MAURITIUS RESEARCH COUNCIL

MOLECULAR AND CLINICAL EFFECTS OF FUNCTIONAL FOOD ON DIABETES AND CARDIOVASCULAR DISEASES

Final Report

April 2013

MAURITIUS RESEARCH COUNCIL

Address:

Level 6, Ebène Heights,
34, Cybercity,
Ebène 72201,
Mauritius.

Telephone: (230) 465 1235
Fax: (230) 465 1239
Email: mrc@intnet.mu
Website: www.mrc.org.mu

This report is based on work supported by the Mauritius Research Council under award number MRC/RUN-1009. Any opinions, findings, recommendations and conclusions expressed herein are the author's and do not necessarily reflect those of the Council.

MOLECULAR AND CLINICAL EFFECTS OF FUNCTIONAL FOODS ON DIABETES AND CARDIOVASCULAR DISEASES

FINAL REPORT

APRIL 2013

SUBMITTED BY:

Professor (Dr) Theeshan BAHORUN

NATIONAL RESEARCH CHAIR

ANDI CENTRE OF EXCELLENCE FOR BIOMEDICAL AND BIOMATERIALS RESEARCH

UNIVERSITY OF MAURITIUS

MOLECULAR AND CLINICAL EFFECTS OF FUNCTIONAL FOODS ON DIABETES AND CARDIOVASCULAR DISEASES

The material is based upon work partly supported by the Mauritius Research Council under the award number MRC/RUN/EXP-1009

TABLE OF CONTENT

INTRODUCTION

This is the final report of the project entitled "Molecular and clinical effects of functional foods on diabetes and cardiovascular diseases". This project was part funded by MRC under the unsolicited research grant scheme – Contract MRC/RUN/EXP-1009

This final report highlights biochemical, molecular and microbiological works supporting the efficacy of green tea and fermented papaya to be used as adjunct therapy in nutritional management programs against diabetes and cardiovascular diseases.

This project is supported by:

-The Ministry of Health and Quality of Life

-Cardiac Centre, SSRN Hospital

-The University of Reunion

-The Tertiary Education Commission

-The University of Mauritius

-Osato Research International

-Apollo Bramwell Hospital

-Touro College of Pharmacy (New York)

-Societé Usinière de Bois Chéri

-Bio Health Ltd

2 Mphil/PhD students are currently enrolled under the project and the outcome in terms of publications has been as follows to date:

J. Somanah, O. I. Aruoma, T. K. Gunness , S.Kowelssur, V. Dambala, F. Murad, K. Googoolye, D. Daus, J. Indelicato, E. Bourdon, T. Bahorun. Effects of a short term supplementation of a fermented papaya preparation on biomarkers of diabetes mellitus in a randomized Mauritian population. *Preventive Medicine, 54. S90–S97 (2012)*

J Somanah, E. Bourdon, T. Bahorun, O.I Aruoma. Fermented papaya preparation reduces growth, hydrophobicity and acid production of*Streptococcus mutans*, *Streptococcus mitis* and *Lactobacillus acidophilus*: implications for oral health in diabetes. *Molecular Nutrition and Food Research* (under review)

J. Somanah, E. Bourdon, P. Rondeau, T. Bahorun, Okezie I.Aruoma. Relationship between a fermented papaya preparation supplementation, erythrocyte integrity and antioxidant status in pre-diabetics. *Food and Chemical Toxicology* (under review)

N. A. Toolsee, O. I. Aruoma, T. K. Gunness, S. Kowlessur, V. Dambala, F. Murad, K. Googoolye, D. Daus, J.Indelicato, P. Rondeau, E. Bourdon, T. Bahorun. Effectiveness of green tea in a randomized human cohort: Relevance to diabetes and its complications. *Biomed Research International* (Under Review)

