavonoids and proanthocyandins which are very effective free radical scavengers and reducing agents as discussed in Soobrattee et al, 2005 and Neergheen et al, 2006(In press)

The total antioxidant content of *M. globosum* is comparable to literature data on wild medicinal plants used in New Mexico, in which the Trolox-based assay was shown to range between 27 and 972 µmol trolox equivalent/ g dry weight respectively (Vanderjagt et al, 2002) compared to 761 µmol Trolox equivalent/ g dry weight observed in the Mauritian endemic plant extract. *M. globosum* also showed a high ferric reducing capacity (603 µmol Fe (II)/g dry weight) approaching that of the *Cassia fistula* pod extract (811 µmol Fe (II)/g dry weight) (Luximon-Ramma et al, 2002; Bahorun et al, 2005).

In conclusion, the present study showed that *M. globosum* extract possessed both antigenotoxic activity and genotoxicity. Whereas chemopreventive compounds display a threshold value, DNA damaging compounds, i.e. mutagens and/or genotoxic carcinogens are theoretically bioactive even at lowest concentrations. Thus the eventual use of the *M. globosum* extract for medical treatment or dietary purposes must take into account the amount of bioavailable flavonols.

Although data pertaining to the bioavailability of *M. globosum* extract are lacking, several reports have shown that polypenolic compounds, in particular the glycosides, can be absorbed directly in the small intestine (Hollman et al 1995). Flavonoids in their glycosidic forms are more bioavailable than their corresponding aglycones (Bagchi et al, 2000). Phenolics have been detected in a wide range of tissues including brain, endothelial cells, heart, kidney, spleen, pancreas, prostate, uterus, ovary, mammary gland, testes, bladder, bone and skin (Manach et al, 2004) and the concentration obtained in these tissues ranged from 30 to 3000 ng aglycone equivalents/g tissue. Data concerning the metabolism and bioavailability of phenolic compounds from plant extracts are extremely primordial for a reasonable evaluation of their potential health effects and for a proper understanding of the biological activities at the tissue and cellular level leading to a comprehensive view on how phenolics influence metabolic pathways and consequently modulate human health.

In order to define the potential risks or benefits related to the consumption of *M. globosum* extracts, detailed *in vivo* studies are necessary. Understanding the mechanisms of the antimutagenic, anticarcinogenic and antioxidant functions of the Mauritian endemic plant *M. globosum* will be important for defining its prophylactic potentials

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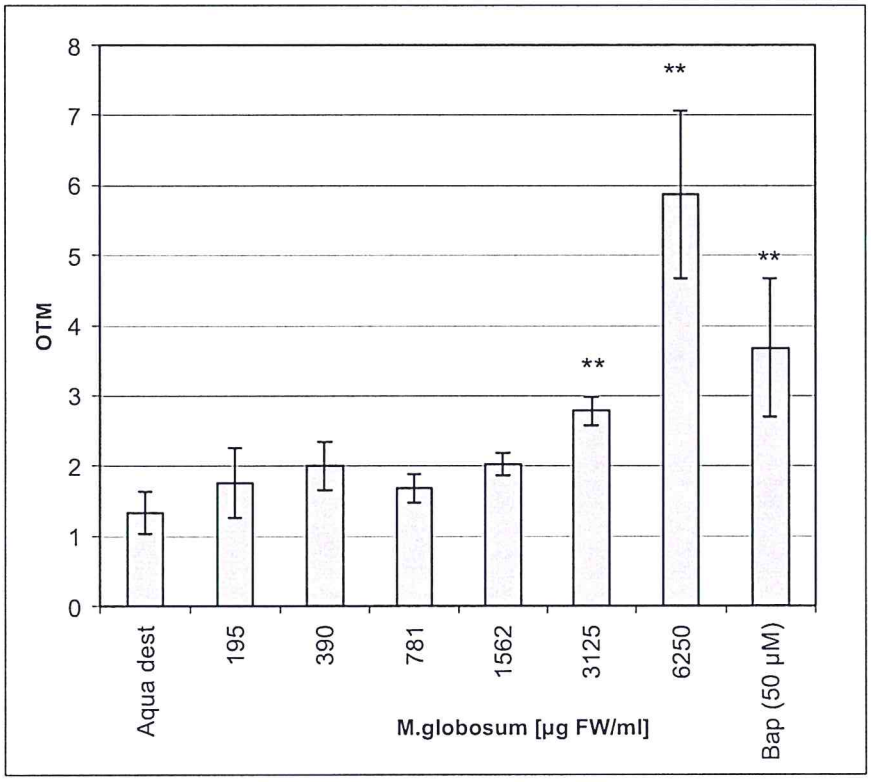
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Revised Figure 2

Figure 2:

DNA migration caused by *M.globosum* extracts (195-6250 µg FW/ml) in HepG2 hepatoma cells using alkaline SCGE [mean values and standard deviation of Olive tail moment (OTM) three independent experiments; OTM: Olive tail moment; *p≤0.01]



Revised Figure 1

Figure 1:

Monimiastrum globosum leaves and twig.

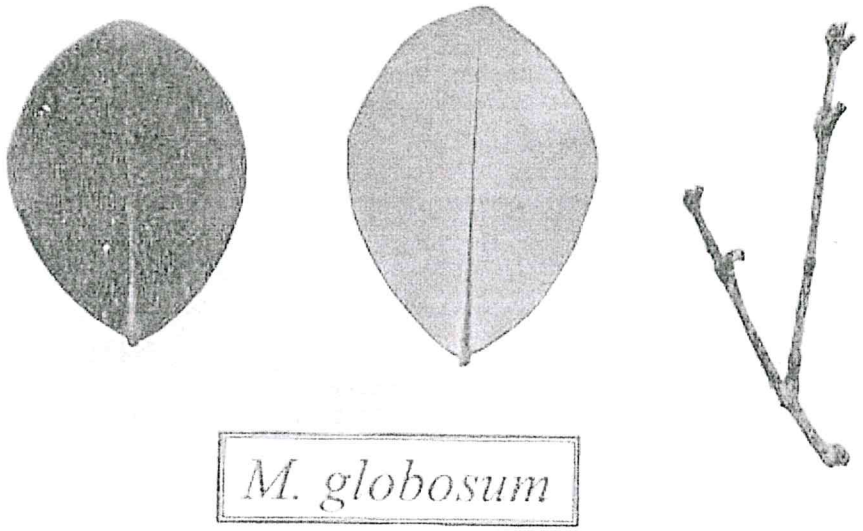
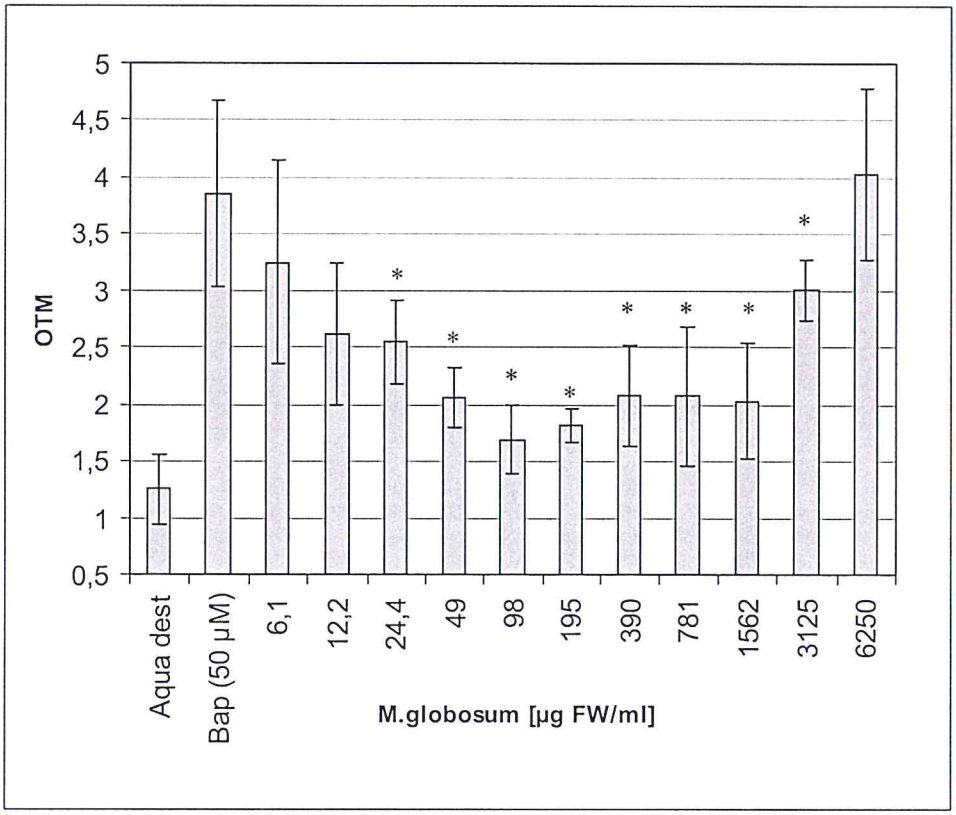


Figure 3:

Prevention of benzo[a]pyrene-induced DNA migration by *M. globosum* extracts (6.1-6250 µg FW/ml) in HepG2 hepatoma cells using alkaline SCGE [mean values and standard deviation of Olive tail moment (OTM) of three independent experiments; * $p \leq 0.05$; significance evaluated against the Bap reference control]



Bioefficacy of Mauritian Endemic Medicinal Plants: Assessment of Their Phenolic Contents and Antioxidant Potentials

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Abstract

The increasing role of free radicals in the etiology and development of a wide range of clinical disorders has indicated that phenolic antioxidants can offer a realistic promise to reduce the incidence of a number of pathologies implicating oxidative stress. In this study the total phenol, flavonoid and proanthocyanidin contents of the Mauritian medicinal plants *Crinum mauritianum* Lodd. (Asteraceae), *Gaertnera psychotroides* DC (Rubiaceae), *Psidia terebinthnina* A. J. Scott. (Asteraceae)[cl1] and *Tylophora coricea* Marais. (Monimiaceae) [cl2]were assessed and contrasted with their antioxidant potentials. The antioxidant propensity was evaluated by the ability of the extracts to scavenge hypochlorous acid and hydroxyl radical and the artificial $ABTS^{\cdot+}$ radical including their abilities to inhibit microsomal lipid peroxidation. The endemic plants *Badula multiflora* A. DC. (Myrsinaceae), *Croton vaughanii* L. (Euphorbiaceae), *Erythroxylum macrocarpum* Lam. (Erythroxylaceae), *Ochna mauritiana* Lam. (Ochnaceae), *Tambourissa cordifolia* Lorence. (Monimiaceae) [cl3]and *Turraea rigida* Vent. (Meliaceae) [cl4]were similarly investigated. *Badula multiflora* (Myrsinaceae) and *Erythoxylum macrocarpum* (Erythroxylaceae) showed highest antioxidant activity in the TEAC and FRAP assay. *Badula multiflora, Ochna mauritiana* (Ochnaceae), *Gaertnera psychotroides* (Rubiaceae) were very potent scavengers of hypochlorous acid and inhibited microsomal lipid peroxidation induced by 2,2'-azobis(2-amidinopropane) hydrochloride (AAPH) suggesting that the inhibition was intrinsically linked to peroxyl radical scavenging. The antioxidant activity of *Gaertnera psychotroides*, *Tylophora coriacea*, *Psidia terebinthina*, *Crinum mauritianum* may account for the therapeutic effects of these extracts in particular in conditions characterised by inflammation and oxidative mechanisms. Whilst these polyphenolic-rich endemics are good sources of natural antioxidants, there is an urgent need for sustainable conservation programmes for their protection.

Key words: Antioxidant; Endemic medicinal plants; Flavonoids; Lipid peroxidation; Proanthocyanidins; Total phenols

Introduction

Considerable attention has been focused on plant resources worldwide as a potential source of pharmacologically active compounds. The African continent is particularly rich in species diversity and it has been reported that Southern Africa contains almost 10% of the world's flora with an estimated 23,404 vascular plant species (Arnold & De Wet, 1993; Cowling & Hilton-Taylor, 1994). A diversified and unique flora also characterizes Mauritius, a tropical island of volcanic origin in the Indian Ocean, which is considered to be part of the African continent. The island of Mauritius forms part of the Mascarene archipelago that consists of several outlying islands of the Indian Ocean namely Réunion, Rodrigues, Agalega, Tromelin. Oceanic islands have been widely discussed for their rich diversity and high degree of endemism particularly because they are far from continental masses thus providing different sets of conditions conducive for speciation. It has been reported that 45% of the flora are strict endemics and include 311 taxa (Guého, 1988). This level of endemicity far exceeds estimates from Réunion and Rodrigues islands both with 35% and other African countries mainly Namibia (17%), Zambia (4.4%), Zimbabwe (2.1%) and Bostwana (0.8%) (Guého, 1988; Maggs et al., [cl5]1998). The sustained use of the flora for phytotherapy dates from time immemorial and the use of medicinal plants play a dominant therapeutic role in the treatment of human ailments in both developing and developed countries. A wide number of the Mauritian endemics have been mentioned in the traditional pharmacopoeia for their medicinal uses against a wide range of physical ailments such as bronchitis, diabetes, asthma, dysentery and inflammatory diseases (Gurib-Fakim, 1991; Bahorun et al., 2003).

Literature data has suggested that the therapeutic potential of medicinal plant lies in the presence of secondary metabolites including alkaloids, terpenoids and polyphenols (Levêque et al., 1996). Plant polyphenols, in particular have been greatly acknowledged for their biological and therapeutic activities including antimicrobial, anti-inflammatory, vasodilatory, expectorant, anti-mutagenic and antioxidant properties (McGregor et al., 1999; Spignoli, 2000; Soobrattee et al., 2005). In addition the regulatory activity of phenolic antioxidants on cell signal transduction pathways mainly the MAPK, JNK kinases, transcription factors mainly NF-κB, c-Jun, c-myc, c-fos have been highly emphasized as a strategy for reducing the incidence of diseases induced by ROS and RNS (Owuor & Kong, 2002; Hou et al., 2004). The prophylactic effects of phenolic antioxidants stem mainly from several epidemiological studies and their ability to arrest the onset of chronic degenerative diseases in animal models and cell cultures (Cao et al., 2004; Dijsselbloem et al., 2004; Kris-Etherton et al., 2002). Literature data abounds in examples where phenolic compounds are the bioactive components responsible for the prophylactic benefits of traditionally used plants sources (Ishige et al., 2001; Ivanova et al., 2005; Spignoli, 2000). For instance, soy isoflavones (genistein, daidzein, biochanin) from soybean also known as phyto-estrogens are applied in hormone replacement therapy (Dijsselbloem et al., 2004) and these phytochemicals also have applications in the prevention of atherosclerosis (Cassidy et al., 2003). (-)-EGCG, the major antioxidative component of green tea has been reported for growth inhibitory effects in cancer cells which appear to be mediated by the induction of apoptosis, the arrest of a specific phase of the cell cycle, the inhibition of aberrant arachidonic acid metabolism and the inhibition of proteasome activities (Fujiki et al, 2003[cl6], Hou et al, 2004).

The Mauritian flora contains significant levels of phenolic compounds and can thus be suitable sources of natural antioxidants (Neergheen et al., 2005). The present study involves the screening of 10 Mauritian endemic plants for their total phenol, total proanthocyanidin and flavonoid contents and their antioxidant activities using five highly reproducible assays (Table 2). Several of these endemics including *Crinum mauritianum*, *Gaertnera psychotroides*, *Psidia terebinthina* and *Tylophora coriaceae* have been discussed for their traditional uses (Table 1). The major impetus of this study involves the declining number of these species and the subsequent lost of potential sources of prophylactic agents. Considering the uniqueness of our flora and their polyphenolic richness and antioxidant propensities, there is an urge to reinforce existing conservation strategies to safeguard the threatened endemics with medicinal and potential therapeutic uses. This paper thus additionally provides for justification to the development of a sustained policy towards conservation.

Materials and methods

Plant material

Mature leaves of *Badula multiflora* A. DC., *Erythroxylum macrocarpum* Lam., *Ochna mauritiana* Lam., *Gaertnera psychotroides* DC., *Tambourissa cordifolia* Lorence, *Croton vaughanii* L., *Turraea rigida* Vent., *Crinium mauritianum* Lodd., *Tylophora coriacea* Marais. and *Psidia terebinthina* A.J. Scott were obtained from Brise Fer forest, Le Mondrain natural reserve and Le Pétrin conservation management area (centre of Mauritius). The leaf specimens were harvested in 2002, immediately weighed and kept at –20°C prior to extraction. The leaf specimens were authenticated by the Mauritius Sugar Industry Research Institute and voucher specimens have been deposited at the Department of Biosciences, Faculty of Science, University of Mauritius.

Extraction

Leaves (50 g) were extracted first with acetone/water (70/30 v/v) (2 x 400 ml) and finally with pure methanol (3 x 400 ml). Filtrates were concentrated *in vacuo* and the resulting aqueous extract was washed with dichloromethane to remove lipids before being freeze-dried. Part of the freeze-dried extract (corresponding to 1 g FW) was dissolved in water for antioxidant assays, this solution being adjusted at a final 1/2 plant fresh weight/volume ratio. An aliquot of the freeze dried extract (corresponding to 1 g FW) was dissolved in absolute methanol for storage at –20°C for subsequent analyses, this solution being adjusted at a final 1/5 leaves fresh weight/volume ratio.

Determination of total phenol content

Total phenol content was determined by the method adapted from Singleton and Rossi (1965) using the Folin-Ciocalteu reagent. To each tube, 0.25 ml of the extract was added followed by 3.75 ml of distilled water and 0.25 ml of Folin-Ciocalteu reagent. After 3 min, 20% sodium carbonate was added. The tubes capped, mixed thoroughly, heated at 40°C for 40 min. The blue coloration was read at 685 nm against a blank standard. Results were expressed in mg of gallic acid/g fresh weight of plant material.

Determination of total proanthocyanidin content

The HCl/Butan-1-ol assay adapted from Porter et al. (1986) was used to quantify the total amount of proanthocyanidins. To each tube, 0.25 ml of the methanol extract was added, followed by 3 ml of butan-1-ol/HCl solution and 0.1 ml of $NH_4Fe(SO_4)_2.12H_2O$ in 2M HCl. The tubes were capped and incubated for 40 min at 95°C. After cooling in the dark, the red

coloration was then read at 550 nm against a blank standard. The amount of proanthocyanidins was expressed in mg of cyanidin chloride/g fresh weight.

Determination of total flavonoid content

The $AlCl_3$ method (Lamaison & Carnet, 1990) was used for the quantification of the total flavonoid content of methanol plant extracts. 1.5 ml of 2% $AlCl_3.6H_2O$ was added to equal volumes of the extract. The mixture was shaken and the absorbance was read at 440 nm. Flavonoid contents were expressed in mg quercetin equivalent/g fresh weight.

Trolox equivalent antioxidant capacity (TEAC)

The ABTS/MnO_2 method (Campos & Lissi, 1997) was used to evaluate the ability of the plant extracts to scavenge the preformed radical cation $ABTS^{\cdot+}$. The $ABTS^{\cdot+}$ radical was generated by a reaction between ABTS (0.5 mM) and activated MnO_2 (1 mM) in phosphate buffer (0.1 M). To 3 ml of the $ABTS^{\cdot+}$ solution, 0.5 ml of plant extracts was added and the decay in absorbance was followed for 15 min at 734 nm. TEAC values were expressed in μmol trolox equivalent /g fresh weight for the plant extracts.

Ferric reducing antioxidant power (FRAP)

The ferric reducing antioxidant power (FRAP) assay gives an indication of the reducing ability of the plant extract. This method was adapted from Benzie and Strain (1996). FRAP reagent was freshly prepared by mixing together 10 mM 2,4,6-tripyridyl triazine (TPTZ) and 20 mM ferric chloride in 0.25 M acetate buffer, pH 3.6. Plant sample (100 μL) was added to 300 μL of water followed by 3 ml of FRAP reagent. The absorbance was read at 593 nm after 4 min

incubation at ambient temperature against a blank of distilled water. A calibration curve of ferrous sulphate (100-1000 μmol/L) was used, and results were expressed in μmol Fe (II)/g fresh weight of plant material.

Hypochlorous scavenging assay

The ability of the plant extract to scavenge hypochlorous acid (HOCl) was assessed. HOCl was prepared by adjusting the pH of a 1% (v/v) solution of NaOCl to 6.2 with dilute sulphuric acid. The working concentration of the stock solution was determined spectrophotometrically by measuring its absorbance at 235 nm and applying a molar extinction coefficient of 100 M^{-1} cm^{-1} (Weiss at al., 1982). The reaction mixture contained taurine (10 mM), HOCl (1 mM), plant extract (variable concentrations), phosphate saline buffer (pH 7.4) in a final volume of 1 ml. The solution was mixed thoroughly and incubated for 10 min at ambient temperature followed by the addition of 10 μl of potassium iodide. A yellow coloration was developed and the absorbance was read at 350 nm. The results were expressed as IC_{50} values (mg FW/ml).

Deoxyribose assay

The hydroxyl radical scavenging potential of the extracts was determined using the deoxyribose assay (Halliwell *et al.,* 1987, Aruoma 1994). The reacting mixture contained in a final volume of 1 ml the following reagents: 200 μl KH_2PO_4-KOH (100mM), 200 μl deoxyribose (15 mM), 200 μl.$FeCl_3$ (500 μM), 100 μl EDTA (1mM), 100 μl sample, 100 μl H_2O_2 (10 mM) and 100 μl ascorbic acid (1 mM). Reaction mixtures were incubated at 37 °C for 1 h.

At the end of the incubation period, 1 ml 1% (w/v) thiobarbituric acid (TBA) was added to each mixture followed by the addition of 1 ml 2.8% (w/v) trichloroacetic acid (TCA). The solutions

were heated in a water bath at 80°C for 20 min to develop the pink coloured MDA-$(TBA)_2$ adduct. As turbidity was encountered, the MDA-$(TBA)_2$ chromogen was extracted into 2 ml butan-1-ol and its absorbance measured at 532 nm. Results were expressed as the percentage inhibition of deoxyribose degradation/g fresh weight of plant material.

Preparation of microsomes

Beef liver microsomes were prepared by tissue homogenization with ice-cold 0.25 M sucrose-10 mM Tris buffer, pH 7.4 with 1 mM EDTA. Microsomal fractions were isolated by removal of the nuclear fractions at 1500 rpm for 5 min and removal of mitochondrial fraction at 5000 rpm for 15 min. The supernatant was then centrifuged in a Beckman Optima™ ultracentrifuge at 100,000 x g for 60 min at 4°C. The pellet was then washed in 0.25 M NaCl, pH 7.5. The membranes were suspended in saline (0.25 M, pH 7.5) and stored in aliquots at -20°C. Microsomal protein was determined by the Lowry-Folin method (Lowry et al, 1951).

2, 2'-azobis(2-amidinopropane) hydrochloride (AAPH)-induced lipid peroxidation

The reaction mixture contained in a final volume of 0.8 ml the following reagents: 200 µl of beef liver microsomes (0.5 mg/ml microsomal protein final concentration) suspended in 0.1 M potassium phosphate buffer, pH 7.5, 400 µl sample and 200 µl 2,2'-azobis(2-amidinopropane hydrochloride) (AAPH) (20 mM) to initiate peroxidation. The mixture was incubated at 37 °C for 1 h and the solution gently shaken at 10 min interval. After incubation, 1.6 ml TCA-TBA-HCl stock solution (15% w/v trichloroacetic acid, 0.375% w/v thiobarbituric acid, 0.25 M HCl) was added. The solution was heated in a boiling water bath for 15 min. After cooling, the precipitate was removed by centrifugation and the absorbance of the resulting supernatant measured at 532 nm.

Statistical Analysis

Results are expressed as mean value ± standard deviation (n = 3). Simple regression analysis was performed to calculate the dose-response relationship of standard solutions used for calibration as well as for the test samples. Linear regression analysis was performed, quoting the correlation coefficient r_{xy}.

Results

Phenolic content of the plant extracts

The total phenol content of the endemic plant extracts measured by the Folin Ciocalteu method ranged between 1 and 78 mg gallic acid/g FW. The highest phenol content was observed in *Badula multiflora* (78 mg/g FW). *Erythroxylum macrocarpum*, *Ochna mauritiana* and *Gaertnera psychotroides* also had elevated levels of phenolics with 40, 34 and 32 mg/g FW respectively. Moderate levels of phenolics were recorded in *Tambourissa cordifolia, Croton vaughanii* and *Turraea rigida* while *Crinium mauritianum* was very poor in phenolics with minimal levels detected under this experimental condition (1.21 mg/g FW). The set of data is presented in Figure 1.

The proanthocyanidin content ranged from 6 to 37 mg/g FW with the highest level measured in *E. macrocarpum. G. psychotroides, O. mauritiana,* B. *multiflora* and *T. cordifolia* had appreciable levels of proanthocyanidins: 30 ± 0.13, 30 ± 0.63, 24 ± 1.27 and 23 ± 0.8 mg/g FW respectively. Amounts less than 10 mg/g FW was measured in *C. vaughanii* and *T. rigida.*

The flavonoid content was estimated within the range of 0.1 to 9.3 mg quercetin equivalent/g FW. *O. mauritiana* was shown to contain the highest level (9 ± 0.18 mg/g FW) followed by *E. macrocarpum* (7 ± 0.12 mg/g FW) and *C. vaughanii* (7 ± 0.16 mg/g FW). Very low amounts were measured in *P. terebinthina* and *C. mauritianum*.

Antioxidant activities of the plant extracts

The free radical scavenging abilities of the plant extracts were evaluated by monitoring the reduction of the $ABTS^{\cdot+}$ radical in the TEAC system and by measuring the scavenging potency towards hypochlorous acid, hydroxyl and peroxyl radicals in the HOCl scavenging, deoxyribose and lipid peroxidation assays respectively. The plant extracts showed high $ABTS^{\cdot+}$ radical scavenging with activities ranging from 3 to 849 µmol trolox equivalent/g FW. *B. multiflora* had the highest measurable activity (849 µmol/g FW) while moderate TEAC values were observed for *E. macrocarpum* (441 µmol/g FW), *G. psychotroides* (358 µmol/g FW) and *O. mauritiana* (302 µmol/g FW). The antioxidant hierarchy observed in the TEAC system was: *B. multiflora* > *E. macrocarpum* > *G. psychotroides* > *O. mauritiana* > *T. cordifolia* > *T. rigida* > *C. vaughanii* > *T. coriacea* > *P. terebinthina* > *C. mauritianum*. *C. mauritianum* extract exhibited a very low scavenging effect.

A similar pattern of activity to TEAC activity was obtained for the plant extracts in the ferric reducing assay. The ferric reducing activity ranged between 6 and 923 µmol/g FW. *B. multiflora* and *E. macrocarpum* showed high ferric reducing power with FRAP values 923 and 517 µmol/g FW respectively while *C. mauritianum* had the least measured activity. The endemic plant extracts were also powerful scavengers of hypochlorous acid. The concentration of extracts able to scavenge HOCl acid by 50% (IC_{50}) under the experimental conditions was found

to be in the following order: *B. multiflora* > *E. macrocarpum* > *C. vaughanii* ≈ *O. mauritiana* > *T. cordifolia* > *G. psychotroides* > *T. rigida* > *T. coriacea* ≈ *P. terebinthina* > *C. mauritianum*. *B. multiflora* and *E. macrocarpom* were strong HOCl scavengers with IC_{50} values of 0.43 and 0.89 mg FW/ml. *C. vaughanii*, *O. mauritiana*, *T. cordifolia*, *G. psychotroides*, *T. rigida* were also very effective scavengers. *P. terebinthina* and *T. coriacea* had comparable activity in the HOCl assay and showed relatively weaker activities (IC_{50} = 13 mg FW/ml).

The ability to inhibit lipid peroxidation initiated by the water-soluble AAPH initiator was assessed using beef liver microsomes as a lipid source. The latter undergo rapid nonenzymatic peroxidation when incubated in the presence of AAPH, which readily decomposes into peroxyl radicals. *B. multiflora*, *O. mauritiana*, *G. psychotroides* and *E.macrocarpom* were effective inhibitors of lipid peroxidation at low concentrations (Figure 2). The calculated IC_{50} values obtained from the plots of concentration-dependent inhibition of oxidation are given in Table 2. No activity was measured under the experimental condition for *C.mauritianum* extract thereby indicating that much higher concentrations of the extract would be required to inhibit microsome lipid peroxidation.

A mixture of $FeCl_3$-EDTA, ascorbate and H_2O_2 at pH 7.4 generates hydroxyl radicals, which can be measured by their ability to degrade the sugar deoxyribose into products that generate a pink chromogen on heating with thiobarbituric acid at low pH (Aruoma, 1994). The activity order measured in the deoxyribose assay was in the following decreasing order: *T.cordifolia* > *P.terebinthina* > *O.mauritiana* ≈ *T. coriacea* > *C.vaughanii* > *B.multiflora* > *E.macrocarpom* > *G.psychotroides* > *T. rigida* > *C.mauritianum* (Figure 3) Crinum *mauritianum* was a very weak inhibitor of deoxyribose damage by hydroxyl radicals generated by the Fenton

system. The maximum activity was observed with *T. cordifolia* plant extract with a 29% inhibition of damage followed by *P. terebinthina* (26%).

Discussion

The high degree of endemism reflects the uniqueness of the Mauritian flora and consequently several of these plant species may be of significant economic importance and medicinal uses. This unique flora has resulted from colonization and adaptive radiation of plant species from different sources. 70% of the phanerogam genera seemed to have been derived from Madagascar and the mainland Africa, 8% from Asia, 12% of pan Indo-Pacific origin and 8% are endemic. Furthermore, since over 50% of all modern clinical drugs are of natural product origin (Farombi, 2003), it can be speculated that the Mauritian endemic flora can be an interesting source of bioactive compounds. Thus it is important for prioritizing sites for conservation in order to protect the dwindling number of the endemic plants species. This prompted the evaluation of the antioxidant efficacy in relation to the phenolic contents of some selected Mauritian endemic plants with medicinal value.

The Folin-Ciocalteu method indicated high phenolic content in some of the endemic plant extracts studied in particular *B. multiflora*, *E. macrocarpum*, *O. mauritiana* and *G. psychotroides*. Substantial levels of proanthocyanidins and flavonoids have also been observed in these plant extracts and the data is indicative of the polyphenolic richness of the Mauritian endemic flora. Tropical plants are tolerant of high levels of environmental stress induced by ultraviolet radiation and pollutants including smoke and this may explain the high levels of phenolic compounds in the endemic plant species studied. Literature data abounds in examples

where plant extracts rich in phenolic compounds and plant-derived constituents are used for their prophylactic benefits (Škerget et al., 2005; Katsube et al., 2004). For instance, some edible plants from Japan particularly *Mallotus japonicus* (akamegashiwa), *Camellia sinensis* (green tea), *Ligustrum japonium* (Japanese privet) and *Diospyros kaki* (astringent persimmon) contained high levels of phenolic compounds, which have been suggested to account for the medicinal properties of these plants (Katsube et al., 2004).

Moreover several bioactive components isolated from plant sources have been proposed for their health-promoting effects, for example grape seed proanthocyanidins has been shown to exhibit cardioprotective ability in animals and humans (Bagchi et al., 2003). There is also continued interest on the application of soy isoflavones as a potential alternative to the synthetic selective estrogen receptor modulators that are currently applied in hormone replacement therapy and also in the prevention and/or delaying of atherogenesis (Dijsselbloem et al., 2004; Cassidy et al., 2003). In addition, the catechins constituents of black tea has recently been found to significantly reduce endothelial dysfunction in patients with coronary artery disease thus suggesting that other flavonoids and polyphenolic components of tea can favorably contribute to vascular health (Widlansky et al., 2005). Plant-derived phenolic compounds are also accepted as chemopreventive agents as they can potentially inhibit several stages of carcinogenesis in vivo, particularly caffeic acid phenethyl ester and cucurmin which have been found to decrease the incidence of intestinal tumours by increasing enterocyte apoptosis (Mahmoud et al., 2000). Although the exact role of polyphenolic compounds in nutrition need to be unravelled, accumulated evidence increasingly suggest that these compounds can potentially be used as therapeutic alternatives and/or to complement existing therapies.

Plant phenolics have been widely described for their antioxidant activities and the mechanism of action of these phenolic antioxidants include mainly their free radical scavenging activities, metal chelating properties, ability to regulate gene expression and acting as co-antioxidants (Bors et al., 1990; Mira et al., 2002; Toyokuni et al., 2003). In this study, the direct scavenging activities of the plant extracts towards the artificially generated $ABTS^{\cdot+}$ radical and biologically relevant ROS were measured. The activities of the extracts in the TEAC assay indicated that the phenolic-rich extracts were potent donors of hydrogen, a property inherent of phenolic compounds (Bors et al., 1990; Rice-Evans and Miller, 1996[cl7]). The activity order observed in the TEAC assay is: *B. multiflora* > *E. macrocarpum* > *G. psychotroides* > *O. mauritiana* > *T. cordifolia* > *T. rigida* > *C. vaughanii* > *T. coriacea* > *P. terebinthina* > *C. mauritianum*. The reducing potential of the plant extracts showed similar pattern of activity as the $ABTS^{\cdot+}$ radical scavenging whilst the hypochlorous acid scavenging activity measured is in the following decreasing order: *B. multiflora* > *E. macrocarpum* > *C. vaughanii* ≈ *O. mauritiana* > *T. cordifolia* > *G. psychotroides* > *T. rigida* > *T. coriacea* ≈ *P. terebinthina* > *C. mauritianum*. Literature data widely reports the free radical scavenging activities of medicinal and edible plants indicating their antioxidant efficacy and potential health benefits (Kang et al., 2003; Lee et al., 2000). A recent study on the endemic plants of Réunion island particularly *Croton mauritianus*, *Psidia argentea*, *Psidia boivinii*, *Psidia salaziana*, *Gaertnera vaginata* has shown appreciable level of free radical scavenging activity (Poulain et al., 2004). These data along with the presently reported results indicate the antioxidant efficacy of the Mascarene flora.

The ability of the plant extracts to protect deoxyribose sugar against hydroxyl radicals generated by the Fenton system was in the decreasing order *T. cordifolia* > *P. terebinthina* > *O.*

mauritiana ≈ *T. coriacea* > *C. vaughanii* > *B. multiflora* > *E. macrocarpum* > *G. psychotroides* > *T. rigida* > *C. mauritianum.* In a similar system, methanol[cl8] extracts of lotus plumule and lotus blossom were potent scavengers of hydroxyl radicals preventing deoxyribose degradation by 60 % (Wang et al., 2003) The data suggests that these Mauritian endemic plants in particular *B. multiflora*, *E. macrocarpum*, *T. cordifolia*, *O. mauritiana, C.vaughanii* are good scavengers of reactive oxygen species. Several of these endemics reported in this study have traditional uses, for instance *Psidia terebinthina* is used for its expectorant properties, used against boils, abscesses, fever, wounds; *Tylophora coriacea* against vomiting while *Crinum mauritianum* and *Gaertnera psychotroides* are considered as an ailment for rheumatic pain (Table 1) (Gurib-Fakim et al., 1995, 1996a,b). The bioactive compounds responsible for these medicinal uses are unknown but there are indications that the phenolic components may play an important role and that some of these medicinal properties may result from the antioxidative potentials of the phytochemicals. Several herbal medicine rich in flavonoids and phenolic compounds are viewed as promising therapeutic drugs for free radical mediated pathologies (Kang et al., 2003; Lee et al., 2000; Soobrattee et al., 2005). Kang et al (2003) reported the substantive antioxidant activity of solvent extracts of traditionally used herbal medicines used in Korea with superoxide, hydroxyl radical and lipid peroxide scavenging activities.

Lipid peroxidation particularly of unsaturated lipids in biomembrane disrupts their important structural and protective function, accompanying pathological events such as chronic inflammatory processes, diabetes, atherogenesis, Alzheimer's disease and aging (Griesmacher et al., 1995; Reaven & Witztum, 1996; Rikans & Hornbrook, 1997; Butterfield and Lauderback., 2002[cl9]). The plant extracts used in this study were potent inhibitors of microsome lipid peroxidation induced by AAPH suggesting that the inhibition is intrinsically linked to peroxyl

radical scavenging. The hierarchy of activity was as follows: *B. multiflora* > *O. mauritiana* > *G. psychotroides* > *E. macrocarpum* > *T. cordifolia* > *T. rigida* > *C. vaughanii* > *T. coriacea* > *P. terebinthina*. Plant extracts and plant-derived antioxidants are receiving wide attention in the food industry and in the biomedical research primarily as a result of their ability to stabilize bulk oils, emulsions and biological membrane against lipid peroxidation (Aruoma et al., 1998; Shahidi, 1997). Thus *B. multiflora*, *O. mauritiana* and *G.psychotroides* can be effective lipid peroxidation inhibitors in food systems as well as prophylactic agents against biological membrane peroxidation. However the data pertaining to the bioactive components responsible for such activity and also the cytotoxicity of the extracts will delineate the future use of these Mauritian endemic plants.

The Mauritian endemic flora acts as a reservoir of putative bioactive compounds and therefore shows tremendous potential for research and development. This indicates that there is need for the implementation of a sustainable development and conservation policy for Mauritius, in particular to protect the native plants from exotic invasive species. Several conservation programmes have been set up worldwide to preserve the dwindling level of biodiversity, in particular the conservation of endemic and medicinal plants of the Thar Desert and the flowering plants of Peru (Khan et al., 2003; Van der Werff & Consiglio, 2004). In Mauritius, existing conservation programmes need to be strengthened to protect the endemic medicinal plants and other plant species which are potential sources of untapped bioactive components. Among the above-mentioned list of plants studied, *Croton vaughanii*, *Tambourissa cordifolia*, *Tylophora coriacea*, *Turraea rigida*, *Psidia terebinthina* and *Badula multiflora* are highly vulnerable species with only five individuals of *Croton vaughanii* reported in the wild. Our study showed the antioxidant effectiveness of these highly endangered plant species thus suggesting that

reinforcement of the existing conservation strategies will allow the safeguard of the Mauritian heritage whilst alternative means of regeneration of the threatened species via tissue culture can also be considered as a strategy to increase the plant population.

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Table 1: List of plant species used in this study; traditional uses and status of species population are included.* Source Gurib-Fakim et al, 1995; 1996a,b.

Plant species	*Common name*	*Family*	*Traditional medicinal property**	*Status of species population*
Badula multiflora	Bois nacre	Myrsinaceae	_	Vulnerable
Crinum mauritianum	-	Amaryllidaceae	Against rheumatic pain	Not threatened
Croton vaughanii	-	Euphorbiaceae	_	Rare
Erythroxylum macrocarpum	Bois piment/Bois de ronde	Erythroxylaceae	_	Not threatened
Gaertnera psychotroides	Bois banane	Rubiaceae	Rheumatism, skin infections	Not threatened
Ochna mauritiana	Bois bouquet banané/Bois jasmin	Ochnaceae	_	Not threatened
Psidia terebinthina	Baume d'Ile platte	Asteraceae	Expectorant properties; used against boils, abscesses, fever, wounds	Vulnerable
Tambourissa cordifolia	Bois tambour	Monimiaceae	_	Rare
Tylophora coricea	Ipéca du pays	Asclepadiaceae	Emetic properties	Rare
Turraea rigida	Bois café marron	Meliaceae	_	Rare

Table 2: Antioxidant propensities of the plant extracts. Results are expressed in [a]µmol trolox equivalent/g fresh weight; [b]µmol Fe(II) equivalent/ g fresh weight; [c]IC_{50} (mg FW/ml)

Plant species	TEAC[a]	FRAP[b]	HOCl scavenging[c]	Lipid peroxidation[c]
Badula multiflora	849 ± 15.4	923 ± 5.1	0.4 ± 0.01	0.092
Erythroxylum macrocarpum	442 ± 35.3	518	0.9 ± 0.08	0.23
Gaertnera psychotroides	358 ± 22.4	380 ± 8.2	1.4 ± 0.22	0.17
Ochna mauritiana	303 ± 22.4	275 ± 48.9	1.1 ± 0.12	0.15
Tambourissa cordifolia	208 ± 6.4	142 ± 3.5	1.2 ± 0.04	0.43
Turraea rigida	170 ± 6.4	170 ± 0.6	1.5 ± 0.08	0.62
Croton vaughanii	161 ± 11.5	141 ± 7.1	1.1 ± 0.02	0.67
Psidia terebinthina	31 ± 0.2	32 ± 3.1	13.0 ± 0.11	2.1
Tylophora coriacea	29 ± 0.6	41 ± 7.8	13.0 ± 0.37	1.82
Crinium mauritianum	3 ± 0.01	6 ± 1.8	59.9 ± 1.49	-

Figure 1: Total phenol, total proanthocyanidin and total flavonoid content of some highly threatened Mauritian endemic plant extracts. Values are expressed in mg/g fresh weight.

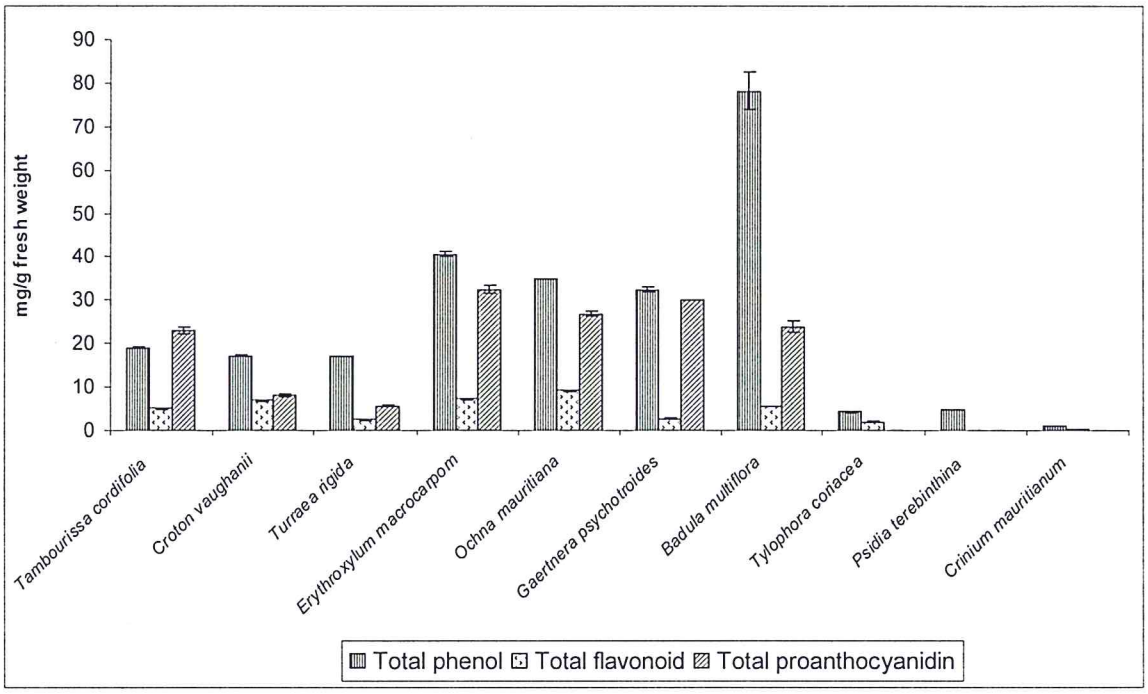


Figure 2: Inhibition of AAPH-induced microsome peroxidation by *B. multiflora* and *O.mauritiana* plant extracts. Concentrations yielding 50% inhibition for *B. multiflora* and *O.mauritiana* were 0.09 and 0.15 mg FW/ml respectively.

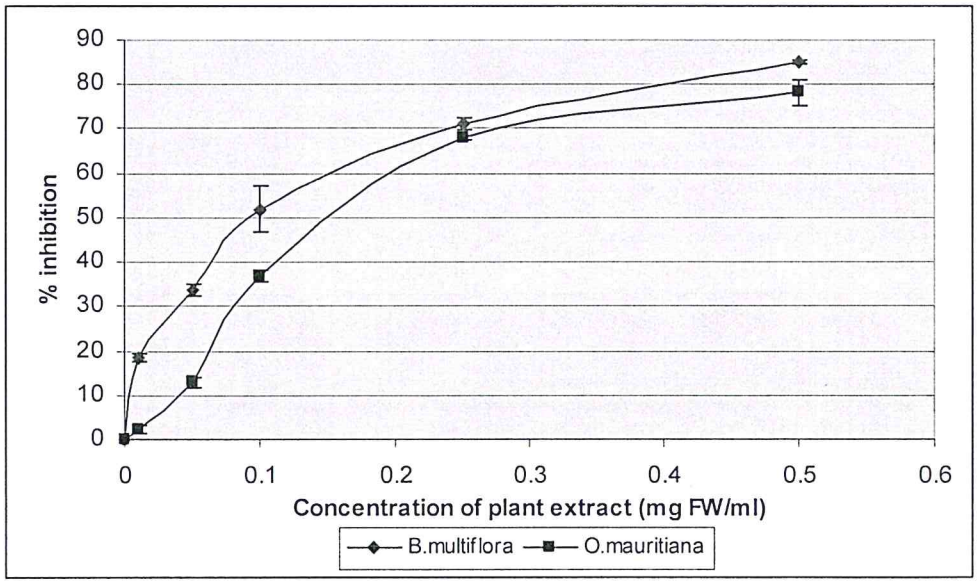
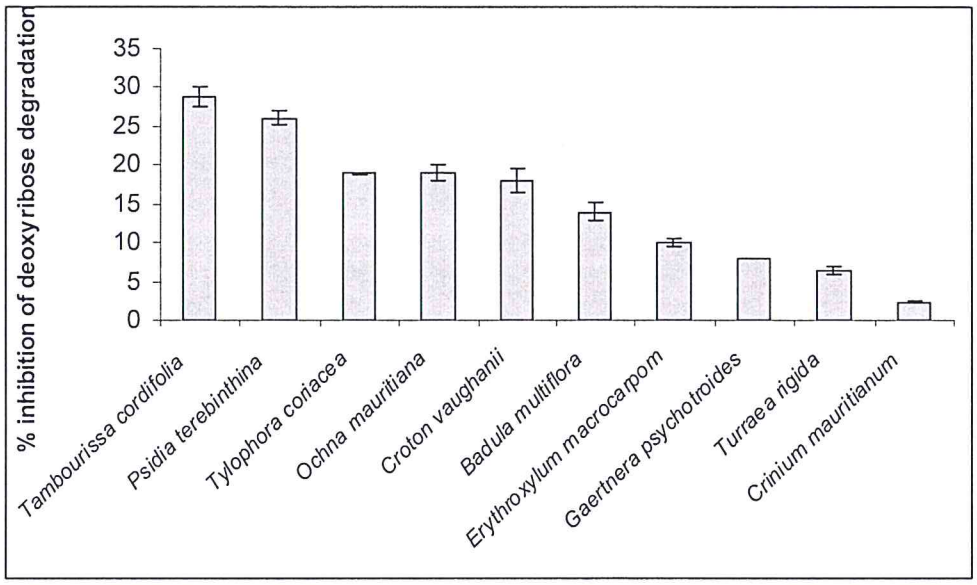


Figure 3: Percentage inhibition of deoxyribose degradation. Results are expressed per mg FW/ml plant extracts.



Manuscript

Phenolics content and antioxidant actions of the Rubiaceae, Ebanaceae, Celastraceae, Erythroxylaceae and Sterculaceae families of Mauritian endemic plants

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Running title: Phenolic contents of tropical endemic plants of Mauritius

Keywords: Mauritian endemic plants; Medicinal plants; Rubiaceae, Ebanaceae, Phenolics; Flavonoids, Antioxidants; Chronic neurodegenerative diseases; functional food science; Biopharmaceuticals

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Abstract

There is continued interest in the assessment of the bioefficacy of the active principles in extracts from a variety of traditional medicine and food plants in order to determine their impact on the management of a variety of clinical conditions and maintenance of health. The polyphenolic composition and antioxidant potential of 13 Mauritian endemic plants of the Rubiaceae, Ebanaceae, Celastraceae, Erythroxylaceae and Sterculaceae family were determined. The phenolics level of the plant extracts varied from 1 to 75 mg/g FW, the maximum level measured in *Diospyros neraudii* (Ebanaceae). *Coffea macrocarpa* showed the highest flavonoids content with 18 ± 0.7 mg/g FW. The antioxidant capacity based on the TEAC and FRAP values were strongly related to total phenolics and proanthocyanidins content, while a weaker correlation was observed with (-) gallic acid. *Erythroxylum sideroxyloides* showed the highest protective effect in the lipid peroxidation systems with IC_{50} of 0.0435 ± 0.001 mg FW/ml in the Fe^{3+}/ascorbate system and 0.05 ± 0.002 mg FW/ml in the AAPH system. *Cassine orientalis, Erythroxylum sideroxyloides*, *Diospyros mellanida* and *Chassalia coriancea* var. johnstonii were weakly prooxidant only at higher concentration greater of 10 g FW/L indicating potential safety. Mauritian endemic plants, particularly the genus *Diospyros*, are good sources of phenolic antioxidants and potential candidates for the development of prophylactic agents.

1. Introduction

Phytochemicals exerting antioxidant actions are largely being recognised as beneficial to human health and disease prevention, possibly by interfering in the processes involved in reactive oxygen and nitrogen species mediated pathologies including coronary diseases, cancer, age-related degenerative brain disorders and infectious diseases (Almeida et al., 2002; Vallejo et al., 2002; Joseph et al., 1999; Aruoma 1998; Aruoma et al., 2003; Gilani et al., 2000; Kähkönen et al., 2001; Bahorun et al., 2003). Epidemiological studies have consistently shown an inverse association between consumption of vegetables and fruits and the risk of cardiovascular diseases and certain forms of cancer (Keli et al., 1996; Geleijnse et al., 1999; Bazzano et al., 2001).

The protective effects may be a result of concerted actions of well-known antioxidants such as vitamin C, vitamin E and β-carotene, plant phenolics. Indeed, phenolic substances are ubiquitously distributed in the plant kingdom and exhibit a wide range of pharmacological and medicinal properties, including anti-inflammatory, anti-carcinogenic, vasodilatory actions (Caroll et al., 1998; Middleton et al., 2000); these protective effects have been mostly ascribed to their free radical scavenging, metal chelating and antioxidant actions. Furthermore, phenolics can exert modulatory actions in cell by interacting with a wide spectrum of molecular targets central to the cell signaling machinery. These include activation of mitogen-activated protein kinase (MAPK) (Chung et al., 2003), protein kinase C (PKC) (Levites et al., 2002), serine/threonine protein kinase Akt/PKB (Lorenz et al., 2003), activation of peroxisome proliferator-activated receptor gamma (PPARγ) (Liang et al., 2001), NF-κB, c-JUN (Manna et al., 2000), as well as modulation of several cell survival/cell-cycle genes (Levites et al., 2002; Weinreb et al., 2003).

Mauritius is a tropical island endowed with a unique flora of which 8 % (corresponding to about 311 species) are strictly endemic. The species *Diospyros neraudii, Diospyros revaughanii, Diospyros tesselleria* and *Diospyros mellanida* are used in folk medicine for the treatment of bacterial, fungal, viral, helminthic, protozoan, and malarial infections

(Gurib-Fakim et al., 1996). *Chassalia coriacea* var. johnstonii has astringency property while *Cassine orientalis* is used against hypertension. *Maytenus pyria* is used against dysentery and exhibits anti-tumoral, anti-inflammatory and anti-leukemic activities while *Erythroxylum sideroxyloides* is used in the treatment of throat infections, fever and renal stones (Gurib-Fakim et al., 1996). In continuation of our research centred on the characterization of the total phenols, catechins, flavonoids and proanthocyanidins of Mauritian endemic plants (Toyokuni et al., 2003; Neergheen et al., 2006), the phenolic constituents of the Rubiaceae, Ebanaceae, Celastraceae, Sterculaceae and Erythroxylaceae family of endemic plants were determined and their potential antioxidant efficacy assessed using established assays (Aruoma 2003). The Mauritian endemic plants and more particularly those of the genus *Diospyros* are good sources of phenolic antioxidants and potential candidates for in vivo studies of antioxidant protection.

2. Materials and methods

2.1. Chemicals

2,2'-azino-bis(3-ethylbenzthioziozline-6-sulphonic acid (ABTS) and (2,4,6-tri(2-pyridyl) s-triazine (TPTZ) were purchased from Sigma Co. (St. Louis, MO, USA). 2,2'-azobis (2-amidinopropane) dihydrochloride (AAPH), trolox C (6-hydroxy-2, 5, 7, 8-tetramethylchroman-2-carboxylic acid), a water-soluble analogue of vitamin E, HPLC grade of (+)-catechin ((+)-C), (-)-epicatechin ((-)-EC), (-)-epigallocatechin ((-)-EGC), (-)-epicatechin-3-gallate ((-)-ECG), (-)-epigallocatechin-3-gallate ((-)-EGCG), procyanidin dimers B1 and B2, myricetin, kaempferol and gallic acid (GA) were purchased from Sigma-Aldrich (Germany). HPLC grade of quercetin and cyanidin chloride were obtained from Extrasynthèse (Genay, France).

2.2. Plant materials

Once collected the mature leaves were weighed, washed with distilled water and stored at –20° C within 2 hours following collection (Table 1). Voucher specimens were deposited at the Mauritius Herbarium, Mauritius Sugar Industry Research Institute, Réduit and at the Department of Biosciences, University of Mauritius.

2.3. Extraction

50 g of plant material was homogenized using a Waring blender in acetone/water (70:30, v/v) (2 x 300 ml) and left to macerate for 24 h at 4 °C. After filtration the residue was macerated in 100% methanol (2 x 300 ml) for 24 h at 4° C. Organic solvents were removed from the collected filtrates *in vacuo* at 37° C. The aqueous phase was centrifuged, washed with dichloromethane to remove lipophilic pigments. The resulting aqueous extract was concentrated *in vacuo* and divided into two equal aliquots. One part was freeze-dried, re-dissolved in methanol at a final 1/5 fresh weight/volume ratio and used for the quantitative phenolics analysis whilst the second part was in the assays to evaluate the antioxidant activity.

2.4. Total phenolic content

The total phenolic content of the extract was estimated using the Folin-Ciocalteu method adapted from Singleton and Rossi (1965). To 0.25 ml of diluted extract, 3.5 ml of distilled water was added followed by 0.25 ml of Folin-Ciocalteu solution (Merck). After 3 mins, 1 ml sodium carbonate (20% w/v) was added. The whole mixture was incubated at 40 °C for 40 mins. A deep blue colouration developed whose absorbance was read at 685 nm. Results are expressed in mg of gallic acid equivalent g^{-1} fresh weight of plant material.

2.5. Total flavonoid content

The $AlCl_3$ method adapted from Lamaison and Carnet (1991) was used for the determination of the total flavonoid content of the extracts. 1.5 ml methanolic extract was added to equal volume of a solution of 2% $AlCl_3.6H_2O$. After proper mixing, the mixture was incubated for 10 minutes at ambient temperature. The absorbance of the solution was read at 440 nm. Flavonoid contents are expressed in mg quercetin equivalent g^{-1} fresh weight of plant material.

2.6. Total proanthocyanidin content

The modified acid/butanol assay of Porter et al. *(1986)* was used to quantify the total proanthocyanidin content. 0.25 ml aliquot of extract was added to 3 ml of a 95% solution

of n-butanol/HCl (95:5 v/v) followed by 0.1 ml of a solution of $NH_4Fe(SO_4)_2.12H_2O$ in 2 M HCl. The tightly capped tubes were incubated for 40 min at 95 °C. Extinction of the cooled extract was read at 550 nm. Results are expressed in mg of cyanidin chloride g^{-1} fresh weight.

2.7. High-performance liquid chromatography (HPLC)

Chromatographic analyses of total and hydrolysed plant extracts were carried out using a HP1100 series HPLC (Germany) equipped with a vacuum degasser, quaternary pump, autosampler, thermostatted column compartment, diode array detector and HP Chemstation for data collection and manipulation. After filtration on Millipore (0.22 µm), 30 µl of extract was injected onto a Waters Spherisorb ODS-2 column (5µm particle size, 80 Å pore size, 4.6 mm id x 150 mm) eluted by an acidified acetonitrile-water gradient. Chromatographic separation was carried out at 25°C with a flow rate of 0.7 ml/min was as follows: 0-30 minutes, 0-15% B in A; 30-50 minutes, 15% B in A; 50-60 minutes, 15-25% B in A; 60-90 minutes, 15-100% B in A; 90-100 minutes, 100-0% B in A (Solvent A : acetonitrile/water, 1/9 v/v, pH 2.6; Solvent B : acetonitrile/water, 1/1 v/v, pH 2.6). The identification and quantification of the compounds were carried out from (i) the retention time and (ii) peak area in comparison with authentic standards.

2.7.1. Quantitative analysis of phenolic acid and flavan-3-ols in the plant extracts

Gallic acid, (+) catechin, (-) epicatechin, (-) epicatechin gallate, (-) epigallocatechin, (-) epigallocatechin gallate, procyanidins B1 and B2 were identified and quantified by comparison with authentic standard in duplicates. Absorption wavelength was selected at 280 nm with a 65 mins running time.

2.7.2. Hydrolysis of flavonoid conjugates and quantitative analysis of aglycones released

Hydrolysis of flavonoid conjugates was carried out essentially as described by Crozier et al. (1997). An aliquot of 1g fresh weight equivalent freeze dried extract was dissolved in 20 ml 60% methanol containing 100 µg morin as internal standard. 5 ml of 6 M HCl was

added such that the resulting solution contained 1.2 M HCl. The mixture was refluxed at 90 °C for two hours. After cooling the flavonoid aglycones were exhaustively extracted using ethyl acetate (2 x 20 ml). The latter phase was evaporated to dryness and taken up in absolute methanol prior to analysis by HPLC. The hydrolysed extract was subjected to HPLC analysis and quercetin, myricetin, kaempferol, apigenin and luteolin were identified and quatified within the plant extracts. Absorption wavelength was selected at 360 nm with a 100 mins running time.

2.8. Trolox equivalent antioxidant capacity (TEAC) assay

The total antioxidant activity of the extracts was assessed by the TEAC assay according to the method of Campos and Lissi (1996). To 3 ml of $ABTS^{+}$ solution, generated by a reaction between ABTS (0.5 mM) and activated MnO_2 (1 mM) in phosphate buffer (0.1 M, pH 7), 0.5 ml diluted plant extract was added. The decay in absorbance at 734 nm was monitored for 15 minutes. Data are expressed in µmol Trolox equivalent g^{-1} fresh weight.

2.9. Ferric reducing antioxidant power (FRAP) assay

The method of Benzie and Strain (1996) was employed to assess the reducing power of the extracts. 100 µl of plant sample was added to 300 µl water followed by 3 ml FRAP reagent. The mixture was incubated at room temperature for 4 minutes and the absorbance was read at 593 nm. Results were expressed in µmol Fe^{2+} g^{-1} fresh weight.

2.10 Scavenging of hypochlorous acid

The ability of the extracts to scavenge hypochlorous acid was assessed essentially as described in Neergheen et al (2006). The results were expressed as IC_{50} values (mg FW/ml).

2.11. Scavenging of hydroxyl radical

The hydroxyl radical scavenging potential of the extracts essentially as described in Halliwell et al (1987) and Aruoma (1994). Results were expressed as the percentage inhibition of deoxyribose degradation/g fresh weight of plant material.

2.12. Copper-phenanthroline (Cu-phen) assay

The Cu-phen assay was conducted essentially as described in Aruoma et al (1993), and Soobrattee et al (2005).

2.13. Liver microsomal preparation

Beef liver microsomes were prepared by tissue homogenization with 4 volumes of ice-cold 0.25 M containing 10 mM Tris buffer and 1 mM EDTA, pH 7.4. Microsomal fractions were isolated by removal of the nuclear fractions after centrifugation at 1500 rpm for 5 minutes and removal of mitochondrial fraction at 5000 rpm for 15 minutes. The microsomal fraction was then sedimented in a Beckman Optima™ ultracentrifuge at 100 000 x g for 60 minutes at 4°C. The pellet was then washed in 0.25 M NaCl, pH 7.5. The membranes, suspended in saline (0.25 M, pH 7.5), were stored in aliquots at -20°C. Microsomal proteins were estimated by the Lowry-Folin method (Lowry et al, 1951), using bovine serum albumin as standard.

2.13.1. Fe^{3+}/Ascorbate induced microsomal lipid peroxidation

The formation of malondialdehyde (MDA) measured as thiobarbituric reactive substances (TBARS), was used to monitor lipid peroxidation as described previously by Aruoma et al. (1993). The reacting mixture contained in a final volume of 1 ml the following reagents: 200 µl phosphate buffered saline (3.4 mM, pH 7.4), 200 µl microsome (0.5 mg/ml microsomal protein), 400 µl of sample (variable concentrations), 100 µl iron (III) chloride (1 mM) and 100 µl ascorbate (1 mM). The mixture was incubated for 1 hour at 37°C. At the end of the incubation period, 100 µl BHT (2% w/v) was added followed by 1 ml 1% (w/v) thiobarbituric acid and 2.8 % (w/v) trichloroacetic acid. Addition of BHT to the reaction mixture minimizes erroneous increases in color due to iron ion-dependent hydroperoxide decomposition during the acid heating stage (Verbunt et al, 1996; Buege and Aust, 1978). The solutions were heated in a water-bath at 80°C for 20 min to develop the MDA-TBA adducts (TBARS). The chromogen was extracted using 2 ml butan-1-ol and its absorbance read at 532 nm. Results are expressed as IC_{50} mg FW/ml.

2.13.2. AAPH induced microsomal lipid peroxidation

AAPH, which produces radicals by thermal decomposition (Niki, 1987), was also used to induce lipid peroxidation. The reaction mixture contained in a final volume of 0.8 ml the following reagents: 200 µl liver microsomes (1.5 mg/ml microsomal protein final concentration) suspended in 0.1 M potassium phosphate buffer (pH 7.5), 400 µl sample and 200 µl AAPH (20 mM). The mixture was incubated at 37°C for 1 hour.

After incubation, 1.6 ml TCA-TBA-HCl solution (15% w/v TCA, 0.375% TBA, 0.25 N HCl) was added. The solution was heated in a boiling water bath for 15 minutes. After cooling, the precipitate was removed by centrifugation and the absorbance of the resulting supernatant measured at 532 nm. Results are expressed as IC_{50} mg FW/ml.

2.14. Statistical analysis.

All spectrophotometric measurements were made on a Unicam spectrophotometer interfaced with Unicam UV Series Vision 32 software (Version 1.22, Unicam Instruments, Cambridge, UK). Results are presented in terms of descriptive as well as inferential statistics. SPSS 13.0 was used to analyse the data. As the sample size of the data set were small, normality test was performed before using parametric tests. Normality tests were based on Shapiro Wilk's test where a P value of < 0.05 was considered significant, hence resulting in non-normal data. Levene's test was used to test for homogeneity of variance. Where both assumptions are violated transformations of data was carried out. The following transformations were applied:

$$\sqrt{x+1} \text{ or } \sqrt{x} \quad ; \frac{1}{\sqrt{x+1}} \text{ or } \frac{1}{\sqrt{x}}; x^2; \frac{1}{(x^2+1)} \text{ or } \frac{1}{x^2}; \log(x) \text{ or } \log(x+1); \ln(x) \text{ or } \ln(x+1); \arcsin\left(\sqrt{x+1}\right); \exp(x)$$

The following non-parametric alternative inferential statistics were used:

Kruskal Wallis test as a non-parametric alternative for one-way ANOVA

Friedman test as a non-parametric alternative for two-way ANOVA

Spearman's rank correlation coefficient

3. Results

3.1. Phenolic content of Mauritian endemic plant extracts

The total phenolic content of the plant extracts varied from 1 to 75 mg/g FW. The genus *Diospyros* had high levels of phenolics with the maximum in *Diopyros neraudii* (75 ± 1.08 mg/g FW) followed by *Diospyros mellanida* (73 ± 0.74 mg/g FW). The genus *Chassalia* had lower levels (<10 mg/g FW). *Coffea macrocarpa, Cassine orientalis* and *Erythroxylum sideroxyloides* contained similar amount of phenolics (24 mg/ g FW) (Figure 1). As most of the data were non-normal (Table 2), 2-way ANOVA on ranked was carried out (Table 3). A highly significant difference in the phytochemicals content (total phenolics, flavonoids and proanthocyanidins) among the species were observed (χ^2-value = 9.256, $P < 0.01$).

Diospyros tesselleria, *Cassine orientalis* and *Diospyros mellanida* had the highest amount of proanthocyanidins with values reaching 21 ± 0.09 mg/g FW, 21 ± 0.36 mg/g FW and 17 ± 2.05 mg/g FW respectively. This was followed by *Diospyros revaughanii* (12 ± 0.81 mg/g FW), *Diospyros neraudii* (11 ± 1.03 mg/g FW) and *Erythroxylum sideroxyloides* (7 ± 0.96 mg/g FW) (Figure 1). The level of B1 dimer varied from 78.58 (*Coffea macrocarpa*) to 3721.61 (*Diospyros mellanida*) (Table 4). *Cassine orientalis*, *Coffea macrocarpa* and *Coffea mauritiana* showed lower levels of the B1 dimer. Although the B1 dimer was not detectable in *Trochetia boutoniana*, this plant had the highest level of the B2 dimer (2345.11 ± 4.54 μg/g FW). Interestingly, *Diospyros tesselleria* contained high levels of the B1 and B2 dimers. The dimers were not detectable in *Chasssalia grandifolia* and *Erythroxylum sideroxyloides* under our experimental conditions.

Total flavonoids in *Coffea macrocarpa*, *Diospyros tesselleria* and *Diospyros revaughanii* were respectively of the order 18 ± 0.7 mg/g FW, 11 ± 0.34 mg/g FW and 11 ± 0.11 mg/g FW. Flavonoid levels in *Coffea mauritiana*, *Chassalia lanceolata* subsps. latifolia, *Diospyros neraudii*, *Diospyros mellanida* and *Erythroxylum sideroxyloides* were in the range 5 to 8 mg/g FW (Figure 1). Although quercetin was in general the predominant flavonol its content varied widely among the different plant species (Table 5).

Diospyros revaughanii and *Diospyros neraudii* had highest levels of quercetin with values of 9878.7 µg/g FW and 9847.1 µg/g FW respectively. *Diospyros tesselleria* showed the highest kaempferol content (4425 µg/g FW). The myricetin content variation pattern was of the following order: *Diospyros revaughanii* > *Maytenus pyria* > *Cassine orientalis* > *Diospyros tesselleria* > *Coffea mauritiana* > *Erythroxylum sideroxyloides* (Table 5). A highly significant difference was observed among the flavonol derivatives (χ^2 = 13.501, P < 0.01) while the difference among the species was significant at 5 % (χ^2 = 16.026, P < 0.01). Newman-Keuls multiple comparison test on ranked data revealed that each of the flavonol derivative measured form a distinct subset as their averages are quite apart (myricetin = 11.385 µg/g FW, kaempferol = 20.92 µg/g FW, quercetin = 27.692 µg/g FW).

The highest level of catechin ((+) C) (the most widely distributed flavan-3ol derivatives in the endemic plants) were measured in *Diospyros mellanida*, *Diospyros tesselleria* and *Chassalia coriacea* var. johnstonii with values of 3892, 3091 and 1094 µg/g FW respectively. Although a lower level of (+) C was measured in *Diospyros neraudii* this flavonol was not detectable in *Chassalia lanceolata* subsps. latifolia and *Maytenus pyria*. The upper concentration of the other flavan-3-ols, (-)-EC, (-)-EGC, (-)-ECG and (-)-EGCG, measured were 1923.96, 1927.34, 2156.34 and 2681.74 µg/g FW respectively. The 'catechin index' (summation of the amounts of individual catechins that indicate the catechin richness of the plant extracts (Bahorun et al 2003; 2004) was in the following order: *D. mellanida* > *D. tessellaria* > *C. macrocarpa* > *D. revaughanii* > *C. orientalis* > *C. mauritiana* > *C. coraiceae* var. johnstonii > *D. neraudii* > *M. pyria* > *C. grandifolia* > *E. sideroxyloides* > *C. lanceolata* subsps. latifolia > *T. boutoniana*. (Table 2). Two-way ANOVA on ranks revealed that there was significant difference in the average contents of the flavon-3-ol derivatives (χ^2 = 17.993, P = 0.006) and among species for these derivatives (χ^2 = 8.214, P = 0.004).

The levels of gallic acid in *Diospyros tesselleria*, *Diospyros mellanida* and *Diospyros revaughanii* were more pronounced when compared with the values measured in *Maytenus pyria*, *Coffea macrocarpa* and *Erythroxylum sideroxyloides* (Table 4).

However gallic acid was not detectable in *Chassalia grandifolia*, *Chassalia coriacea* var. lanceolata, *Diospyros neraudii* and *Trochetia boutoniana* under our experimental conditions.

3.2. Antioxidant activity of Mauritian endemic plant extracts

The antioxidant activities of the endemic plant extracts on the basis of TEAC and FRAP values, and their abilities to scavenge HOCl and hydroxyl radicals are shown in Table 6. The TEAC values of the plant extracts varied from 24 to 989 μmol/g FW. The *Diospyros* species showed the highest activity which was in the order *D. tesselleria* > *D. revaughanii* > *D. neraudii* > *D. mellanida*. The other plant extracts exhibited moderate activity while the *Chassalia* species and *T. boutaniana* had low TEAC values. A very high correlation was obtained when TEAC values were compared with the total phenolics ($r = 0.95$, $P < 0.01$) (Figure 2) and proanthocyanidins ($r = 0.87$, $P < 0.01$) content while weaker correlation was observed with the flavonoid ($r = 0.67$, $P < 0.01$) content.

The FRAP and HOCl data paralleled the TEAC values. The *Diospyros* species were characterised by the highest antioxidant activity, *T. boutoniana* and the *Chassalia* species showed the lowest scavenging potential while the remaining plant species exhibited moderate activity. The reducing potential of the extracts in the FRAP assay showed very high correlation with the total phenolics ($r = 0.94$, $P < 0.01$) (Figure 2) and proanthocyanidin ($r = 0.87$, $P < 0.01$) content while lower correlation was obtained with the flavonoid ($r = 0.66$, $P < 0.05$), 'catechin index' ($r = 0.71$, $P < 0.01$) and gallic acid ($r = 0.67$, $P < 0.05$) content. The ability of the extract to react with HOCl showed good correlation with the total phenolics ($r = 0.85$, $P < 0.01$), proanthocyanidin ($r = 0.74$, $P < 0.01$) and ECG ($r = 0.65$, $P < 0.05$) content.

The ability of the extracts to scavenge hydroxyl radicals was assessed using the deoxyribose assay (Halliwell et al., 1987; Aruoma, 1994). Lower % activities were observed and this may be attributed to solubility restrictions. The absolute rate constants of reactions with the hydroxyl radical could not be calculated as the exact molecular entity of the phenolics responsible for the scavenging effects are not known, the order of

activity was *Cassine orientalis* > *Coffea macrocarpa* > *Chassalia lanceolata* var. latifolia = *Coffea macrocarpa* > *Diospyros neraudii* > *Diospyros revaughanii* >> *Trochetia boutoniana* (Table 6). There was no correlation of hydroxyl radical scavenging ability with the total phenolics and total proanthocyanidins contents. However a good relationship was observed with the (-)-EC content (r = 0.77, $P < 0.01$). *Diospyros tesselleria* extract showed weak prooxidant activity and this was further examined in the copper-phenanthroline assay.

The ability of the plant extracts to inhibit microsomal lipid peroxidation induced by Fe^{3+}/ascorbate and the water soluble AAPH were also evaluated by measuring TBARS. *Erythroxylum sideroxyloides* was the most potent inhibitor of microsomal lipid peroxidation with IC_{50} 0.0435 mg FW/ml (Fe^{3+}/ascorbate system) and 0.05 mg FW/ml (AAPH system) (Table 7). The *Diospyros* species were also very effective in protecting the microsomes against peroxidation in both systems with IC_{50} values ranging from 0.09 mg FW/ml to 0.3 mg FW/ml. Although *Coffea macrocarpa* had a very high antioxidant capacity in both systems ($IC_{50} \leq 0.115$ mg FW/ml) that of *Coffea mauritiana* was low. Extracts from the *Chassalia* species were the least effective in inhibiting microsomal lipid peroxidation. The activities of the extracts in the lipid systems were related to their total phenolic and proanthocyanidin content (AAPH: $r = 0.60$, $P < 0.03$; Fe^{3+}/ascorbate: r 0.72, $P < 0.01$).

The potential pro-oxidant activity of the extracts was evaluated by measuring levels of oxidative damage to the sugar moiety of DNA chelated to the copper-phenanthroline complex. When the effect by ascorbate (240 μM) is taken as an arbitrary 100%, it is clear that the prooxidant actions were moderate only for a restricted number of extracts and only at high concentrations of 10g FW/L, e.g. *Cassine orientalis, Erythroxylum sideroxyloides*; *Diospyros mellanida* and *Chassalia coriancea* var. johnstonii (Table 8).

4. Discussion

There is continued interest in the assessment of the bioefficacy of the active principles in extracts from a variety of traditional medicine and food plants in order to determine their

impact on the management of a variety of clinical conditions and maintenance of health (Scartezzini and Speroni, 2000; Aruoma et al., 2003). The *Diospyros* species had the highest phenolics content. *Coffea macrocarpa*, *Cassine orientalis* and *Erythroxylum sideroxyloides* contained relatively similar phenolics level while *Trochetia boutoniana* and plants of the genus *Chassalia* showed the lowest phenolic content (< 10 mg/g FW).

In a study by Kähkönen et al. (1999) the content of phenolics in 92 edible and non-edible plant materials (berries, fruits, vegetables, herbs, cereals, tree materials, plant sprouts and seeds), levels of (0.2 – 1.3) mg GAE/g DW in cereals, (0.4 – 6.6) mg GAE/g DW in vegetables, and (12.4 – 50.8) mg GAE/g DW in berries were reported. Skerget et al (2005) observed that the phenolic content of olive tree (*Olea europaea*), St. John's wort (*Hypericum perforatum*), hawthorn (*Crataegus laevigata*), oregano (*Origanum vulgare*) and laurel (*Lauris nobilis*) leaves varied between 99.7 and 186 g GAE/kg of extract. These data are highly corroborative of our data depicting the importance the endemic plants studied as good sources of dietary phenolics.

The level of proanthocyanidins measured in the plant extracts were equally high with the *Diospyros* species showing greatest values. The observed order in terms of contents was *Diospyros tesselleria* > *Diospyros mellanida* > *Diospyros revaughanii* > *Diospyros neraudii*. The *Cassine orientalis* had levels of proanthocyanidins (21 mg/g FW) which was similar to the level in *Diospyros tesselleria*. In addition to total proanthocyanidins several flavan-3-ol derivatives, namely (+)-C, (-)-EC, (-)-EGC, (-)-ECG and (-)-EGCG, have been quantified by HPLC. These substances are widely discussed with regards to their neuroprotective, cardioprotective and anti-carcinogenic effects (Dufresne and Farnworth 2001; Mandel and Youdim 2004; Soobrattee et al 2005).

The level of flavonoids were less pronounced in the endemic plants however concentrations of 18 mg/g FW was detected in *Coffea macrocarpa* and the levels in the *Diospyros* species was in the range 7 mg/g FW and 11 mg/g FW. *Chassalia grandifolia* and *Trochetia boutoniana* showed the lowest flavonoid content (1 mg/g FW) in the study whilst interestingly, flavonoids could not be detected in *Maytenus pyria*. Qualitative and

quantitative determination of individual flavonoid glycosides remains difficult due to lack of reference compounds commercially and due to the large number of flavonoid derivatives, e.g for quercetin alone more than two hundred different glycosides are known. Thus hydrolysis of the flavonoid conjugates to their corresponding aglycones is a more practical approach for quantitative analysis of flavonoids. Of the aglycones quantified quercetin and kaempferol were ubiquitously distributed in all the extracts, however their levels varied significantly (χ^2 = 13.501, P = 0.001). In line with literature data, quercetin was the most abundant flavonol. Marked quercetin levels were observed in *Diospyros revaughanii*, *Diospyros neraudii*, *Cassine orientalis* and *Erythroxylum sideroxyloides*. The content of kaempferol was more pronounced in *Diospyros tesselleria* (4425.5 μg/g FW) whilst *Diospyros revaughanii* had the highest myricetin content (5318.7 μg/g FW). Flavonol derivatives, such as quercitrin, rutin and astilbin, from *Hypericum perforatum* L have been shown tohave antidepressant activity in different model systems (Jensen et al 2001; Noldner and Schotz 2002). The antioxidant actions of quercetin and myricetin have been suggested to be responsible for the beneficial effects of *Ginkgo biloba* on brain neurons subject to ischemia (Oyama et al., 1994). It was interesting that of the one of the flavone *Ginkgo biloba*, apigenin was detected only in *Erythroxylum sideroxyloides* (330.9 μg/g FW).

The mechanism of the reaction involving antioxidants is complex and depends on the physico-chemical parameters of the test reagents and substrates. For this reason, use of single test system is widely discouraged (Frankel and Myer 2000; Aruoma, 2003). Although several screening approaches have been proposed, an estimation of the antioxidant potential by using the TEAC, FRAP, oxygen radical absorption assay (ORAC), the DPPH assay, total oxyradical scavenging capacity (TOSC), the deoxyribose assay, assays involving oxidative DNA damage and assays involving reactive nitrogen intermediates (e.g. $ONOO^-$) can provide preliminary information, giving a basis for further characterization procedures (Aruoma, 2003). The present study reveals that the antioxidant capacities of the Mauritian endemic plants were comparable or even higher than those cited in the literature. The plant extracts studied had varied antioxidant potencies which was of the order *Diospyros* species >> *Cassine orientalis* >

Erythroxylum sideroxyloides > *Coffea macrocarpa* > *Maytenus pyria* >> *Chassalia coriacea* var. johnstonii > *Coffea mauritiana* > *Chassalia lanceolata* subsps. latifolia >> *Trochetia boutoniana* > *Chassalia grandifolia.* Cook et al. (1998) reported that the antioxidant activities of 61 West African indigenous food plants were in the range of 3.9 to 26.3 μmol trolox/g DW. Investigating the antioxidant potential of five anti-viral medicinal plants used in China, Chen et al (2005) observed that the TEAC values varied between 26.1 and 61.7 mM Trolox at a concentration of 10 mg/ml. The antioxidant capacity of Mauritian exotic fruits was found to vary between 1 and 47 μmol trolox/ g FW while the FRAP values ranged from 0.3 to 34 μmol/ g FW (Luximon-Ramma et al, 2003; Bahorun et al 2004).

Screening the antioxidant activities of 20 traditional Mediterranean and Chinese medicinal herbs, Schinella et al. (2002) observed that most of the extracts scavenged about 30 % of the $OH^{\cdot}$ in the deoxyribose system. Although our data compares well with this, only the extracts from *Cassine orientalis* showed the most significant potential to scavenge hydroxyl radicals in the deoxyribose assay.

Erythroxylum sideroxyloides was the most potent inhibitor of microsomal lipid peroxidation followed by the *Diospyros* species and *Coffea macrocarpa.* The order of activity in both AAPH and microsomal lipid peroxidation systems were similar thus suggesting that the inhibitory activity of the plant extracts were intrinsically linked to peroxyl radical scavenging. This was confirmed by Mann Whitney U test, which showed that there was no significant difference between data obtained using the two peroxidation systems ($Z = -0.131$, $P = 0.895$). Although *Erythroxylum sideroxyloides* had a lower phenolic content than the *Diospyros* species, the higher antioxidant capacity demonstrated clearly indicates that the nature of phenolics rather than the amounts was responsible for antioxidant activity in this particular system. This is in agreement with the reports of Kähkönen et al (1999) and Shahidi and Marian (2003), where the differences in antioxidant activities of plants extracts were suggested to be due to different structures of plant extracts from phenolic acids and flavonoids as well as their derivatives.

The TEAC, FRAP and HOCl data strongly correlated with the total phenolics and proanthocyanidins content. The association between the antioxidant potential of the extracts and the proportion of phenolics present as flavonoids and proanthocyanidin dimers were less evident despite the fact that these compounds are generally known to be potent antioxidants. Such positive relationship between phenolics content and antioxidant activity has also been reported in vegetables (Bahorun et al 2004), fruits (Luximon-Ramma et al., 2003), juices (Gil et al., 2000), teas (Luximon –Ramma et al, 2005), wines (Burns et al., 2000), fresh and processed edible seaweeds (Jimenez-Escrig et al., 2000) and cell cultures (Bahorun et al., 2003). The antioxidant activity in mushrooms has also been related to their phenolic content (Mau et al., 2002).

The antioxidant properties of phenolic compounds are widely discussed for their positive role in human nutrition and disease prevention, but focus on prooxidant activity abounds. The primary concern is that these compounds can redox cycle metal ions, generate ROS and accelerate oxidative damage of DNA, protein and carbohydrate in vitro; the ROS generated is $OH^{\cdot}$ or a species with similar oxidative potential (Sakihama et al, 2002). At a concentration of 10 g FW/L, *Cassine orientalis* showed the highest prooxidant activity followed by *Erythroxylum sideroxyloides*. However, the extent of prooxidant activity was much lower than that of ascorbic acid. Similarly, Munasinghe et al. (2001) reported that at high concentrations (20 –125 μg/ mL) extracts from *Terminalia arjuna* and *Vitex negundo,* used in Ayurvedic formulations, appeared to stimulate deoxyribose damage. It is unlikely that the plant extracts will have prooxidant activity in vivo due to the low physiological level of phenolic compounds (high nanomolar or low micromolar) (Aruoma, 1994, 2003; Halliwell et al, 2000).

5. Conclusion

Phenolic compounds are particularly attractive as prophylactic agent in the management of ROS-mediated disorders due to their pleiotropic effects: free radical scavenging, metal chelating, antioxidant, phytoestrogen and modulation of cell signalling pathways. Literature data abounds in reports where medicinal plant and herbal extracts, containing a wide spectrum of phenolic antioxidants, are described as functional foods and

nutraceuticals for maintaining good health and combating disease. Mauritian endemic plants, particularly the *Diospyros* species, contain significant level of phenolic compounds and exhibited higher antioxidant activity than commonly consumed vegetables and fruits which are considered good sources of natural antioxidants. In the light of the present study it can be concluded that plants of the genus *Diospyros* holds much promise as potential source of beneficial prophylactic antioxidants, however their effects at molecular levels needs to now be evaluated.

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Abbreviations

FRAP: ferric reducing antioxidant power
FW: fresh weigh
g: gram
M: molar
MDA: malondialdehyde
min: minutes
ROS: reactive oxygen species
TBARS: thiobarbituric acid reactive substances
TEAC: trolox equivalent antioxidant capacity
µg: microgram

Figure(s)

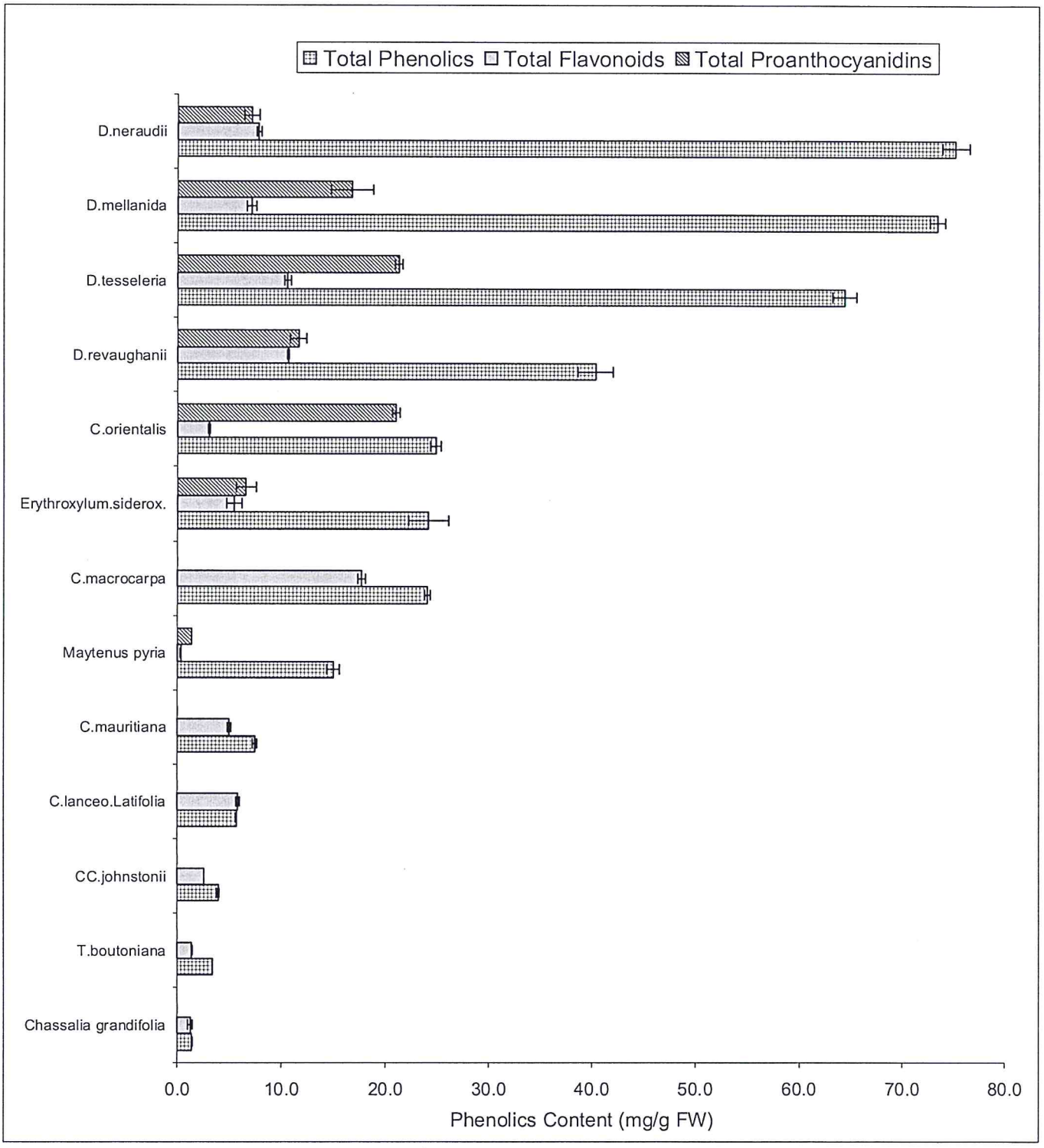


Figure 1. Total phenol[a], flavonoid[b] and proanthocyanidin[c] content of Mauritian endemic plants. Data expressed as mean values ± standard eror (n = 3). [a] mg gallic acid/g FW; [b] mg quercetin/g FW; [c] mg cyaniding chloride/g FW.

Figure(s)

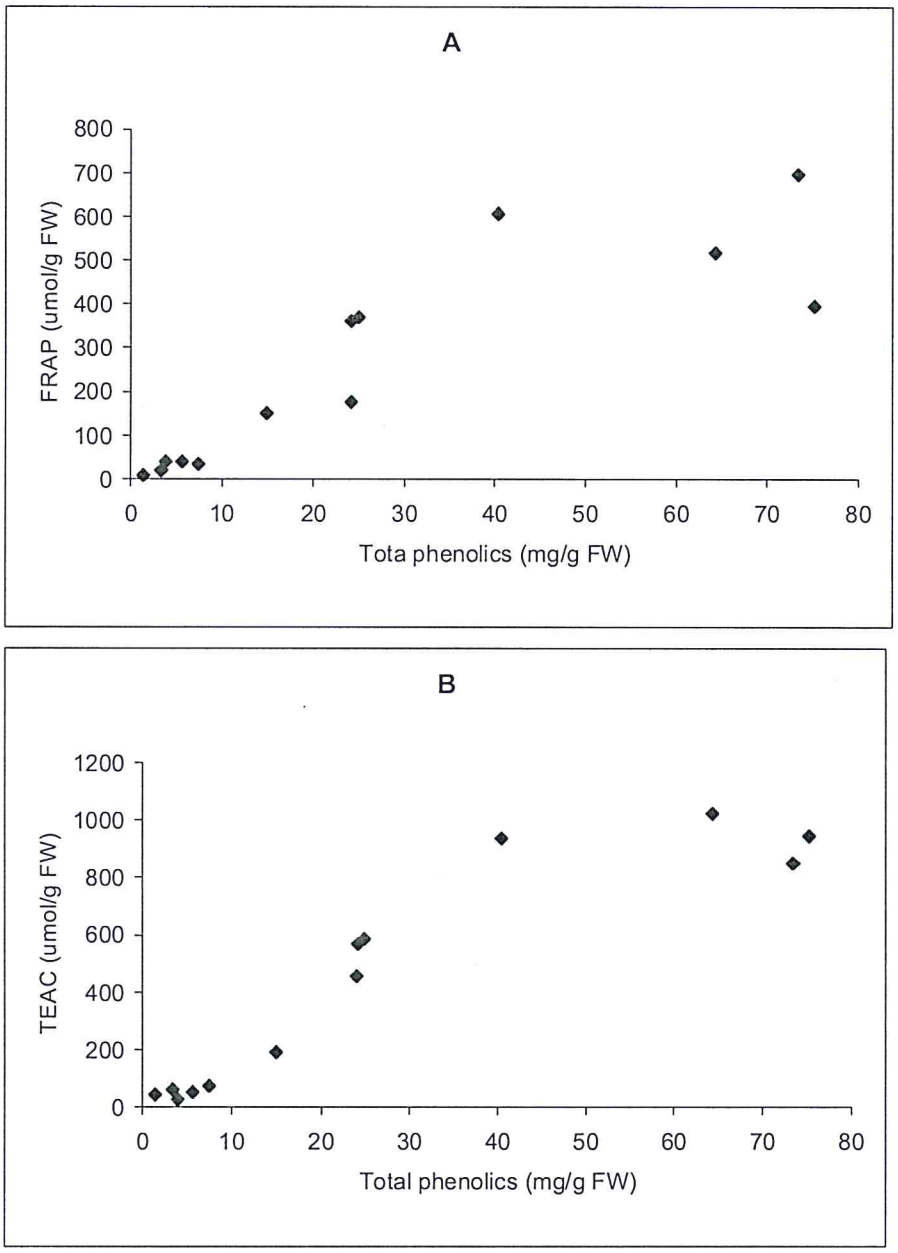


Figure 2. Effects of total phenolics on (A) FRAP and (B) TEAC antioxidant capacities of Mauritian endemic plants. Correlation between total phenolics and antioxidant activities was assessed; with FRAP, $r = 0.94$ and for TEAC, $r = 0.95$.

Table(s)

Table 1. Details of Mauritian endemic plants studied.

Family	*Plant Species*	*Vernacular name*	*Site of collection*
Rubiaceae	*Chassalia grandifolia*	Bois corail	Brise Fer forest
Rubiaceae	*Chassalia coriacea* var. johnstonii	Bois corail	Brise Fer forest
Rubiaceae	*Chassalia lanceoloata* subsps. latifolia	Bois corail	Brise Fer forest
Ebanaceae	*Coffea mauritiana*	Café maron	Magenta nature reserve
Ebanaceae	*Coffea macrocarpa*	Café maron	Magenta nature reserve
Ebanaceae	*Diospyros neraudii*	Ébène	Le Pétrin Conservation management area
Ebanaceae	*Diospyros revaughanii*	Ébène	Le Pétrin Conservation management area
Ebanaceae	*Diospyros tesselleria*	Ébène	Magenta nature reserve
Ebanaceae	*Diospyros mellanida*	Ébène	Magenta nature reserve
Celastraceae	*Cassine orientalis*	Bois d'olive	Magenta nature reserve
Celastraceae	*Maytenus pyria*	Bois a poudre	Magenta nature reserve
Erythroxylaceae	*Erythroxylum sideroxyloides*		Magenta nature reserve
Sterculaceae	*Trochetia boutoniana*	Boucle d'oreille	Réduit

Table 2. Summary of Normality test

Phenolics	Shapiro Wilk's Test		
	Statistic	df	Significance
Total phenolics	0.836	13	0.019
Total flavonoids	0.912	13	0.198*
Total proanthocyanidins	0.777	13	0.004
TEAC	0.838	12	0.027
FRAP	0.861	12	0.050*
HOCl	0.797	12	0.009
Deoxyribose	0.961	12	0.801*
Fe^{3+}-ascorbate	0.738	13	0.001
AAPH	0.686	13	0.000
Kaempferol	0.825	13	0.014
Myricetin	0.458	13	0.000
Quercetin	0.900	13	0.133*
(+) C	0.649	13	0.000
(-) EC	0.737	13	0.001
(-)ECG	0.636	13	0.000
(-) EGCG	0.735	13	0.001
(-) EGC	0.643	13	0.000
Catechin Index	0.846	13	0.025
B 1	0.528	13	0.000
B 2	0.825	13	0.014
Gallic acid	0.801	13	0.007

* Data for element is normal ($P > 0.05$)

Table(s)

Table 3. Summary table of Analysis of Variances

Type of Analysis	Source	df	F-statistic	Significance
2-Way ANOVA (on ranks)	Total phenolic content	2	15.084	0.000
	Species	12	5.737	0.000
2-Way ANOVA (on ranks)	Flavon-3-ol derivatives	9	4.815	0.000
	Species	12	5.807	0.000
2-Way ANOVA (on ranks)	Flavonols	2	15.636	0.000
	Species	12	2.729	0.018
2-Way ANOVA (on ranks)	Antioxidant activity	3	50.234	0.000
	Species	11	1.114	0.382

Table(s)

Table 4. Flavan-3ols derivatives content of Mauritian endemic plants. Values are expressed in µg/g FW.

Plant species	(+) C	(-) EC	(-) ECG	(-) EGCG	(-) EGC	Catechin index	B 1	B 2	Gallic acid
Chassalia grandifolia	336.88 ± 1.46	_	_	_	26.87 ± 0.41	363.75		_	_
Chassalia coriacea var. johnstonii	1093.73 ± 4.75	978.14 ± 3.28	_	501.88 ± 3.95	_	2573.75	_	880.07 ± 1.71	110.84 ± 1.46
Chassalia lanceoloata subsps. Latifolia	_	_	_	_	108.89 ± 1.67	108.89	1199.87 ± 1.60	365.67 ± 7.09	_
Coffea mauritiana	354.35 ± 1.54	634.88 ± 2.55	_	1585.15 ± 2.89	148.78 ± 2.27	2723.16	_	271.72 ± 5.27	61.20 ± 0.81
Coffea macrocarpa	545.75 ± 2.37	1798.21 ± 7.23	2156.34 ± 13.15	631.82 ± 4.98	189.70 ± 2.91	5321.82	78.58 ± 0.1	766.64 ± 1.49	19.22 ± 0.25
Diospyros neraudii	84.58 ± 0.37	248.29 ± 1.33	522.68 ± 4.25	165.38 ± 1.30	_	1020.93	393.97 ± 0.52	_	_
Diospyros revaughanii	179.61 ± 0.78	721.15 ± 3.87	1642.69 ± 13.36	567.94 ± 4.47	_	3111.39	_	157.41 ± 3.05	228.34 ± 3.01
Diospyros tesseleria	3091.11 ± 13.41	_	_	2681.74 ± 2.12	1324.35 ± 2.28	7097.2	_	1702.25 ± 3.29	349.87 ± 4.61
Diospyros mellanida	3892.22 ± 16.89	_	436.84 ± 3.56	915.07 ± 7.2	1927.34 ± 9.52	7171.47	3721.61 ± 3.29	713.61 ± 1.38	266.03 ± 3.51
Cassine orientalis	436.60 ± 1.89	1923.96 ± 8.59	_	_	734.40 ± 1.25	3094.96	_	818.58 ± 1.58	114.58 ± 1.51
Maytenus pyria	_	_	358.56 ± 1.82	_	42.55 ± 0.65	401.11	647.87 ± 0.86	_	40.24 ± 0.53
Erythroxylum sideroxyloides	173.97 ± 0.75	_	_	_	48.94 ± 0.75	222.91	_	_	19.00 ± 0.25
Trochetia boutoniana	106.67 ± 0.46	_	_	_	_	106.67	_	2345.11 ± 4.54	_

Table(s)

Table 5. Flavonols (kaempferol, myricetin and quercetin) content of Mauritian endemic plant extracts. Values are expressed in µg/g FW.

Plant species	Flavonols		
	Kaempferol	Myricetin	Quercetin
Chassalia grandifolia	113.0 ± 2	_	115.4 ± 4
Chassalia coriacea var. johnstonii	484 ± 10	_	2717.0 ± 94
Chassalia lanceoloata subsps. latifolia	455.7 ± 9	_	37.5 ± 1
Coffea mauritiana	92.1 ± 2	265.9 ± 9	1259.3 ± 43
Coffea macrocarpa	3315.3 ± 67	_	4729.6 ± 163
Diospyros neraudii	3918.4 ± 79	_	9847.1 ± 339
Diospyros revaughanii	3131.0 ±64	5318.7 ± 171	9878.7 ± 340
Diospyros tesseleria	4425.5 ± 90	400.9 ± 13	4227.3 ± 145
Diospyros mellanida	1360.9 ± 28	_	3913.6 ± 135
Cassine orientalis	1401.3 ±28	599.2 ± 19	7927.4 ± 273
Maytenus pyria	102.1 ± 2	872.1 ± 28	900 ± 31
Erythroxylum sideroxyloides	251.1 ± 5	238.7 ± 8	5995.9 ± 286
Trochetia boutoniana	821.4 ± 17	_	816.7 ± 28

Table(s)

Table 6. Antioxidant activities of Mauritian endemic plants

Species	TEAC [a] (µmol /g FW)	FRAP [b] (µmol /g FW)	HOCl [c] (IC_{50} mg FW/ml)	Deoxyribose Assay [d] (%scavenged/g FW)
Chassalia grandifolia	43 ± 4.52	7 ± 0.14	13.84 ± 0.28	14.5 ± 0.5
Chasslia coriacea var. johnstonii	24 ± 1.09	39 ± 0.43	4.20 ± 0.21	20.8 ± 1
Chassalia lanceolata subsps. latifolia	50 ± 0.68	38 ± 0.14	5.77 ± 0.16	23.85 ± 3
Cofffea mauritiana	73 ± 3.67	34 ± 1.8	6.36 ± 0.14	23.99 ± 2
Coffea macrocarpa	447 ± 7.07	175 ± 1.95	0.78 ± 0.05	26.58 ± 0.98
Diospyros neraudii	911	396 ± 8.81	0.33 ± 0.02	16.75 ± 1.2
Diospyros revaughanii	945 ± 15.71	607 ± 25.6	0.21 ± 0.01	20.2 ± 1.5
Diospyros tesselleria	989 ± 9.43	517 ± 13.98	0.31 ± 0.03	Prooxidant
Diospyros mellanida	818 ± 6.29	696 ± 23.52	0.32 ± 0.02	17.5 ± 0.5
Cassine orientalis	584 ± 5.24	368 ± 11.79	2.27 ± 0.01	27.15 ± 0.64
Maytenus pyria	190 ± 0.87	151 ± 2.68	0.31 ± 0.04	10.4 ± 2.1
Erythroxylum sideroxyloides	567 ± 5.24	361 ± 17.62	0.49 ± 0.10	12.51 ± 0.88
Trochetia boutoniana	60 ± 2.69	20 ± 1.21	7.22 ± 0.6	5.09 ± 0.69

All analyses were mean of triplicates measurements ± standard deviations. [a] Results expressed in units µmol trolox equivalent/g fresh weight. [b] Values are expressed in units of µmol Fe (II)/ g fresh weight. [c] IC_{50} is defined as the concentration sufficient to obtain 50 % of a maximum scavenging activity. [d] Results are expressed as % inhibition of deoxyribose oxidation by 1 g FW/L plant extract.

Table(s)

Table 7. Concentration of plant material that caused 50% inhibition of microsomal lipid peroxidation at a fixed concentation of lipid source. The IC_{50} values were determined from a calibration curve for each plant extract.

Plant species	IC_{50} mg FW/ml	
	Fe^{3+}/ascorbate system	AAPH system
Chassalia grandifolia	8.2 ± 0.071	9.8 ± 0.028
Chassalia coriacea var. johnstonii	9.9 ± 0.067	9.4 ± 0.033
Chassalia lanceoloata subsps latifolia	13 ± 0.080	7.2 ± 0.05
Coffea mauritiana	9.1 ± 0.071	1.3 ± 0.041
Coffea macrocarpa	0.086 ± 0.001	0.115 ± 0.002
Diospyros neraudii	0.148 ± 0.001	0.165 ± 0.001
Diospyros revaughanii	0.14 ± 0.004	0.09 ± 0.001
Diospyros tesselleria	0.105 ± 0.004	0.165 ± 0.001
Diospyros mellanida	0.166 ± 0.002	0.3 ± 0.008
Cassine orientalis	0.188 ± 0.002	0.17 ± 0.006
Maytenus pyria	0.147 ± 0.030	0.139 ± 0.037
Erythroxylum sideroxyloides	0.0435 ± 0.001	0.05 ± 0.002
Trochetia boutoniana	6.5 ± 0.071	4.19 ± 0.026

Table(s)

Table 8. Effect of plant extracts on copper 1-10 phenanthroline dependent DNA damage

Plant extract (g FW/L)	Extent of DNA damage (A_{532} nm)	Plant extract (g FW/L)	Extent of DNA damage (A_{532} nm)
None	0.000	*Diospyros revaughanii*	
		0.1	0.005
		0.5	0.012
Ascorbic acid (240 μM)	0.179	1.0	0.017
		5.0	0.026
		10	0.037
Chassalia grandifolia		*Diospyros tesselleria*	
0.1	0.000	0.1	0.002
0.5	0.000	0.5	0.010
1.0	0.006	1.0	0.023
5.0	0.013	5.0	0.036
10	0.017	10	0.057
Chasslia coriacea var. johnstonii		*Diospyros mellanida*	
0.1	0.002	0.1	0.003
0.5	0.008	0.5	0.010
1.0	0.015	1.0	0.019
5.0	0.052	5.0	0.048
10	0.075	10	0.072
Chassalia lanceolata subsps. latifolia		*Cassine orientalis*	
0.1	0.000	0.1	0.000
0.5	0.004	0.5	0.007
1.0	0.007	1.0	0.025
5.0	0.013	5.0	0.093
10	0.025	10	0.157
Cofffea mauritiana		*Maytenus pyria*	
0.1	0.000	0.1	0.005
0.5	0.000	0.5	0.012
1.0	0.000	1.0	0.027
5.0	0.000	5.0	0.040
10	0.015	10	0.053
Coffea macrocarpa		*Erythroxylum sideroxyloides*	
0.1	0.000	0.1	0.003
0.5	0.006	0.5	0.009
1.0	0.019	1.0	0.020
5.0	0.034	5.0	0.060
10	0.063	10	0.085
Diospyros neraudii		*Trochetia boutoniana*	
0.1	0.000	0.1	0.000
0.5	0.003	0.5	0.005
1.0	0.009	1.0	0.008
5.0	0.021	5.0	0.015
10	0.043	10	0.026

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Eugenia pollicina leaf extract: A rich source of polyphenolic antioxidants for potential use as prophylaxis and food preservation

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Abstract

In an attempt to investigate the potential use of a Mauritian endemic plant, *Eugenia pollicina*, as a source of natural antioxidants for use as prophylactics and food preservatives, the polyphenolic profile of a leaf extract of the plant was assessed. High levels of phenolics, flavonoids and proanthocyanidins were obtained. The levels of flavan-3-ol derivatives quantified by HPLC were in the order of (-)-epicatechin (EC) > (-)-epigallocatechin gallate (EGCG) > (+)-catechin (C) > (-)-epicatechin gallate (ECG).

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Procyanidin B2 and B1 dimers were also measured with amounts ranging from 1 and 3 mg g^{-1} FW respectively. The *in vitro* antioxidant activity of the leaf extract was assessed using various established methods. The Trolox equivalent antioxidant capacity (TEAC) and ferric reducing antioxidant power (FRAP) values were 796 μmol g^{-1} FW and 302 μmol g^{-1} FW respectively. *E. pollicina* extracts also strongly inhibited the $FeCl_3$ and ascorbate-dependent microsome lipid peroxidation, a function that is linked to their flavonoid contents. The extracts did not show prooxidant damage to DNA when compared with the effect of a reference 240 μM ascorbate in the copper-phenanthroline-dependent DNA damage assay. *E. pollicina* extracts inhibited lipid peroxidation in the 30% (v/v) olive- and soybean- oil-in-water emulsions and was effective in slowing down the formation of hydroperoxides in the unstripped oil emulsions during 13 days storage at 40°C as determined by the peroxide, conjugated diene and para-anisidine values. Thus, the Mauritian endemic plant, *E. pollicina* is a potential source of antioxidants relevant to the maintenance of oxidative stability of the food matrix, cosmetics and/or pharmaceutical preparations.

Keywords: Food preservatives, prophylactic, *Eugenia pollicina*, flavonoids, proanthocyanidins, *in vitro*, antioxidant efficacy, emulsions, soybean oil, olive oil.

1. Introduction

Oxidative stress mechanisms caused by reactive oxygen species (ROS) have been implicated in the development of several degenerative diseases such as atherosclerosis, stroke, myocardial infarction, trauma, arthritis, ischemia/reoxygenation injury and cancer [1-5]. Moreover, in foods, oxidative damage to lipids, proteins, sugars and vitamins can produce undesirable volatile compounds, destroy essential fatty acids, amino acids and vitamins, and produce carcinogens [6]. Autoxidation reactions result in a negative impact on flavor thereby decreasing the acceptability of food products to consumers [7]. Because of these highly undesirable consequences, the reduction of ROS in both human and food systems is highly desirable.

Recently, there has been an impetus in research for the development of natural antioxidant sources that can be used as prophylactics following an increase in awareness that natural components having antioxidative potency can play a crucial role in health promotion and disease prevention. In the food industry also, the attention of manufacturers has shifted from synthetic to natural antioxidants as although synthetic antioxidants have to date been economically used to effectively control oxidation and prolong the shelf life of foods [8], their safety has been questioned due to their high volatility and instability at elevated temperatures and their suspected carcinogenicity [9].

In evaluating the potential antioxidant functions of biofactors in natural plant extracts as prophylactic agents or food additives it is important to employ a number of analytical techniques since the antioxidant potency can differ substantially according to the physical

63 and chemical parameters of the systems used for their characterization [10-12]. It is also
64 important to fully assess the levels of active phenolic components present in crude extracts
65 and the interaction of plant extracts with other antioxidants in order to comprehensively
66 understand the antioxidant mechanism of natural polyphenols in food [13].
67
68 In this study, the phenolic profile and antioxidant activity of the leaf extract of a rare
69 Mauritian endemic plant species, *Eugenia pollicina*, vernacular name "Bois de clous", has
70 been assessed. The plant belongs to the Myrtaceae family and has no reported
71 pharmacological and traditional uses. A previous study has shown that the mature leaves
72 contain high amounts of flavonoids most probably due to the tropical status of the plant
73 with a high level of environmental stress induced by ultraviolet radiation. The effect of
74 *Eugenia pollicina* extract on the promoter activities of antioxidant enzymes has been
75 studied and results showed that the promoter activity of Cu, Zn-SOD (Cu, Zn-superoxide
76 dismutase) was proportionally associated with the phenolic content of plant extracts in
77 COS7 cells. There was an inverse association with the catalase promoter activity [14]
78 indicating that the prophylactic potentials of the extract might reside in its abilities to
79 modulate the expression of antioxidant enzyme genes. In the present investigation, the
80 antioxidant potency of *E. pollicina* leaf extract was assessed using various established
81 assays: Trolox equivalent antioxidant capacity (TEAC), ferric reducing antioxidant power
82 (FRAP), scavenging of hypochlorous acid, inhibition of microsomal lipid peroxidation,
83 inhibition/promotion of oxidative DNA damage [15] and assays involving the autoxidation
84 of oil-in-water emulsion systems during storage at 40°C. The results obtained in this study

would constitute the basis for the potential use of the leaf extract as a source of natural antioxidants.

2. Methods and materials

2.1. Standards and chemicals

2,2'-Azino-bis(3-ethylbenzthiozoline-6)-sulfonic acid (ABTS), 2,4,6-Tri(2-pyridyl)-*s*-triazine (TPTZ) and Butylated hydroxytoluene (BHT) were purchased from Sigma (St. Louis, MO, USA). Para-anisidine was purchased from Acros Organics (New Jersey, USA). Trolox C (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) was purchased from Sigma-Aldrich (Deisenhofen, Germany). Quercetin, (+)-catechin, (-)-epicatechin, (-)-epigallocatechin, (-)-epigallocatechin-3-gallate, procyanidin dimers B1 and B2, chlorogenic acid, and cyanidin chloride (HPLC grade) were from Extrasynthèse (Genay, France). HPLC solvents were of HPLC grade. All other reagents used were of analytical grade. Soybean oil (Moroil™) and extra virgin olive oil (Olivor™) were bought from a local supermarket in Mauritius.

2.2. Preparation of plant total extract

Mature leaves were used as they have been reported to contain higher levels of secondary metabolites. The leaves of several plants were collected at Brise-fer forest, situated on the central plateau of Mauritius, and pooled together for extraction. 100g of crushed mature leaves were macerated at 4°C for 24 h in 300 mL acetone/water (70/30 v/v) and then filtered. The filtrate was collected and the residue was extracted with 300 mL acetone/water (70/30 v/v) followed by an exhaustive extraction with 300 mL of 100%

methanol. The mixture was then filtered and the collected filtrates were concentrated at low pressure. The crude aqueous extract thus obtained was thoroughly washed with dichloromethane to remove lipids and chlorophylls. The total aqueous extract was concentrated under pressure and freeze-dried. The freeze-dried powder was dissolved in methanol or distilled water prior to analysis.

2.3. Determination of total phenolic content

Total phenol content was determined by the method adapted from Singleton and Rossi [16] using the Folin-Ciocalteu reagent. An aliquot (0.25 ml) of the extract was added to 3.75 ml of distilled water in a test tube, followed by 0.25 ml of Folin-Ciocalteu reagent. After 3 minutes, 0.75 ml of 20% sodium carbonate was added. Tubes contents were vortexed and heated at 40°C for 40 minutes. The blue coloration was read at 685 nm against a blank standard. Results were expressed in mg of gallic acid g^{-1} fresh weight of plant material.

2.4. Determination of total proanthocyanidin content

The HCl/Butan-1-ol assay, adapted from Porter *et al.* [17] was used to quantify the total amount of proanthocyanidins. An aliquot (0.25 ml) of the methanolic extract was added to 3 ml of *n*-BuOH-HCl solution (95:5 v/v) in stoppered test tubes followed by 0.1 ml of $NH_4Fe(SO_4)_2.12H_2O$ in 2M HCl. The tubes were capped and incubated for 40 minutes at 95°C. After cooling in the dark, the absorbance of the red coloration was read at 550 nm against a blank standard. The amount of proanthocyanidins was expressed in mg of cyanidin chloride g^{-1} fresh weight.

131 *2.5. Determination of total flavonoid content*

132 The $AlCl_3$ method [18] was used for the determination of the total flavonoid content of the
133 methanolic leaf extract. 1.5 ml of 2% $AlCl_3.6H_2O$ dissolved in methanol was added to
134 equal volumes of the extract. The mixture was shaken and the absorbance was read at 440
135 nm after 10 minutes incubation at room temperature. Flavonoid contents were expressed in
136 mg quercetin equivalent g^{-1} fresh weight.

137

138 *2.6. High-performance liquid chromatography (HPLC)*

139 HPLC analyses of the leaf extracts were carried out using a Hewlett Packard (Waldbronn,
140 Germany) 1100 series liquid chromatography system equipped with a vacuum degasser,
141 quaternary pump, autosampler, thermostatted column compartment and diode array
142 detector. Leaf extracts (30µL) filtered on Millipore (0.22µm) were injected on a Waters
143 Spherisorb ODS-2 column (5µm particle size, 80 Å pore size) eluted by an acidified
144 acetonitrile-water gradient. Elution with a flow rate of 0.7 ml/min at 25°C was as follows:
145 0-30 min, 0-15% B in A; 30-50 min, 15% B in A; 50-60 min, 15-25% B in A; 60-90 min,
146 15-100% B in A; 90-100 min, 100-0% B in A (Solvent A: acetonitrile/water, 1/9 v/v, pH
147 2.6; solvent B: acetonitrile/water, 1/1 v/v, pH 2.6). The absorption wavelength was
148 selected at 280 nm for proanthocyanidins and phenolic acids and 360 nm for flavonoids.
149 (+)-catechin, (-)-epicatechin, (-)-epicatechin gallate, (-)-epigallocatechin gallate,
150 procyanidin B1 and B2 dimers and gallic acid were identified and quantified by comparison
151 with authentic samples at an absorption wavelength of 280 nm.

152

153 *2.7. Hydrolysis of flavonoid conjugates*

154 Flavonoid conjugates were hydrolysed as described in Harborne [19]. An aliquot of 1g
155 fresh weight equivalent of freeze-dried extract, dissolved in 2 ml water, was added to an
156 equal volume of 2 M HCl. The extract was refluxed at 100°C for 100 min. After cooling
157 the flavonoid aglycones were extracted using 2 x 4 ml of ethyl acetate. The latter phase
158 was evaporated to dryness and taken in absolute methanol prior to analysis by HPLC.
159
160 *2.8. Measurement of antioxidant activity*
161 *2.8.1. TEAC assay*
162 The trolox equivalent antioxidant capacity (TEAC) of the total leaf extract was measured in
163 terms of radical-scavenging ability according to the method of Campos and Lissi [20].
164 Results were expressed as μmol Trolox equivalent g^{-1} fresh weight.
165
166 *2.8.2. FRAP assay*
167 The ferric reducing antioxidant power (FRAP) assay was carried out as recommended by
168 Benzie and Strain [21]. Results were expressed as μmol Fe^{2+} g^{-1} fresh weight
169
170 *2.8.3. Lipid Peroxidation assay*
171 Beef liver microsomes were prepared by tissue homogenization with ice-cold 0.25 M
172 sucrose-10 mM Tris buffer, pH 7.4 with 1 mM EDTA. Microsomal fractions were isolated
173 by removal of the nuclear fractions after centrifugation at 1500 rpm for 5 minutes and
174 removal of mitochondrial fraction at 5000 rpm for 15 minutes. The supernatant was then
175 centrifuged in a Beckman Optima™ ultracentrifuge at 100 000 X g for 60 minutes at 4°C.
176 The pellet was then washed in 0.25 M NaCl, pH 7.5. The membranes were suspended in

177 saline (0.25 M, pH 7.5) and stored in aliquots at -20°C. Microsomal protein was
178 determined by the Lowry-Folin method [22]. The formation of malondialdehyde (MDA),
179 measured as thiobarbituric reactive substances (TBARS), was used to monitor lipid
180 peroxidation. The reacting mixture contained in a final volume of 1 ml the following
181 reagents: 200 µl phosphate buffered saline (3.4 mM, pH 7.4), 200 µl microsome (0.5mg ml^{-1}
182 microsomal protein), 400µl of sample (variable concentrations), 100 µl iron (III) chloride
183 (1mM) and 100 µl ascorbate (1mM). The mixture was incubated for 1 hour at 37°C. At the
184 end of the incubation period, 100 µl 2% (w/v) BHT was added followed by 1 ml 1% (w/v)
185 thiobarbituric acid and 2.8 % (w/v) trichloroacetic acid. Addition of BHT to the reaction
186 mixture minimizes erroneous increases in color due to iron ion-dependent hydroperoxide
187 decomposition during the acid heating stage [23,24]. The solutions were heated in a water-
188 bath at 80°C for 20 min to develop the MDA-TBA adducts (TBARS). The chromogen was
189 extracted using 2 ml butan-1-ol and its absorbance read at 532 nm. Results are expressed
190 as IC_{50} mg FW ml^{-1}.
191
192 *2.8.4. Copper-phenanthroline assay*
193 The Cu-phen assay was conducted essentially as described by Aruoma [24,25]. Reaction
194 mixture contained in a final volume of 1.2 ml, the following reagents in order of addition
195 indicated: 100 µl of 1.8 mM 1,10-phenanthroline (weighed crystals were initially dissolved
196 in 50 µl ethanol and then taken up in water), 480 µl of 250 µM copper (II) chloride, 300 µl
197 DNA (1.68 mg/ml) and 120 µl of 100mM KH_2PO_4-KOH buffer at pH 7.4. 100 µl of
198 ascorbate (stock solution: 2.88 mM) or extracts were added to initiate the reaction. The
199 tubes were incubated at 37°C for 1 hour. At the end of the incubation period 100 µl 0.1 M

200 EDTA was added to stop the reaction. The extent of DNA doxy-sugar damage was
201 assessed by adding 1 ml of 1% (w/v) TBA and 1 ml 25 % (v/v) HCl followed by 15
202 minutes incubation at 80°C. The pink chromogen formed was extracted into butan-1-ol and
203 the absorbance measured at 532 nm.
204
205 *2.8.5. Assessment of antioxidant activity in oil-in-water emulsions*
206 The antioxidant activity of the total extract was assessed in oil-in-water emulsions prepared
207 with two different unstripped vegetable oils namely soybean oil and extra virgin olive oil.
208 The unstripped oil (30%) was emulsified in 50 ml beakers with water (70%) containing
209 sodium acetate buffer (0.1M, pH 5.40) and Tween 20 (0.1%), by sonication using an
210 ultrasonic homogenizer (Soniprep 150, Sanyo Gallenkamp PLC, UK). 0.5 ml of a 1%
211 methanolic solution of BHT or total extract was added to the unstripped oil prior to
212 sonication to obtain 0.02% m/m BHT or 0.02% total extract (TE) concentration in the
213 emulsions (25g). The emulsions (25 g) were prepared in duplicate, covered with nescofilm
214 and stored at 40°C. In order to monitor hydroperoxide formation, 3.5 ml emulsion samples
215 were taken before and at regular intervals during storage, transferred into 15 ml centrifuge
216 tubes and frozen at -50°C. The samples were thawed at room temperature and centrifuged
217 at 3700 rpm for 10 minutes at 10 °C prior to the chemical analyses. The isolated oil
218 fractions were then used for peroxide value, conjugated diene value and para-anisidine
219 value determinations [26-28].
220
221 *2.9. Statistical analyses*

For the TEAC, FRAP, HOCl scavenging, copper-phenanthroline and lipid peroxidation assays simple regression analysis was performed to calculate the dose-response relationship of standard solutions used for calibration as well as test samples. Results are expressed as mean value ± standard deviation (n=3). Peroxide values, conjugated diene values and para-anisidine values in the tables and figures are expressed as mean ± standard deviation.

The time taken (number of days) for the conjugated diene value of the emulsions to increase by 25% was calculated from exponential curves generated using Microsoft Excel. A randomized block design was applied on the data obtained with 2 treatments (BHT, plant extract) and one control at 2 levels (soybean oil and olive oil) with 2 replicates each. 2-way ANOVA was carried out to determine any significant (i) difference among treatments and control, (ii) difference between oils and (iii) interaction between oils and treatments. All levels of significance were set at 5%.

3. Results

The total leaf extract of *E. pollicina* contains high levels of total phenolic, and comparable amount of total flavonoids and proanthocyanidins (Table 1). HPLC analysis of the plant extract showed the presence of several flavan-3-ol derivatives quantified in the following decreasing order: (-)-EC > (-)-EGCG > (+)-C > (-)-ECG (Table 1). The "catechin index" (summation of the amount of individual catechins) [9] was 9.7 mg g^{-1} FW in *E. pollicina* leaf extract and the levels of procyanidin dimers varied between 1 and 3 mg g^{-1} FW. Indeed the high levels of quercetin and kaempferol aglycones suggest that significant levels of flavonol glycosides are present in the plant extract (Table 1).

245

246 A TEAC value of 796 ± 33.3 μmol g^{-1} FW and a FRAP value of 302 ± 1.51 μmol g^{-1} FW
247 were measured in the aqueous leaf extract. Beef liver microsomes undergo rapid
248 peroxidation when incubated with $FeCl_3$ and ascorbate at pH 7.4. *E. pollicina* strongly
249 inhibited lipid peroxidation of the liver microsomes in a concentration dependent manner
250 (Fig. 1) with a calculated IC_{50} value of 0.012 mg FW ml^{-1}. The ability of the leaf extract to
251 exert prooxidant actions was assessed using the copper-phenanthroline assay. The extracts
252 tested at concentrations <10 g FW L^{-1} was not prooxidant when compared with the
253 reference effect of ascorbate (240μM) (Table 2). The antioxidant activities of the standard
254 compounds, detected by HPLC in the leaf extract, were assessed in the different systems
255 (Table 3).
256

257 Peroxide values in unstripped oil emulsions are known to increase with storage (Fig. 2 and
258 3). *E. pollicina* leaf extract was effective in slowing down hydroperoxide formation in
259 unstripped soybean oil emulsions during 13 days storage at 40°C as demonstrated by
260 peroxide values in Fig. 2. The mean peroxide value recorded for control emulsions after 13
261 days, was more than twice the value obtained for emulsions containing the plant extract
262 (Control: 50.99 meq/kg; *E. pollicina* leaf extract: 20.27 meq/kg).
263

264 The increase in conjugated diene value during 13 days storage at 40°C was higher for
265 control emulsions compared to emulsions with 0.02% *E. pollicina* leaf extract or 0.02%
266 BHT (Table 4). The time taken for unstripped soybean oil emulsions to attain a 25%
267 increase in conjugated diene value decreased in the following order ($p>0.05$): BHT (11.97

± 0.460 days) >*E. pollicina* leaf extract (8.46 ± 1.500 days) >control (4.22 ± 0.912 days). At the end of the storage period, control emulsions contained the highest amount of aldehydes (principally 2-alkenals) with a p-anisidine value of 6.00 ± 0.368 compared to emulsions with BHT and *E. pollicina* leaf extract which obtained p-anisidine values of 3.79 ± 0.170 and 4.05 ± 0.926 respectively.

Unrefined olive oil contain impurities that combine to account for the high initial peroxide values of the unstripped olive oil emulsions. International standards specify values of up to 20 meq/kg for extra virgin olive oils [29]. Hydroperoxide formation was faster in control emulsions compared to emulsions containing 0.02% BHT or 0.02% *E. pollicina* leaf extract during 17 days of storage at 40°C. The conjugated diene data (Table 5) show a similar pattern. The time taken for the emulsions to achieve a 25% increase in conjugated diene value during storage at 40°C showed that whilst emulsions with BHT and emulsions with *E. pollicina* extract did not differ significantly ($p<0.05$) (BHT: 15.49 ± 0.290 days, *E. pollicina*: 15.90 ± 0.290 days), control emulsions took significantly less time ($p>0.05$) (8.05 ± 0.078 days) to achieve this increase. The para-anisidine values show that the levels of aldehydes were higher for control emulsions (13.18 ± 0.827) than emulsions containing BHT (2.88 ± 0.495) or *E. pollicina* total extract (3.12 ± 0.049), after 17 days of storage at 40 °C.

The outcome of the 2-way ANOVA applied on the time taken by emulsions to obtain a 25% increase in conjugated diene value indicated a very highly significant difference between treatments including control ($F=111.58$; $df=2$; $P<0.001$). The least significant

291 difference (LSD) test showed highly significant differences among each pair of treatment
292 combinations ($P<0.01$). A very highly significant difference between oils was also
293 obtained ($F=126.19$; $df=1$; $P<0.001$) whereby olive oil emulsions took 13.14 days to attain
294 a 25% increase in conjugated value compared to soybean oil emulsions which took 8.21
295 days. The interaction between treatments and oils was significant ($F=8.20$; $df=2$; $P<0.05$).
296 In order to analyze the nature of this interaction, a graph was generated as shown in Fig. 4.
297 The figure shows that BHT was equally effective in inhibiting oxidation in both oil
298 systems. In the case of unstripped olive oil emulsions there is only a slight increase
299 ($p<0.05$) in time taken for 25% increase in conjugated diene value when the leaf extract
300 was used instead of BHT. However, in the case of soybean oil emulsions, there was a large
301 decrease in time taken when the leaf extract was used in place of BHT. The increasing
302 slopes of lines C_1 and C_2 reveal an overall positive effect of total extract of *Eugenia*
303 *pollicina* on the oils used to prepare emulsions. The leaf extract had a greater antioxidative
304 activity in unstripped olive oil emulsions than in unstripped soybean oil emulsions.
305

306 ## 4. Discussion

307 The total leaf extracts of *Eugenia pollicina* exhibited high antioxidant and free radical
308 scavenging activities in the various independent antioxidant systems used. This antioxidant
309 efficacy can be ascribed to its rich polyphenolic content. HPLC analysis showed the
310 significant presence of catechins namely (+) C, (-) EC, (-) ECG, (-) EGCG, (-) EGC and
311 procyanidin dimers B1 and B2. These flavan-3-ol derivatives are reported in the literature
312 to contribute to the powerful antioxidative potency of tea extracts [30,31] by acting as
313 effective scavengers of free radicals [30] and chelators of metal ions [32]. Procyanidins

314 have also been shown to limit free radical formation by inhibiting enzymes [33]. The
315 antioxidant activity of catechins is determined by the B-ring catechol structure and is
316 further enhanced in gallocatechins by the 5' hydroxyl group on the B ring [34,35].
317
318 *E. pollicina* leaf extract showed high TEAC and FRAP values (796 μmol g^{-1} fresh weight
319 and 302 μmol g^{-1} fresh weight respectively). In order to compare the TEAC and FRAP
320 values of the extract with those reported in the literature, the values were recalculated in
321 terms of dry weight. The TEAC value of *E. pollicina* (2202 μmol trolox equivalent g^{-1} dry
322 weight) is indicative of the strong scavenging activity of the leaf extracts compared to the
323 antioxidant activities of some medicinal plants in New Mexico which range between 27 and
324 972 μmol trolox equivalent g^{-1} dry weight [36]. *E. pollicina* also showed a high ferric
325 reducing capacity (840 μmol Fe (II) g^{-1} dry weight) which is comparable to the very
326 antioxidative *Cassia fistula* pod extract (811 μmol Fe (II) g^{-1} dry weight) [37]. In the
327 copper-phenathroline assay *E. pollicina* at a concentration of up to 10 g FW L^{-1} was not
328 able to stimulate DNA damage to the same extent as the reference 240 μM ascorbic acid.
329 The ability of plant phenolics to exhibit pro-oxidant properties is an undesirable effect of
330 these compounds [38-40]. Indeed several phenolic antioxidants could accelerate oxidative
331 damage to DNA, proteins and carbohydrates *in vitro* [38,39] and this can be counteracted
332 by the addition of albumin in the incubation medium [41]. DNA cleavage in copper-
333 phenanthroline system is due to localized generation of hydroxyl radicals on DNA with
334 damage predominantly directed to the bases [42]. In the context of the food matrix, DNA
335 damage caused by incorporated phenolic antixidants may not be significant in terms of food

336 stability, but it may of importance if constituents of these products are absorbed through the
337 gut [24,25,41]. However, adequate endogenous antioxidant defense may prevail.
338
339 *E. pollicina* inhibited beef liver microsomal peroxidation induced by the Fe^{3+}/ascorbate
340 with a low IC_{50} value of 0.012 mg FW ml^{-1}. Bahorun *et al* [43] compared the antioxidant
341 activities of extracts of *Crataegus monogyna* and reported IC_{50} values for flavanols (namely
342 catechins and oligomeric proanthocyanidins), (-) epicatechin and procyanidin B2 dimer that
343 are similar to the data presented here. Indeed the low IC_{50} value that was obtained for (-)-
344 epicatechin strongly suggests that its antioxidant capacity together with that of catechins
345 and procyanidin dimers were responsible for the protection against lipid peroxidation
346 observed. It is reassuring that there continues to be interest in characterization of the
347 antioxidant potency of plant extracts. Some brief examples are worthwhile. The aqueous
348 extracts of *Ocimum sanctum* had significant protection against lipid peroxidation with a
349 calculated IC_{50} of 0.08 mg ml^{-1} [44]. The extracts from *Evolvulus alsinoides*, *Sida*
350 *cordifolia* and *Cynodon dactylon* three Indian medicinal plants traditionally used for the
351 management of neurodegenerative diseases were equally protective with calculated IC_{50} of
352 0.089, 0.127 and 0.608 mg/ml respectively [45].
353
354 *E. pollicina* leaf extracts protected both unstripped soybean oil emulsions and unstripped
355 extra virgin olive oil emulsions against lipid autoxidation. Natural antioxidants exert
356 interfacial affinities between air-oil and oil-water interfaces that affect their activity in bulk
357 oil and emulsion systems [46,47]. For antioxidants to be effective in emulsion systems,
358 they need to be hydrophobic or less polar in nature such that they become oriented in the

359 oil-water interfaces protecting the oil phase from oxidation [47]. Hydrophilic antioxidants,
360 on the other hand, become diluted in emulsion systems and are less effective due to
361 dissolving in the aqueous phase [48]. Based on the above, the antioxidant activity of *E.*
362 *pollicina* leaf extracts in the emulsion systems can be ascribed to the pooled effect of the
363 relatively less polar polyphenols such as (-)-epicatechin and procyanidin dimers. A mixture
364 of catechin isomers present in tea extracts was also found by Roedig-Penman and Gordon
365 [49] to confer a very effective protection to unstripped sunflower oil emulsions during
366 storage at 30°C. Moreover, tea catechins were reported to reduce the formation of
367 peroxides more effectively than α-tocopherol or BHA in a β-carotene/linoleate model
368 system [50] and a fish muscle model system[51].
369
370 The two emulsion systems tested in this study showed a differential resistance to
371 autoxidation with olive oil emulsions being more resistant than soybean oil emulsions.
372 This difference in oxidation levels reached by the emulsions can be ascribed to the inherent
373 oxidative stability of the unstripped oils used, as depicted by the results obtained for the
374 control emulsions. The fatty acid composition plays an important role in the susceptibility
375 of oils to oxidation [52]. According to Gomez-Alonso *et al.* [53], the higher oxidative
376 stability of virgin olive oil, compared to that of other vegetable oils, is due to its high
377 monounsaturated oleic acid content whilst soybean oil which is composed of large amounts
378 of polyunsaturated fatty acids is more susceptible to oxidation [52]. Apart from the fatty
379 acid composition of the oils, both the quantitative and qualitative aspect of inherent natural
380 antioxidants present is an important factor that significantly influences the resistance of the
381 oils to oxidation. Soybean oil antioxidant activity can mainly be attributed to the presence

382 of high amounts of δ- and γ- tocopherols [53,54] both of which possess higher antioxidant
383 efficiencies as compared to α-tocopherol [55]. The stability of extra virgin olive oil, on the
384 other hand, is mostly due to its high concentration of polyphenols in the polar fraction of
385 the oil and partly to the high concentration of tocopherols of which more than 90% is α-
386 tocopherol [56].
387
388 Apart from the inherent antioxidants of extra virgin olive oil confering greater stability to
389 the olive oil emulsions, the total extract of *E. pollicina* exhibited greater antioxidant activity
390 in unstripped olive oil emulsions than in unstripped soybean oil emulsions. It is interesting
391 to note here that whilst there was no significant interaction between BHT and the oils, *E.*
392 *pollicina* exerted different antioxidant activity in the two oil systems tested indicating an
393 interaction between the leaf extract and the oils. Rice-Evans *et al.* [57] suggested that plant
394 extracts, when added to lipid-containing foods, can reduce the loss of α-tocopherol. The
395 existence of an antioxidant interaction between the flavonols, (-)-epicatechin and (+)-
396 catechin, and α-tocopherol in homogeneous solutions of peroxidating methyl linoleate was
397 also established by Pedrielli and Skibsted [13]. Moreover, a strong synergistic effect has
398 been reported between procyanidins from *Vitis vinifera* seeds and vitamin E in PUFA
399 protection in micellar systems [58]. It was proposed that plant phenolics could regenerate
400 α-tocopherol from tocopheryl free radical by donating an electron or a hydrogen atom [13,
401 58]. Thus, the positive interaction observed between the extract of *E. pollicina* and the oils
402 could be due to a synergism between the flavanol derivatives of the leaf extract and α-
403 tocopherol which is present in both extra virgin olive oil and soybean oil but at different
404 concentrations. Moreover, the flavanol derivatives of the extract could have acted

405 synergistically with the polar polyphenols found in extra virgin olive oil to provide greater
406 oxidative stability to the olive oil emulsions.
407
408 These results suggest that mature leaves of *Eugenia pollicina* are potential source of natural
409 antioxidant for application in food matrices. However, as with all non-food sources, the
410 potential toxicity of the extract need to be assessed and a safety level established before its
411 use as a food additive or functional food ingredient.
412

413 ## Acknowledgements

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Figure 1

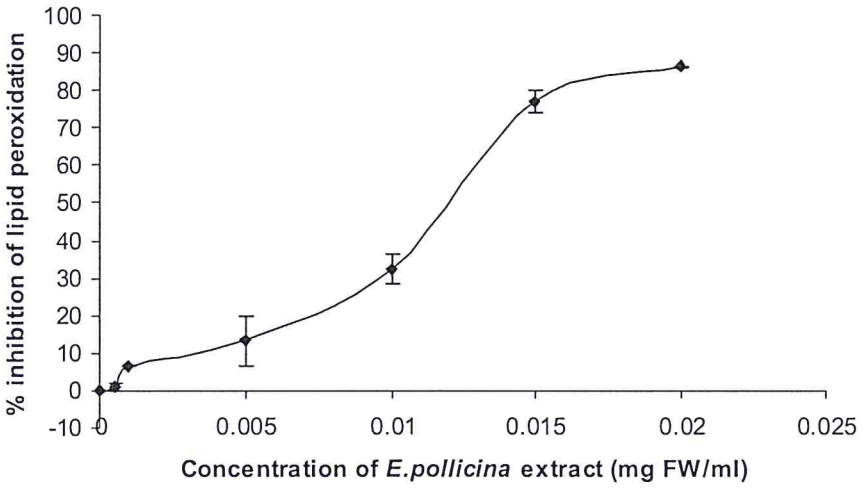


Fig. 1. Inhibition of Fe^{3+}/Ascorbate dependent microsome lipid peroxidation by *E.pollicina* leaf extract.

Figure 2

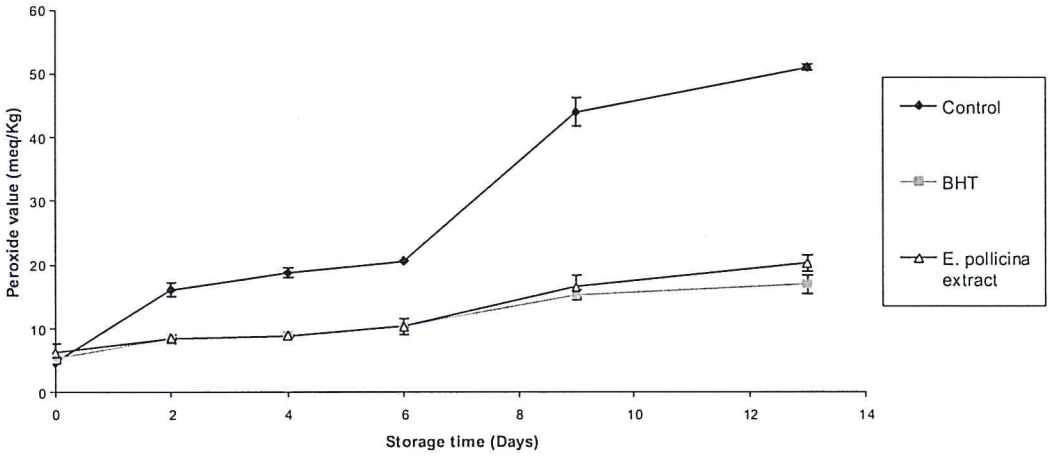


Fig. 2. Effect of a total extract of mature leaves of *Eugenia pollicina* and BHT on the oxidation of unstripped soybean oil emulsions stored at 40°C, assessed by the peroxide value (meq/kg). Control values are without antioxidant or plant extract. BHT and the leaf extract were tested at a concentration of 0.02% w/v.

Figure 3

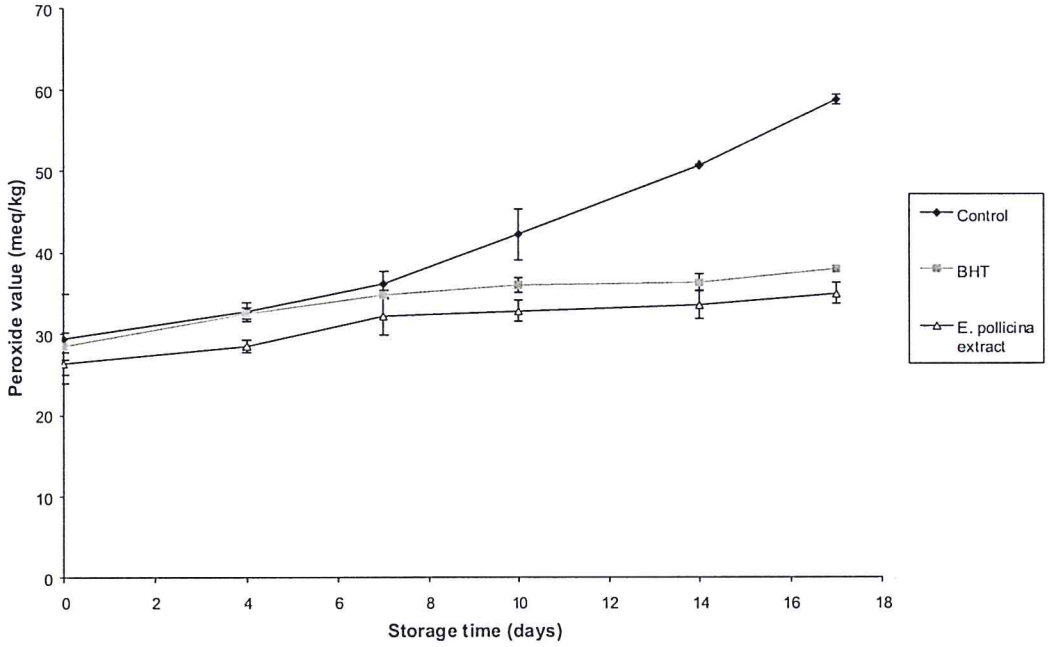


Fig. 3. Effect of a total extract of mature leaves of *Eugenia pollicina* and BHT on the oxidation of unstripped extra virgin olive oil emulsions stored at 40°C, assessed by the peroxide value (meq/kg). Control values are without antioxidant or plant extract. BHT and the total extract were tested at a concentration of 0.02% w/v.

Figure 4

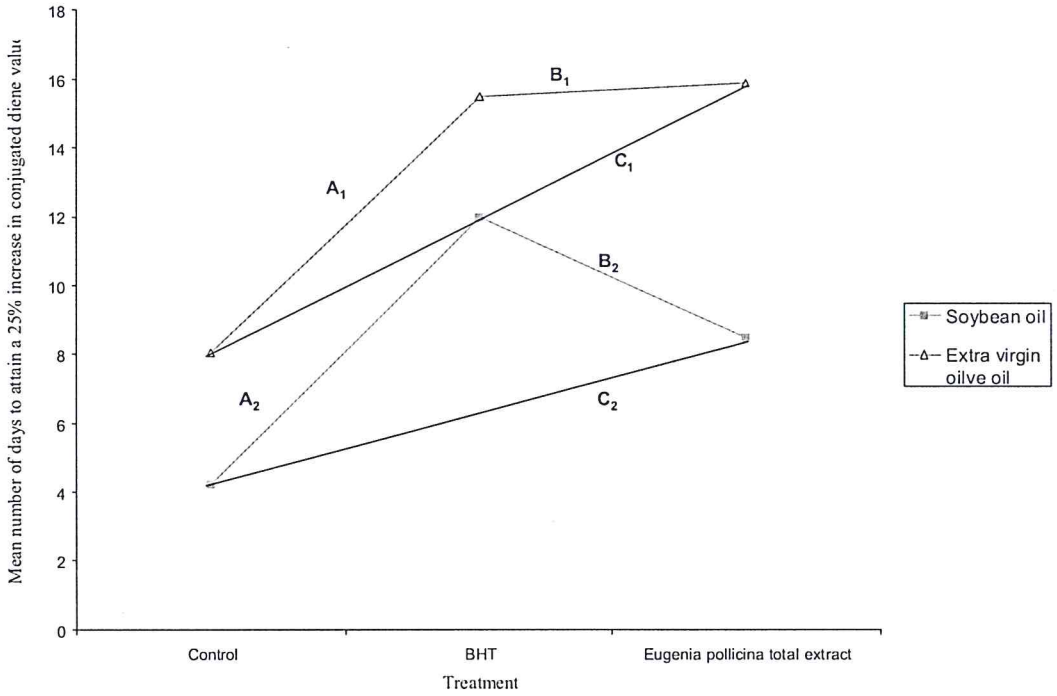


Fig. 4. Interaction graph showing relationships between oil samples and BHT/ leaf extract of *E. pollicina*. Control values are without antioxidant or plant extract. BHT and the total extract were tested at a concentration of 0.02% m/m.

Table 1

Table 1

The total phenol content, total proanthocyanidin and flavonoid content of the rude extract of *E.pollicina* and the quantitative analysis of catechins and its derivatives in the extracts.

	Amount detected(mg g^{-1} FW)[a]
Total phenol content	77 ± 0.78
Total proanthocyanidin content	12 ± 2.02
(+)-catechin	1.5 ± 0.01
(-)-epicatechin	3.6 ± 0.32
(-)-ECG	1.2 ± 0.01
(-)-EGCG	3.4 ± 0.02
Procyanidin B1	3.0 ± 0.04
Procyanidin B2	1.0 ± 0.01
Total Flavonoid content	12 ± 0.41
Quercetin (in hydrolysed extract)	2.6 ± 0.1
Kaempferol (in hydrolysed extract)	1.5 ± 0.07

[a] Values are mean ± standard deviation (n=3)

Table 2

Extent of DNA damage measured in the copper-phenanthroline assay

Substance	Ascorbic acid (240 μM)	*Ep*-0.1[a]	*Ep*-0.5[a]	*Ep*-1.0[a]	*Ep*-5.0[a]	*Ep*-10.0[a]
DNA damage (A_{522})	0.179	0.013	0.024	0.037	0.048	0.061

[a] Concentration of plant extract in g FW/L.

Table 3

Table 3. Antioxidant capacities of the reference compounds as measured by TEAC, FRAP and inhibition of lipid peroxidation

Phenolic compound	(+) C	(-) EC	(-) ECG	(-) EGCG	(-) EGC	B1	B2	Gallic acid
TEAC (mmol Trolox/L)	3.2 ± 0.21	3.6 ± 0.35	4.2 ± 0.16	4.4 ± 0.28	3.9 ± 0.09	6.6 ± 0.45	7.6 ± 0.21	3.6 ± 0.075
FRAP (mmol Fe^{2+}/L)	2.5 ± 0.12	2.9 ± 0.09	7.0 ± 0.35	8.0 ± 0.004	4.0 ± 0.21	11.9 ± 0.46	8.6 ± 0.31	5.3 ± 0.25
Inhibition of microsomal lipid peroxidation (IC_{50} μM)	39.14 ± 2.1	39.82 ± 3.3	-	32.31 ± 3.45	46.4 ± 1.89	14.2 ± 2.67	13.5 ± 1.03	39.3 ± 2.32

Values are expressed as mean ± standard deviation of triplicate measurements. (+) C: (+) Catechin; (-) EC: (+) Epicatechin; (-) ECG: (-) Epicatechin gallate; (-) EGCG: (-) Epigallocatechin gallate; (-) EGC: (-) Epigallocatechin; B1: Procyanidin B1; B2: Procyanidin B2.

Table 4

Table 4

The effect of a total extract of mature leaves of *Eugenia pollicina* and BHT on the oxidation of unstripped soybean oil emulsions during storage at 40°C, assessed by conjugated diene value (g/100g)

Sample	Control [b]	BHT[c]	*E. pollicina* extract [c]
Day 0	0.405 ± 0.0071	0.392 ± 0.0021	0.391 ± 0.0156
Day 2	0.442 ± 0.0028	0.438 ± 0.0057	0.429 ± 0.0177
Day 4	0.469 ± 0.0064	0.455 ± 0.0057	0.431 ± 0.0212
Day 6	0.515 ± 0.0078	0.460 ± 0.0085	0.433 ± 0.0219
Day 9	0.591 ± 0.0283	0.483 ± 0.0035	0.493 ± 0.0078
Day 13	0.850 ± 0.1195	0.516 ± 0.0064	0.567 ± 0.0028

Values are expressed as mean ± standard deviation for 4 measurements . [b] Control values are without antioxidant or plant extract. [c] BHT and the plant extract were tested at a concentration of 0.02% w/v.

Table 5

Table 5

The effect of a total extract of mature leaves of *Eugenia pollicina* and BHT on the oxidation of unstripped extra virgin olive oil emulsions during storage at 40°C, assessed by conjugated diene value (m/m)

Sample	Control [b]	BHT[c]	*E. pollicina* extract [c]
Day 0	0.405 ± 0.0071	0.392 ± 0.0021	0.391 ± 0.0156
Day 2	0.442 ± 0.0028	0.438 ± 0.0057	0.429 ± 0.0177
Day 4	0.469 ± 0.0064	0.455 ± 0.0057	0.431 ± 0.0212
Day 6	0.515 ± 0.0078	0.460 ± 0.0085	0.433 ± 0.0219
Day 9	0.591 ± 0.0283	0.483 ± 0.0035	0.493 ± 0.0078
Day 13	0.850 ± 0.1195	0.516 ± 0.0064	0.567 ± 0.0028

Values are expressed as mean ± standard deviation for 4 measurements . [b] Control values are without antioxidant or plant extract. [c] BHT and the plant extract were tested at a concentration of 0.02% w/v.

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Neuroprotection by bioactive components in medicinal and food plant extracts

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Abstract

Neurodegenerative diseases of the human brain comprise a variety of disorders that affect an increasing percentage of the population. Some of these are age dependent (e.g. Alzheimer's and Parkinson's diseases) and some are infection dependent, e.g. human immunodeficiency virus (HIV/AIDS). The vulnerable brain regions in HIV/AIDS individuals include the dentate nucleus in the cerebellum, the red nucleus, substantia nigra (SN) in the mid-brain, the subthalamic nucleus, thalamic fasciculus in the diencephalons, the globus pallidus and striatum (or neostriatum, which consists of caudate and putamen) in the forebrain. Lesion in these regions may lead to progressive dementia, which is similar to what is observed in Alzheimer's disease and Parkinson's disease. The entry of calcium into the cytoplasm of cells at concentrations that can activate oxidative enzymes such as phospholipase A_2 and xanthine oxidase, deplete cells of cysteine and glutathione, cause mitochondrial release of free radicals and cell death. Glutamate and its receptors are key molecular elements at the interface between neurons and glia. Dietary factors can modulate physiological functions (including brain function) thereby increasing the economic productivity of a population as a function of health. A greater understanding of the molecular mechanisms of neuroprotection, oxidative stress and immune function will facilitate definition of the prophylactic potentials of diet, nutritional/food supplements, medicinal plants and herbal extracts.
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Keywords: Neuroprotection; Flavonoids; Excitotoxicity; Medicinal plants; HIV/AIDS dementia; Stroke; Parkinson's disease; Alzheimer's disease

1. Introduction

Inflammation and oxidative stress are key components of the pathology of the chronic neurodegenerative conditions (in particular Alzheimer's, Down syndrome, multiple sclerosis, etc.) and in human immunodeficiency virus (HIV/AIDS) dementia but the molecular mechanisms of antioxidant actions in altering, reversing and/or halting the associated neuronal and behavioural deficits are yet to be fully elucidated. Indeed the World Health organisation (WHO) has initiated global initiatives on neurology and public health in an effort to increase public awareness of the prevalence, severity and cost of neurological disorders with a view to identify possibilities for prevention [1]. The report of Olden et al. [2] of the National Institutes Environmental and Health Sciences, USA, discusses the partnership for research in Parkinson's disease encompassing patients, researchers, clinicians

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and sponsors with a view to explore more mechanism based research to understand putative environmental triggers for the disease.

The human immunodeficiency virus invades the central nervous system early after infection where it later gives rise to cognitive, motor, and behavioural manifestations in children and adults [3–5]. The vulnerable brain regions in HIV/AIDS individuals include the dentate nucleus in the cerebellum, the red nucleus, substantia nigra (SN) in the mid-brain, the subthalamic nucleus, thalamic fasciculus in the diencephalons, the globus pallidus and striatum (or neostriatum, which consists of caudate and putamen) in the forebrain. Lesion in these regions leads to progressive dementia similar to what is observed for Parkinson's disease and Alzheimer's disease. Among individuals that have HIV dementia as the first AIDS-defining illness, a period of cognitive stability during the asymptomatic phase of HIV infection appear to be followed by a gradual and relatively modest decline in psychomotor performance. However, different patterns of cognitive decline are seen among those patients who developed dementia following other AIDS-defining illnesses [3]. The neuropathological substrate of HIV-related brain disorders is mainly represented by cerebral atrophy affecting various structures including the neocortex with a loss of up to 30% of the neocortical neurones in brains from AIDS patients, an effect that occurs mainly in the fronto-orbital cortex when compared with the parietal cortex. Although large neurones are reported to be more sensitive to HIV infection compared with small neurones, loss of large neurones appears to have no relationship with either the presence of clinical dementia or HIV encephalitis [3]. By contrast, the hippocampal formation of AIDS patients display marked morphological changes, in spite of the maintenance of their total number. These alterations probably lead to dysfunction of the hippocampal circuitries and, thus, might contribute to explaining the dementia features that occur under this condition. Other indirect effects may involve the release of neurotoxins from activated astrocytes and microglia. There may also be the direct effect of HIV-related proteins (e.g. glycoprotein 120 (gp120)) on neurones involving chemokine receptors [6]. The major types of cells targeted by HIV-1 following infection are the CD4-positive dendritic cells in the skin, mucosa, lymphocytes and resident macrophages in many tissues, particularly the brain (microglia). Macrophages and dendretic cells are infected first with the T-lymphocytes becoming the main targets later in the disease as increasingly virulent strains of HIV-1 emerge, accelerating the destruction of the immune system [5,7].

The precise characteristics of the neurological problems depend on several factors, which can include the severity of the disease, age of the patient, nutritional status, etc. The fundamental approach (Fig. 1) [8,9] advocates the functional relationship of diet, supplements, functional foods on health and disease management, needs to embrace the management of dementia and the attain restoration of immune functions. The defining objective will be to probe critically the bioefficacy and neuroefficacy of dietary constituents in foods/supplements.

2. Oxidative stress, flavonoids and chemokine network in neuroinflammation

The rate and selectivity of reactions involving free radicals depend on the concentrations of the species, delocalization of the single electron of the radical (thus increasing its life time), and on the absence of weak bonds in any other molecules present with which the radical could interact. Most biological molecules however, are non-radicals containing only paired electrons. Free radicals of importance in living organisms include hydroxyl ($OH^{\bullet}$), superoxide ($O_2^{\bullet -}$), nitric oxide ($NO^{\bullet}$), and peroxyl ($RO_2^{\bullet}$). Peroxynitrite ($ONOO^-$), hypochlorous acid (HOCl), hydrogen peroxide (H_2O_2), singlet oxygen $^1\Delta g$ (often written as $^1O_2$) and ozone (O_3) are not free radicals but can easily lead to free radical reactions in living organisms. The terms 'reactive oxygen species' (ROS) is often used to include both the radical and non-radical species [10,11]. Similarly, the terms reactive nitrogen species (RNS) refer to the nitrogen containing species. Oxidative stress is the term referring to the imbalance between generation of reactive oxygen species and the activity of the antioxidant defences. Severe oxidative stress can cause cell damage and death. The focus of this review is on the diseases of neurodegeneration and HIV/AIDS and the potential use of prophylactic dietary and nutritional antioxidants. The importance of oxidative stress injury is dependent on

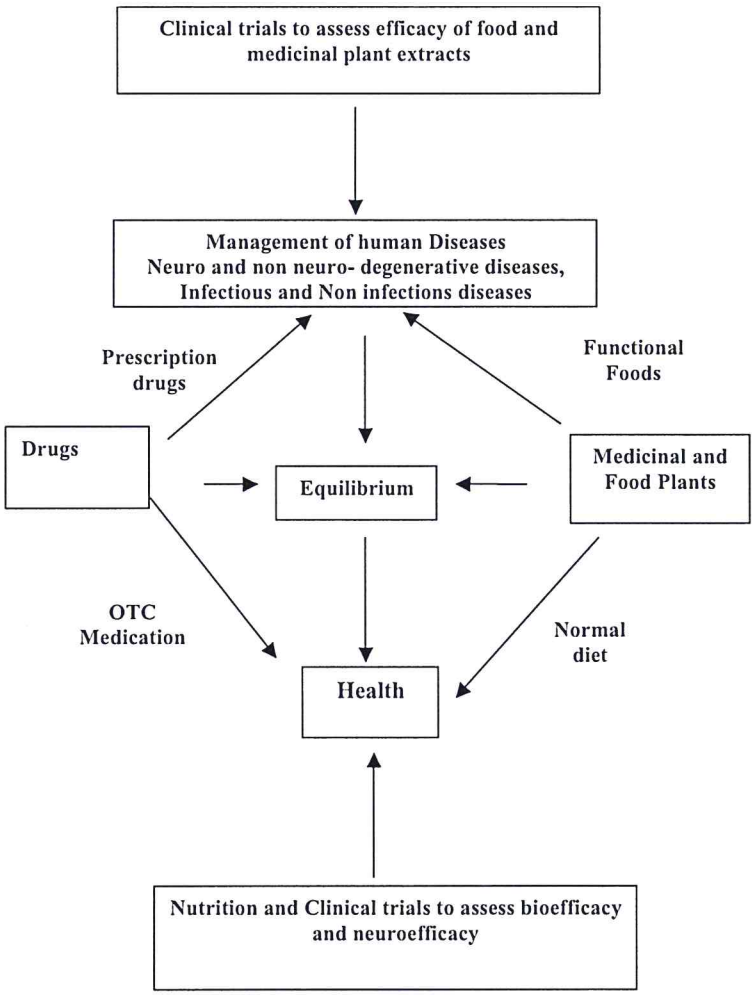


Fig. 1. A strategy to facilitate definition of the prophylactic potentials of diet, nutritional/food supplements and medicinal plants and herbal extracts. Research directed towards the understanding the role of plant/herbal extracts and dietary antioxidants in human health should be complemented with the development and validation of biological markers.

the molecular target, the severity of the stress and the mechanism by which the oxidative stress is imposed i.e., drug induced, Fenton chemistry, trauma, enzyme activation (e.g. nitric oxide synthase activity) and the cellular transduction mechanisms which may affect the expression of certain proteins including the DNA repair enzymes [12].

Several classes of dietary compounds have been suggested to reduce the risk of some cancers, especially those of the gut, and there is some evidence that consumption of certain foods leads to a reduction in biomarkers of oxidative damage. The active principles in extracts from a variety of plants and seeds from tropical and sub-tropical regions of the world continue to be of interest for use in complementary medicine supplements. In the main, most of the extracts contain important Vitamins, Vitamin E, Vitamin C, β-carotene, flavonoids and other polyphenols that become bio-available upon consumption [13–16].

Flavonoids are polyphenolic compounds and structurally have variations in the C ring that characterizes the different types namely, flavonols, flavones, isoflavones, flavonones, flavanol and anthocyanins. Some of the examples of the simple chemical structures in each of the classes of the groups are shown in Table 1. The antioxidant activities of these compounds are widely reported in the literature [17–20]. Many biological functions of these molecules such

Table 1
Chemical structures of flavonoids and some examples

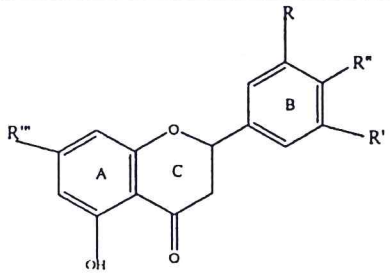
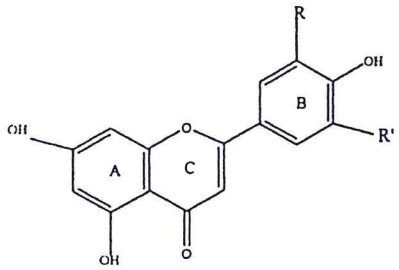
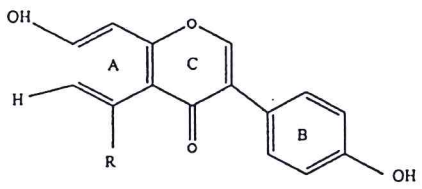
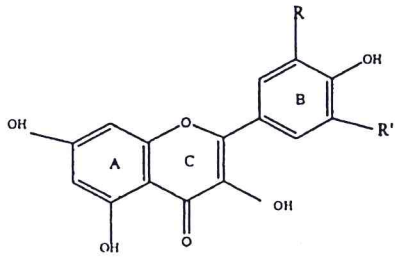
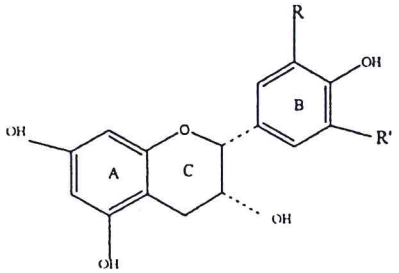
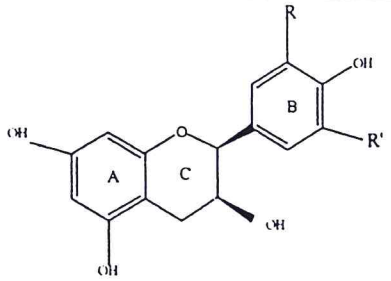
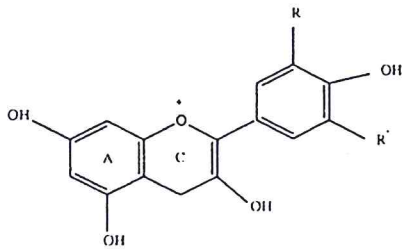
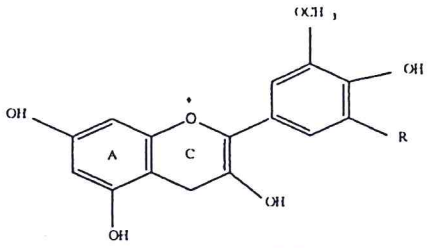
Classes	Structural formula	Examples
Flavanones		R=R^{I}=H, R^{II}=R^{III}=OH; Naringenin R=OH, R^{I}=H, R^{II}=R^{III}=OH; Eriodyctiol R=R^{I}=OH, R^{II}=R^{III}=OH 5^{I-}OH–Eriodyctiol
Flavones		R=R^{I}=H; Apigenin R=OH, R^{I}=H; Luteolin R=R^{I}=OCH_3; Tricetin
Isoflavones		R=OH; Genistein R=H; Daidzein
Flavonols		R=R^{I}=H; Kaempferol R=OH, R^{I}=H; Quercetin R=R^{I}=OH; Myricetin
Flavanols		R=OH, R^{I}= H; (−)–Epicatechin R=R^{I}=OH; (−)Epigallocatechin R=OH, R^{I}=H; (+)–Catechin R=R^{I}=OH; (+)–Gallocatechin

Table 1 (*Continued*)

Classes	Structural formula	Examples
		R=OH, R^1= H; (+)–Epicatechin R=R^1=OH; (+)Epigallocatechin R=OH, R^1=H; (−)–Catechin R=R^1=OH; (−)–Gallocatechin
Anthocyanidins		R=OH, R^1= H; Cyanidin R=R^1=OH; Deiphinidin R=R^1=OCH_3; Malvidin R=R^1=H; Pelargonidin
		R=H; Peonidin R=OH; Petunidin

as anti-mutagenic, anti-carcinogenic and anti-ageing amongst others, at least partially stems from their antioxidant and free radical scavenging activity [21–24]. Rosemary (*Rosmarinus officinalis* Linn) is a common household plant grown in many parts of the world and is used for flavouring food, as a beverage, in cosmetics as well as in many folk medicines for its choleretic, hepatoprotective and antitumourigenic activity. Rosemary extracts inhibits HIV infection in vitro [25] and are powerful lipid-soluble antioxidants [26], a function ascribed to the phenolic compounds carnosol and carnosic acid. There are no toxicity concerns for rosemary since the herb is widely consumed and adverse effects and/or side effects would be rare. The rosemary components are bio-available [27] and have been shown to alleviate carbon tetrachloride-induced acute hapatotoxocity [28], as well as preventing the free radical dependent damage to muscarinic acetylcholine receptor (mAChR). The mAChR just like the NMDA glutamate subtype receptor is involved in memory and learning [29]. There is current interest in our laboratory to evaluate the molecular mechanisms of the neuroprotective potentials of rosemary extracts and it bioactive components.

Several investigators have sought to establish the antiviral activities of flavonoids and the work of Mahmood et al. [30,32] have largely addressed the ability of flavonoids to inhibit specific antibody binding to gp120 have been studied by assessing their abilities to prevent binding of monoclonal antibody 358 [31]. Antibody 358 is known to neutralise virus infectivity by binding to the variable region V3 loop of gp120, thus compounds binding to this region may have anti-HIV properties [30]. The flavonoids (numbered 5, 23, 24, 25 and 34–38), inhibited binding of antibody 358 and they had no effect on the interaction of monoclonal antibodies 323 and 360 specific for the C- and N-terminal regions of gp120 (Table 2)

Table 2
Inhibition of antibody 358-binding to gp120, anti-HIV activity and cytotoxicity of flavonoids

Compound (The numbers identify compounds referred to in the text)[a]	Concentrations tested (μg/ml)	Inhibition of the binding of antibody 358 to gp120 (%)	*EC_{50}	*TC_{50}
1: Chrysin, 5,7-dihydroxy-flavone	50	0	20	50
5: Myricetin, 3,3′,4′,5,5′,7-hexahydroxy-flavone	10	90	2	40
	1	50		
7: Kaempferol, 3,4′,5,7-tetrahydroxy-flavone	100	0	4	70
11: Quercetin, 3,3′,4′,5,7-pentahydroxy-flavone	50	7	20	100
	5	0		
22: (−)Epigallocatechin	100	0	Inactive	>100
23: (−)Epicatechin, 3,3′,4′,5,7-penta-hydroxyflavan	50	96	2	>100
	5	44		
	0.5	17		
24: (+)Catechin	50	59	4	>100
	5	24		
25: (−)Epicatechin-3-O-gallate	10	98	1	>100
	2	78		
	0.4	11		
34: 3,5,7,8,4′-pentahydroxy-flavanone	50	73	40	>100
	10	73		
	2	66		
35: Taxifolin, 3,5,7,3′,4′-pentahydroxy-flavanone	16	86	50	100
	3.2	64		
	0.65	49		
36: Taxifolin-7-O-B-D-glucopyranoside**	250	81	100	350
	50	81		
	10	73		
37: Prunin-7-O-B-D-glucopyranoside**	80	77	40	>100
	16	40		
	3.2	21		
38: Coccinoside B, 5,7,2′,5′-tetrahydroxy-7-O-B-D-glucopyranosyl-flavanone**	80	85		>100
	16	80		
	3.2	61		

The respective numbers identified by name here are referred to in the text. *EC_{50}, concentration (μg/ml) which reduces by 50% the production of gp120 in infected C8166 cells. TC_{50}, concentration (μg/ml) which causes 50% cytotoxicity to uninfected C8166 cells. 'Inactive' indicates that EC_{50} and TC_{50} values are indistinguishable. AZT was included as positive control which showed high activity with EC_{50} of 0.01–0.03 μM and TC_{50} of 2–3 × 10^3 μM in C8166 cells. Compounds showing moderate activity against acute infection of C8166 cells were also tested in chronically infected H9 cells, which provides a more sensitive test of their effects on maturation of infectious virions. **7-O-B-D are the sugar substituted derivatives of the same compounds. The sources of the compounds in the data presented are chrysin (Sigma), myricitin (Sigma), kaempferol (*Rosa damascena*), quercetin (*Rosa damascena*), (−) epigallocathechin (*Croton draconoides*), (−)epicatechin, 3,3′,4′,5,7-penta hydroxyflavan (*Detarium microcarpum*), (+) catechin (*Detarium microcarpum*), (−)epicatechin-3-O-gallate (*Detarium microcarpum*), 3,5,7,8,4′-pentahydroxyflavanone (*Cuscuta reflexa*), Taxifolin, 3,5,7,3′,4′-pentahydroxy flavanone (*Cuscuta reflexa*), taxifolin-7-O-B-D-glucopyranoside (*Cuscuta reflexa*), prunin-7-O-B-D-glucopyranoside (*Cuscuta reflexa*), coccinoside B, 5,7,2′,5′-tetrahydroxy-7-O-B-D-glucopyranosyl flavanone (*Cuscuta reflexa*). As AZT has no effect on viral infectivity from chronically infected cells, protease inhibitor Ro-31-8959 was used as positive control (EC_{50}, 0.001 μg/ml and TC_{50} 10 μg/ml). The active compounds elicited a comparable effectiveness against HIV-2 and SIV-infections of C8166 cells. (Mahmood et al., 2000 with permission).

[a] The numbers identify compounds referred to in the text.

[31,32]. The flavones (numbered 5, 8, 10 and 11), flavan (numbered 25), flavanones (numbered 32, 33) inhibited gp120/CD4 interaction in a dose dependent manner, suggesting that they may act at an early stage of virus infection and inhibit virus binding or fusion with cells [32,33]. Flavans exhibited the greatest selective anti-HIV-1 activity (Table 2). The galloyl derivative, (−) epicatechin-3-O-gallate (numbered

25), consistently exhibited the greatest activity, EC_{50} of 1 μg/ml and selectivity index >100 which was somewhat more active than (−) epicatechin (numbered 23). There were differences between isomers, (+) catechin (numbered 24) was less active than (−) epicatechin, whereas (−) gallocatechin was inactive in contrast to (+) gallocatechin. Substitution of the hydroxyl group at position 7 of flavan 24 by a gallate group caused a reduction in anti-HIV activity [32].

The anti-HIV activity and cytotoxicity (Table 2) of flavanoids can be measured by incubating dilutions of compounds with C8166 (human T-lymphoblastoid cells) infected with HIV-1_{IIIB} or HIV-1_{MN} and H9 human T-cell lymphoma cells chronically infected with HIV-1_{IIIB}, for 5–6 days at 37 °C. The inhibition of infection can be monitored by examining syncytia formation, by measuring the cell viability using the XTT–Formazan method and by estimating viral antigen gp120 using ELISA or finally by titrating progeny virus released. The last two assays are most sensitive and are widely used for calculating EC_{50} values (Table 2) [32]. The small differences in the structure can influence their interaction with different viral proteins and their specificity and mode of action for inhibiting HIV-infection in vitro [32]. For example, a difference of one OH group in ring B is responsible for making kaempferol and chrysin specific for binding to protease and aromadendrin to gp120. An additional OH group at the 3′ position of ring B causes quercetin, epicatechin-3-O-gallate and taxifolin to be less specific in their interactions with HIV proteins. The sugar-substituted derivatives of flavanoids had little effect on enzymes but caused a significant reduction in gp120/CD4 interaction by binding to gp120. Since the mechanisms of action of some of the flavanoids are different from that of nucleoside analogues. The protease inhibitor kaempferol, was studied in combination with AZT and the results indicated that the inhibitory effect was additive [33].

3. Aids dementia as a form of excitotoxicity: potential management using herbal remedies and dietary factors

HIV-infected macrophages/microglia as well as macrophages activated by HIV-I envelope protein gp120 appear to secrete excitants/neurotoxins [3,6,38]. These substances may include arachidonic acid, platelet-activating factor, free radicals, glutamate, cysteine, cytokines (TNF, IL1-β, IL-6), and certain unidentified factors emanating from activated macrophages and possibly reactive astrocytes. The over-activation of *N*-methyl-D-aspartate (NMDA) receptor-operated channels results in excitoxicity causing excessive influx of Ca^{2+} which leads to neuronal injury. This pathway for neuronal susceptibility appears to be operative similar to that observed in stroke, trauma, epilepsy, and several neurodegenerative diseases, including Huntington's disease, amyotrophic lateral sclerosis, and neuropathic pain. The *N*-methyl-D-aspartate subtype of glutamate receptor, have many important role in normal and pathologic brain functions, including synaptic transmission, learning and memory as well as neuronal development.

The pathogenic mechanisms underlying AIDS dementia raises the possibility that several noxious substances (many of which are released by HIV-infected or activated macrophages, microglia, and astrocytes) may play a role in the generation of the clinical dementia syndrome. These toxins may include HIV-1 proteins (gp120 and gp41, which together constitute the entire envelope protein, gp160, or TAT, Nef, and possibly others) and substances released from activated macrophages and astrocytes (glutamate-like neurotoxic molecules, amines, free radicals, cytokines and eicosanoids). The mechanism underlying this indirect form of neuronal injury is related to excessive influx of Ca^{2+} in response to the noxious factors released from immune-activated, HIV-infected, or gp120-stimulated brain macrophages and microglia, which produce excessive stimulation of NMDA receptors (reviewed in [6]). Although gp120 and tat can induce directly neurotoxicity, nanomolar or micromolar amounts of protein are generally required in order to see these effects. However, picomolar or femptomolar amounts that can result in the indirect form of neurotoxicity involving excitotoxicity, that may be actually present in the brain [3,6]. Further, the induction of inflammatory mediators can be regulated by the activation of redox-sensitive transcription factors Activator Protein-1 (AP-1), (c-Fos/c-Jun) and nuclear factor-kappa B (NF-κB) stimulated in response to oxidants and TNF. TNF increases AP-1 binding via the MAP kinase (stress activated protein kinase/JNK) signalling pathway, and activates NF-κB

via the Iκ-B kinase pathway [34,35]. TNF also affects the local tissue oxidant/antioxidant balance and is important in mediating glutatamate toxicity. For example, the autocrine/paracrine TNF-α-dependent signalling leading to prostaglandin formation not only control glutamate release and astrocyte communication but also causes their derangement when activated microglia co-operates to dramatically enhance release of cytokines [36]. Indeed TNF-α is known to increase in both the brain and in the cerebrospinal fluids of parkinsonian patients [37]. As observed by Olden et al. [1], the presence of reactive microglia and associated cytokines have been observed in the substantia nigra in post-mortem samples obtained from Parkinson's disease patients. Moreover, viral infection and its resultant injury are thought to be a trigger for Parkinson's disease development in some cases. At present, it is unclear whether inflammatory processes reflect a primary etiologic event or a secondary response to neurodegeneration. Clearly, the role of inflammation in PD needs to be better understood. Here then is the complex nature of the scientific work that lies ahead, particularly in diseases with clearly defined neuroinflammation (e.g. Alzheimer's disease and HIV/AIDS dementia). L-Glutamate is the principal excitatory neurotransmitter in the central nervous system and glutamate receptor mediated excitotoxicity is known as a causative factor in a variety of neurological disorders. There are two classes of glutamate receptors [39]:

- Ionotrophic receptors. There are three types so defined on the basis of selective agonists namely *N*-methyl-D-aspartate, *a*-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid (AMPA) and kianic acid.
- Metabotrophic receptors.

Glutamate receptors (ionotropic and/or metabotropic) in normal neurotransmission is non-toxic but its prolonged activation and excitation leads to neurotoxicity. Research based on the molecular mechanisms of the neurotoxicity of the excitatory glutamate agonist *N*-methyl-D-aspartate could enable the understanding the complex mechanisms and facilitate development of neuroprotective agents based on dietary antioxidants. In this vein, use of animal models is beginning to enable the underpinning of the identification of potential dietary neuroprotectants [40–42].

Lee et al. [40] assessed the protective effects of vegetable extracts including red and white cabbage (*Brassica oleracecea var. capitata* L.), green and red mustard leaf (*Brassica japonica* L), parsley (*Sedum sarmentosum* L.) and rough aster (*Aster scaber* L.) on oxidative stress caused by NMDA in the brain of mice. Administration of some of the extracts modulated the NMDA-dependent decreases in the brain levels of glutathione, however, rough aster, parsely and red cabbage showed the greatest neuroprotective capacity [40].

The retinal-vitreal system is as in vivo model for studying neurotoxicity and possible therapeutic strategies for dietary antioxidants and micronutrients [41,42]. The mammalian retina itself is an integrated part of the central nervous system. It is peripherally located and therefore highly accessible experimentally. The retina has a biochemically and structurally well-defined glial and neuronal population within the CNS. The retinal system is in a closed environment making it advantageous for accurate assessment of the effectiveness of specific chemical compounds that are known to be neuroprotective or neurotoxic to glia cells or neurons. Free radicals are produced as a consequence of over-stimulation of the NMDA glutamate subtype receptor and the resulting oxidative damage. NO reacts with superoxide anion at a near diffusion-limited rate and the reaction product peroxynitrite is a potent oxidant that can nitrate tyrosine residues of structural proteins. Neurofilament, a protein that provides structural stability to neurones, is one of the target proteins of peroxynitrite and the resulting nitration results in disrupted neurofilament assembly and thus neuronal damage. Interestingly, ergothioneine absorbed from plant foods with mushroom as the best source is neuroprotective [41] (Fig. 2) and an excellent scavenger of peroxynitrite [43]. Thus the evaluation of tropical mushrooms as potential source of natural ergothioneine is suggested. Similar observation has been reported for the antioxidant beverage effective micro-organisms EM-X [41], a widely available health beverage in Asia. EM-X is derived from fermentation of unpolished rice, papaya and sea weeds with grouped effective micro-organisms of lactic acid bacteria, yeast and photosynthetic bacteria [44]. EM-X contains over 40 of minerals; antioxidants, such as flavonoids, kaempferol, panaxin, quercetin, lycopene, oryzanolum, ascorbic acid,

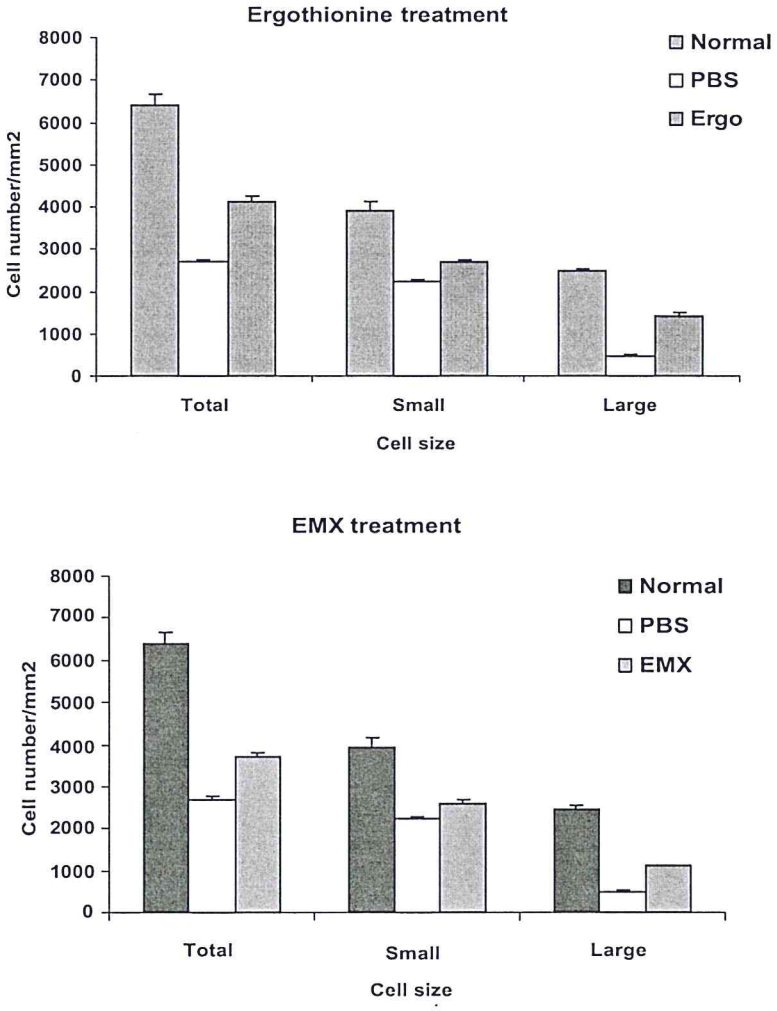


Fig. 2. The effect of NMDA treatment and its protection by ergothioneine. The neurons counted were divided into two groups with somata smaller than 6 μm, or equal to or larger than 6 μm in diameter. The great majority of neurons larger than 6 μm are retinal ganglion cells. Neurons with smaller somata are primarily non-ganglion cells or displaced amarcine cells. Closed bars show normal animals after NMDA injection, shaded bars show cell numbers after intravitreal injection of NMDA followed by PBS i.p., stripped bars show cell numbers after NMDA and ergothioneine or EM-X i.p. Note significant reduction in cell loss after treatment with ergothioneine. ($P < 0.001$ as compared to PBS in respect with the right hand bar for each group).

tocopherol and ubiquinone. The biological functions of EM-X includes enhancement of the activities of the human T- and B-lymphocytes and natural killer cell, anti-HIV activity [45], increases the serum superoxide dismutase, decreases the malondialdelhyde on the D-galactose-induced aging in mice and prolongs the life of fruit flies [46], and has antihyperglycemic effects on the animals [47]. EM-X inhibits oxidant-induced interleukin-8 release and the peroxidation of phospholipids [48] and modulates oxidative damage in the kidney and liver of rats by protecting unsaturated fatty acids [49].

4. Neuroprotective potentials of dietary antioxidants and herbal extracts

One of the vulnerable brain regions in HIV/AIDS individuals is the substantia nigra in the mid-brain [3–5]. The pathological hallmarks of idiopathic PD

are very consistent, i.e., specific loss of dopaminergic neurones connecting the substantia nigra to the striatum [1,2,50]. Activation of these neurons control movement and loss of the neurones results in bradykinesia, rigidity and resting tremor. The evidence for the loss of dopamine (DA) in PD comes from the reported decrease in the number of cell bodies in the SN, decreases in the dopamine content, number of DA-transporter sites and a decrease in vesicular monoamine transporters in the striatum. The mechanism of neuronal cell death in PD may be associated with increased oxidative stress and evidence for this has come mainly from post-mortem studies on the PD brain indicating increased levels of lipid, DNA and protein oxidation products [50].

Oxidative stress in PD is also associated with increased neuronal iron or decreased antioxidant (e.g. glutathione) capacity. The concentrations of GSH and other sulfhydryl compounds are decreased in the blood, liver and CNS of HIV-infected patients [51]

Table 3
Typical examples of phytochemical composition of medicinal and food plants that are endemic plants (from refs. [60,61])

Plant names (Families)	Phytochemicals present	Traditional uses
Psiadia sp. (Asteraceae)	Phenols, flavonoids, saponins, tannins	Pulmonary infections, asthma, fever, coughs
Canarium paniculatum (Burseraceae)	Phenols, anthocyanins, tannins, terpenes, coumarins	Rheumatism, skin ulcerations
Senecio ambavilla (Asteraceae)	Phenols, flavonoids, tannins	Rheumatism, gout, urinary infections, renal infections
Grangeria borbonica (Chrysobalanaceae)	Phenols, flavonoids, tannins	Stomach pains, asthma
Cnestis glabra (Connaraceae)	Flavonoids, tannins, proanthocyanidins, saponins	Fever
Erythroxylum hypericifolium	Flavonoids, saponins, tannins	Fever, renal stones, throat infections
E. laurifolium	Phenols, flavonoids, saponins, tannins	Fever, renal stones, throat infections
E. sideroxyloides (Erythroxylaceae)	Phenols, flavonoids, saponins, tannins, triterpenes, alkaloids	Fever, renal stones, throat infections
Antidesma madagascariensis	Phenols, flavonoids, proanthocyanidins, saponins, tannins	Fever, astringent, diabetese, dysentery, jaundice
Phyllanthus casticum	Phenol, flavonoids, proanthocyanidins, tannins, saponins	Fever, diarrhoea, dysentery, urino-genital infections
P. phyllireifolius (Euphorbiaceae)	Phenols, flavonoids, proanthocyanidins, tannins	Colics, diuretic, emmenagogue, cystitis
Aphloia theiformis (Flacourtiaceae)	Phenols, flavonoids, proanthocyanidins, saponins, tannins	Dysentery, fever, rheumatism, gastro-intestinal infections, jaundice
Foetidia mauritiana (Lecythidaceae)	Alkaloids, proanthocyanidins, saponins, tannins	Laxative, emmenagogue, purgative, diuretic
Leea guinensis (Leeaceae)	Phenols, flavonoids, proanthocyanidins, tannins	Oedemas, antiseptic, colds
Turraea casimiriana (Meliaceae)	Phenols, flavonoids, proanthocyanidins, saponins, tannins	Boils, hypotensive, emmenagogue
Tambourissa sp. (Monimiaceae)	Flavonoids, proanthocyanidins, saponins, tannins	Dermatitis, emmenagogue
Ficus reflexa (Moraceae)	Phenols, proanthocyanidins	Throat infections
Embelia angustifolia (Myrsinaceae)	Alkaloids, phenols, flavonoids, saponins	Liver complaints, dysentery, urinary tract infections
Scutia myrtina (Rhamnaceae)	Phenols, flavonoids, proanthocyanidins, saponins, tannins	Diarrhoea, dysentery, astringent, antidote for toxic fish poisoning
Chassalia coriaceae (Rubiaceae)	Anthocyanin heterosides, saponins, triterpenes, iridoids flavonoids	Astringent
Dodonaea viscosa	Flavonoids, saponins, tannins, proanthocyanidins	Rheumatism, gout, throat infections, contusions
Doratoxylon apetalum	Flavonoids, saponins, tannins, proanthocyanidins	Venereal diseases, skin infections
Molinae alternifolia (Sapindaceae)	Phenols, flavonoids, flavones, proanthocyanidins, saponins, tannins	Dysentery, throat infections
Mimusops maxima (Sapotaceae)	Phenols, flavonoids, flavones, proanthocyanidins, tannins	Diarrhoea, dysentery, astringent
Premna corymbosa (Verbenaceae)	Alkaloids, saponins, flavonoids, phenols	Coughs, influenza

and such a low GSH content is associated with poor survival in HIV-infected patients. The decreased concentration of GSH is involved in various disorders such as inflammation, apoptosis of CD4+ T-lymphocytes and neural cells, and a decrease in the detoxification of antiretroviral molecules. The neurotoxic effects of human HIV-1 transcription factor TAT has been shown to require a polyamine sensitive-site on the NMDA receptor [52], again suggesting that prophylactic agents able to modulate excitotoxic reactions will be beneficial [39–41].

The work of Levites et al. [53] described the attenuation of 6-hydroxydopamine (6-OHDA)-induced NFκB activation of and cell death by green and black tea extracts on human neuroblastoma SH-SY5Y cells and suggested that green tea inhibited both NFκB nuclear translocation and binding activity of NFκB by 6-OHDA. The neuroprotection was attributed to the antioxidant and iron chelating actions of the polyphenolic constituents of the tea extracts. Eleveated catalytic metal ions particularly copper ions have been reported in the cerebellum, median putamen and substantia nigra of PD brains [54]. Normal PD values of 21±10:29±7 nmol/g tissue (cerebellum), 21±16:56± 30 nmol/g tissue (median putamen) and 53 ± 48:63 ± 48 nmol/g tissue (substantia nigra) based on the assessment of 20–30 mg of post-mortem human brain tissues from the various regions were reported. Severe oxidative stress may not completely deplete systemic GSH in vivo, and therefore other mechanisms could play a part [55]. GSH biosynthesis might be compromised by several HIV components via modulation of key enzymes involved in the GSH cycle [3]. In addition, chronic inflammation of the CNS, together with activation of microglia, which is commonly observed in HIV-infected individuals, might be associated with abnormalities in GSH content, depending on GSH concentration, GSH demand and the generation of ROS and NO. Such deleterious effects could be mediated mainly by the nuclear transcription factor-κB (NF-κB), the expression of which represents a key event in the regulation of the balance between survival and death in CNS cells. The interrelationships of the status of endogenous thiols and the dietary antioxidant/herbal extracts are critical to unravelling the complex mechanisms involved. Dietary components can meaningfully contribute towards the management of the biochemical, physiological and behavioural features of PD, Alzheimer's diseases and HIV/AIDS [9,16,23,56,57]. Much scientific work remains to be done in this area and in particular to understand the link between genetics, oxidative stress and neuronal degeneration [9,57–59] and use of traditional herbal medicine (Table 3), including oriental herbal medicine and food plants.

5. Conclusion

It is clear that dietary antioxidants/herbal extracts can significantly contribute to the modulation of the complex mechanisms of neurodegenerative diseases. Given their potential contribution to immune modulation, use of traditional medicine and food plant extracts in the management of the neurological disorders and infectious diseases is of great interest. Understanding the molecular mechanisms of neuroprotection, oxidative stress and immune function will facilitate definition of prophylactic potentials. Research needs to proceed in parallel with efforts to demonstrate clinical efficacy of the secondary metabolites in traditional medicine and food plants.

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Antioxidant and Neuroprotective Potentials

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I. RNS/ROS AND OXIDATIVE STRESS

Free radicals are formed from molecules by bond breaking such that each fragment keeps one electron (free radicals may also be formed by collision of the nonradical species by a reaction between a radical and a molecule, which must then result in a radical, as the total number of electrons is odd), by cleavage of a radical to give another radical and by oxidation or reduction reactions. Aerobic organisms, including man, animals, and plants, are constantly challenged by reactive nitrogen species (RNS) and reactive oxygen species (ROS). These are either synthesized endogenously, e.g., in energy

metabolism and by the immune defense system of the body, or produced as reactions to exogenous exposures such as cigarette smoking, imbalanced diet, exhaustive exercise, environmental pollutants, and food contaminants (1–3). Reactive nitrogen species is a collective term that includes nitric oxide ($NO^{\bullet}$) and nitrogen dioxide ($NO_2^{\bullet}$) radicals, as well as nonradicals such as peroxynitrite ($OONO^-$), nitrous acid (HNO_2), and dinitrogen trioxide (N_2O_3). Reactive oxygen species is also a collective term that includes both oxygen radicals, e.g., peroxyl ($ROO^{\bullet}$), superoxide ($O_2^{\bullet-}$), and hydroxyl ($OH^{\bullet}$) radicals and the nonradicals, e.g., singlet oxygen ($^1O_2$), hypochlorous acid (HOCl), and hydrogen peroxide (H_2O_2). Intricate and diverse defense systems exist in vivo (2–5). For example, antioxidant protection is afforded by the antioxidant enzymes such as superoxide dismutases, catalase, and glutathione peroxidase, as well as by low molecular weight antioxidants such as glutathione, NADH, carnosine, uric acid, melatonin, α-lipoic acid, bilirubin, some of them are endogenously produced and some are provided through dietary intake (ascorbic acid, tocopherols, ergothioneine, carotenoids, quinones, phenolics). Links continue to be made between oxidative stress and increased risk of chronic diseases such as cancer, cardiovascular diseases, eye diseases such as cataract, and age-related macular degeneration, immune and neurodegenerative disorders, as well as for the general ageing process (2,6,7).

II. NEURODEGENERATIVE DISORDERS

Approximately 15% of the population over the age of 65 are afflicted with Alzheimer's disease (AD) (8) and 1% by Parkinson's disease (PD) (9). Neurodegenerative disorders are associated with various degrees of behavioral impairments that significantly decrease the quality of life (10). The brain may be particularly vulnerable to ROS, in part due to the reasons in what follows.

i. The brain consumes ~20% of the total body oxygen but comprises <2% of total body weight.

ii. The brain contains high levels of unsaturated fatty acids.
iii. The brain may have reduced endogenous antioxidants.
iv. The brain has limited capacity for regeneration.
v. Iron accumulates in brain-specific regions (i.e., red nucleus, substantia nigra pars reticularis, globus pallidus). Iron-binding proteins (ferritin) may be relatively deficient in the brain (11), so that the high concentrations of ascorbic acid may present a pro-oxidant environment.

Oxidative stress has been implicated in the pathogenesis of neurodegenerative disorders, such as cerebral ischemia/reperfusion injury and trauma (12), as well as chronic conditions such as amyotrophic lateral sclerosis (ALS) and PD (6,13–16). That ROS and RNS are implicated (17) has given impetus to the research focus directed at medicinal and food plants (18–20).

III. ALZHEIMER'S DISEASE

The disease AD is a progressive degenerative brain disease and is the commonest cause of adult onset dementia. There are deficits in cognitive function that cause amnesia (loss of memory), aphasia (impairment of language), apraxia (inability to do motor tasks despite intact motor function), and agnosia (inability to recognize, despite intact sensory functions). Psychiatric symptoms and behavioral disturbances, such as depression, personality change, delusions, hallucinations, and misidentifications, become apparent. Early manifestation of the disease include difficulties with daily living activities such as handling money, using telephone, driving, and later, difficulties with dressing, feeding, and toileting. Patients eventually lose interest in their surroundings and become confined to wheelchair or bed. The final stages of the disease, marked by mental emptiness and loss of control of all body functions, may not occur until 5–10 years after onset.

The major microscopic alterations in AD are senile plaques (SP) and neurofibrillary tangles (NFT) formation, selective neuronal loss and shrinkage, neuropil thread formation, synapse loss, and amyloid angiopathy. The NFT and SP represent an accumulation of intraneuronal and extracellular filamentous protein aggregates, respectively. Hyperphosphorylated tau is the major protein in NFT. Amyloid β peptide, derived from the amyloid precursor protein (APP), is the major protein in SP and amyloid angiopathy. The AD brain is also characterized by neuronal cell loss and changes in neuronal morphology. This is reflected by a decreased brain weight and by atrophy of the cortex. Neuronal loss is most notable in the hippocampus, frontal, parietal, and anterior temporal cortices, amygdala, and the olfactory system (21). Neuronal cell loss occurs in the nucleus basalis, a large cholinergic system at the base of the forebrain, and this may account for the severe cholinergic deficiency in the cortex of AD patients (22). The cell loss that is likely in the locus ceruleus may also account for the reduction in brain level of norepinephrine AD patients. In the hippocampus, the most prominent zones that are affected are the CA1 region, the subiculum, and the entorhinal cortex. The entorhinal cortex receives major innervation from the neocortex, basal forebrain, and amygdala. The large neurons of the entorhinal cortex (layer II), which project to the subiculum and the dentate gyrus by means of the perforant pathway, are prominent sites of tangle formation in AD. The major hippocampal output—to mammillary bodies, hypothalamus, and dorsomedial thalamus—arises from axons of the pyramidal cells that exit the hippocampus via the fornix. Thus, severe pathology occurs in those neuronal populations that receive input to the hippocampus or provide hippocampal efferents. The limbic system, including the olfactory bulbs, olfactory cortices, amygdala, cingulate gyrus, and hypothalamus, may also be affected, and this may explain some of the abnormal behavioral characteristics of some AD patients. The vulnerable brain regions in HIV/AIDS individuals include the denta nucleus in the cerebellum, the red nucleus and substantia nigra in the midbrain, the subthalamic nucleus and thalamic fasciculus in the diencephalons, and the globus pallidus and striatum (or neostriatum, which consists of caudate and putamen) in the forebrain. It is easy to see why lesions in these regions may lead to progressive dementia, which is similar to what is observed in AD and PD (18).

III.A. Risk Factors for AD

Although AD is generally thought to be a multineurotransmitter deficiency disease, there seems to be a genetic basis for its development. At least three different genes have thus been far associated with early-onset (<60 years of age) forms of the disease and have a direct causal effect: the APP gene located on chromosome 21 (23) and presenilin genes 1 (S 182) (24) and 2 (E5-1) (25) located on chromosomes 14 and 1, respectively. The apolipoprotein E-4 (Apo E-4) gene located on chromosome 19, the α_2-macroglobulin gene located on chromosome 12, and other unidentified genes may determine susceptibility in late-onset forms and sporadic cases (26,27). Risk of AD increases and the age of onset decreases with the number of Apo E-4 allele. Up to 90% of individuals homozygous for Apo E-4 have a chance of developing AD by the age of 80. However, the Apo E-2 genotype appears to be somewhat protective for AD (28). An increasing number of factors, including hypertension, smoking, alcoholism, low level of education, diabetes mellitus, migraine, high LDL cholesterol, head injury, diet, and depression, are associated with the development of AD (29–31) and oxidative stress (18,32). The salient neuropathological features include primary involvement (with atrophy) of neocortical association areas, the hippo campus and entorhinal cortex, amygdala, and nucleus basalis of Meynert, accompanied by more variable involvement of the medial nucleus of the thalamus, dorsal tegmentum, locus ceruleus, paramedian reticular areas, and lateral hypothalamic nuclei (21). By definition, involved regions must contain numerous neuritic plaques (focal structures consisting of an amyloid β protein core surrounded by degenerating nerve terminals) and NFT (neuronal cell bodies filled with paired helical filaments consisting mainly of tau protein) (33,34). These neuropathologic hallmarks typically appear first in the hippocampal CA1 field and subiculum,

with increasing involvement of the association cortex, the basal nucleus, and other structures, as the disease progresses (21). The most striking neurochemical alteration in the cerebral cortex is the loss of the cholinergic markers, choline acetyltransferase, and acetylcholinesterase (35,36). In addition, there are deficits in serotonin (37), noradrenaline (22), somatostatin (38), and corticotrophin-releasing factor (39). Although several hypotheses have been offered to account for the etiology of AD, oxidative stress play a crucial role in the disease pathogenesis as is evident from the following discussions (14,18,19,40–48). These include increased brain iron, aluminum and mercury, all of which are capable of stimulating ROS generation. There are lipid peroxidation and decreased polyunsaturated fatty acids in AD brain, increased 4-hydroxynonenals in AD ventricular fluid (40–42), increased protein carbonyl content in the inferior parietal lobule, increased levels of both nuclear and mitochondrial 8OH-dG, which are particularly striking in the parietal cortex (43), increased DNA strand breaks in cerebral cortex, diminished energy metabolism and cytochrome *c* oxidase activity in the AD brain, advanced glycation end products (AGE), malondialdehyde (MDA), carbonyls, peroxynitrite, heme oxygenase-1 and SOD-1 in NFT; AGE, heme oxygenase-1, SOD-1 in sp, and amyloid β peptide, which is capable of generating ROS and its mediation of neuronal degeneration and death (14,18,19,44–48).

IV. ANTIOXIDANTS AS PROPHYLACTIC AGENTS IN THE MANAGEMENT OF AD

There is no set treatment for the reversing or halting neuronal degeneration in AD. It is worth pointing out that the FDA approved the use of cholinesterase inhibitors (anticholinesterase drugs in clinical practice—donepezil, rivastigmine, and galantamine) (49), which have demonstrated limited palliative value. There is interest in the use of antioxidants (e.g., phenolics) as potential therapeutic strategy. Markers of oxidative stress represent an early indicator of oxidative stress

in AD susceptible neurons, often appearing before the detection of any other pathology; antioxidant therapies are thus a promising avenue for treatment.

The administration over a 2-year period of the trivalent iron-chelating agent, desferrioxamine, slows the clinical development of AD (50). The therapeutic importance is directed to the removal of iron and possibly inhibition of ROS formation. The beneficial effects of vitamin E (α-tocopherol), selegiline (MAO B inhibitor), and *Ginkgo biloba* (Egb 761) have been suggested. For example, Sano et al. (51) found that effects of α-tocopherol and selegiline in 324 patients with moderately severe AD showed no significant improvement on cognitive tests, but they did observe significant delays in the time of the following occurrences: death, institutionalization, loss of the ability to perform basic activities of daily living, and severe dementia. Several studies (18,19,45,47,52,53) involving neuronal cultures have shown that vitamin E inhibits Aβ-induced lipid peroxidation, protein oxidation, free radical formation, and cell death.

Human lacks the enzyme L-gulono-γ-lactone oxidase that is necessary for the biosynthesis of vitamin C, and therefore must obtain ascorbate from dietary sources. Ascorbate is a water-soluble antioxidant present primarily as a monovalent anion at physiological pH. Ascorbate soluble in the aqueous phase can reduce the tocopheryl radical formed when vitamin E scavenges a lipid radical within the membrane (54). Plasma ascorbate levels have been found to be decreased in AD patients when compared with control patients, in levels corresponding to dementia (13,55,56). More interestingly, CSF levels of ascorbate were found to be decreased in AD patients when compared with control subjects that may hinder the reduction of tocopheryl radical back to tocopherol (57). The synergistic vitamins C and E were chosen in a study in which 400 IU vitamin E and 1000 mg vitamin C were given daily to patients (58). The combination of vitamins E and C increased vitamins E and C levels in the plasma and CSF, making CSF and plasma lipoproteins less susceptible to in vitro oxidation. The plasma and CSF of patients given only vitamin E were not protected against in vitro oxidation. This study highlights

the concept of synergism between antioxidants, in this particular case between vitamins C and E. Aruoma has advocated that bioactive components in plant food could possess that are complementary in a synergistic manner.

α-Lipoic acid (LA) is a low molecular weight dithiol antioxidant that is an important cofactor in multienzyme complexes in the mitochondria. α-Lipoic acid is readily available from the diet, absorbed through the gut, and easily passes through the blood–brain barrier. In addition, LA is synthesized in the mitochondria of plants (59). As an antioxidant, LA and its reduced form, dihydrolipoic acid, are capable of quenching ROS and RNS such as hydroxyl radicals, peroxyl radicals, superoxide, hypochlorous acid, and peroxynitirite and chelating metals such as Cd^{2+}, Fe^{3+}, Cu^{2+}, and Zn^{2+} (60,61). α-Lipoic acid has been suggested to interact with other antioxidants such as glutathione, ubiquinol, thioredoxin, vitamin C, and indirectly with vitamin E, regenerating them to their reduced forms (62). Studies are beginning to show that LA improves behavior and diminishes markers of soxidative stress in rats that fed a diet supplemented with LA (63,64).

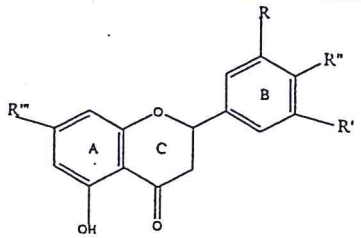
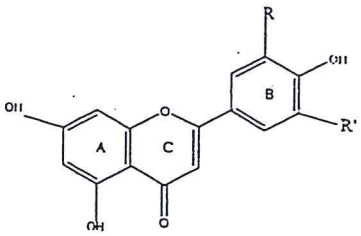
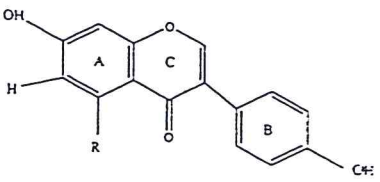
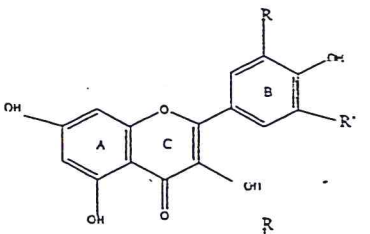
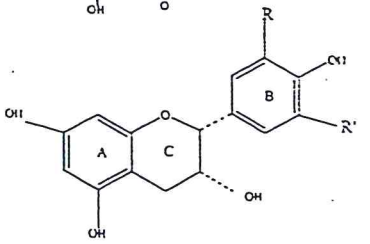
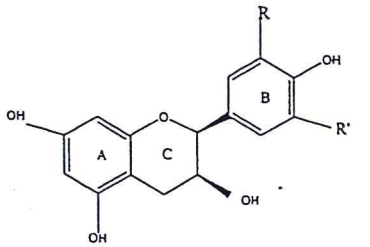
IV.A. Phenolics in the Treatment of AD

The potential neuroprotective effects of phenolics against the neuronal deficits associated with aging or age-related neurodegenerative diseases are of increasing interest (18,65). Cellular studies (66), examining the potential mechanisms of neuroprotection by flavonoids in preventing neuronal cell death caused by oxidized low-density lipoprotein-induced oxidative stress, have identified three different mechanisms: flavonoids can prevent cell death after glutamate injury by scavenging ROS, maintaining the correct GSH levels and inhibiting Ca^{2+} influx, which represents the last step in cell death cascade. These properties, coupled with the anti-inflammatory properties attributed to some phenolics (67), render this class of compounds suitable for application where oxidative stress, together with inflammation and antioxidant defense depletion, takes place, such as AD.

Modern scientific studies on the biological activity of extracts from dried *Ginkgo biloba* leaves started 20 years ago, even though the beneficial effects of these natural substances were known for 5000 years in traditional Chinese medicine. The ginkgo extracts that are currently used for medicinal purposes contain 24% flavonoids and 6% terpenoids. The antioxidant effects of flavonoids combined with the anti-inflammatory properties of the terpenoids bilobalide and ginkgolides A, B, C, M, and J, terpenoid antagonists of platelet-activating factor, make these natural extracts plausible to use in AD, characterized by both oxidative damage (15,47) and inflammation (68). In ROS-exposed mice (69), apoptosis was significantly reduced after pretreatment with ginkgo extracts. In several cell lines, treatment with ginkgo led to increased endogenous glutathione (70). Extensive studies on ginkgo extracts showed their ability to protect brain neurons from oxidative stress (71), to inhibit apoptosis in cell culture (72), and to rescue PC12 neuronal cells from Aβ-induced cell death (73).

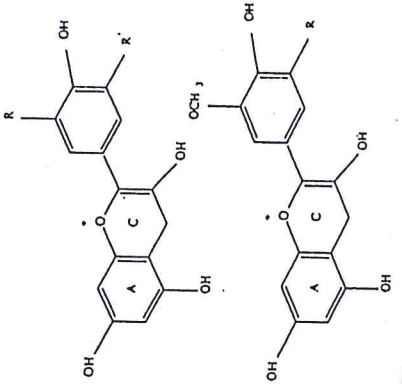
Tea is widely advocated as beneficial prophylactic agents from the standpoint that antioxidant phenolics are highly abundant in tea leaves. The main flavonoids present in green tea are catechins, in particular epigallocatechin gallate (EGCG), in the amount of 30–130 mg per cup of tea. Other phenolic compounds, such as quercetin, kaempferol, and myricetin and their glycosides, are found in lower concentration Table 1 shows some representative structures. The different properties exhibited by these compounds have been tested by a variety of studies in cell lines. Tea catechins have been shown to possess anticarcinogenic (69), antiallergic (70), and antiapoptotic properties. In hippocampal neurons, tea phenolics show a protective effect against ischemic insult (74), whereas neurotoxicity induced by Aβ (1–42), whose deposition in the brain accompanies neuronal loss in AD, was attenuated in the presence of EGCG (75). The protective antioxidant effect of these natural compounds was also confirmed by other studies in synaptosomes (76). Furthermore, studies involving tea phenolics found that intracisternal injection of epicatechin improved memory impairment induced by intracisternal glucose oxidase.

Table 1 Classes and Structural Formula of Flavonoids

Classes	Structural formula	Examples
Flavanones		R = R^1 = H, R^{11} = R^{111} = OH; Naringenin R = OH, R^1 = H, R^{11} = R^{111} = OH; Eriodyctiol R = R^1 = OH, R^{11} = R^{111} = OH; 5^1-OH-Eriodyctiol
Flavones		R = R^1 = H; Apigenin R = OH, R^1 = H; Luteolin R = R = R^1 = OCH_3; Tricetin
Isoflavones		R = H; Daidzein R = OH; Genistein
Flavonols		R = R^1 = H; Kaempferol R = OH, R^1 = H; Quercetin R = R^1 = OH; Myricetin
Flavonols		R = OH, R^1 = H; (–)-Epicatechin R = R^1 = OH; (–)-Epigallocatechin R = OH, R^1 = H; (+)-Catechin R = R^1 = OH; (+)-Gallocatechin
Flavonols		R = OH, R^1 = H; (+)-Epicatechin R = R^1 = OH; (+)-Epigallocatechin R = OH, R^1 = H; (–)-Catechin R = R^1 = OH; (–)-Gallocatechin

(*Continued*)

Table 1 Classes and Structural Formula of Flavonoids (*Continued*)

Classes	Structural formula	Examples
Anthocyanidins		R = OH, R^1 = H; Cyanidin R = R^1 = OH; Delphinidin R = R^1 = OCH_3; Malvidin R = R^1 = H; Pelargonidin
		R = H; Peonidin R = OH; Petunidin

The flavonoids contained in blueberries, mainly anthocyanins, have been extensively studied in vitro and in vivo to assess their action in several pathologies (77). In aged rats, blueberry extracts were effective in reversing age-related decline with cognitive, motor, and neuronal effects (78). Phenolics were capable of enhancing red blood cell resistance to oxidative stress in vitro and in vivo, supporting the idea of a protective role of these substances in ROS-mediated, age-related neurological decline. Although there are no studies on the action of blueberry extracts in ameliorating AD cognitive decline, improvement on behavior and memory has been reported in transgenic mice with APP/PS-1 double mutations, a model for AD (79).

Effective micro-organism-X (EM-X) is an antioxidant drink derived from the ferment of unpolished rice, papaya, and seaweeds with effective micro-organisms (EM) selected from actobacillaceae, saccharomycetes, funguses, actinomyces, and photosynthetic bacteria. This cocktail drink is widely available in south east Asia and has applications as supplements in the clinic for the treatment of cancer, hypertension, various allergies, diabetes, and tuberculosis. EM-X contains over 10 minerals, α-tocopherol, lycopene, ubiquinone, ascorbic acid, saponin, and flavonoids, such as quercetin, quercetin-3-*O*-glucopyranoside, and quercetin-3-*O*-rhamnopyranoside. EM-X increases the serum levels of superoxide dismutase, modulates immunological functions in animals, decreases MDA levels in D-galactose-induced aging in mice, and prolongs survival time of mice at high temperatures, as well as under hypoxic conditions (80). Aruoma and colleagues (81) observed that pretreatment with EM-X significantly reduces retinal neurodegeneration following NMDA injection into the vitreous body. Neuroprotection against the toxicity of 6-hydroxydopamine has also been reported for EMX (82). Although the exact molecular entities responsible for the antioxidant action were unclear, the neuroprotective effect stems, at least in part, due to its flavonoids and other antioxidant contents (vitamin E, saponin, ascorbic acid, etc.).

Resveratrol showed a protective effect against oxidative insult in PC12 cells (83) and protects rat brain against excitotoxic damage (84) and the hippocampal neurons against nitric

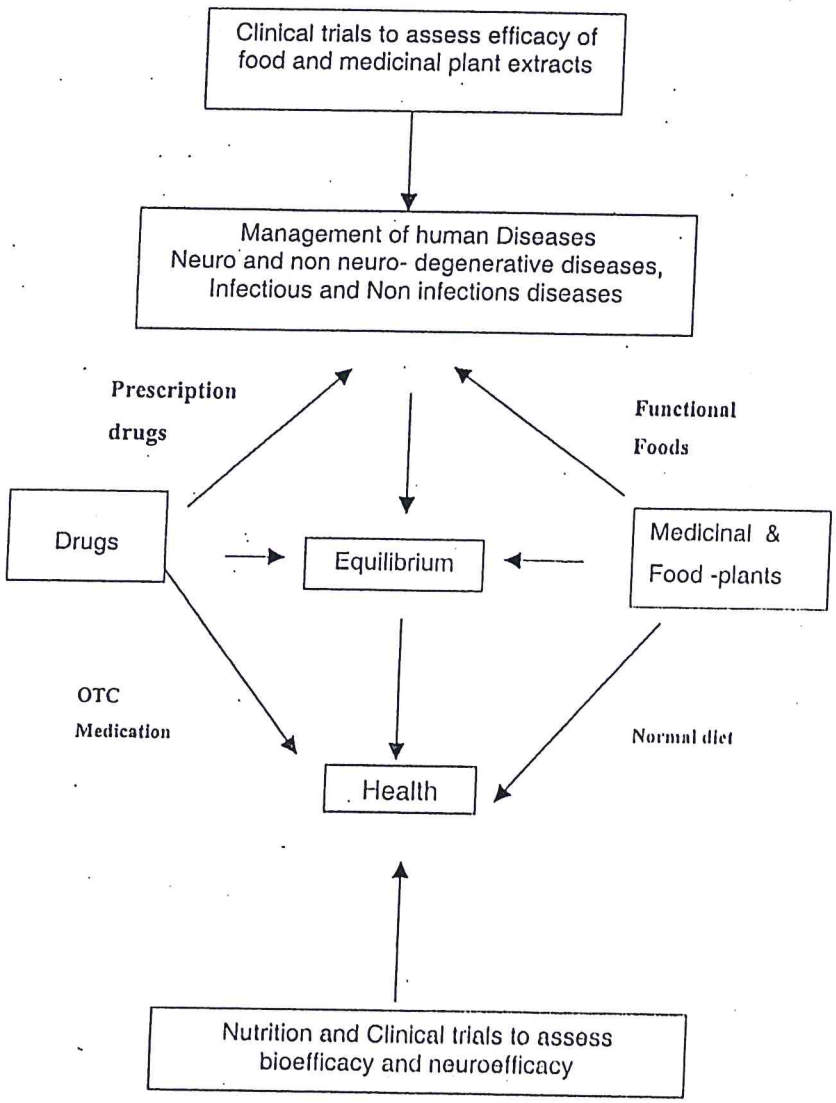


Figure 1 A strategy to facilitate definition of the prophylactic potentials of diet, nutritional/food supplements, medicinal plants, and herbal extracts 18).

oxide toxicity (85). A French study (86) on a community of persons of age ≥65, whose only consumption of alcoholic beverages was represented by red wine in the amount considered moderate (3–4 glasses per day), analyzed the incidence of dementia and AD compared with nondrinkers. After adjusting for age, sex, education, and other factors, the study showed an inverse relationship between moderate wine drinking and AD incidence. The intake of flavonoids and the risk of dementia will remain a unique research area in biomedicine (18,87,88). Finally, a compound that is targeted for neuronal protection should be able to cross the highly selective blood–brain barrier (20,81,82,87,88). Thus, in addition to boosting the endogenous antioxidant status, dietary phenolics can also potentiate cognitive function and memory performance. A strategy to facilitate the definition of the prophylactic potentials of diet, nutritional/food supplements, medicinal plants, and herbal extracts is proposed in Fig. 1 (18). Research directed towards the understanding of the role of plant/herbal extracts and dietary antioxidants in human health should be complemented with the development and validation of biological markers.

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Resveratrol—A Unique Polyphenolic Antioxidant Present in Grape Skins and Red Wine—Is a Preventive Medicine Against a Variety of Degenerative Diseases

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ABSTRACT

Resveratrol abundantly present in grapevines is found in grape skins, wines, especially red wine, and peanuts. Recent studies suggest that resveratrol has a number of health benefits including its potential role as cardioprotective,

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Phenolics as potential antioxidant therapeutic agents: Mechanism and actions

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Abstract

Accumulating chemical, biochemical, clinical and epidemiological evidence supports the chemoprotective effects of phenolic antioxidants against oxidative stress-mediated disorders. The pharmacological actions of phenolic antioxidants stem mainly from their free radical scavenging and metal chelating properties as well as their effects on cell signaling pathways and on gene expression. The antioxidant capacities of phenolic compounds that are widely distributed in plant-based diets were assessed by the Trolox equivalent antioxidant capacity (TEAC), the ferric reducing antioxidant power (FRAP), the hypochlorite scavenging capacity, the deoxyribose method and the copper-phenanthroline-dependent DNA oxidation assays. Based on the TEAC, FRAP and hypochlorite scavenging data, the observed activity order was: procyanidin dimer > flavanol > flavonol > hydroxycinnamic acids > simple phenolic acids. Among the flavonol aglycones, the antioxidant propensities decrease in the order quercetin, myricetin and kaempferol. Gallic acid and rosmarinic acid were the most potent antioxidants among the simple phenolic and hydroxycinnamic acids, respectively. Ferulic acid displayed the highest inhibitory activity against deoxyribose degradation but no structure–activity relationship could be established for the activities of the phenolic compounds in the deoxyribose assay. The efficacies of the phenolic compounds differ depending on the mechanism of antioxidant action in the respective assay used, with procyanidin dimers and flavan-3-ols showing very potent activities in most of the systems tested. Compared to the physiologically active (glutathione, α-tocopherol, ergothioneine) and synthetic (Trolox, BHA, BHT) antioxidants, these compounds exhibited much higher efficacy. Plant-derived phenolics represents good sources of natural antioxidants, however, further investigation on the molecular mechanism of action of these phytochemicals is crucial to the evaluation of their potential as prophylactic agents.
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Keywords: Phenolics; Flavonoid; Proanthocyanidin; Antioxidant; Structure–activity relationship; Therapeutic agents; Mechanism of action; Antioxidant medicine

1. Introduction

There is emerging interest in the use of naturally occurring antioxidants for the preservation of foods

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and in the management of a number of pathophysiological conditions, most of which involve free radical damage. The implication of oxidative and nitrosative stress in the etiology and progression of several acute and chronic clinical disorders has led to the suggestion that antioxidants can have health benefits as prophylactic agents. Epidemiological studies have consistently shown an inverse association between consumption of vegetables and fruits and the risk of cardiovascular diseases and certain forms of cancer [1,2]. Although the protective effects have been primarily attributed to the well-known antioxidants, such as Vitamin C, Vitamin E and β-carotene, plant phenolics may also play a significant role. Moreover, restrictions over the use of synthetic antioxidants BHA and BHT in food further strengthen the concept of using naturally occurring compounds as antioxidants [3].

Phenolic compounds or polyphenols constitute one of the most numerous and ubiquitously distributed group of plant secondary metabolites, with more than 8000 phenolic structures currently known. Natural polyphenols can range from simple molecules (phenolic acids, phenylpropanoids, flavonoids) to highly polymerised compounds (lignins, melanins, tannins), with flavonoids representing the most common and widely distributed sub-group [4]. Phenolics are widely distributed in the plant kingdom and are therefore an integral part of the diet, with significant amounts being reported in vegetables, fruits and beverages [5–7]. Although the dietary intake of phenolics varies considerably among geographic regions, it is estimated that daily intake range from about 20 mg to 1 g, which is higher than that for Vitamin E [8]. Polyphenolics exhibit a wide range of biological effects including antibacterial, anti-inflammatory, antiallergic, hepatoprotective, antithrombotic, antiviral, anticarcinogenic and vasodilatory actions [9]; many of these biological functions have been attributed to their free radical scavenging and antioxidant activity.

Molecular studies have revealed that phenolics can exert modulatory actions in cell by interacting with a wide spectrum of molecular targets central to the cell signaling machinery. These include activation of mitogen-activated protein kinase (MAPK), protein kinase C (PKC), serine/threonine protein kinase Akt/PKB, phase II antioxidant detoxifying enzymes, downregulation of pro-inflammatory enzymes (COX-2 and iNOS) through the activation of peroxisome proliferator-activated receptor gamma (PPARγ), regulation of calcium homeostasis, inhibition of phosphoinositide 3-kinase (PI 3-kinase), tyrosine kinases, NF-κB, c-JUN, as well as modulation of several cell survival/cell-cycle genes [10]. The redox status of the cell has profound effect on the cell signaling pathways, in particular the MAP kinase cascade (Fig. 1). Oxidative insults can also perturb the cellular energy homeostasis by disrupting the mitochondrial integrity. ROS can induce permeabilization of the mitochondrial membrane resulting in the release of apoptosis initiating factors (AIF), such as cytochrome *c*, DIABLO/smac and dissipation of the mitochondrial membrane potential ($\Delta\psi_m$) [11]; these processes mark the point of no return in the cell death process. Although the exact mechanism involved is not fully elucidated, it seems to be mediated by translocation of Bad from the cytosol to the mitochondria where it dimerizes with Bcl-2 and Bcl-XL thereby neutralizing their mitochondrial stabilizing effect [12]. Phenolics may prevent oxidative stress induced mitochondrial transition pore complex opening by decreasing production of Bax and Bad protein, favouring an increase in Bcl2–BclXL/Bax–Bak ratio [12]. Thus, in addition to their antioxidant capacity, phenolics may exert protective effect by selectively inhibiting or stimulating key protein in the cell signaling cascades.

Phenolics are particularly attractive as prophylactic agents due to their high prevalence in the diet and also due to their pluripharmacological effects. The antioxidant activities of more than 30 commercially available phenolic compounds have been measured in the present study, physiologically active and synthetic standards have also been included for comparative purpose. The compounds were selected on the basis of their chemical structural characteristics, availability and prevalence in food plants. For the in vitro characterisation of antioxidant propensities use of single test system is widely discouraged as the mechanisms of reactions involving antioxidants are complex and depend on the physico-chemical parameters of the test reagents and substrates. There is an emerging view that a combination of rapid, sensitive and reproducible methods should be used whenever an antioxidant activity screening is designed and there should be great caution in extrapolating the in vitro data [13]. Consequently for the evaluation of the antioxidant capacities of the phenolic compounds five independent and highly

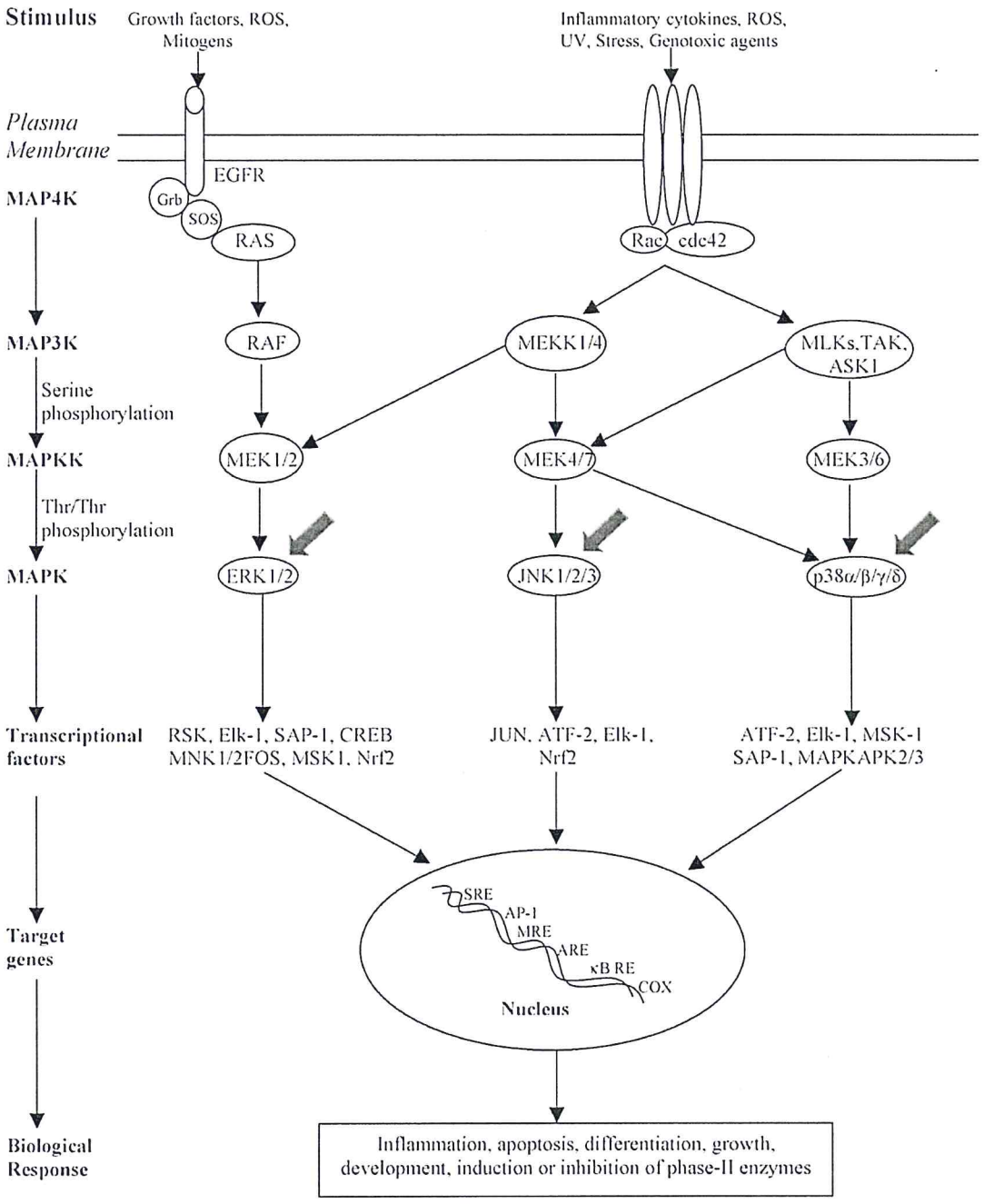


Fig. 1. Mitogen-activated protein kinase (MAPK) signaling cascade. Phenolics may modulate activation of MAPK (indicated by solid arrow) either directly or indirectly by interacting with target molecules upstream. ARE, antioxidant response element; ASK, apoptosis-signal-regulating kinase; ATF, activating transcription factor; CREB, cAMP–response element binding protein; EGFR, epidermal growth factor receptor; Erk, extracellular receptor kinase; JNK, Jun N-terminal kinase; MAPKAPK, MAPK-activated protein kinase; MAP2K, MAPK kinase; MAP3K, MAPKK kinase; MAP4K, MAPKKK kinase; MLK, mixed-lineage kinase; MRE, metal response element; MSK, mitogen- and stress-activated kinase; Nrf, nuclear factor-E2-related factor; Ras, small G-protein; RSK, ribosomal S6 protein kinase; SAP-1, SRE accessory protein; SRE, serum response element; TAK, transforming growth factor β-activated kinase.

reproducible systems, namely TEAC, FRAP, HOCl, deoxyribose and copper-phenanthroline assays, have been used.

2. Material and methods

2.1. Reagents and standards

2,2′-Azino-bis(3-ethylbenzthioziozline-6-sulphonic acid (ABTS), (2,4,6-tri(2-pyridyl) *s*-triazine (TPTZ), butylated hydroxytoluene (BHT) and butylated hydroxyanisol (BHA) were purchased from Sigma Co. (St. Louis, MO, USA). Trolox C (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), a water-soluble analogue of Vitamin E, was purchased from Sigma–Aldrich (Germany). HPLC grade of (+)-catechin ((+)-C), (−)-epicatechin ((−)-EC), (−)-epigallocatechin ((−)-EGC), (−)-epicatechin-3-gallate ((−)-ECG), (−)-epigallocatechin-3-gallate ((−)-EGCG), procyanidin dimers B1 and B2, myricetin, kaempferol, myricitrin, quercetin, quercitrin, isorhamnetin, isoquercitrin, hyperoside, rhamnetin, isovitexin, naringenin, genistein, luteolin, apigenin, orientin, vitexin, cyanidin chloride and gallic acid (GA) were obtained from Extrasynthese (Genay, France). Ellagic acid was from Fluka Biochemika. All other reagents used were of analytical grade.

2.2. TEAC assay

The ABTS/MnO_2 method [14] was used to evaluate the ability of the plant extracts to scavenge the preformed radical cation $ABTS^{\bullet+}$. The $ABTS^{\bullet+}$ radical was generated by a reaction between ABTS (0.5 mM) and activated MnO_2 (1 mM) in phosphate buffer (0.1 M). To 3 mL of the $ABTS^{\bullet+}$ solution, 0.5 mL of plant extracts was added and the decay in absorbance was followed for 15 min at 734 nm. Calculations were made with respect to a dose–response curve of Trolox and the TEAC values were expressed in millimolar.

2.3. Ferric reducing antioxidant power

The ferric reducing antioxidant power (FRAP) assay gives an indication of the reducing ability of the plant extract. This method was adapted from Benzie and Strain [15]. FRAP reagent was freshly prepared by mixing together 10 mM 2,4,6-tripyridyl triazine (TPTZ) and 20 mM ferric chloride in 0.25 M acetate buffer, pH 3.6. Test sample (100 μL) was added to 300 μL of water followed by 3 mL of FRAP reagent. The absorbance was read at 593 nm after 4 min incubation at ambient temperature against a blank of distilled water. A calibration curve of ferrous sulphate (100–1000 μmol/L) was used, and results were in millimolar.

2.4. Hypochlorite scavenging assay

This assay was adapted from Weiss et al. [16]. The reaction mixture contained taurine (150 mM), sodium hypochlorite (600 mM), reference compounds (variable concentrations) and phosphate saline buffer (pH 7.4) in a final volume of 1 mL. The solution was mixed thoroughly and potassium iodide (2 M) was added. A yellow coloration was developed and the absorbance was read at 350 nm. The analyses were made in triplicates and the results were expressed as IC_{50} values.

2.5. Deoxyribose assay

The reacting mixture for the deoxyribose assay [17,18] contained in a final volume of 1 mL the following reagents: 200 μL KH_2PO_4–KOH (100 mM), 200 μL deoxyribose (15 mM), 200 μL $FeCl_3$ (500 μM), 100 μL EDTA (1 mM), 100 μL ascorbic acid (1 mM), 100 μL H_2O_2 (10 mM) and 100 μL sample. Reaction mixtures were incubated at 37 °C for 1 h.

At the end of the incubation period, 1 mL 1% (w/v) thiobarbituric acid (TBA) was added to each mixture followed by the addition of 1 mL 2.8% (w/v) trichloroacetic acid (TCA). The solutions were heated in a water bath at 80 °C for 20 min to develop the pink coloured MDA-$(TBA)_2$ adduct. As turbidity was encountered, the MDA-$(TBA)_2$ chromogen was extracted into 2 mL butan-1-ol and its absorbance measured at 532 nm. The rate constants of reaction were calculated from competition plots obtained with varying concentrations of the antioxidant in the assay mixtures.

2.6. Copper-phenanthroline (Cu-phen) assay

The Cu-phen assay was conducted essentially as described by Aruoma [18]. Reaction mixture

contained in a final volume of 1.2 mL, the following reagents in order of addition indicated: 100 μL 1,10-phenanthroline (1.8 mM stock solution made up in water having initially dissolved the crystals in 50 μL ethanol), 480 μL copper(II) chloride (250 μM), 300 μL DNA (1.68 mg/mL) and 120 μL KH_2PO_4–KOH buffer at pH 7.4 (100 mM). Ascorbate (stock solution: 2.88 mM) or test samples (100 μL) were added to initiate the reaction. The tubes were incubated at 37 °C for 1 h. At the end of the incubation period 100 μL 0.1 M EDTA was added to stop the reaction. DNA damage was assessed by adding 1 mL 1% (w/v) TBA and 1 mL 25% (v/v) HCl followed by 15 min incubation at 80 °C. The pink chromogen formed was extracted into butan-1-ol and the absorbance measured at 532 nm. Results were expressed as the absorbance at 532 nm.

2.7. *Statistical analysis*

Results are expressed as mean value ± standard deviation ($n = 3$). Simple regression analysis was performed to calculate the dose–response relationship of standard solutions used for calibration as well as for the test samples. Linear regression analysis was performed, quoting the correlation coefficient r_{xy}.

3. Results

The antioxidant activities of some standard pure compounds were assessed using the TEAC, FRAP, deoxyribose assay, the assay involving hypochlorite and the Cu-phen assay (Table 1). Several phenolic subclasses including phenolic acids, hydroxycinnamic acids, coumarins, flavones, flavonones, flavonols, flavan-3-ols, other naturally occurring compounds and some synthetic antioxidants were evaluated for their antioxidant capacity in the aforementioned in vitro assays.

The scavenging potencies of the pure compounds as assayed by the TEAC method ranged from 0.13 to 7.58 mM with procyanidin B2 exhibiting the highest $ABTS^{\bullet+}$ scavenging activity (7.58 ± 1.05 mM). B1 was also characterised by high TEAC value (6.55 ± 1.1 mM). Relatively high antioxidant activity was reported among the flavan-3-ols with (−)-EGCG showing very elevated TEAC value (4.39 ± 0.25 mM). The antioxidative order of the catechins in the TEAC assay was (−)-EGCG > (−)-ECG > (−)-EGC > (−)-EC > (+)-C. However, the phenolic and hydroxycinnamic acids were marked by low TEAC values except gallic acid (3.62 ± 0.12 mM) and rosmarinic acid (4.50 ± 0.38 mM). Among the flavonol aglycones, quercetin and myricetin exhibited moderate activities with 3.68 ± 0.12 and 3.07 ± 0.23 mM, respectively, while kaempferol was weaker in activity. The flavonol glycosides showed lower scavenging capacity compared to the aglycones. Naringenin, cyanidin chloride and genistein had low TEAC values (<1 mM). In accordance with literature data BHA had weak TEAC activity whilst BHT, vitexin and coumarin had no measurable antioxidant radical scavenging activity in the TEAC assay. Ascorbic acid, ergothioneine and glutathione were also poor scavengers of the $ABTS^{\bullet+}$ radical.

The highest FRAP values was obtained with cyanidin chloride (12.28 ± 1.17 mM) followed by B1 (11.85 ± 0.07 mM). 3-Coumaric acid, hydroxybenzoic acid, coumarin and BHT were very poor reducing agent in the FRAP assay. Gallic acid and rosmarinic acid showed strong reducing potential with 6.21 ± 0.95 and 5.25 ± 0.18 mM, respectively. In line with the TEAC values, B1 and B2 were potent reducing agents as indicated by the high FRAP values recorded. The flavan-3-ols exhibited strong antioxidant activity in the FRAP assay and similar activity order was observed for the catechins in both FRAP and TEAC assay. Among the flavonols with high FRAP values were myricitrin (7.91 ± 0.73 mM) followed by quercetin (7.39 ± 0.74 mM). Lower antioxidant activities was observed with the flavones, flavanones, isoflavones and the synthetic antioxidants except BHA.

The procyanidin dimers were potent scavengers of ClO^- with low IC_{50} values (Table 1). The flavan-3-ols were strong scavengers with activities in the decreasing order of (−)-ECG ≈ (−)-EGCG > (+)-C > (−)-EC > (−)-EGC. The simple phenolic and hydroxycinnamic acids were weak scavengers except rosmarinic acid, which had antioxidant activity comparable to procyanidin B2. The highest scavenging activity amongst the flavonols was observed with quercetin while the glycosides showed lower activities. Cyanidin chloride and ergothioneine had moderate IC_{50} values. The other compounds had relatively weak scavenging activities in this particular assay.

Table 1
Antioxidant capacities of the reference compounds as measured by TEAC, FRAP, HOCl scavenging and deoxyribose assay

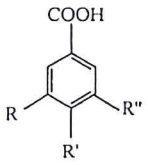
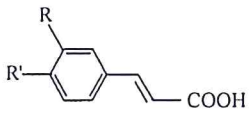
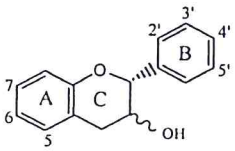
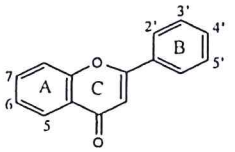
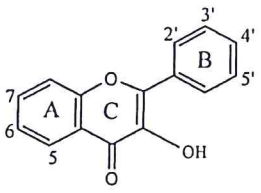
Reference compounds	General structure	TEAC[a]	FRAP[b]	HOCl scavenging[c]	Deoxyribose assay[d]
Simple phenolic acids					
Gallic acid		3.62	5.25	281.1	–
Ellagic acid		1.05	4.39	499.49	3.57×10^{12}
Hydroxybenzoic acid		0.13		591.95	1.2×10^{12}
Hydroxycinnamic acids					
Rosmarinic acid		4.50	6.21	23.55	1.82×10^{12}
Chlorogenic acid		1.45	3.22	82.04	–
Ferulic acid		0.98	1.33	222.65	1.55×10^{12}
3-Coumaric acid		0.17	–	1878.17	0.95×10^{12}
Coumarins					
Coumarin		–	–	2750.44	0.55×10^{12}
Flavan-3-ols					
(−)-Epigallocatechin gallate		4.39	8.04	26.11	3.10×10^{12}
(−)-Epicatechin gallate		4.23	7.03	24.67	1.59×10^{12}
(−)-Epigallocatechin		3.86	4.01	67.44	1.61×10^{12}
(−)-Epicatechin		3.58	2.90	23.87	4.37×10^{12}
(+)-Catechin		3.16	2.47	52.06	2.93×10^{12}
Oligomeric procyanidin					
B2		7.58	8.63	21.32	–
B1		6.55	11.85	15.12	7.19×10^{12}
Flavones					
Luteolin		2.23	2.23	91.19	4.52×10^{12}
Apigenin		2.01	2.01	330.46	3.44×10^{12}
Orientin		1.05	2.81	151.92	2.55×10^{12}
Vitexin			0.56	1028.68	3.45×10^{12}
Flavonols					
Quercetin		3.68	7.39	85.5	5.80×10^{12}

Table 1 (*Continued*)

Reference compounds	General structure	TEAC[a]	FRAP[b]	HOCl scavenging[c]	Deoxyribose assay[d]
Myricetin		3.07	4.58	105.07	5.10×10^{12}
Isoquercitrin		2.72	5.52	77.88	2.90×10^{12}
Isorhamnetin		2.69	0.26	688.85	2.08×10^{12}
Hyperoside		2.33	4.89	105.01	4.18×10^{12}
Quercitrin		1.75	5.31	–	4.51×10^{12}
Rhamnetin		1.27	0.07	864.29	1.70×10^{12}
Kaempferol		1.03	1.95	173.41	6.44×10^{12}
Myricitrin		0.89	7.91	81.52	1.75×10^{12}
Isovitexin		0.15	1.00	795.06	2.80×10^{12}
Flavanones					
Naringenin		0.13	0.44	204.34	1.40×10^{12}
Anthocyanidins					
Cyanidin chloride		0.96	12.28	55.79	1.65×10^{12}
Isoflavonoids					
Genistein		0.32	0.46	207.02	1.46×10^{12}
Synthetic antioxidants					
Butylated hydroxytoluene		–	0.04	1705.15	–
Butylated hydroxyanisole		1.11	3.05	219.63	–
Trolox		1.00	1.91	557.83	2.44×10^{12}
Others					
Ascorbic acid		1.42	2.26	427.62	–
Ergothioneine		0.87	0.89	70.68	0.98×10^{12}
Glutathione		0.60	0.73	669.59	–

All analyses were mean of triplicates measurements ± standard deviations.

[a] Results expressed in units mmol trolox/L.

[b] Values are expressed in units of mmol Fe (II)/L.

[c] The concentration (μM) sufficient to obtain 50% of maximum scavenging activity (IC_{50}).

[d] Rate constant ($M^{-1}s^{-1}$) of reaction with $OH^{\bullet}$ obtained by use of the deoxyribose assay.

The activity of the reference compounds in the deoxyribose assay followed a different trend compared to that observed in the TEAC, FRAP and the assay involving hypochlorite. The percentage inhibition of deoxyribose degradation due to $OH^{\bullet}$ scavenging varied widely and ranged between 1.6 and 68%. Highest activity was measured with (+)-C followed by (−)-EGC and (−)-EGCG. The phenolic acids on the other hand exhibited moderate activities. The synthetic antioxidants, BHA and Trolox also showed moderate activities in inhibiting deoxyribose degradation. Procyanidin dimers had varied activities with procyanidin B1 being more active than the B2 dimer. The calculated rate constants of reaction with $OH^{\bullet}$ in the deoxyribose assay are given for the compounds however (Table 1).

Although cyanidin chloride was characterised by moderate activity in the Cu-phen assay, none of the other reference compounds showed prooxidant activity at a concentration of 500 μM.

4. Discussion

During the last decade natural antioxidants, particularly phenolics, has been under very close scrutiny as potential therapeutic agents against a wide range of ailments including neurodegenerative diseases, cancer, diabetes, cardiovascular dysfunctions, inflammatory diseases and also aging (Table 2). The medicinal actions of phenolics is mostly ascribed to their antioxidant capacity, free radical scavenging, chelation of redox active metal ions, modulation of gene expression and interaction with the cell signaling pathways.

Table 2
Mechanism of actions of phenolic compounds in various pathophysiological conditions

Phenolic compounds	Pathology	Mechanism of actions	References
Quercetin, Kaempferol, genistein, resveratrol	Colon cancer	Suppresses COX-2 expression by inhibiting tyrosine kinases important for induction of COX-2 gene expression	[47]
Catechins	Neurodegenerative diseases	Enhance activity of SOD and catalase	[31]
(−)-EGCG	Neurodegenerative conditions	Decreases the expression of proapototic genes (bax, bad, caspase-1 and -6, cyclin dependent kinase inhibitor) thus maintaining the integrity of the mitochondrial membrane	[32]
(−)-EGCG	Cancer, diabetic retinopathy, chronic inflammation	Suppression of angiogenesis by inhibiting growth factor triggered activation of receptors and PKC. Downregulation of VEGF production in tumour cells. Repression of AP-1, NF-κB and STAT-1 transcription factor pathways. Inhibits capillary endothelial cell proliferation and blood vessel formation.	[24–26]
Proanthocyanidin (GSPE)	Cardiovascular disorders	Inhibitory effects on proapoptotic and cardioregulatory genes. Modulating apoptotic regulatory bcl-X_L, p53 and c-myc genes	[22]
Ferulic acid	Diabetes	Decrease lipid peroxidation and enhances the level of glutathione and antioxidant enzymes	[50]

The free radical scavenging and antioxidant activities of phenolics are dependent upon the arrangement of functional groups about the nuclear structure. Both the number and configuration of H-donating hydroxyl groups are the main structural features influencing the antioxidant capacity of phenolics [19,20]. Among the reference compounds studied, the greatest antioxidant activity was observed in the dimeric procyanidin B1 and B2. B2 exhibited highest scavenging activity in the TEAC assay (7.58 mM) while B1 showed highest ferric reducing (11.85 mM) and hypochlorite scavenging activity (lowest IC_{50}). The high antioxidant activity of the procyanidin dimers is attributed to their hydroxyl functions that are potent hydrogen donators (Fig. 2). Furthermore, the conjugated double bonds allow electron delocalisation across the molecule thus stabilizing the phenoxyl radical [21]. Several studies have highlighted the cardioprotective effect of proanthocyanidins. Grape seed proanthocyanidin extract (GSPE), a mixture of 75–80% oligomeric proanthocyanidin and 3–5% monomeric proanthocyanidin has proved its efficacy against the incidence of ischemia-reperfusion injury, apoptosis of cardiomyocytes and reduction of foam cell development. The mechanistic pathways of cardioprotection exerted by the proanthocyanidin rich extract included: (1) potent hydroxyl and other free radical scavenging abilities; (2) antiapoptotic, antinecrotic and antiendonucleolytic potentials; (3) modulatory effect on apoptotic regulatory bcl-X_L, p53 and c-myc genes; (4) cytochrome P450 2E1 inhibitory activity; (5) inhibitory effects on proapoptotic, cardioregulatory genes c-JUN and JNK-1 and (6) inhibition of vasoconstriction of vascular smooth muscle and endothelium [22,23].

Among the monomeric flavan-3-ol, the hierarchy of antioxidant activities decreased from (−)-EGCG > (−)-ECG > (−)-EGC > (−)-EC > (+)-C. The decreasing solubility and reduced number of hydroxyl groups might be contributive to this observation. The high antioxidant activity of the gallates, (−)-EGCG and (−)-ECG, is explained by the presence of galloyl moiety attached to flavan-3-ol at the 3 position, adding three more hydroxyl groups. The 3′4′5′-trihydroxyl groups on ring B are responsible for the high activity of (−)-EGC compared to (+)-C and (−)-EC. These observations are consistent with previously reported studies based on the structure–activity relationships of the flavan-3-ols [27,28]. Flavanols are also potent antioxidants in lipid systems where they reduce oxidative modifications of membranes by restricting the access of oxidants to the bilayer and the propagation of lipid oxidation in the hydrophobic membrane matrix [29]. Several studies have shown that catechins have a protective role in neurodegeneration.

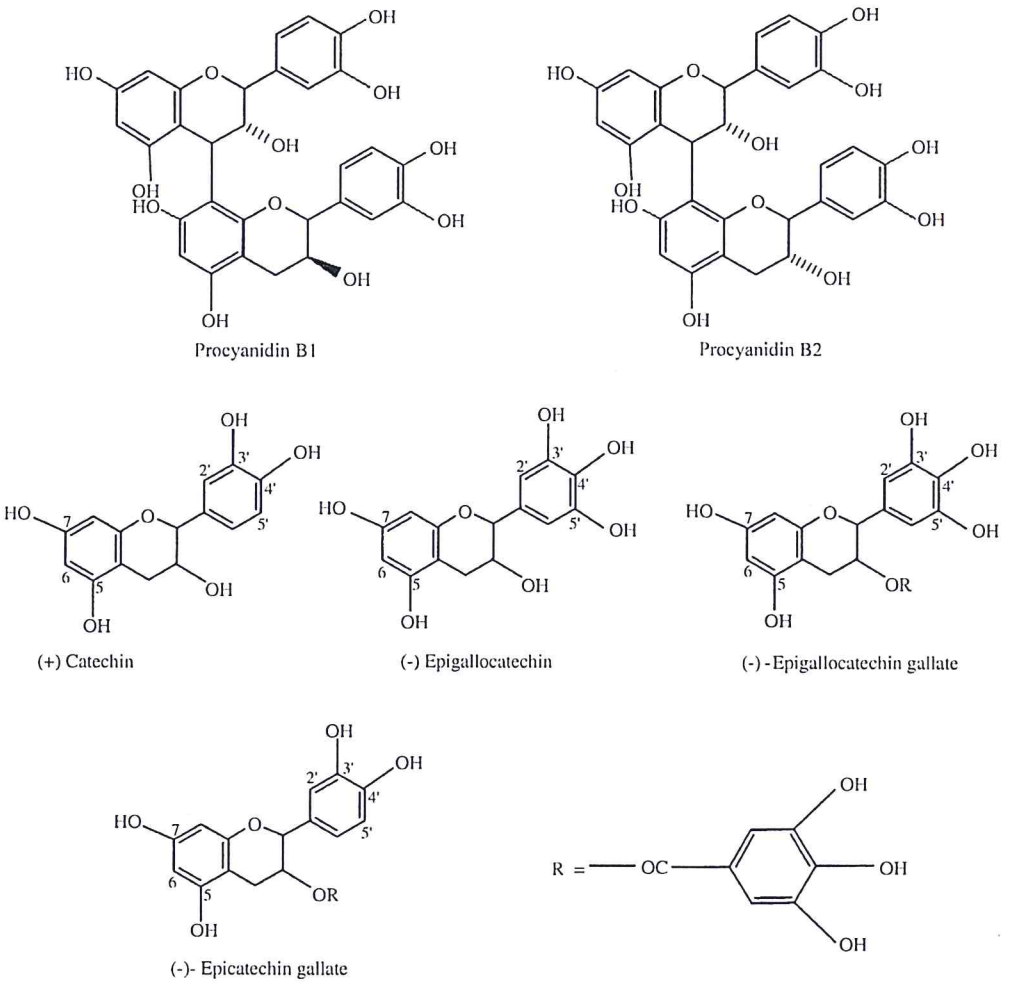


Fig. 2. Structure of the multiply-hydroxylated procyanidins and catechins.

Pretreatment with (−)-EC attenuated neurotoxicity induced by oxidized low-density lipoprotein in mouse-derived striatal neurons, as evidenced by apoptotic DNA fragmentation and caspase-3 activation [30]. (−)-EGCG enhance the activity of superoxide dismutase (SOD) and catalase in mouse striatum thus suggesting that flavan-3-ols can also exhibit their neuroprotective effect via regulation of gene expression [31]. Levites et al. [32] reported that (−)-EGCG decreased the expression of proapoptotic genes Bax, Bad, caspase-1 and -6, cyclin-dependent kinase inhibitor p21, cell-cycle inhibitor gadd45, fas-ligand and TNF-related apoptosis-inducing ligand TRAIL, in SH-SY5Y neuronal cells. The decline in bax expression by (−)-EGCG may favour the increase in the ratio of bcl-2/bcl-XL to bax/bad proteins, thereby contributing to mitochondrial stability and regulation of mitochondrial permeability transition pore (mPTP) [33] (Table 2).

The antioxidant activities of the flavonol aglycones was relatively high with an activity order of quercetin > myricetin > kaempferol. The antioxidant capacity in this particular class of flavonoid is also related to the number of hydroxyl groups present. The presence of a third hydroxyl group in the B ring at C-5 position does not enhance the effectiveness of myricetin compared to quercetin with simply an *o*-diphenolic structure. This is supported by the findings that the presence of three hydroxyl groups on the aromatic nucleus does not improve antioxidant efficiency. The low antioxidant activity of kaempferol among the

flavonol aglycones could be attributed to the presence of a single hydroxyl group in the B ring which apparently makes little contribution even in the presence of the conjugated double bond system and the 3-OH group. Similar results have been mentioned in the literature data [27]. The free radical scavenging by flavonoids is highly dependent on the presence of a free 3-OH. Both flavonol and flavone have identical hydroxyl configuration but, however, the absence of the 3-OH functional group in the flavone explain the lower antioxidant activity of latter. Thus, blocking the 3-OH group in the C ring of quercetin as a glycoside while retaining the 3′,4′-dihydroxystructure in the B ring as in hyperoside and quercitrin decreases the antioxidant activity significantly as revealed by the TEAC, FRAP and hypochlorite scavenging values (Table 1). Burda and Gleszek [34] also reported that substitution of 3-OH by a methyl or glycosyl group decreases the activity of quercetin and kaempferol against β-carotene oxidation in linoleic acid. In addition to a reduction in the number of OH group, removal of the 3-OH affect the conformation of the molecule. The torsion angle of the B ring with respect to the rest of the molecule strongly influences free radical scavenging ability. Flavonols and flavanols with a 3-OH are planar, while the flavones and flavanones, lacking this feature, are slightly twisted. Planarity permits conjugation, electron dislocation, and a corresponding increase in flavonoid phenoxyl radical stability [35]. Eliminating this hydrogen bond effects a minor twist of the B ring, abrogates coplanarity, conjugation and electron delocalisation capacity, thereby compromising scavenging ability. Due to this intramolecular hydrogen bonding, the influence of a 3-OH is potentiated by the presence of 3′,4′-catechol, explaining the potent antioxidant activity of flavan-3-ols and flavonols [36].

Ishige et al. [37] reported that three structural features of flavonol were crucial for neuroprotective effect: the presence of the hydroxyl group on the C3 position, an unsaturated C ring, and hydrophobicity. The importance of the C3 hydroxyl group for antioxidant and free radical scavenging activities has also been observed in the present study. The neuroprotective effect of flavonol is also substantially increased by the presence of the catechol structure. The unsaturation of the C ring in flavonoid is essential for protection from glutamate toxicity. Quercetin with the unsaturated C ring showed protective action while flavanones and flavan-3-ols are totally ineffective [37]. Studying the neuroprotective effects of flavonoids in PC 12 model, Dajas et al. [38] reported that the three aforementioned structural features were critical for cytoprotective activities.

Absence of a C3 hydroxyl group from the flavonol basic moiety decreased the antioxidant efficacy of the flavones. Luteolin with the catechol and 5,7 hydroxylated groups was more potent than apigenin, orientin and vitexin. The latter was characterised by very low ferric reducing capacity and no scavenging activity probably due to the presence of a sugar moiety at the C-8 position which decreased its antioxidative efficacy. Similarly naringenin was a weak antioxidant due to the presence of a single 3′ hydroxyl group and the characteristic saturated heterocyclic C ring, which led to consequent lack of conjugation between the A and B rings, in contrast to fiavones and flavonols. Vaya et al. [39] highlighted very similar activities when a series of 20 flavonoids were assessed for their DPPH scavenging potential. Although characterised by low antioxidant activity, several studies have shown that isoflavone can act as phytoestrogens. It appears that phytoestrogens, including apigenin and genistein, bind preferentially to the β-subtypes receptor leading to gene induction, although at a lower affinity that estrogens [40]. Such agents have been suggested to protect against hormone-related cancers, such as breast and prostate, and menopausal symptoms [40].

Cyclooxygenase (COX) is a key enzyme in the biosynthesis of prostaglandin from arachidonic acid. COX exists in two major isoforms: COX1 and COX2. The over expression of COX2 has been associated with inflammation-mediated pathologies, such as neurodegeneration and cancer [41,42]. In response to a variety of endogenous and exogenous ligands (e.g. NSAIDs) the peroxisome proliferator-activated receptor gamma (PPARγ), a receptor and nuclear transcription factor, can down regulate the expression of COX [43]. Flavones and isoflavones can also act as PPARγ ligand, however, this activation is structure-dependent: the C2–C3 double bond of the C ring and hydroxyl groups at position 5 and 7 on ring A and at 4′-position on B ring are important for activity while the presence of a 3′ hydroxyl group on the B ring lead to decrease PPARγ activation [44]. Flavonoids may also suppress COX gene expression through interaction with the cell signaling pathways, namely NF-κB [45], protein kinase C [46] and tyrosine kinase [47].

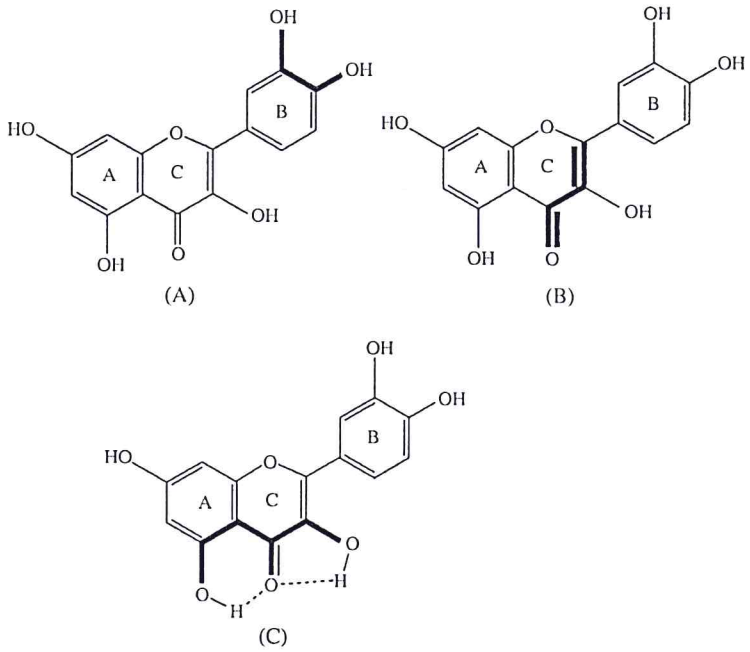


Fig. 3. Structural groups for radical scavenging.

In line with literature data the present study corroborate with the view that the following structural features of flavonoid are important for antioxidant and free radical scavenging activities: (A) an *o*-diphenolic group (in ring B), (B) a 2–3 double bond conjugated with 4-oxo function and (C) hydroxyl groups in positions 3 and 5 (Fig. 3).

Considering the benzoic acid derivatives, the hydroxycinnamic acids exhibited higher antioxidant capacities than the simple phenolic acids. Among the simple phenolic acids assayed, hydroxybenzoic acid showed no antioxidant activity in most of the different antioxidant assays. This is consistent with the electron withdrawing potential of the single carboxyl functional group attached to the aromatic ring, dampening the H-donation capacity of the hydroxyl group. Gallic acid, with 3-hydroxyl groups, was characterised by the highest antioxidant activity. The antioxidant activities of the hydroxycinnamic acids were in the order: rosmarinic acid > chlorogenic acid > caffeic acid > ferulic acid > coummaric acid. These results are consistent with data reported by Cuvelier et al. [48] who suggested that the presence of CH=CH–COOH group in cinnamic acids ensures greater efficiency than the COOH group in benzoic acids. The present findings also support contention from earlier studies conducted by Castelluccio et al. [49] where the order of effectiveness in inhibiting LDL oxidation mirrored this sequence. Comparing the mono- and diphenolic acid, combination of two acid-phenols as in rosmarinic acid lead to an increase in antioxidant efficacy [48]. Balasubashini et al. [50] reported the beneficial effect of ferulic acid on oxidative stress in diabetic subjects. A significant decrease in the levels of TBARS, hydroperoxides and free fatty acids were observed in the liver along with an increase gluthathione and antioxidant enzymes content particularly gluthathione peroxidase, superoxide dismutase and catalase. Caffeic acid derivatives were reported to inhibit several enzymes, including 5-lipoxygenase, aldose reductase, protein kinase C, that are involved in pathological processes, such as allergic diseases, inflammation, cataracts and neuropathies. Their activity was found to be dependent on their antioxidant function, amphiphilic characteristics and partial affinity for intracellular membrane systems [51].

The flavonoids were marked by greater antioxidant activities compared to BHT, BHA and Trolox. Ascorbic acid lacking a phenolic structure had low activities in the assays tested. Similar low antioxidant efficacies

were observed for ascorbic acid compared to certain dietary polyphenols [52]. However, great variability in antioxidant activity was seen among the reference compounds as well as different patterns of antioxidant capacity depending on the method used. This justifies the use of a multimethod approach for the evaluation of the antioxidant propensities of particular compounds. The reference compounds had lower percentage activities in their ability to protect against $OH^{\bullet}$ dependent deoxyribose degradation and this may reside on solubility restrictions.

It is critical to bear in mind that the reported antioxidant activities is based on phenolic derivatives as present in plants using in vitro models. However, several studies have shown that phenolics are extensively metabolized in vivo, mainly during transfer across the small intestine, by colonic microflora and in the liver, resulting in significant alteration in their redox potentials [53,54]. After undergoing phase I deglycosylation, the phenolic aglycones are converted to glucuronides, sulphates and *o*-methylated derivatives during phase II metabolism [55]. Thus, to delineate the prophylactic potential of phenolic compounds, it is essential to screen the efficacy of these phenolic derivatives as bioavailable in vivo using cell systems and animal models [13]. The physiological significance of dietary antioxidants depends on their mechanism of absorption and biotransformation, thus warranting further investigations on the bioavailability of the polyphenols. Furthermore, elucidating the mechanisms of action of these compounds in several physiological processes including cellular signal transduction, cell proliferation and differentiation, apoptosis, inflammation may yield important insights into their prophylactic uses.

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CHARACTERIZATION OF THE PHENOLIC PROFILE OF ENDEMIC MAURITIAN *CHASSALIA* SPECIES AND ASSESSMENT OF THEIR ANTIOXIDANT ACTIVITIES

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ABSTRACT

Accumulating biochemical, clinical and epidemiological evidence supports the chemopreventive effects of phenolic antioxidants against oxidative-mediated disorders. The current investigation examined the phenolic composition and antioxidant propensities of eight endemic Chassalia species of the Rubiaceae family, some of which being on the verge of extinction. The total phenolic content of the plant extract ranged from 662 to 3627 mg / 100g DW. The highest flavonoid level was observed in C lanceolata subspp. latifolia 1906 mg / 100g DW while the highest proanthocyanidin content was measured in C. capitata with 281 mg / 100g DW. TLC and HPLC analyses of the total extracts showed the presence of several flavonoid glycosides, namely rutin, hyperoside, isoquercitrin, kaempferol glucoside, quercitrin, and chlorogenic acid. Based on the TEAC and FRAP assays the antioxidant potential of the extracts decreased in the following order: C. boryana > C. lanceolata subsp. latifolia > C. capitata > C. coriaceae var. coriaceae > C. coriaceae var. johnstonii > C. grandifolia > C. petrinensis > C. lanceolata subsp. lanceolata. For both antioxidant assays marked correlation was obtained with total phenolics ($r > 0.8$), flavonoid ($r > 0.75$) content, while weaker correlation was observed with proanthocyanidin content ($r \geq 0.5$). The present study suggests that some of the endemic Chassalia species can be potential sources of natural antioxidants, thus warranting urgent conservation program.

Keywords: *Chassalia*, Mauritian endemic plants, conservation program, phenolic, flavonoid, antioxidant activities, TEAC, FRAP

INTRODUCTION

Cellular redox state is recognised as a critical component of stress-induced cellular responses and disease. Although the level of reactive oxygen species (ROS) is usually kept in check by the body's antioxidant system, these entities when produced in excess are capable of damaging cellular components and accumulating evidence suggests that they may contribute to various diseases (Southorn and Powis, 1988). ROS are not only associated with lipid peroxidation leading to pathological conditions, but are also involved in the deterioration of food products resulting in the alteration of organoleptic properties (Kocchar, 1993).

Like animals, plants also have developed a strategy to preserve their cellular integrity or to overcome the deleterious effects of ROS in situation of oxidative stress. They react by enhancing the expression of multiple, synergistic, antioxidant mechanisms, which include the synthesis of primary and secondary metabolites. Among these natural substances, polyphenolics are a particularly attractive class ubiquitously distributed in the plant kingdom (Haslam, 1998). Interest in plant phenolics has increased greatly because of their roles as antioxidants and scavengers of free radicals and their potential effects on human health (Bors et al., 1990; Salah et al., 1995). Recent studies have revealed that phenolics can exert modulatory actions in cell by interacting with a wide spectrum of molecular targets central to the cell signaling machinery (Chung et al., 2003; Levites et al., 2002).

The Mascarene Islands originally covered by dense tropical forests have experienced the rapid transformation of native ecosystems and extinction of endemic species since their colonisation by man around the early sixteenth century (MacDonald et al., 1991). In Mauritius, the indigenous vegetation consists of only small relict patches in addition to the ten nature reserves covering some 585 ha. (Lorence and Sussman, 1986). With 45% (corresponding to about 311 species) of endemic plants, our small volcanic island is rightly considered as being the hotspot of endemism among the Mascarene

Islands. Among all the species recorded, 153 are classified as endangered, of which, 113 (73.5%) are endemic. 54 species are currently known with 10 individuals or less, of which 40 are endemic (Page 1995). Some of these critically endangered species include *Hyophorbe amaricaulis* (1 specimen), *Diospyros angulata* (1 specimen) and *Badula reticulata* (1 specimen) amongst others (Page 1995).

Members of the *Chassalia* genus, noted for their beautiful coral-shaped flowers, have unreservedly rarefied with species like *Chassalia boryana* being reduced to only one individual in the wild (D. Florens & G. d'Argent, 2001,Personal Communication; Page, 1995). These plants, that once were common under-storey shrubs, form part of those remnants that are now degraded and over-run by numerous exotic plants, like *Lignum robustrum*, and *Psidium cattleianum* Sabine, and introduced animals (Baker, 1877; Lorence and Sussman, 1986). Like for most other Mauritian endemic species, very little data are available on the phytochemical and prophylactic potential of the *Chassalia* species. In line with our research interest on Mauritian endemic species and with the looming danger of these plants to become extinct, the present study was aimed towards the screening of their phytochemical profile and the assessment of their antioxidant potentiality for prophylaxis with the view to providing further justification to ongoing conservation program.

MATERIALS AND METHODS

Plant material

Mature leaves were collected from Le Pétrin Conservation management area (**Table 1**), Mauritius. They were weighed, washed with distilled water and stored at -20° C within 2 hours following collection. Voucher specimens were deposited at the Mauritius Herbarium, Mauritius Sugar Industry research Institute, Réduit and at the Department of Biosciences, University of Mauritius.

Table1 Details of Mauritian endemic *Chassalia* species studied

Specimen Collection	Nature Reserves	Harvest Dates
Chassalia boryana	Mondrain	June 11 2001
Chassalia coriacea var *coriacea*	Perrier	October 4 2001
Chassalia coriacea var *johnstonii*		
Chassalia capitata	Pétrin	November 16 2001
Chassalia petrinensis		
Chassalia grandifolia	Brise Fer (Old Plot)	
Chassalia lanceolata subsp *lanceolata*		
Chassalia lanceolata subsp *latifolia*		November 2 2001

Extraction

Extraction of plant material was carried out according to Bahorun et al., (1994).

Analysis of Total Phenolic Content

The total phenolic content of the extract was estimated using the Folin-Ciocalteu method adapted from Singleton and Rossi (1965).

Total Flavonoid Content

The AlCl3 method adapted from Lamaison and Carnet (1990) was used for the determination of the total flavonoid content of the extracts.

Total Proanthocyanidin Content

The modified acid / butanol assay of Porter et al., (1986) was used to quantify the total proanthocyanidin content.

Thin Layer Chromatography (TLC)

Flavonoids and phenolic acids from the total extracts as well as standards were separated by TLC using ethyl acetate: formic acid: water (8:1:1 v/v) solvent system adapted from Wagner et al. (1984) quoted from Lamaison et al., (1990).

High-performance liquid chromatography (HPLC)

Chromatographic analyses of total and hydrolysed plant extracts were carried out using a HP1100 series HPLC as described by Bahorun et al., (1994). The identification and quantification of the compounds were carried out from

(i) the retention time and
(ii) peak area in comparison with authentic standards.

Quantitative analysis of phenolic acid and flavan-3-ols in the plant extracts

Gallic acid, (+) catechin, (-) epicatechin, (-) epicatechin gallate, (-) epigallocatechin, (-) epigallocatechin gallate, procyanidins B1 and B2 were identified and quantified by comparison with authentic standard in duplicates. Absorption wavelength was selected at 280 nm with a 65 mins running time.

Hydrolysis of flavonoid conjugates and quantitative analysis of aglycones released

Hydrolysis of flavonoid conjugates was carried out essentially as described by Crozier et al., (1997).

Trolox Equivalent Antioxidant Capacity (TEAC) assay

The total antioxidant activity of the extracts was assessed by the TEAC assay according to the method of Campos and Lissi (1996).

Ferric Reducing Antioxidant Power (FRAP) assay

The method of Benzie and Strain (1996) was employed to assess the reducing power of the extracts.

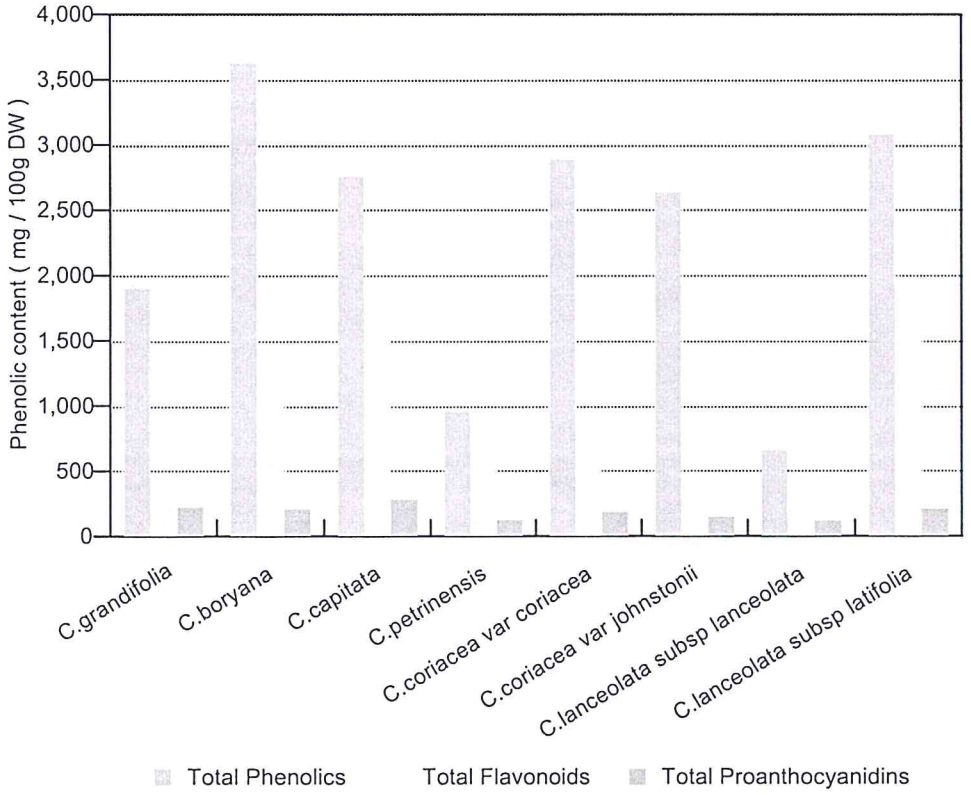
STATISTICAL ANALYSIS

Results are expressed as mean value ± standard deviation (n = 3). Simple regression analysis was performed to calculate the dose-response relationship of standard solutions used for calibration as well as for the test samples. Linear regression analysis was performed, quoting the correlation coefficient rxy.

RESULTS AND DISCUSSION

In recent years, there has been a surge towards the use of phytochemicals with antioxidant activity. Natural antioxidants have applications not only in the modern phytotherapy but also in the food and cosmetic industries. The present study evaluates the phenolic profile and antioxidant capacity of eight *Chassalia* species endemic to Mauritius. *Chassalia boryana* and *Chassalia lanceolata* subsp *latifolia* contained the highest level of phenolics with 3 627 mg and 3 077 mg GAE / 100 g dry material respectively (**Figure 1**). It is noteworthy that the two variants, *Chassalia coriacea* var *coriacea* and *Chassalia coriacea* var *johnstonii*, along with *Chassalia capitata* had comparatively the same amount of total phenols with 2 888 mg, 2 657 mg and 2 759 mg GAE / 100 g DW respectively. On the other hand, *Chassalia grandifolia*, *Chassalia petrinensis* and *Chassalia lanceolata* subsp *lanceolata* contained the lowest levels with only 1901 mg, 954 mg and 662 mg GAE / 100 g DW respectively. In situating the phytochemical content of *Chassalia* species in the context of literature data, the present

Figure 1: Total Phenolic, flavonoids and proanthocyanidins content of Mauritian endemic *Chassalia* species



study shows that the *Chassalia* species contain moderate to high level of phenolic compounds. Using similar methodology, Kähkönen et al., (1999) determined the phenolic content of 92 edible and non-edible plant materials (berries, fruits, vegetables, herbs, cereals, tree materials, plant sprouts and seeds) and reported levels of (0.2 – 1.3) mg GAE / g DW in cereals, (0.4 – 6.6) mg GAE / g DW in vegetables, and (12.4 – 50.8) mg GAE / g DW in berries. Herbal extracts contained (9.1 – 23.1) mg GAE / g DW whereas for the non-edible tree materials examined (excluding pine cork and birch bark) relatively high contents of phenolics (17.5 – 155.3 mg GAE / g DW) were reported (Kähkönen et al. ,1999). Katsube et al., (2004) indicated that the phenolic content of 25 Japanese medicinal plants screened varied between 4.4 µmol and 188.5 µmol EGCG equivalent / g of sample.

Assessment of the flavonoid subclass show that *Chassalia lanceolata* subsp *latifolia*, *Chassalia capitata* and *Chassalia boryana* had maximum flavonoids amounting to 1906 mg, 1853 mg and 1453 mg / 100 g DW respectively. The other species had comparatively lower flavonoid content (< 1000

mg / g DW). The flavonoid levels of the two variants differed considerably; the amount quantified in *Chassalia coriacea* var *coriacea* (845 mg / 100 g DW) being nearly twice as high as that in *Chassalia coriacea* var *johnstonii* (486 mg / 100 g DW). The lowest flavonoid concentration was measured in *Chassalia lanceolata* subsp *latifolia* with only 149 mg / 100 g dry matter.

Analysis of proanthocyanidins (**Figure 1**) revealed that these compounds had very limited distribution in all the eight *Chassalia* species and these were found to occur in the range of 120 mg to 281 mg / 100 g DW. The highest levels were noted in *Chassalia capitata* (281 mg / 100 g DW) and the lowest in *Chassalia lanceolata* subsp *lanceolata* (120 mg / 100 g DW).

TLC is a rapid and useful technique for the identification of individual compounds from complex mixtures. Using this methodology, our data show that hyperoside was present in all species except *C. grandifolia*, while only trace amounts of isoquercitrin were observed in the *C. boryana* (**Table 2**). Although a high concentration of kaempferol-3-glucoside was detected in *C.boryana*, lower levels were observed in *C. capitata*, *C. lanceolata* subsp *latifolia* and *C. lanceolata* subsp *lanceolata*. Quercitrin was present only in the two variants. Furthermore, an unidentified polar phenolic with an Rf of 0.07 was prominent in all the species.

Table 2 Thin Layer Chromatography data showing the distribution of leaf flavonoids in Mauritian endemic *Chassalia* species
(System: Ethyl acetate / Formic acid / Water: 8:1:1 (v / v / v); detection: DPBAE 360 nm)

Species / Rf		Phenolic Acid and Flavonoid Compounds Detected					
		Rutin	Chlorogenic acid	Hyperoside	Isoquercitrin	K3G	Quercitrin
		(-0.35)	(0.48)	(0.63)	(0.66)	(-0.73)	(0.83)
L1	C. grandifolia	++	+	-	-	-	-
L2	C. boryana	+++	+++	++	-	+++	-
L3	C. capitata	+++	+++	++	+	++	-
L4	C. petrinensis	++	++	++	-	-	-
L5	C. coriacea var coriacea	+++	+++	+	-	-	++
L6	C. coriacea var johnstonii	+++	+++	++	-	-	++
L7	C. lanceolata subsp lanceolata	++	++	++	-	tr	-
L8	C. lanceolata subsp latifolia	+++	+++	+++	-	++	-

Note: (+++): Very prominent; (++): Prominent; (+): Present; tr: Trace; (-): not detected

HPLC data confirmed the TLC results obtained (**Table 3**). In addition flavan-3-ol derivatives, namely (+) catechin, procyanidin dimers B1 and B2, were also identified in the extracts. In a previous study on the Mauritian endemic *Trochetia* genus several flavan-3-ols were also detected (Lai Fang et al., 2000) with however their level being much higher in the *Chassalia* species. It is noteworthy that the high antioxidant activities of teas, red wine and cacao products are ascribed mainly to the presence of flavan-3ol derivatives (Miura et al., 2000). Other studies have also shown that flavanols have a protective effect in neurodegeneration (Choi et al., 2001; Levites et al., 2003; Mandela and Youdim 2004). Pretreatment with (-) EC attenuated neurotoxicity induced by oxidized low-density lipoprotein in mouse-derived striatal neurons, as evidenced by apoptotic DNA fragmentation and caspase-3 activation (Schroeter et al., 2001). (-) EGCG protects against 6-OHDA and MPP+ induced human neuroblastoma cell damage (Levites et al., 2002). The *Chassalia* species therefore represent a potential source of prophylactic flavan-3ol derivatives.

Table 3 HPLC data showing the distribution of some leaf flavonoids in the *Chassalia* species

R_T / SPECIES		*C. grandifolia*	*C. boryana*	*C. capitata*	*C. petrinensis*	*C.coriacea var coriacea*	*C.coriacea* var *johnstonii*	*C.lanceolata* subsp *lanceolata*	*C.lanceolata* subsp *latifolia*
Procyanidin B1	(15.210)	+	Tr	-	++	-	-	+++	++
(+) Catechin	(18.951)	+	-	tr	+++	-	-	-	-
Chlorogenic Acid	(21.428)	tr	++	++	+	tr	tr	++	+++
Procyanidin B2	(25.244)	-	-	-	tr	-	-	+++	+++
Isoquercitrin	(67.573)	-	Tr	-	+	+	++	++	+
Hyperoside	(66.462)	++	Tr	tr	+	++	+++	+	Tr

Note: (+++): Very prominent; (++): Prominent; (+): Present; tr: Trace; (-): not detected

The TEAC values of the extracts varied from 41 µmol trolox / g DW (*C. lanceolata* subsp *lanceolata*) to 379 µmol trolox / g DW (*C. boryana*) while the FRAP values ranged from 497 mmol Fe2+ / g DW (*C. lanceolata* subsp *lanceolata*) to 3 743 mmol Fe2+ / g DW (*C. boryana*) (**Table 4**). Based on the TEAC and FRAP assays the antioxidant activities of the extracts was in the following order: *C. boryana* > *C. lanceolata* subsp. *latifolia* >> *C. capitata* > *C. coriaceae* var. *coriaceae* > *C. coriaceae* var. *johnstonii* > *C. grandifolia* >> *C. petrinensis* > *C. lanceolata* subsp. *lanceolata*. Furthermore, the two variants – *C. coriacea* var *coriacea* and *C. coriacea* var *johnstonii* had approximately the same

Table 4 Antioxidant activities of Mauritian endemic *Chassalia* species

Plant Species	TEAC	FRAP
C.grandifolia	157.40 ± 1.0	1 053.28 ± 2.2
C.boryana	379.52 ± 5.4	3 743.01 ± 12.2
C.capitata	268.27 ± 2.8	1 762.24 ± 5.7
C.petrinensis	80.94 ± 0.4	563.72 ± 6.1
C.coriacea var *coriacea*	185.29 ± 2.4	1 407.24 ± 53.2
C.coriacea var *johnstonii*	174.06 ± 2.8	1 285.55 ± 32.1
C.lanceolata subsp *lanceolata*	41.85	497.24 ± 1.0
C.lanceolata subsp *latifolia*	378.27 ± 2.0	3 314.69 ± 1.9

All analyses are mean of triplicate measurements ± standard deviations.
[a] Results expressed in µmol trolox equivalent / g DW.
[b] Values are expressed in units of µmol Fe (II) / g DW.

redox capacity. Comparing the antioxidant acitivity of the *Chassalia* genus with other plants in the literature, the present study clearly shows that some of the endemic *Chassalia* species are relatively good antioxidants. Cook et al., (1998) reported that the antioxidant activities of 61 West African indigenous food plants were in the range of 3.9 to 26.3 µmol trolox equivalent / g DW. Ivanova et al., (2005) screened the phenolic content and antioxidant activity of 21 plants used in the Bulgarian phytotherapy for the treatment of respiratory, gastrointestinal and other inflammatory disorders; the TEAC value of the plant extracts ranged from 0.09 mM to 7.05 mM trolox equivalent.

Linear regression analysis showed a very strong correlation between the antioxidant activities and the total phenolic (TEAC: r = 0.90; FRAP: r = 0.84) and flavonoid (TEAC: r = 0.85; FRAP: r = 0.76) content, while a lower correlation was observed with the proanthocyanidin content (TEAC: r = 0.66; FRAP: r = 0.50). Using the TEA0C and FRAP assays, Luximon et al. (2002) reported that antioxidant activity of the *Cassia fistula* (Indian Labernum) was strongly related to its total phenolics (TEAC: r = 0.99; FRAP: r = 0.95) and proanthocyanidin content (TEAC: r = 0.98; FRAP: r = 0.90). Using different lipid peroxidation model, Hsieh and Yen (2000) observed a direct relationship between the polyphenolic content and antioxidant activities of *Eucommia ulmoides* (Du Zhong) leaf extracts.

Similar positive correlation between phenolic content and antioxidant activities has also been reported in medicinal plants (Katsube et al., 2004), fruits (Luximon-Ramma et al., 2003), tea (Luximon-Ramma et al., 2005), vegetables (Velioglu et al., 1998; Bahorun et al., 2004) and cell cultures (Bahorun et al. 2003).

There is general consensus that a number of assays should be used when assessing the antioxidant activity as no one method can give a comprehensive prediction of antioxidant efficiency. A combination of rapid, sensitive and reproducible methods should be used whenever an antioxidant activity screening is designed. This 'screening' approach can be used to rule out direct antioxidant activity in vivo: a compound that is poorly effective in vitro will not be any better in vivo. Although several screening approach have been proposed, the trolox equivalent antioxidant capacity (TEAC) and the ferric reducing antioxidant power (FRAP) have been used in the present study to evaluate the antioxidant potential of the plant extracts. Their simplicity, reliability and reproducibility make these assays very popular as evidenced by literature data.

CONCLUSION

The accelerating rate at which the world's botanical resources are being depleted threatens many plant species with extinction at a magnitude unparalleled in human history. Likewise, several Mauritian endemic plants are facing a great challenge for survival due to prolific exotic plant and animal competitors. Their disappearance will deprive us the golden opportunity to exploit biologically active compounds which they might harbour; this notion is clearly illustrated with the endemic *Chassalia* genus. The present study shows that some of the *Chassalia* species, particularly *C. boryana* and *C. lanceolata* subsp *latifolia*, are characterised by high phenolic content and potent antioxidant efficacy; thus making them potential candidate to be considered as functional food, nutraceuticals and nutriceuticals. Although the *in vitro* studies carried out facilitate the primary evaluation of the plant extracts, *in vivo* settings are essential to delineate their prophylactic potential; hence further research is needed in this regard.

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Free Radicals and Antioxidants in Cardiovascular Health and Disease

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ABSTRACT: Current hypotheses favour the concept that lowering oxidative stress can have a health benefit. Free radicals can be overproduced or the natural antioxidant system defenses weakened, first resulting in oxidative stress, and then leading to oxidative injury and disease. Cardiovascular disease is one example of this process. This disorder continues to be the major cause of premature death worldwide. Oxidation of human low-density lipoproteins is considered an early step in the progression and eventual development of atherosclerosis, one of the leading causes to cardiovascular dysfunction. Compelling support for the involvement of free radicals in disease development originates from epidemiological studies showing that an enhanced antioxidant status is associated with reduced risk of several diseases. Dietary nutraceuticals such as vitamins C, E and polyphenolics and reduction of cardiovascular disease incidence are a notable example. This paper reviews the biology of ROS/RNS, their pathways through which they relate to the pathology of cardiovascular disease and discusses the putative roles that antioxidants, including phenolics, may play in controlling oxidative stress and reduce the incidence of cardiovascular disease.

KEY WORDS: Oxidative stress, cardiovascular disease, atherosclerosis, inflammation, cell signaling and transduction mechanisms, antioxidants, dietary phenolics.

INTRODUCTION: Cardiovascular disease (CVD) is the leading cause of death in the United States, Europe and Japan[1] and is poised to become the most significant health problem worldwide. According to the World Health Organisation (WHO), an estimated 17 million people die of CVDs, particularly heart attack, stroke and heart failure, every year. In Mauritius deaths due to cardiovascular dysfunctions have kept on increasing for the last 10 years attaining the 51% mark in 2004[2]. Cardiovascular disease is of multifactorial etiology associated generally to a variety of risk factors for its development including hypercholesterolaemia, hypertension, smoking, diabetes, poor diet, stress and physical inactivity amongst others. During the last few decades, research data has prompted a passionate debate as to whether oxidation, or specifically, oxidative stress mediated by free radicals/reactive oxygen species (ROS)/reactive nitrogen species (RNS), is a primary or secondary cause of many chronic diseases. As a result, scientific resources have focused to a large extent on the role that antioxidants could play to delay or prevent oxidative stress and consequently the incidence of chronic disorders. This article will review the biology of ROS/RNS, their pathways through which they relate to the pathology of cardiovascular disease. We shall also discuss the roles that antioxidants may play in controlling oxidative stress and reduce the incidence of CVDs.

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BASIC CONCEPTS OF FREE RADICALS, REACTIVE NITROGEN SPECIES, REACTIVE OXYGEN SPECIES AND OXIDATIVE STRESS:

A free radical is any chemical species (capable of independent existence) possessing one or more unpaired electrons, an unpaired electron being one that is alone in an atomic or molecular orbital. Free radicals are formed from molecules via the breakage of a chemical bond such that each fragment keeps one electron (free radicals may also be formed by collision of the non radical species by a reaction between a radical and a molecule -which must then result in a radical since the total number of electrons is odd), by cleavage of a radical to give another radical and, finally via redox reactions [3,4,5]. Radicals are generally less stable than non-radical species, although their reactivity varies **(Table 1)**.

Free radicals and reactive oxygen/nitrogen species of importance in living organisms include hydroxyl ($OH^{\cdot}$), superoxide ($O_2^{\cdot-}$), nitric oxide ($NO^{\cdot}$), nitrogen dioxide ($NO_2^{\cdot}$) and peroxyl ($ROO^{\cdot}$). Peroxynitrite ($OONO^-$), hypochlorous acid (HOCl), hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$), ozone (O_3), nitrous acid (HNO_2) and dinitrogen trioxide (N_2O_3) are not free radicals but can easily lead to free radical reactions in living organisms. The term 'reactive oxygen species' (ROS) and 'reactive nitrogen species' (RNS) is a collective term that includes not only the radicals but also the non-radicals. Oxidative stress is the term referring to the imbalance between generation of reactive oxygen species and the activity of the antioxidant defences.

Humans and other aerobes are able to tolerate oxygen (O_2) because, at the same time that organisms were evolving electron-transport chains and other enzyme systems to utilize this molecule, antioxidant defenses to protect against the toxic effects of O_2 were evolving in parallel. The aerobic life-style offers great advantages, but is fraught with danger. ROS/RNS are constantly generated in vivo via two main types of processes. ROS/RNS can arise from accidental generation; this encompasses such mechanisms as 'leakage' of electrons onto O_2 from mitochondrial electron transport chains; nuclear membrane, endoplasmic reticulum (xenobiotic metabolism, prostaglandin synthesis) and hepatocytes (detoxification) contain electron transport systems, cytochrome P-450 and b_5, which produce free radicals[6]. Accidental generation also includes the direct reaction of autoxidisable molecules with molecular O_2, generating superoxide free radical. The major biological process leading to $O_2^{\cdot-}$ generation is the electron transport associated with mitochondrial membrane; ubiquinone-cytochrome *b* is the most important site of $O_2^{\cdot-}$ production. It has been estimated that about 1-3% of O_2 respired is converted to $O_2^{\cdot-}$, a rate that increases during periods of increased energy metabolism. $O_2^{\cdot-}$ also is produced by phagocytic cells (neutrophils, monocytes, macrophages, eosinophils) and helps them to inactivate viruses and bacteria. When these cells encounter a phagocytable particle, their O_2 consumption increases tremendously ('respiratory burst') with the activation of a membrane-located enzyme (NADPH-oxidase) which catalyse the reduction of O_2 into $O_2^{\cdot-}$. $O_2^{\cdot-}$ participate in the production of very reactive chemical species such as $OH^{\cdot}$, hypochlorite and chloramines **(Figure 1)**. The importance of ROS production by the immune system is clearly exemplified by patients with granulomatous disease[7]. These patients have defective membrane-bound NADPH oxidase system thus cannot produce $O_2^{\cdot-}$, resulting in multiple and persistent infection, especially *Staphylococcus aureus*. $O_2^{\cdot-}$ is also generated by a variety of cytosolic and membranes-bound enzymes, including xanthine oxidase, cytochrome 450 complex and phospholipase A_2.

Many biomolecules undergo autooxidation reaction on contact with O_2 producing ROS, for example catecholamines, tetrahydrofolates and reduced reduced flavins react directly with O_2 to form $O_2^{\cdot-}$[8]. Several sugars, including glucose, react with proteins to produce oxygen radicals. It has been suggested that years of exposure of body tissues to elevated blood glucose in diabetic patients can result in them suffering an 'oxidative stress' that may contribute to the side effects of hyperglycaemia [9].

H_2O_2 is a nonradical. It resembles water in its molecular structure and is very diffusible within and between cells. H_2O_2 is able to diffuse across biological membranes, whereas $O_2^{\cdot-}$ does not. As well as arising from dismutation of $O_2^{\cdot-}$, H_2O_2 is produced by the action of several oxidase enzymes *in vivo*, including amino acid oxidases and the enzyme xanthine oxidases [10]. Xanthine oxidase catalyses the oxidation of hypoxanthine to xanthine, and of xanthine to uric acid; oxygen is simultaneously reduced both to $O_2^{\cdot-}$ and to H_2O_2. Xanthine oxidase is present in many mammalian tissues, especially in the gastrointestinal tract [11]. Phagocytic cells generate substantial amount of H_2O_2 that is responsible

for the cytotoxic action observed in localized tissue inflammation. Peroxisomes contain several ROS generating enzymes including glucose oxidase, amino acid oxidase, xanthine oxidase, glycollate and urate oxidase, as well as flavoprotein oxidases [12]. The major by-product of these oxidases is H_2O_2, thus explaining the high level of catalase present in peroxisomes which detoxifies H_2O_2 to H_2O. Much of the toxicity of $O_2^{\cdot-}$ and H_2O_2 involves formation of $OH^{\cdot}$ which is the most reactive free radical in vivo with an estimated half-life of about 10^{-9} sec In the presence of H_2O_2, superoxide act as the precursor of hydroxyl radical ($OH^{\cdot}$) **(Figure 1)**. This reaction is very slow and cannot occur in the absence of catalyst; iron ion acts as catalyst for this reaction. The superoxide anion reduces Fe^{3+} to Fe^{2+}. In the presence of Fe^{2+}, H_2O_2 readily decompose into $OH^{\cdot}$ and OH^{-}. This reaction is known as the *Fenton reaction* [13]. Thus, the simultaneous presence of superoxide anion, H_2O_2, and iron ion lead to the production of hydroxyl radical. Copper ions also react with H_2O_2 to form $OH^{\cdot}$. Hydroxyl radical may also be formed by exposure of living organisms to ionising radiation which causes fission of O-H bonds in water, to give $H^{\cdot}$ and $OH^{\cdot}$ [14].

The bioavailability of metal ions is strictly control under normal physiological condition. After absorption from the gut, metal ions are complexed to transport proteins (transferrin, ceruloplasmin). Excess metal ions are stored coupled to storage proteins such as ferritin, haemosiderin. Hydroxyl radical generation can take place when the homeostasis is altered. For example, tissue injury may cause the release of metal ions from damaged cells, contributing to a worsening of the injury. Hydroxyl radical reacts at a diffusion-controlled rate with almost all molecules in living cells. Hence, when hydroxyl radical is formed *in vivo*, it damages whatever it is generated next to, as hydroxyl radical cannot migrate any significant distance within the cell. $OH^{\cdot}$ attacks proteins, DNA, polyunsaturated fatty acid (PUFA) and many other biological molecules.

HOCl is produced by the neutrophil-derived enzyme myeloperoxidase at sites of inflammation and when activated neutrophils infiltrate reoxygenated tissue[15]. The enzyme oxidizes chloride ions in the presence of hydrogen peroxide **(Figure 1)**. HOCl is not a free radical, but it is a potent chlorinating and oxidizing agent. HOCl reaction with cholesterol causes chlorohydrins that could disrupt cell membranes, leading to cell lysis and death. On the basis of this observation, the cholesterol chlorohydrins have been suggested to be potential biomarkers for oxidative damage associated with neutrophil/monocyte activation[16]. HOCl can attack many other biological molecules. Thiols and thioethers are particularly reactive and other compounds, including ascorbate, urate, pyridine nucleotides and tryptophan are oxidized by HOCl. The main biological chlorination reactions are with amine groups to give chloramines; with tyrosyl residues to give ring chlorinated products; with unsaturated lipids to give chlorohydrins and ring chlorination of cytosine resides in nucleic acids [17].

$NO^{\cdot}$ is formed from the oxidation of L-arginine by nitric oxide synthase (NOS) of which three isoforms are known. $NO^{\cdot}$ has a variety of functions, including memory formation, synaptic plasticity and synaptogenesis. It is thought that the endothelium derived-relaxing factor (EDRF) produced by vascular endothelium, which is an important mediator of vascular responses induced by several pharmacological agents (including brandykinin) is identical to $NO^{\cdot}$ [18,19].

Excess $NO^{\cdot}$ is cytotxic, both directly (e.g. by combining with tyrosine) and indirectly, by forming $ONOO^{-}$. Since $NO^{\cdot}$ relaxes smooth muscle in blood vessel walls resulting in lower blood pressure, $O_2^{\cdot-}$ by removing $NO^{\cdot}$ can be a vasoconstrictor. Thus, excess vascular $O_2^{\cdot-}$ production could contribute to hypertension and vasospasm[20]. $ONOO^{-}$ formed in blood vessel walls may aggravate atherosclerosis by depleting antioxidants and causing peroxidation of LDL. Furthermore, nitration of tyrosine by $ONOO^{-}$ may interfere with cell signal transduction [21]. A role for $NO^{\cdot}$ has also been demonstrated in such human diseases as malaria where $NO^{\cdot}$ appears to be partly involved in resistance to malarial infection, in cardiovascular disease, acute inflammation, cancer, neurodegenerative diseases, and diabetes. Moreover $NO^{\cdot}$ has been implicated in adult respiratory distress syndrome, septic shock, hypertension, thrombosis, renal failure, AIDS encephalopathy, bronchospasm, stroke and male impotence [22].

Table 1 Chemical Reactions of Free Radicals*

Unimolecular Radical Reactions

Reactions result from the instability of the first formed radical. The radicals may completely decompose or rearrange before reaction with other molecules or radicals present.

***Decomposition*:**	reaction in which the radical decomposes to give a stable molecule and a new radical
***Rearrangement*:**	1. breaking of an adjacent C-C bond in a cyclic system with concomitant formation of a new bond, usually carbonyl and a new isomeric radical 2. migration of an atom, via intramolecular abstraction by the radical center, thus creating a new, isomeric radical.

Radical-Molecule Interactions

***Addition to unsaturated systems*:**

1. Addition of a radical to an olefinic double bond to give a new saturated, radical. Typical reaction is the radical induced polymerization of olefins.
2. Addition of a radical to an aromatic double bond. This intermediate step is widespread in free radical chemistry, e.g. in the radical substitution of aromatic compounds (homolytic aromatic substitution). The net overall reaction is displacement of an aromatic substituent by a radical:

$$AR — X + Y^{\cdot} \rightarrow AR — Y + X^{\cdot}$$

***Abstraction or displacement: S_H2 reactions*†**

– Biomolecular reaction involving homolytic attack of a radical on a molecule. The radical attacks a univalent atom, usually a terminal halogen or hydrogen in an abstraction reaction to give rise to a new radical, e.g.

$$Ph^{\cdot} + CBrCl_3 \rightarrow {}^{\cdot}CCl_3 + PhBr$$

– Homolytic substitution at multivalent atoms also occurs but both do not normally occur at saturated carbon centers.

Reaction with oxidizing agents

Radicals readily undergo 1-electron oxidations with oxidizing reagents of suitable redox potential to give positive ions. Example is the Meerwein reaction, which involves the oxidation of cinnamyl derived radicals by cupric ions:

$$Ph\dot{C}HCHRCO_2\,Et + Cu^{2+} \rightarrow PhC^{+}HCHRCO_2Et + Cu^{+}$$

Radical-Radical Interactions

Dimerization or radical coupling

Localized radicals (methyl, phenyl radicals) react readily with little chance of dimerization. Only delocalized radicals have a high probability of dimerization in solution. Thus,

$$R'^{\cdot} + R'''^{\cdot} \rightarrow R' - R''$$

When R' = R", the reaction is dimerization and when R' ≠ R" the reaction is radical coupling or combination.

Radical disproportionation

Involves collision of the radicals resulting in the abstraction of an atom, usually hydrogen, by one radical from the other. This leads to the formation of two stable molecules, with the atom abstracted being β to the radical center:‡ e.g. the disproportionation of two phenylethyl radicals to give styrene and ethylbenzene.

* The reader is referred to Moad and Solomon [5] for an extensive overview of the reaction sequences highlighted in this table and an examination of the complex chemistry of organic radicals.

† S_H2 stands for substitution homolytic biomolecular.

‡ The disproportionation reaction derives its driving force from the formation of two newstrong bonds and from the fact that the β-CH bonds in radicals are usually weak.

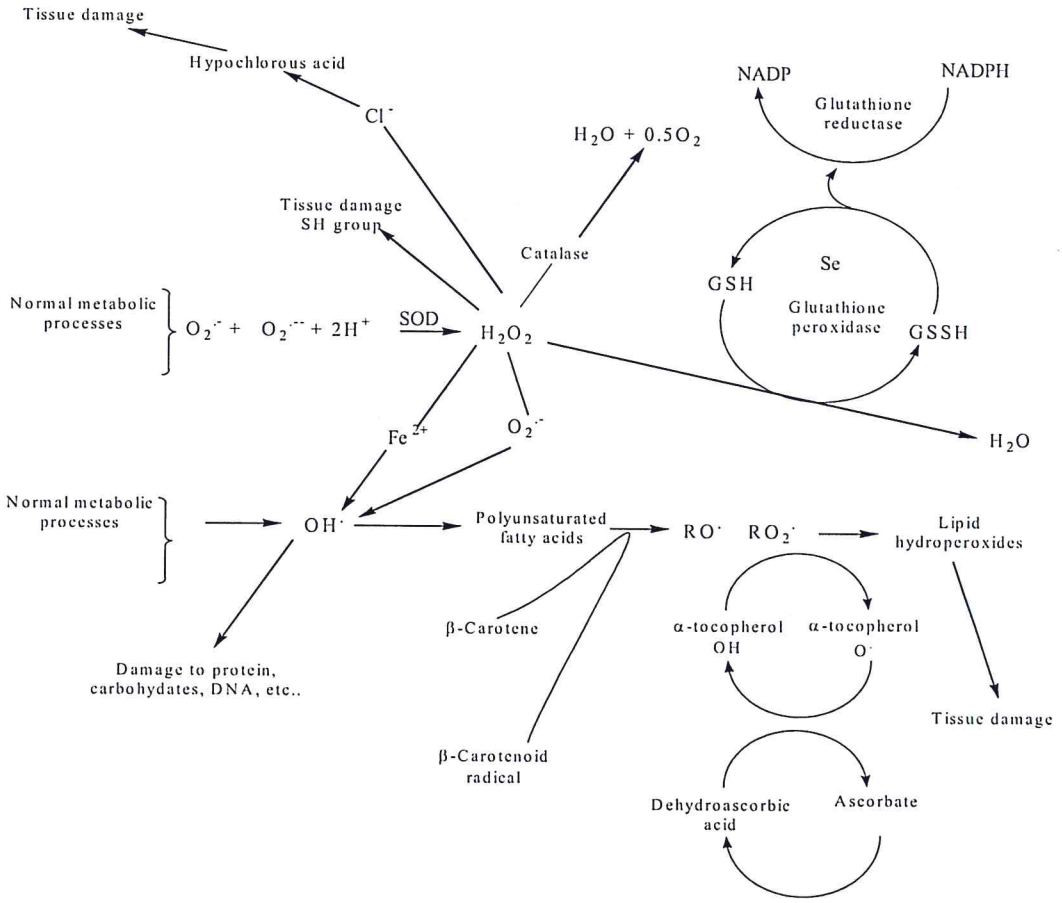


Figure 1: Inter-relationship between reactive oxygen species and antioxidants.

REACTIVE OXYGEN SPECIES AND THE CARDIOVASCULAR SYSTEM:

Reactive oxygen species have been considered deleterious to cell function and there is good evidence to suggest that they play a role in the pathophysiology of cardiac disease states. However, direct cause and effect relationships have not been clearly delineated. The increase in the generation of ROS under several pathophysiological conditions, that seem to be related to inflammatory processes, is still to be comprehensively understood; this may be due to difficulties in defining their site of origin. Impaired mitochondrial reduction of molecular oxygen may be an intracellular source. Secretions by phagocytic white blood cells, dysfunctional endothelial cells, or the auto-oxidation of catecholamines may be the extracellular sources. ROS may also result from cellular injury due to exposure to ionizing radiation, ultraviolet rays, cigarette smoking or other air pollutants. Besides their deleterious effects, ROS are also now being recognized as important regulators of cell function and modulators of cell signalling pathways.

ROS in pathophysiology of Heart disease:

One of the strategies used to assess the role of oxidative stress in the pathogenesis of cardiac dysfunction has been to expose isolated cardiac tissues to a defined oxidation stress condition and study the resulting effects [23-31]. Further in vivo and ex vivo studies have provided precious evidence supporting the role of oxidative stress in a number of conditions (atherosclerosis, ischemia-reperfusion injury, hypertension, catecholamine-induced cardiomyopathy, diabetic cardiomyopathy, cardiac hypertrophy and congestive heart failure etc...) leading to severe cardiovascular dysfunctions. In this review the

role of ROS in atherosclerosis is being emphasized as, besides being considered as the major cause of morbidity and mortality[32] its outcome is also linked to other conditions leading to cardiovascular disorders. The role of ROS in other above-mentioned conditions has been extensively reviewed and the reader is referred to a number of excellent reports[33, 34].

Most cardiovascular events are secondary to atherosclerosis, a disease of the arteries involving a local thickening of the vessel wall.

A stroke or myocardial infarction occurs when the lumen of the vessel becomes completely occluded, usually by a thrombus forming at the site of a plaque. Atherosclerotic lesions are thought to be initiated by emigration of monocytes into the arterial inner core (tunica intima), recruited by adhesion molecules, possibly in response to arterial endothelium injury[35]. A variety of factors have been implicated in causing this initial injury, including mechanical damage from flow stress worsened by high blood pressure, viral infection (herpes viruses and cytomegalovirus), exposure to blood-borne toxins such as xenobiotics from cigarette smoke and elevated levels of normal metabolites, such as glucose, homocysteine or cholesterol[36]. Although a high level of plasma cholesterol is considered to trigger atherosclerosis, the oxidation of cholesterol seems to be a necessary step. In fact, uptake of oxidized low-density lipoprotein ($_{ox}$LDL) was shown to be an early event leading to the development of atherosclerosis **(Figure 2)**. $_{ox}$LDL and oxidized lipoproteins have been reported to stimulate $O_2^{\bullet-}$ formation leading to apoptosis of cells in the umbilical vascular wall; this was prevented by treatment with antioxidants SOD and catalase[37]. In cultured human coronary artery smooth muscle cells, low levels of $_{ox}$LDL stimulate the extracellular matrix synthesis indicating the involvement of oxidative stress in the pathogenesis of atherosclerosis[38]. High levels of $_{ox}$LDL were apoptotic implicating the additive role of ROS in increased plaque vulnerability; this effect was reduced by probucol and catalase [38]. Patients with atherosclerosis and hypercholesterolemia showed higher susceptibility of LDL to oxidation in comparison to patients treated with lipid-lowering agents such as lovastatin and probucol[39].

In the atherosclerotic lesion produced in the rabbit aorta, significant increases in the iron content were observed suggesting that iron-catalysed free radical reactions may be associated with the development of atherosclerosis[40]. The occurrence of intracellular Ca^{2+}-overload has been proposed as a mechanism of injury due to oxidative stress because human endothelial cells subjected to oxidative stress showed an increase in the level of intracellular Ca^{2+} and plasma membrane blebbing[41]. Endothelial dysfunction may play an important role in the atherosclerotic process because in patients with atherosclerosis, the antioxidants, probucol and ascorbic acid, improved the endothelium-dependent relaxation suggesting the involvement of ROS in endothelial dysfunction[42]. Increased production of $O_2^{\bullet-}$ has been implicated in the impaired endothelium-dependent relaxation in cholesterol fed rabbits and was suggested to be an early event in the hypercholesterolemic atherosclerotic process[43]. Oxidative inactivation of $NO^{\bullet}$ by superoxide has been proposed as a plausible explanation for endothelial dysfunction[44]. When exposed together, $O_2^{\bullet-}$ and $NO^{\bullet}$ react with each other three times faster than the reaction rate of $O_2^{\bullet-}$ with either Mn^{2+} and Cu^{2+}/Zn^{2+}-SOD[45]. Therefore, $O_2^{\bullet-}$ would preferentially react with $NO^{\bullet}$ rather than SOD and cause inactivation of NOd. In human atherosclerotic arteries, the production of endothelial nitric oxide synthase (the enzyme catalysing $NO^{\bullet}$ formation) as well as $NO^{\bullet}$ has been shown to be depressed[46]. SOD was shown to protect the inactivation of $NO^{\bullet}$ in the canine coronary artery [47]. The generation of $O_2^{\bullet-}$ was thought to be due to the activation of the vascular and endothelial enzyme NADH/NADPH oxidase[42]. Moreover, an increase in NADH/NADPH oxidase-dependent vascular $O_2^{\bullet-}$ was reported in hypercholesterolaemic rabbits[48]. Oxidation of $NO^{\bullet}$ by $O_2^{\bullet-}$ results in the formation of peroxynitrite which could initiate lipid peroxidation or play a role in the oxidation of lipoproteins[49,50]. Both of the above may be important steps in the development of atherosclerosis.

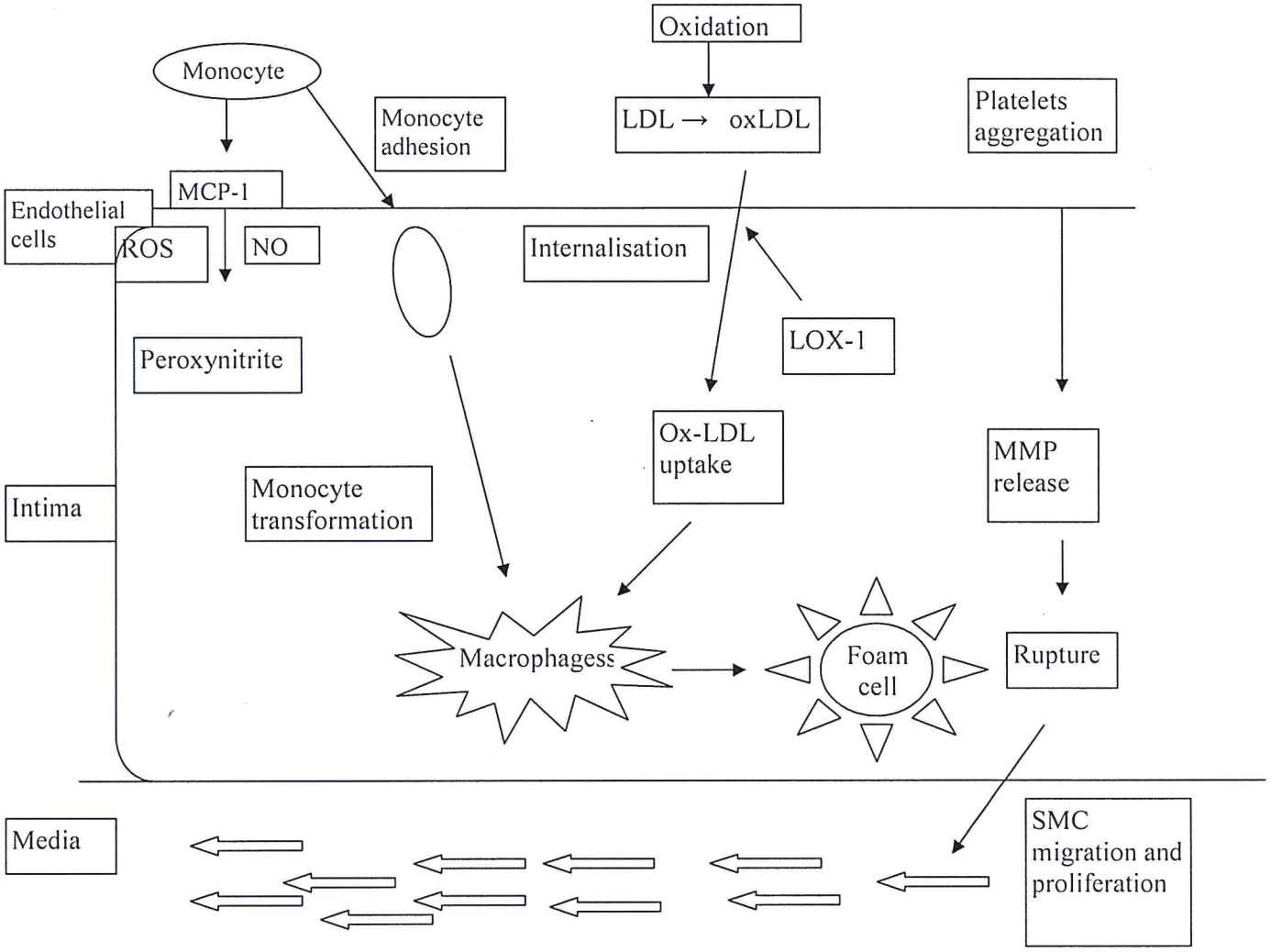


Figure 2: ROS and atherosclerosis: increased production of ROS may affect four fundamental mechanisms that contribute to atherosclerosis (i) oxidation of LDL to oxLDL; (ii) endothelial cell dysfunction; (iii) vascular smooth muscle cells migration and proliferation as well as MMPs release; (iv) monocyte adhesion and migration as well as foam cell development due to uptake of ox-LDL.

ROS in Mediated Signal Transduction Pathways in Cardiovascular disorders:

The cardiovascular system is a highly complex, well organised system in which signal transduction plays critical physiological and pathophysiological roles (**Figure 3**). The cellular elements of the heart and vascular wall are equipped with an array of specific receptors and with complex intracellular machinery that facilitates and drives appropriate responses to extracellular stimuli. All vascular cell types, including endothelial cells, smooth muscle cells, adventitial fibroblasts, and resident macrophages, produce ROS[51-55]. Of particular importance in the vasculature are superoxide ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2), since these ROS act as inter- and intra-cellular signaling molecules. The major source of ROS in the vascular wall is non-phagocytic NADPH oxidase, which is regulated by vasoactive agents

(Ang II, ET-1, thrombin, serotonin), cytokines (IL-1, TNFa), growth factors (PDGF, IGF-1, VEGF) and mechanical forces (cyclic stretch, laminar and oscillatory shear stress). High levels of low-density lipoprotein (LDL), especially in the form of oxidized low-density lipoprotein (ox-LDL), have also been shown to increase intracellular ROS generation. Under physiological conditions, vascular production of ROS and the consequent activation of redox-dependent signaling pathways and induction of redox-sensitive genes are tightly regulated. However, in pathological conditions, such as in hypertension, atherosclerosis, hyperlipidemia, hyperhomo-cysteinemia, and diabetes, where generation of ROS is increased and the renin angiotensin system may be upregulated, these redox-sensitive events may contribute to cellular processes involved in vascular dysfunction and structural remodeling [56-58].

Redox signalling has been suggested in vascular smooth muscle proliferation, atherosclerosis, angiogenesis, cardiac hypertrophy, fibrosis[59]. Modulation of intracellular signaling pathways (MAPKs), and the subsequent activation of downstream redox sensitive transcription factors like NF-κB, HIF-1, AP-1 results in alterations in gene and protein expression[60] that significantly enhance cardiac dysfunction. Increased bioavailability of vascular ROS leads to VSMC growth, migration, collagen deposition, and altered MMP activity, important factors in arterial remodeling in cardiovascular disease [56-58]. In endothelial cells, oxidative excess induces apoptosis and aniokis (cell shedding), leading to endothelial cell loss and resultant impaired endothelial function. In addition, oxidative stress stimulates activation of transcription factors (e.g., NFkB and AP-1) and pro-inflammatory genes (cytokines, interleukins), upregulation of adhesion molecules (e.g., ICAM, VCAM, PECAM), stimulation of chemokine production (e.g., MCP-1) and recruitment of inflammatory cells (monocytes, macrophages), critical processes involved in vascular inflammation and injury[61]. Increased vascular $^{\cdot}O_2^-$ and H_2O_2 also impair endothelium-dependent relaxation, increase contractile reactivity and alter vascular tone. These effects may be mediated directly by elevating cytosolic Ca^{2+} concentration or indirectly by reducing concentrations of the vasodilator $NO^{\cdot}$. A common link between free radicals and the aforementioned pathological condition is the disrupted intracellular signal transduction networks and this suggests a rationale for targeting these pathways in chemoprevention.

ANTIOXIDANTS AND THEIR RELEVANCE TO CARDIOVASCULAR DISEASE:

An antioxidant has been defined as "any substance that, when present at low concentrations compared with those of an oxidizable substrate, significantly delays or prevents oxidation of that substrate"[38]. When ROS/RNS are generated in vivo, their actions are opposed by intricate and coordinated antioxidant lines of defence systems[61]. These include enzymatic and non-enzymatic antioxidants that keep in check ROS/RNS level and repair oxidative cellular damage **(Figure1)**. The major enzymes, constituting the first line of defence, directly involved in the neutralization of ROS/RNS are: superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) **(Figure 1)**[61]. SOD is a cytoplasmic and mitochondrial enzyme, which accelerate the dismutation of superoxide. There are three forms of SOD: an extracellular and an intracellular copper/zinc (Cu/Zn) and a mitochondrial, manganese (Mn) SOD. All three forms catalyse the dismutation of $O_2^{\cdot-}$ to H_2O_2. Because SOD enzymes generate H_2O_2, they work in collaboration with H_2O_2-removing enzymes. CAT, an exclusively peroxisomal enzyme in most tissues, converts H_2O_2 to water and O_2. However, the most important H_2O_2-removing enzymes are the selenoprotein GPx enzymes. GPx enzymes remove H_2O_2 by using it to oxidize reduced glutathione (GSH) to oxidized glutathione (GSSG). Glutathione reductase, a flavoprotein enzyme, regenerates GSH from GSSG, with NADPH as a source of reducing power **(Figure 3)**. Glutathione peroxidase also catalyse the reduction of unstable hydroperoxides at the expense of GSH [62].

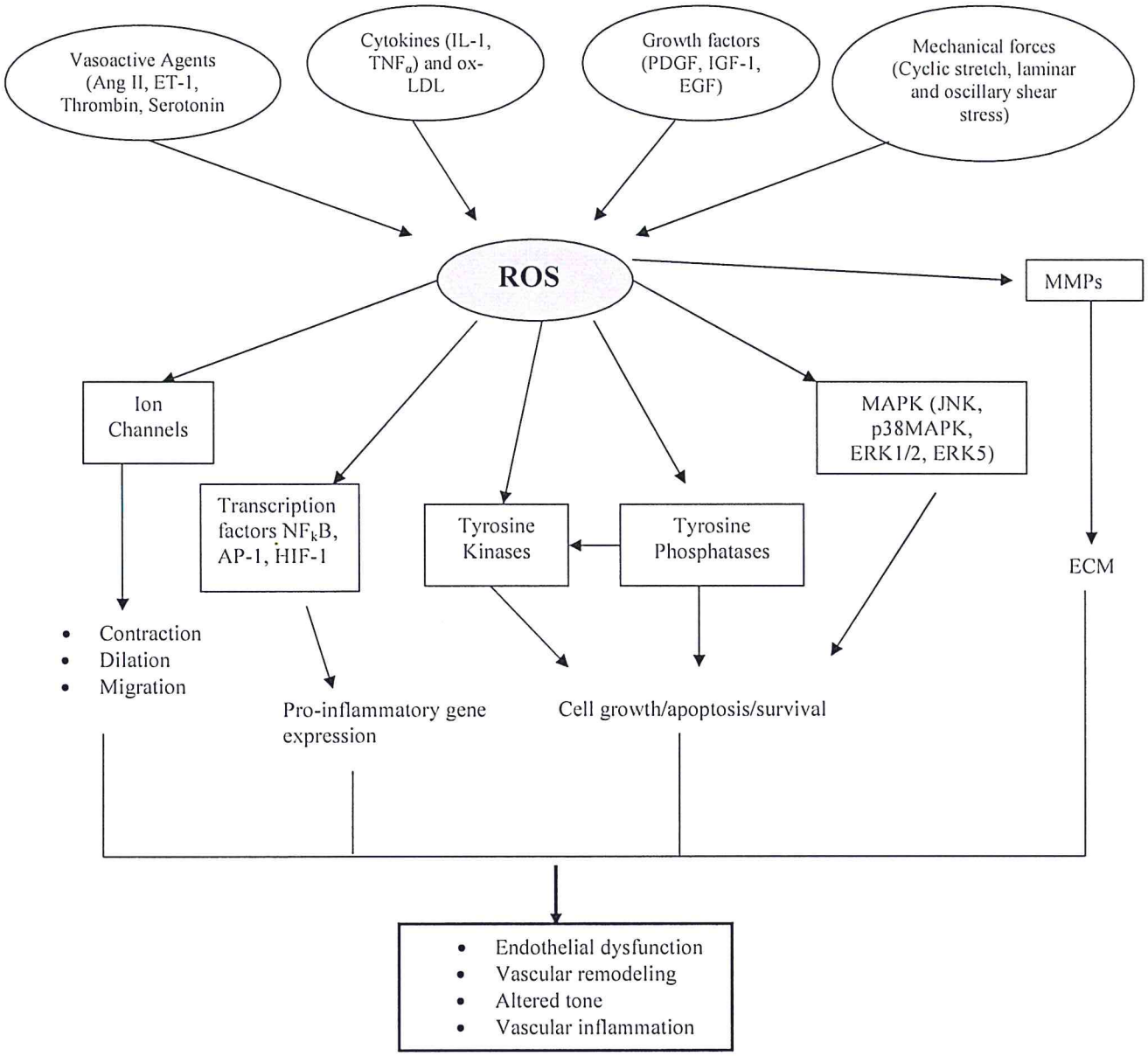


Figure 3: Redox-dependent signaling pathways in vascular smooth muscle cells: The increase ROS which is produced from NSDPH oxidase may modify the activity of tyrosine kinases, such as Src, Ras, JAK2, Pyk2, PI3K, and EGFR, as well as mitogen-activated protein kinases (MAPK), particularly p38MAPK, JNK and ERK5. ROS may inhibit protein tyrosine phosphatase activity, further contributing to protein tyrosine kinase activation. ROS also influence gene and protein expression by activating transcription factors, such as NF□B activator protein-1 (AP-1) and hypoxia-inducible factor-1 (HIF-1). ROS stimulate ion channels, such as plasma membrane Ca^{2+} and K^{+} channels, leading to changes in cation concentration. Activation of these redox-sensitive pathways results in numerous cellular responses which, if uncontrolled, could contribute to hypertensive vascular damage.

The second line of defence is represented by radical scavenging antioxidants such as vitamin C, vitamin A and plant phytochemicals like phenolics (emphasised later in this review) that inhibit the oxidation chain initiation and prevent chain propagation [63]. This may also include the termination of a chain by the reaction of two radicals. The repair and de novo enzymes act as the third line of defense by repairing damage and reconstituting membranes. These include lipases, proteases, DNA repair enzymes and transferases [64].

A number of studies have been conducted to explore the roles of various antioxidants in cardiovascular diseases. Diphenylene iodonium (DPI), a potent inhibitor of NADH/NADPH oxidase enzyme has been shown to suppress p38 MAP kinase-mediated VSMC hypertrophy *in vitro*[65]. Furthermore, it was reported that *N*-acetyl-*L*-cysteine (NAC), a radical scavenger and intracellular glutathione precursor, inhibited endothelin-induced ROS generation, JNK activation and VSMC proliferation[66]. Probucol is a modestly potent LDL-lowering agent with powerful anti-oxidant properties that effectively inhibits the oxidative modification of LDL, independent of its lipidlowering effect. By exerting anti-oxidant effect, it may inhibit VCAM-1 and MCP-1 expression and inhibit human aortic SMC proliferation as well as atherogenesis [67].

The HMG-CoA reductase inhibitors, also known as statins, are potent lipid-modifying agents. There is overwhelming evidence from clinical studies that reducing plasma LDL levels with statins, results in a markedly lower risk of cardiovascular events related to atherosclerosis[68]. Recent studies in patients with established CAD show that these agents can cause a modest regression of atherosclerotic lesions. It has been suggested that the antiatherosclerotic effect of statins may be independent of their LDL-lowering effect [69,70].

AT1R blockers and ACE inhibitors are widely used to treat patients with hypertension and/or congestive heart failure by blocking the effect of Ang II or its formation. Recent studies show that Ang II is also a strong stimulus for ROS generation[71-73] and AT1R blockers as well as ACE inhibitors inhibit the expression of pro-atherogenic factors by decreasing ROS production in vascular endothelial cells and in animal models[74-77]. A large number of studies have provided direct evidence showing the antiatherosclerotic effects of these agents[78-80]. Statins and AT1R blockers exert synergistic effects on the inhibition of atherosclerotic lesions in the apo E-knockout mice placed on a high cholesterol diet[81].

PPAR-γ ligands, which are widely used in the treatment of type II diabetes, have been identified as potent antioxidants[82]. By suppressing NADPH oxidase expression and reducing intracellular ROS production, these agents inhibit the expression of several proatherogenic proteins and apoptosis in vascular endothelial cells and SMCs[83,84]. Experimental studies show that thiazolidinedione, a potent PPAR-γ ligand, reduces the size and number of atherosclerotic lesions in the vessel wall by modulating foam cell formation and inflammatory responses of macrophages[85].

Vitamins E and C have been demonstrated to reduce the progression of atherosclerosis. Trolox C, a water-soluble vitamin E analogue, and vitamin C (ascorbic acid) abolished the stimulatory effect of Ang II on JNK and p38 activity in VSMC[86]. Intake of vitamin E decreased the incidence of cardiovascular events in the population of ischemic heart disease patients in the Cambridge Heart Antioxidant Study (CHAOS)[87]. Clinical studies show that while these anti-oxidant vitamins do not reduce endpoints related to atherosclerosis, they improve endothelial function by increasing local NO bioavailability and, therefore, endothelium-dependent vasodilation. It has been reported that high dose of vitamin C infusions improved endothelial dysfunction in patients with renovascular hypertension[88]. However, a GISSI-3 study[89] and HOPE study[90] could not show significant beneficial effects of vitamin E in the secondary prevention of coronary artery disease. A Heart Protection Study (HPS) in the UK also could not demonstrate any benefits of vitamin E, vitamin C, and β-carotene combined antioxidants therapy in a large number of high-risk people[91].

There is increasing interest in phenolics stemming from the context of the "French paradox"[92]. This paradox refers to the correlation of a high-fat and high-cholesterol diet with a lower incidence of coronary heart disease found in Mediterranean cultures and contrasted with a higher incidence of coronary heart disease among most Western cultures. It has been shown that the French paradox may be attributable to regular consumption of red wine and that the unique antiatherogenic effects of red wine reside in the action of polyphenols. Phenolic compounds or polyphenols constitute one of the most numerous and ubiquitously distributed group of plant secondary metabolites, with more

than 8000 phenolic structures currently known. Natural polyphenols can range from simple molecules (quinones, phenolic acids,) to highly polymerised compounds (lignins, melanins, tannins), with flavonoids such as flavonols, flavones, isoflavones, flavonones, flavanols and anthocyanins representing the most common and widely distributed sub-group [93] **(Table 2)**. Phenolics are therefore an integral part of the diet, with significant amounts being reported in vegetables, fruits, teas and traditional plants[94-97]. Although the dietary intake of phenolics varies considerably among geographic regions, it is estimated that daily intake range from about 20 mg to 1 g, which is higher than that for Vitamin E[98]. Epidemiological evidence indicates that consumption of fruit, vegetables and teas may reduce the risk of cardiovascular disease and it is increasingly suggested that this may due to their antioxidants that include ß-carotene, vitamin C, vitamin E and polyphenolics. Dietary antioxidant phenolics may quench reactive oxygen and nitrogen species and, hence potentially modify pathogenic mechanisms relevant to cardiovascular disease. The effectiveness of a dietary antioxidant will depend on a number of factors, such as which ROS or RNS is being scavenged, how and where they are being generated and the accessibility of the antioxidant to possible sites of damage[99]. Many phenolic compounds have been shown to have antioxidant activity in vitro [100] and several observational studies support their role in potentially protecting against cardiovascular disease[101,102]. However, not all epidemiological studies have found a protective effect of dietary phenolics against heart disease[102, 103]. In vitro studies have used various systems to oxidize LDL and then measure prevention of oxidation after inclusion of phenolic compounds like cinnamic acids [104]. A range of other compounds such as ellagic acid, dimethydiisoeugenol, phenolic rich extracts derived from olive oil, wine, grape, apple and blackcurrent juices have been shown to act as potent antioxidants inhibiting LDL oxidation *Ex vivo* and/or increasing plasma antioxidant capacity[99] .Tea polyphenols have the inherent capacity to inhibit the development of atherosclerotic lesions by down-regulating genes controlling lipid metabolism, cytokine production and cellular activity within the arterial wall namely genes coding for PPAR-γ, CD36, LXR-α, C-myc coupled with the up-regulation of genes coding for LDL-R and PPAR-α at the transcriptional level[105]. Grape seed proanthocyanidin extract (GSPE), a mixture of 75-80% oligomeric proanthocyanidin and 3-5% monomeric proanthocyanidin has proved its efficacy against the incidence of ischemia-reperfusion injury, apoptosis of cardiomyocytes and reduction of foam cell development. The mechanistic pathways of cardioprotection exerted by the proanthocyanidin rich extract included (1) potent hydroxyl and other free radical scavenging abilities; (2) anti-apoptotic,anti-necrotic and anti-endonucleolytic potentials; (3) modulatory effect on apoptotic regulatory bcl-X_L, p53 and c-myc genes; (4) cytochrome P450 2E1 inhibitory activity; (5) inhibitory effects on proapoptotic, cardioregulatory genes c-JUN and JNK-1 and (6) inhibition of vasoconstriction of vascular smooth muscle and endothelium[106,107].

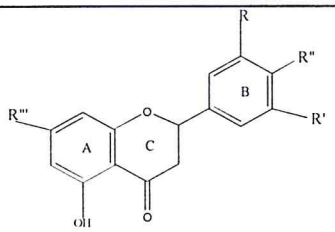
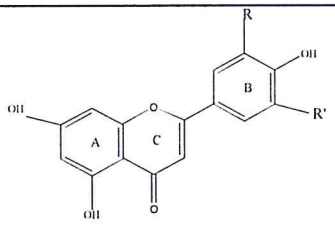
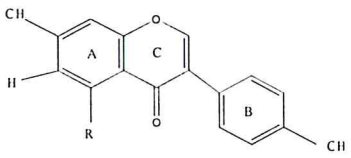
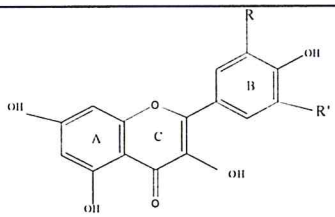
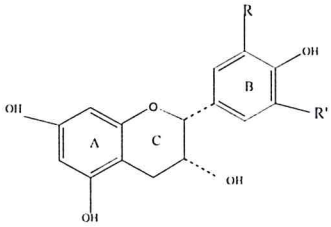
CONCLUSION:

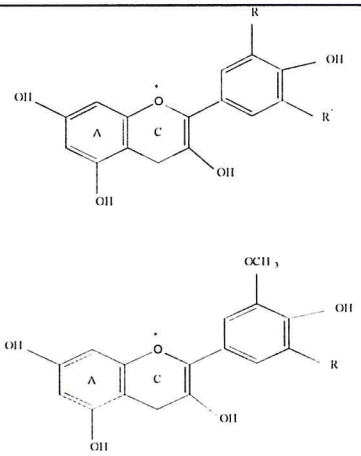
The implication of oxidative stress in the etiology of several chronic and acute degenerative disorders suggests that antioxidant therapy represents a promising avenue for treatment. Strategies for the intervention and prevention of cardiovascular disease require an understanding of the basic molecular mechanism (s) by prophylactic agents (synthetic antioxidants, dietary antioxidant factors from food plants and medicinal plants) that may potentially prevent or reverse the promotion or progression of the disease. It remains unequivocal that emerging scientific support for health claims and identification of active functional ingredients needs to be balanced by addressing toxicological concerns[108]. The real proof of efficacy for existing or novel compounds/extracts should emanate from a demonstration of clinical efficacy on defined therapeutic categories[109]. In this respect, the outcome of one such trial conducted on the Mauritian population on the effects of Mauritian black tea on markers of oxidative stress leading to cardiovascular disease is currently much awaited.

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Table 2: Chemical structures of flavonoids and some examples

Classes	Structural Formula	Examples
Flavanones		R=R^{I}=H, R^{II}=R^{III}= OH; Naringenin R= OH, R^{I}=H, R^{II}=R^{III}= OH; Eriodyctiol R=R^{I}=OH, R^{II}=R^{III}= OH; 5^{I}-OH- Eriodyctiol
Flavones		R=R^{I}=H; Apigenin R= OH, R^{I}= H; Luteolin R=R^{I}=OCH_3; Tricetin
Isoflavones		R = H; Daidzein R = OH; Genistein
Flavonols		R=R^{I}=H; Kaempferol R= OH, R^{I}= H; Quercetin R=R^{I}=OH; Myricetin
Flavanols		R= OH, R^{I}= H; (+)-Epicatechin R=R^{I}=OH; (+)-Epigallocatechin R=OH, R^{I}=H; (–)-Catechin R=R^{I}=OH; (–)-Gallocatechin

Anthocyanidins		R= OH, R^1= H; Cyanidin R= R^1= OH; Delphinidin R= R^1 = OCH_3; Malvidin R= R^1= H; Pelargonidin R= H; Peonidin R= OH; Petunidin

LIST OF ABBREVIATIONS:

Ang II: Angiotensin II; AP-1: activator protein 1; AT1R: Angiotensin II type 1 Receptor; ECM: extracellular matrix; ERK: extracellular receptor kinase; ET-1: Endothelin-1; HOCl: Hypochlorous acid; HMG-CoA: 3-hydroxy 3-methylglutaryl coenzyme A; ICAM: intercellular adhesion molecule; IGF-1: insulin-like growth factor; IL-1: Interleukin-1; JNK: Jun N-terminal kinase; LDL: low density lipoprotein; MAPK: Mitogen-activated protein kinase; MCP-1: monocyte chemoattractant protein-1; MMPs: matrix metalloproteinases; NF-κB: nuclear factor κB; oxLDL: oxidized low-density lipoprotein; PDGF: platelet-derived growth factor; PECAM: platelet endothelial cell adhesion molecule; PPAR-γ: peroxisome proliferator-activated receptor-γ; Ras: small G-protein; RNS: reactive nitrogen species; ROS: reactive oxygen species; SOD: superoxide dismutase; TNFα: tumor necrosis factor alpha; VCAM: vascular cell adhesion molecule; VEGF: vascular endothelial growth factor; VSMC: vascular smooth muscle cell.

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Review

Chemopreventive actions of polyphenolic compounds in cancer

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Abstract. Oxidative stress and associated mechanisms involving inflammation, aberrant signaling pathways and gap junction intercellular communication is increasingly associated with the pathogenesis of various chronic degenerative disorders such as atherosclerosis, neurodegeneration and cancer. Consumption of fruits, vegetables and beverages like teas continue to be suggested to have the capacity to reduce the incidence of cancer. The bioactive compounds including phenolics may be responsible for the chemopreventive effects. While the free radical scavenging and antioxidant properties of phenolics are well established, emerging literature reports suggest that their chemopreventive effects may also be ascribed to their ability to modulate components of cell signaling pathways. This paper reviews the potential chemoprevention role of phenolics with a focus on cellular signal transduction mechanisms and prevention of gap junction intercellular communication relevant to cancer.

Keywords: Cancer chemoprevention, inflammation, cell signaling, redox biochemistry, MAPK, NFκB, gap junction intercellular communication, phenolics

1. Introduction

Cancer is the second leading cause of death, after cardiovascular diseases, in the United States and in many Western and developed/developing countries. Despite many therapeutic advances in the understanding of the processes in carcinogenesis, there has been a constant rise in the cancer prevalence/mortality rate during the past half-century and statistics indicate that men are largely plagued by lung, colon, rectum and prostate cancer whilst women increasingly suffer from breast, colon rectum and stomach cancer [1]. Although the etiology of cancer may be multifactorial (e.g. diet, genetic, environment), there is wide recognition that reactive oxygen and nitrogen species (ROS/RNS) play a pivotal role in the pathophysiological process. ROS/RNS have been shown to be carcinogenic and may exert their deleterious effects by (i) causing DNA damage. (ii) alter cell signaling pathways (MAPK, NFκB, AP-1, PLA, ASK 1). and (iii) modulate gene expression (proto-oncogene, tumour suppressor gene).

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Table 1
Dietary sources of chemopreventive agents known to suppress carcinogenesis

Food source	Bioactive components
Fruits and Vegetables	
Tomatoes	Lycopene, vitamin C
Grapes	Catechins, resveratrol
Tea, chocolate	Catechin, epicatehcin, epigallocatachin, epigallocatechin gallate
Turmeric	Curcumin
Aloe	Emodin
Pomegranate	Ellagic acid
Citrus fruit (peel)	D-limonene, perillyl alcohol, geraniol, menthol, carvone
Citrus fruit	Tangeretin, nobiletin, rutin, vitamin C
Peanuts	Taxifolin
Artichoke	Silymarin
Onions, garlic, scallions, chives	Diallyl sulphide, allyl methyl trisulphide, allicin, ajoene
Horseradish	Sinigrin
Red chilli	Capsaicin
Strawberries, raspberries, blackberries	Caffeic acid, ferulic acid, ellagic acid
Cereals, pulses	Genistein
Mustard seed	Sinigrin, phenolic acids
Honey-bee propolis	Caffeic acid, caffeic acid phenethyl ester
Carrots	α- and β-carotene, lignin
Soybean	Genistein, daidzein, phenolic acids, tocopherols
Plant extracts	
Pinus pinaster	Ferulic acid, proanthocyanidin, pycnogenol
Curcuma longa L.	Curcumin, esculetin
Rosmarinis officinalis	Carnosic acid, rosemarinic acid

Carcinogenesis is a multistage, multimechanism process, involving the interaction of genetic, developmental, sexual, dietary, lifestyle and environmental factors [2]. However, evidence from *in vitro* and *in vivo* laboratory studies, clinical trials and epidemiological investigations show that plant-based diets have protective effect against various cancers. Indeed, it has been suggested that about 7–31% of all cancer worldwide could be reduced by diets high in fruits and vegetables [3]. Such foods are the sources of a myriad of bioactive compounds that have wide variations in their chemical structures and functions (Table 1), possessing potential chemopreventive properties [4,6]. However, a clear demonstration of their bioefficacy in clinical trials remains [7]. Lycopene, a potent antioxidant carotenoid in tomatoes and fruits such as guava, watermelon and papaya, may protect against various forms of cancer, including prostate, cervix, stomach, bladder and colon cancer. Several studies have shown that organosulfur compounds in garlic and onions, isothiocyanates in cruciferous vegetables (such as broccoli, cabbage, cauliflower), and monoterpenes in citrus fruits, cherries, and herbs have anticarcinogenic activities. Several epidemiological studies have consistently shown an inverse association between consumption of fruits and vegetables and the risk of cardiovascular diseases and certain forms of cancer [5]. Although the protective effects have been primarily attributed to the well-known antioxidants, such as vitamin C and vitamin E, plant phenolics may also play a significant role. It is the scope of this review to outline the role of reactive oxygen and nitrogen species in cellular functions and to emphasize their importance in signal transduction pathways associated with cancer. From data obtained from cell culture systems, animal models and clinical studies, the prophylactic importance of a number of phenolic compounds in cancer prevention and gap junction intercellular communication mechanisms is explicated.

2. Phenolics

Phenolics are particularly attractive as prophylactic agents due to their pluripharmacological effects including antibacterial, anti-inflammatory, antiallergic, hepatoprotective, antithrombotic, antiviral, neuroprotective, vasodilatory and anticarcinogenic actions [4–7], which may be attributed to their free radical scavenging and antioxidant activity. Other functions include deactivation of carcinogens, inhibition of phase I and induction of phase II metabolizing enzymes, partial agonists and antagonists of oestrogen receptor, inhibition of enzymes that promote carcinogenesis and expression of mutated genes, as well as promoting detoxification of xenobiotics [8–10].

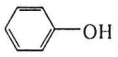
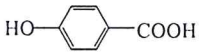
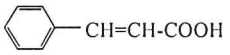
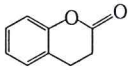
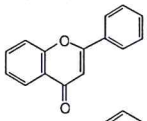
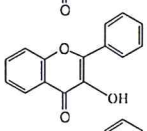
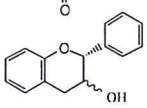
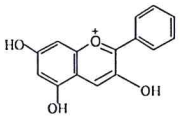
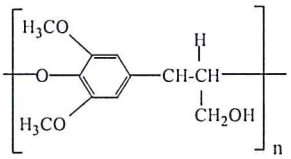
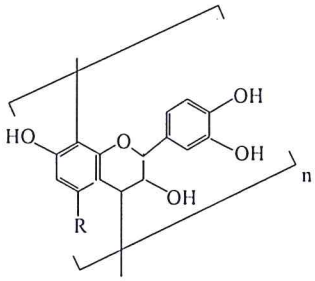
Natural polyphenols can range from simple molecules to highly polymerised compounds with flavonoids representing the most common and widely distributed sub-group with more than 4000 structures described [11]. Flavonoids and other plant phenols share a common origin: the amino acid phenylalanine. Consequently these compounds stem from a common building block in their carbon skeleton, the phenylpropanoid unit, C_6-C_3. This biosynthetic pathway leads to the formation of a wide range of plant phenols: benzoic acids (C_6-C_1), cinnamic acids (C_6-C_3), coumarins (C_6-C_3), flavonoids (C_6-C_3-C_6), proanthocyanidins $(C_6\text{-}C_3\text{-}C_6)_n$ and lignins $(C_6\text{-}C_3)_n$ (Table 2). Phenolics are widely distributed in the plant kingdom and are therefore an integral part of the diet, with significant amounts being reported in vegetables, fruits, and beverages [12–14]. The primary phenols in cereals and legumes are flavonoids, phenolic acids and tannins, while flavonols and flavones are the most abundant phenolics in vegetables and fruits respectively [11]. Although the dietary intake of phenolics varies considerably among geographic regions, it is estimated that daily intakes range from about 20 mg to 1 g, which is higher than that for vitamin E [15]. It is now being recognized that phenolics can exert modulatory actions in cells by interacting with a wide spectrum of molecular targets central to the cell signaling machinery. These include modulation of mitogen-activated protein kinase (MAPK), protein kinase C (PKC), serine/threonine protein kinase Akt/PKB, phase II antioxidant detoxifying enzymes, downregulation of pro-inflammatory enzymes (COX-2 and iNOS) through the activation of peroxisome proliferator-activated receptor gamma (PPARγ), regulation of calcium homeostasis, inhibition of phosphoinositide 3-kinase (PI3K), tyrosine kinases, NFκB, c-JUN, as well as modulation of several cell survival/cell-cycle genes [16,17], (Fig. 1).

3. ROS/RNS, cell signaling and carcinogenesis

Although once considered as 'unavoidable' by-products of aerobic metabolism, ROS/RNS serve important physiological functions. ROS participate in the defense against intrusion of foreign bodies and they also act as modulators of internal biological processes, including cell-cell adhesion, cell proliferation, inflammation, signal transduction, transcription, or apoptosis. However their accumulation beyond the immediate needs of the cell results in damage to biomolecules and alteration of the cell signaling machinery leading to pathophysiological conditions.

Low levels of ROS/RNS are constantly generated as an integral component of normal cellular function. Growing evidence indicates that cellular redox plays an essential role not only in the cell survival but also in cellular signaling systems. Redox regulation can occur at multiple levels in the signaling pathways, from receptor to cellular protein. ROS generation can activates a variety of receptors (such as tyrosine kinase, serine/threonine kinase, G protein-coupled receptors) leading to the stimulation of downstream signaling components including, MAPK, protein kinase C (PKC), phospholipase C-γ (PLC-γ). The activation of PLCγ by ROS/RNS elevates the cellular Ca^{2+} levels through the mobilization

Table 2
Main classes of phenolics compounds

Class	Basic Skeleton	Basic structure	Examples
Simple phenols	C_6		Cresol, thymol
Benzoic acids	C_6-C_1		Gallic acid, vanillic acid
Cinnamic acids	C_6-C_3		*p*-coumaric acid, ferulic acid
Coumarins	C_6-C_3		Umbelliferone, aesculetin
Flavone	C_6-C_3-C_6		Apigenin, luteolin, chrysin
Flavonol			Quercetin, kaempferol, myricetin
Flavan-3ols			Catechin, epicatechin, epigallocatechin
Anthocyanin			Cyanidin, malvidin, delphinidin
Lignins	$(C_6$-$C_3)_n$		
Proanthocyanidins	$(C_6$-C_3-$C_6)_n$		Proanthocyanidin, prodelphinidin

of intracellular Ca^{2+} stores and/or through the influx from extracellular space [18]. Changes in Ca^{2+} levels modulate various other signaling paths (calmodulin-dependent signaling, PLKC-α) and has also been implicated in apoptosis. NFκB activation is another well-studied model of redox regulation which

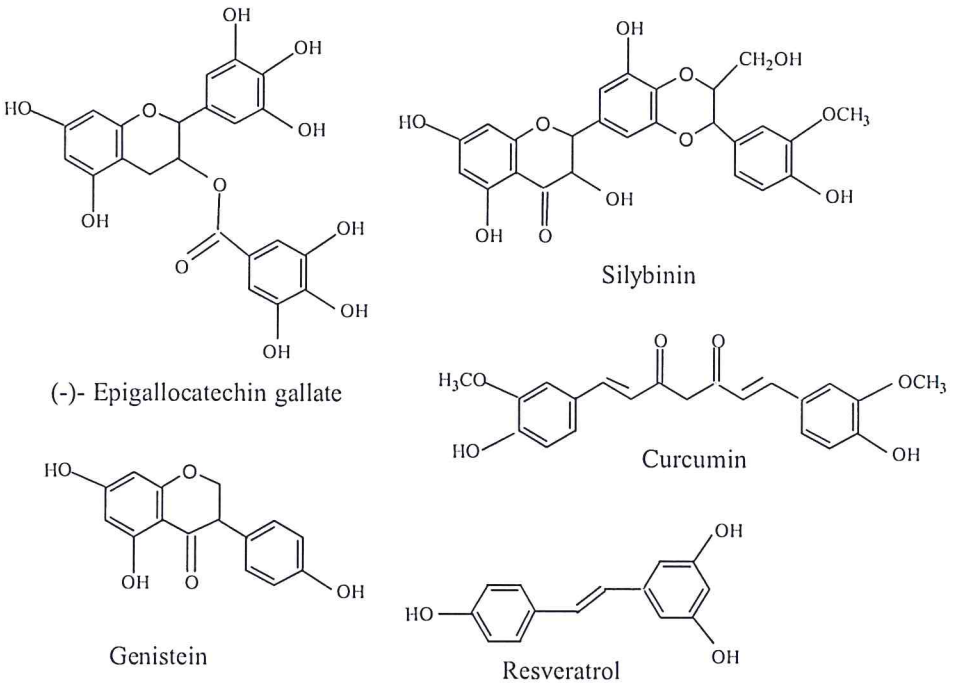


Fig. 1. Chemical structures of phenolics with cancer chemopreventive effects.

may occur via two different mechanisms: (i) ROS/RNS enhanced proteolytic degradation of the NFκB inhibitor IκB. (ii) enhancement of upstream signaling cascades, in particular IκB kinase. Apoptosis is a special form of programmed cell death that plays a crucial role in normal development. Moderate oxidative stress induces apoptosis through the activation of JNK, p53, PI3-K, p85, APO-1/Fas/CD95 receptors [19,20].

ROS production is a common feature of tumour promoters (e.g. 12-*O*-tetradecanoylphorbol-13-acetate – TPA, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, okadaic acid, peroxisome proliferators, steroidal oestrogen, phenobarbital, chlordane and aroclor and UV) and it is implicated in the multistep process of carcinogenesis. Based on the DNA damage pattern, it has been suggested that H_2O_2, which crosses biological membranes easily, can penetrate to the nucleus and react with iron or copper ions to form $OH^{\cdot}$. Although iron and copper ions are normally carefully sequestered in the body, into proteins such as ferritin, transferrin, caeruloplasmin and metallothionein oxidative stress may however cause their release. Alternatively, oxidative stress may also trigger DNA damage by initiating a series of metabolic events within the cell that lead to activation of nuclease enzymes, which cleave the DNA backbone [21,22]. There are three main pathways leading from initial DNA damage by ROS/RNS to subsequent mutations: (1) chemical modification of DNA bases causing a change in their hydrogen bonding specificity; (2) damage to polymerase-specific 'hot spots', which is a likely major contributor to DNA polymerase-mediated mutagenesis; (3) conformational change in the DNA template that diminishes the accuracy of replication by DNA polymerases [21,22].

Although links have been established between oxidative stress and cancer mediated through damage to biomolecules particularly DNA, a number of chemical carcinogens have also been identified as enhancers of neoplastic transformation through mechanism that do not involve direct damage to DNA. Many of these

'nongenotoxic tumor carcinogens' selectively induce the proliferation and/or inhibition of apoptosis in preneoplastic cells [2]. ROS/RNS can contribute to carcinogenesis through the modulation of various cell signaling pathways. Among the main signaling pathways known to be activated by oxidative insults are the MAPK cascades, AP-1, phosphoinositide 3-kinase (PI3-K)/Akt pathway, nuclear factor κB (NFκB), p53, phospholipase C-γ 1 (PLC-γ1), janus protein tyrosine kinase (Jak)/signal transducers and activators of transcription (Stat) pathway, and the heat shock response. Modulation of the different signaling pathways will affect specific gene expression and in general activation of MAPK, PI3-K/Akt, PLC-γ1, Jak/Stat and the heat shock response exert a growth stimulatory effect while phosphorylation of p53, JNK and p38 are more commonly associated with apoptosis [23].

4. Mitogen activated protein kinases (MAPKs)

The redox status of the cell has profound effect on the cell signaling pathways, in particular the MAPK cascade with cell survival or death as a possible consequence. MAPKs, also called extracellular responses kinases (ERKs), are involved in the transduction of extracellular and intracellular stimuli into cellular responses through the phosphorylation of cytosolic or nuclear target proteins and activation of transcription factors, that consequently modulate gene expression. MAPKs can be divided into 3 major subgroups: extracellular signal-regulated kinase-1 (ERK-1, $MAPK^{ERK1/p44}$) and ERK2 ($MAPK^{ERK2/p42}$), c-Jun N-terminal kinases (JNK1/2/3) and the p38 kinases (p38$\alpha/\beta/\gamma/\delta$), all 3 pathways being redox-sensitive [24]. Ultimate targets of MAPKs are several transcriptional regulators such as NFκB, ATF2, CHOP/GADD153, MEF2c, SAP-1, p62TCF/Elk-1, activator protein-1 (AP-1), serum response factor (SRF) and the proto-oncogenes Fos, Myc, and Jun as well as members of the steroid/thyroid hormone receptor super family of proteins; activated MAPKs function as modulators for differentiation, proliferation, cell death, and survival [25].

MAPKs are a group of closely related families of Ser/Thr kinases and for maximal activity the MAPKs requires dual phosphorylation of the tyrosine and threonine residues within the catalytic domain by their respective upstream kinase. MAPKs are, however, not direct substrate for receptor tyrosine kinase (RTKs) but are rather activated by an additional class of kinases, increasing the complexity and diversity of the MAPKs signaling [26]. The simplified core of the MAPKs cascade is a three-tiered signaling module involving the sequential activation/phosphorylation of MAPKKK, MAPKK and MAPK (Fig. 2). Besides the upstream kinases, the activation of MAPK is dependent on the activity of MAPK phosphatases (MKPs) that inactivate MAPK and therefore play an important role in the dynamics of MAPK signaling [24]. Phosphorylated ERK1/2 can activate transcription factors such as mitogen- and stress-activated kinase-1 (MSK1), Elk1, SAP1, Mnk 1/2, pp90 ribosomal S6 kinase (RSK), Stat3 [27]. RSK phosphorylates the Bcl-2 family member Bad, thereby inhibiting its proapoptotic activity. RSK and MSK1 are also potent activators of CREB, a transcription factor for Bcl-2 and, therefore an important factor for cell survival [25].

The role of protein tyrosine kinases in carcinogenesis has been implicated through abnormal EGF receptor signaling. Mutated EGF receptors that are constitutively activated or natural proto-oncogenes (e.g. c-erB-2/neu), that have high levels of signaling capacity may be associated with the development of neoplasia in animals and humans [28]. Thus agents that block EGF receptor signal transduction components may be effective chemopreventive agents against cancer. Several chemopreventive phytochemicals, such as genistein, curcumin and resveratrol, can inhibit the activation of growth factor receptors. Genistein is a potent inhibitor of protein tyrosine kinase and have been suggested to be responsible for the lower rate of breast cancer observed in Asian women consuming high soy diet [29]. EGCG was found to

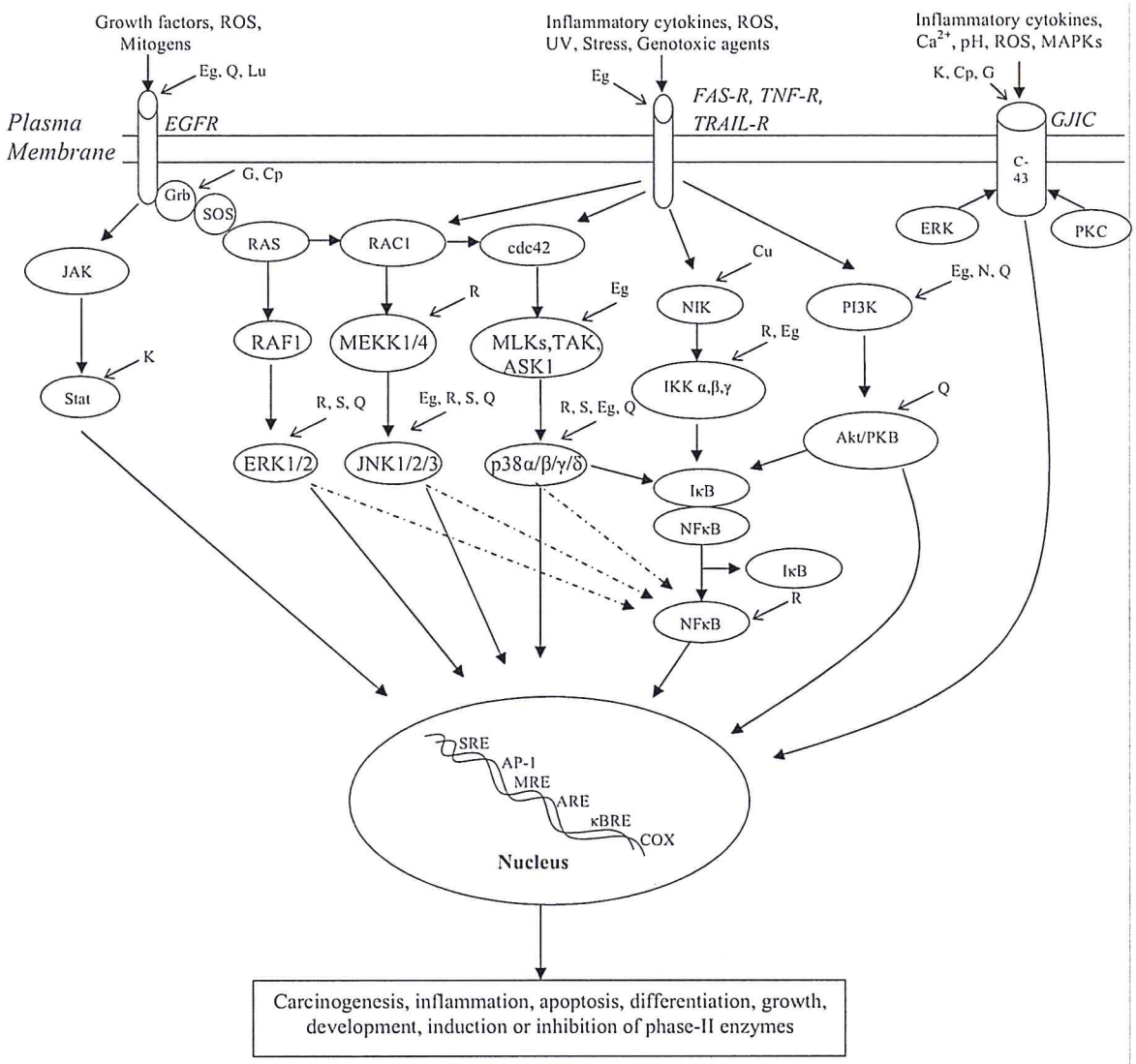


Fig. 2. Schematic representation of the intracellular mitogen-activated protein kinase (MAPK), Jak/Stat, PI3K and NFκB signaling cascades. Potential sites where dietary phenolics may exert modulatory action are indicated. Cp: Caffeic acid phenyl ester, Cu: curcumin, Eg: EGCG, G: Genistein, K: Kaempferol, Lu: Luteolin, N: Narigenin, Q: Quercetin, R: resveratrol; S: Silybinin. (See text for full explanation).

inhibit EGFR autophosphorylation in YCU-N861 and YCU-H891 head and neck carcinoma cell and MDA-MB231 breast carcinoma cell [30,31]. The subsequent downstream activation of MAPK, Stat and Akt were also inhibited by EGCG treatment. The inhibition of EGFR-dependent Stat activation result in a downregulation of VEGF synthesis in cancer cells [31]. Thus, blocking of EGFR signaling by EGCG would potentially inhibit both cancer cell proliferation and angiogenesis. The inhibitory actions of these phytochemicals on growth factor receptors activation have several other potential advantages to patients with late stage cancers. Blockade of EGFR may predispose the cancer cells to apoptosis. Inhibition of

EGFR disables the protein's capacity to provide the cancer cell the matrix independent survival support it requires for proliferation and metastasis [32].

Tea is a popular beverage worldwide. It contains a number of bioactive chemicals and is particularly rich in phenolics. The major tea catechins include (+)-C, (−)-EC, (−)-EGC, (−)-ECG and (−)-EGCG. Of these flavan-3ol derivatives, EGCG is the most abundant and thought to contribute to the beneficial effects of tea such as antioxidant capacities and anticancer. Tea and some of its constituents have a very broad spectrum of cancer chemopreventive activity. Tea has been shown to inhibit tumorigenesis in many animal models, including those cancer of the skin, lung, oral cavity, esophagus, stomach, small intestine, colon, liver, pancreas, bladder and prostate [29]. EGCG inhibited TPA- or EGF-induced transformation of mouse epidermal cell line JB6, and this activity was associated with the inhibition of JNK phosphorylation. Chung et al. [33,34] reported that EGCG treatment inhibited the phosphorylation of MEK1/2, ERK1/2 and ELK-1 as well as c-Jun in 30.7b Ras12 cells (H-ras transformed JB6 cells). Furthermore, EGCG decreased the association between Raf-1 and MEK1 and it competitively inhibited the phosphorylation of ELK-1 by ERK1/2 by competing for the binding site on ERK1/2. Similarly, tea phenolics applied as topical hydrophilic ointment or through drinking water, prevented the UVB-induced activation of ERK1/2, JNK and p38 in the skin of SKH-1 hairless mice [35].

In addition to its implication in DNA damage and tumour initiation, UVB radiation leads to tumour promotion where UVB-induced oxidative stress and activation of various signaling cascades, including receptor and non-receptor tyrosine and serine/threonine kinases, play a major role on cell growth and proliferation leading to clonal expansion of UVB-initiated cells into skin tumours [36]. Silybinin, a naturally occurring flavonoid from milk thistle (*Silybum marianum*), has shown protective effects in several tumorigenesis models including human prostate, breast, cervical as well as chemically-induced skin carcinoma [37]. Studying its mode of action in SKH-1 hairless mouse skin, Gu et al. [38] reported that silybinin inhibits the activation of all 3 MAPKs, namely ERK1/2, JNK1/2 and p38. A down-regulation of the PI3k pathway, with impaired phosphorylation of the downstream signaling molecule Akt, which is also known to play a significant role in tumorigenesis [39].

Silybinin inhibits erbB1 activation leading to the down-regulation of Shc and ERK1/2 pathway in human prostate carcinoma DU145 cells [37]. This impairment of mitogenic signaling selectively induces Kip1/p27 followed by its increased binding to CDKs, causing an inhibition of their enzyme activity. This leads to an impairment of pRb (protein retinoblastoma) hyperphosphorylation, thereby keeping it hypophosphorylated and bound to E2F. Together, these effects cause an arrest in cell cycle progression followed by cell growth inhibition [37].

Naringenin, a flavanone abundant in citrus fuits, inhibits the phosphorylation of p44/p42 MAPK and the PI3k/Akt pathway in MCF-7 breast cancer cells [40]. Studies have shown that cancer cells proliferate faster in response to high levels of glucose [41]. Breast cancer tissues overexpress GLUT1 and the insulin-responsive glucose transporter GLUT4 [42]. Insulin increase cellular glucose uptake by promoting the fusion of cytosolic GLUT4-containing vesicles with the plasma membrane, a process mediated via the PI3K pathway [43]. Naringenin may inhibit MCF7 proliferation by lowering glucose uptake via impairment of the MAPK and PI3K/Akt pathway.

Cell cycle control is a highly regulated process that involves a complex cascades of events mediated through the modulation of the cell cycle-regulatory proteins, including cyclins, cyclin-dependent kinases (CDKs), cyclin-dependent kinases inhibitors (CDKIs), p53 and pRb. Mitogenic signaling cascades also regulate cell cycle progression, possibly by activating CDKs that together with their catalytic subunit cyclins hyperphosphorylate pRb. This phosphorylation releases a number of factors, including the E2F family of transcription factors that promote the expression of several genes, the products of which are important for entering the S phase and for DNA replication. The activities of CDKs are positively regulated

by cyclins and negatively by CDKIs [44]. CDKIs fall into two classes: (1) $p21^{(Cip1/Waf1/Cap20/Sdi1/Pic1)}$, $p27^{(Kip1)}$, and $p57^{(Kip2)}$, having a preference for CDK2 and CDK4/cyclin complexes; (2) $p16^{INK4A}$, $p15^{INK4B}$, $p18^{INK4C}$ and $p19^{INK4D}$, that are specific for CDK4 and CDK6/cyclin complexes [45]. Impairment of a growth stimulatory signaling pathway (such as erbB1, raf, MAPK) has been shown to induce the expression of CDKIs such as $p21^{Cip1}$ and $p27^{Kip1}$ [46,47]. $p^{21Cip1/Waf1}$ is a potent inhibitor protein of CDK/cyclin complexes, which play an important role in G1 and G2 arrest by inhibiting CDK2/cyclin E and CDC2/cyclin B activities respectively. An induced CDKI binds to and subsequently inhibits cyclin – CDK activity, which interferes with hyperphosphorylation of pRb by keeping it in the hypophosphorylated form and bound to E2F, thereby blocking cell proliferation and inducing cell growth arrest [48–50].

EGCG has been shown to induce apoptosis, cell growth inhibition and cell cycle deregulation in DU145 human prostate cancer cells [51]. The same authors observed that treatment of LNcaP cells with EGCG promotes the expression of growth inhibiting genes and repressed genes that belong to the G-protein signaling network. According to Na and Surh [52], EGCG-induced cell cycle dysregulation is achieved by the following mechanisms: (i) upregulation of cyclin dependent kinase inhibitors. (ii) suppression of protein expression of cyclin D1, cyclin E, cdk2, cdk4, and cdk6. (iii) enhancement of the binding of cyclin D1 to $p21^{waf1}$ and $p27^{kip1}$. (iv) inhibition of binding of cyclin E to cdk2. (v) downregulation of phosphorylation of retinoblastoma tumour suppressor protein.

Flavonol derivatives exist mainly in fruits, vegetables and teas as sugar conjugates; the main aglycones being quercetin, myricetin and kaempferol. Several studies have shown that quercetin dose-dependently inhibited the proliferation of different cancerous cell lines, including cancer of the colon (HT-29, COLO 201 and LS-174T), breast (MCF-7 ADRr) and ovary (OVCA 433) [53–55]. Yoshida et al. [56] reported that quercetin reversibly blocked human leukemic T-cells in late G_1 phase of the cell cycle by suppressing the expression of the growth-related histone genes H4, cyclin A and B and p34cdc2. Quercetin blocked the cell cycle at G_0/G_1 phase in HT-29 and other human colon cancer cell lines by binding to type-II estrogen-binding sites [53]. In MCF-7 breast cancer cells quercetin inhibited cell cycle progression through transient M phase accumulation, subsequent G_2 arrest and by induction of apoptosis [57].

Chrysin (5,7-dihydroxyflavone), an apigenin analog, present at high levels in honey and propolis exhibits antioxidant and anti-inflammatory effects and possess cancer chemopreventive activity. Chrysin inhibited rat C6 glioma cells at the G1 phase in a dose-dependent fashion [45], here the expression of $p21^{Waf1/Cip1}$ was significantly increased while levels of CDK2 and CDK4 were reduced in chrysin-treated cells. This suggests that chrysin may exert its growth-inhibitory effects through induction of $p21^{Waf1/Cip1}$ leading to the observed inhibition of CDKs and subsequent cell cycle arrest. EGCG induces cell cycle arrest in the G_0/G_1 phase. In MCF7 breast cancer cells, this arrest is characterized by hypophosphorylation of pRb and overexpression of the CDKIs $p21^{Waf1}$ and $p27^{Kip1}$ while in prostate carcinoma cell lines growth arrest is accompanied by an increase in p53 expression and an induction of $p21^{Waf1}$ [58].

5. Nuclear factor kappa B (NFκB)

NFκB is an inducible and ubiquitously expressed transcription factor, normally sequestered in the cytoplasm of non-stimulated cells and must translocate into the nucleus to regulate effector gene expression. NFκB is one of the key regulatory molecules in oxidative-stress-induced cell activation. As a transcription factor it plays an important role in the intracellular regulation of immune response, inflammation, anti-apoptotic and cell cycle regulation. Several genes that mediate tumorigenesis and metastasis are regulated by NFκB [59–61].

Activation of NFκB results from the release of IκB, through phosphorylation of IκB at Ser-32 and Ser-36 by an ubiquitin-dependent protein kinase followed by ubiquitination at nearby lysine residues and proteolytic degradation [62]. Phosphorylation of IκBα is a critical step in the pathway leading to NFκB activation, and this step is catalyzed by an IκBα kinase (IKK) complex consisting of IKKα, IKKβ and IKKγ (formerly known as NFκB essential modifier or NEMO). IKK is acted upon by a number of upstream signal protein, including NFκB inducing kinase (NIK), NFκB activating kinase (NAK), atypical protein kinase C, Akt/protein kinase B, proteins related to IKK, receptor activator for NFκB and its ligand (RANK and RANKL), but also mitogen activated protein kinase kinases (MAPKK), mitogen activated protein kinase/extracellular signal regulated kinase kinases (MEKK) and the phosphatidyl inositol kinase [61].

Several reports indicate that NFκB is activated by various carcinogens and tumor promoters such as benzo-α pyrene, NNK, UV radiation and phorbol esters. Activation of NFκB lead to the enhanced expression of several cell survival/antiapoptotic genes such as Bcl-2, Bcl-3, Bcl-xl, c*IAP1*, c*IAP2*, x*IAP*, TRAF1, TRAF2, SOD and A20. NFκB also regulate the expression of several genes that are involved in tumor cell invasion and angiogenesis; these include cell adhesion molecules (ICAM-1, VCAM-1, ELAM-1), COX2, iNOS, uPA, MMP-9, MMP-2, VEGF, chemokines and inflammatory cytokines [60]. Furthermore, NFκB has been shown to antagonize the function of p53 [63]. Since the constitutive activation of NFκB has been associated with proliferation and survival of certain tumours, its inhibition by dietary components may contribute to chemoprevention [17,64–66].

Tea phenolics, in particular EGCG has been shown to inhibit the constitutive activation of NFκB in head and neck carcinoma cells. This inhibition may be due to the inhibition of IKK-catalyzed phosphorylation of I-κB [66]. Treatment of A431 epidermoid carcinoma cells with EGCG, inhibited NFκB activation in a does- and time-dependent fashion [67]. Similarly, Hastak et al. [68] reported that EGCG induced apoptosis in human prostate carcinoma LNCaP cells via downregulation of NFκB, thereby decreasing the expression of Bcl-2 and upregulation of p53, resulting in the activation of downstream targets, $p21^{waf1}$ and Bax. The latter alterations caused a change in the ratio of Bax/Bcl-2 in favour of cell death, followed by activation of caspases 9, 8 and 3.

Curcumin, the major yellow pigment in tumeric, curry and mustard, has been reported to possess both antioxidant and anti-inflammatory activity and exhibits profound anticarcinogenic activities in many animal tumour models [29]. Topical application of curcumin blocked the initiation of tumour formation in mouse skin by both benzo-α-pyrene and DMBA. Curcumin also inhibited TPA-induced inflammation, hyperplasia, proliferation, COX-2 activation and ROS generation in the same mouse skin model [26]. Treatment of several human gastrointestinal cell lines with curcumin suppressed expression of COX-2, prostaglandin E2 synthesis and AP-1 DNA binding induced by TPA [69]. Curcumin decrease the TNF-α-induced gene expression through suppression of both AP-1 and NFκB by blocking the degradation of the inhibiting IκBα. Blockade of TNF-α-induced degradation of IκB by curcumin is likely to be mediated via down-regulation of NIK and such downstream kinases ad IKKα and IKKβ [70]. Singh and Aggarwal [68] reported that curcumin inhibited NFκB activation in cultured human myeloid monoblastic leukemia cell line stimulated with TPA, TNF-α or hydrogen peroxide. In this study, curcumin not only abrogated TNF-α-induced phosphorylation and subsequent degradation of IκB, but also inhibited nuclear translocation of p65 and down-modulated AP-1 binding [71].

Resveratrol, found principally in skin of grapes, has been reported to be effective in all 3 major stages of carcinogenesis (i.e. initiation, promotion and progression). The antitumour activity of resveratrol can be attributed to its antioxidant function, the ability to prevent carcinogen activation and subsequent carcinogen DNA-adduct formation, and to enhance carcinogen detoxification through induction of phase

II xenobiotic metabolizing enzymes [72]. Pretreatment of MCF-7 cells and DMBA-induced rat mammary tumours with resveratrol inhibited TNF-α induced DNA binding activity of NFκB [73]. Furthermore, resveratrol down-regulated the expression of COX-2 and matrix metalloproteinase (MMP)-9, which are NFκB-regulated gene products suggesting a protective role in tumour promotion and progression. Resveratrol is also categorized as a phytoestrogen and has been reported to antagonize the growth-stimulatory effect of 17-β-estradiol (E_2) [71].

Capsaicin is the principal pungent and irritating constituents present in hot red and chili peppers. According to the study conducted by Morre et al. [74], capsaicin repressed the growth of several transformed cells, including HeLa, ovarian carcinoma, mammary adenocarcinoma and HL-60 cells. The inhibition of cell survival was accompanied by induction of apoptosis as revealed by the characteristic changes of nuclear DNA. The same investigators have subsequently reported similar effects of capsaicin in human and mouse melanoma cell lines [75,76]. Treatment of HL-60 cells with capsaicin reduced TPA-induced activation of NFκB and AP-1 [77]. Similarly, topical application capsaicin to the dorsal skin of female ICR mice strongly inhibited the TPA-induced activation of these transcription factors [78].

6. Gap junctional intercellular communication (GJIC)

Gap junction channels play an important role in intercellular communication by allowing the direct exchange of cytoplasmic components between adjacently coupled cells. These channels allow the passage of small molecules, up to a molecular mass 1 kDa, such as ions, individual amino acids, short polypeptides, nucleotides, second messengers (Ca^{2+}, IP_3, cAMP) and other metabolites. GJIC regulates growth control, differentiation and apoptosis. Deficient GJIC has been associated with a variety of diseases, including some forms of neuropathy, hereditary deafness, cataracts, skin diseases, heart disease and cancer [2,79].

Gap junction channels are constructed from transmembrane proteins that form structures called connexons. When the connexons in the plasma membranes of two cells in contact are aligned, they form a continuous aqueous channel, which links the two cell interiors allowing the direct cytoplasmic passive diffusion of ions and other molecules. A connexon is composed of a ring of six identical protein subunits called connexins, each of which contains four transmembrane helices. About 20 different connexin polypeptides are known and the most widespread one is connexin43 (Cx43) [80]. These gap junctions function physiologically in multicellular organisms to synchronize electronic or metabolic functions within tissues including regulation of growth, transmission of developmental signals, coordination of muscle contraction and maintenance of metabolic homeostasis. Gap junction channels are regulated in response to various stimuli including growth factors, inflammatory mediators, cytokines, hormones, cell adhesion and extracellular matrices that upon interaction with a cell trigger various intracellular signals such as modulation of Ca^{2+}, pH, redox state, activation of transcription factors, activation of kinases [81].

Chemical tumour promoters (phorbol esters, DDT, phenobarbital, saccharin) and various oncogenes (*ras, raf, neu, src, mos*) down-regulate GJIC while antitumour promoters (retinoids, carotenoids, flavonoids), tumour suppressor genes and antioncogene drugs (lovastatin) up-regulate GJIC [2]. In sustained absence of functional connexin, tumorigenesis is enhanced. The correlation between neoplastic transformation and reduced GJIC has led to the hypothesis that reduced cell-cell communication is a critical step in multistage carcinogenesis [79].

Phosphorylation has been implicated in the regulation of gap junctional communication at several stages of the connexin 'lifecycle', such as the trafficking, assembly/disassembly, degradation, as well as, the gating of gap junction channels. Several kinases including protein kinase A, protein kinase

C, $p34^{cdc2}$/cyclin B kinase, casein kinase 1, MAPK and $pp60^{src}$ kinase can phosphorylate at 12 of the 21 serine and two of the six tyrosine residues of the Cx43 [79]. Activation of many growth factor tyrosine kinase receptors, namely epidermal growth factor (EGF), plalelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF) and insulin or insulin-like growth factor (IGF), result in the phosphorylation of Cx43 and subsequent GJC disruption [82]. The mechanism of inhibition of GJIC by the EGF involves the activation of the MAPK. Binding of ligand to EGF receptor tyrosine kinases lead to the activation of the Ras/Raf/MEK/ERK signaling pathways and induces serine, but not tyrosine, phosphorylation on Cx43. Cx43 is a cellular target of activated MAPK and is phosphorylated at the consensus MAPK phosphorylation sites (Ser^{255}, Ser^{279}, and Ser^{282}) [83]. Phosphorylation of Cx43 results in the disruption of GJC. Phosphorylation of Cx43 promotes channel closure by decreasing P_0 channel open probability rather than by diminishing channel unitary conductance [82].

Since many tumorigenic cells have been reported to be communication-deficient, therefore recovery of their GJIC capacity among themselves or with their normal counterparts could normalize their phenotype. Modulation of specific signaling pathways in view to enhance GJIC represents potential chemopreventive strategy. Green tea extract, carotenoids and flavonoids inhibit neoplastic transformation and tumour cell growth and block neoplastic transformation by increasing connexin expression and gap junction formation in target tissues or block the inhibitory effects of tumour promoters on GJIC [81].

Epicatechin, a major flavan-3ol derivative in cocoa and green tea, exhibited stimulatory effects on GJIC in WB-F344 cell treated with TPA [84]. Phosphorylation of gap junction proteins causes an alteration in GJIC due to structural changes of the protein which results in a translocation from the plasma membrane into the cytosol [85]. TPA-mediated phosphorylation of Cx43, through activation of PKC, may be responsible for the inhibitory effects of this phorbol ester on GJIC, probably due to disturbances in Cx43 trafficking. These inhibitory effects of TPA is largely suppressed by the epicatechin and genistein [84].

Stat are a key-signaling molecule for many cytokines and growth factor receptors and is involved in differentiation and apoptosis [86,87]. They can be activated by phosphorylation through Jak or cytokine receptors or protein tyrosine kinase receptors. Stat3 is constitutively activated in many human epithelial malignancies [88]. Stat proteins contribute to cell survival and growth by preventing apoptosis through increased expression of anti-apoptotic proteins such as Bcl-2 and Bcl-xl. Kaempferol is a flavonoid widely distributed in the plant kingdom and present in high concentration in cruciferous vegetables such as broccoli and onion [11]. Several cancer chemopreventive phytochemicals have been shown to inhibit the Jak/Stat mediated signaling processes. Nakamura et al. [89] observed that kaempferol-treated KNC colon cancer cells had lower stat3 activity, restored Cx43 phosphorylation and functional GJIC. The activation of Src-homology domain (SH2) containing protein tyrosine phosphatases such as SHP-2, a negative regulator of Jak activity was shown to be one of the mechanisms by which curcumin could inhibit Stat signaling [90].

Caffeic acid phenethyl ester (CAPE), a phenolic antioxidant of propolis from honeybee hives, exerts anti-viral, anti-inflammatory and differentially suppresses the growth of numerous human cancer cells as well as inhibits tumour promoter-mediated processes [91,92]. Na et al. [93] reported that the effects of CAPE on WB-ras2 cells, which are GJIC deficient and tumorigenic, include the up-regulation of GJIC, suppression of anchorage independent growth, restoration of normal phosphorylation of Cx43 and re-establishment of normal gap junction plaques on the cell membrane. Additionally, CAPE inactivate $p21^{ras}$ by interacting with accessory factors regulating $p21^{ras}$, such as GTPase activating protein and inhibition of PKC activity through interaction with PLC, resulting in the restoration of GJIC [93].

7. Conclusion

While the damaging effects of ROS/RNS on DNA have been extensively studied, the role of aberrant signal transduction in carcinogenesis in relation with oxidative/nitrosative stress is being gradually unraveled implicating the manipulation of these signaling pathways as potential strategy for therapeutic interventions. Phenolic compounds are attractive as chemopreventive agent since they form an integral part of the diet. Emerging evidence suggests that the antioxidant activity of phenolics are unlikely to be the sole mechanism responsible for their protective action, in part explained by their extensive metabolism *in vivo* and low physiological level compared to ascorbic acid and α-tocopherol, which are present at much higher concentration. Thus development of optimized functionality of flavonoids that are more bioavailable represents significant steps in nutraceutical technology. Nevertheless, according to bioavailability studies, the concentration of phenolic derivatives encountered *in vivo* (30 to 3000 ng aglycone equivalents/g tissue) are sufficiently high to exert modulatory actions through interaction with various signal transduction pathways such as MAPK, NFκB, Jak/Stat, PKC and PI3K/Akt [15, 94]. Seminal research reports are now beginning to delineate the absorption of phenolics enabling an understanding of their bioefficacy and neuroefficacy [95–98]. However further studies are required to decipher the target molecules in the cellular signaling network affected by phenolic metabolites as bioavailable *in vivo*; this will facilitate definition of their potential as chemopreventive agent to be used as adjuncts to current cancer therapies [65,99].

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