1. SUMMARY OF STUDY

Phytochemicals as antioxidant prophylactic agents in functional foods displayed a sustainable prevention for various metabolic ailments. Recent studies examining the oral and renoprotective activities of nutraceuticals have emphasized their supporting role in the management of diabetes and its complications. In this context, the current study encompasses the characterization of green tea phytochemicals, its effects on the energy metabolism of HEK-293 cell and on erythrocytes peroxidation. In the same pipeline, the anti-microbial effects of FPP® have been assessed on *Streptococcus mutans*, *Streptococcus mitis* and *Lactobacillus acidophilus*. A randomized clinical trial has also been conducted at the Cardiac Centre, SSRNH Hospital, to assess how FPP® could affect carbonyl accumulation in pre-diabetic patients. Our previous reports have illustrated the bioefficacy of green tea and FPP® to modulate distinctive markers of diabetes mellitus and cardiovascular diseases in a randomized pre-diabetic Mauritian cohort. Results suggested that 6 g of FPP® and 3 cups of green tea day for a period of 14 weeks would improve the health status of pre-diabetic patients. However, prevention and therapeutic strategies required a profound understanding about how dietary antioxidants interact with reactive radical species, which might be a causative agent of metabolic syndrome. Consequently, a free radical-induced hemolysis assay has been designed to understand how FPP® could scavenge peroxyl radicals *in vitro*.

2. RATIONALE OF STUDY

Non-communicable diseases have become an issue of global concern. The United Nations has recognized diabetes mellitus and its related complications to be a silent epidemic of the 21st century that has created a catastrophic scenario that threatens the current global healthcare systems. With more than 285 million people diagnosed with diabetes in 2010, this figure is expected to surge past 438 million, or 7.8 % of the global adult population, by 2030. While total mortality steadily falls in developing nations, diabetes and diseases of the cerebral and cardiovascular system remain the fourth leading causes of mortality. Mauritius ranks 3rd in the region of Africa with the highest prevalence amongst its population, with a toll of 22.6 % and 21.2 % deaths related to diabetes and cardiovascular diseases respectively (Health Status Report, 2007) a situation which is deemed to worsen worldwide if immediate health interventions are not put into action.

Interest into the phenomenon of oxidative stress as a target of disease prevention and therapy has arisen as oxidative stress is a recognized hallmark in the spread of degenerative diseases such as diabetes (Araki *et al.*, 2010), cancer (Visconti and Grieco, 2009), chronic inflammation and cardiovascular disorders (Gleissner *et al.*, 2007)). In light of the tremendous costs of diabetes, both in terms of monetary resources and of human suffering, it is highly desirable to have practical nutraceuticals that would reduce the risk and curb the progression of diabetes. The present study backs up existing framework, oriented towards the dietary management of neurodegenerative diseases, nephropathy and cardio-related complications associated with oxidative stress during type 2 diabetes.

3. BACKGROUND INFORMATION

Diabetes mellitus is a group of disorders marked by hyperglycemia and is caused by absolute or relative insulin deficiency, sometimes associated with insulin resistance. It has multiple etiologies and segregates into two major forms. Insulin Dependent Diabetes Mellitus (IDDM, Type 1), an auto-immune disease where the body's defense system affects β-cells of the pancreas and Non-Insulin Dependent Diabetes Mellitus (NIDDM, Type 2), a metabolic disorder characterized by insulin resistance and deficiency. In 2004 Robertson has postulated that hyperglycaemia generates reactive oxygen species (ROS) via a number of pathways (Figure 1).

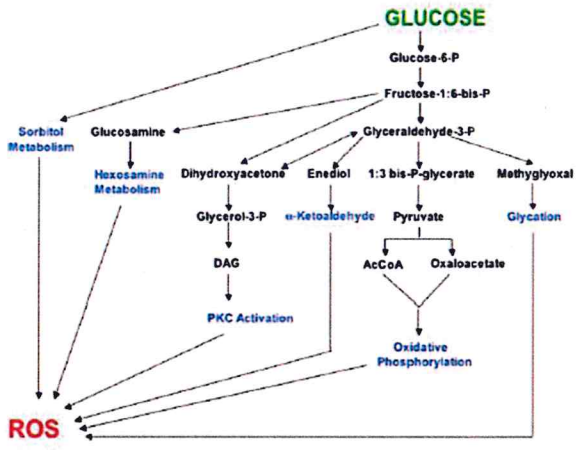


Figure 1. Pathways along which glucose metabolism can form ROS (DAG-Diacylglycerol; PKC-Protein Kinase C; AcCoA-Acetyl-CoA) (Robertson, 2004).

Under physiological condition, glucose primarily undergoes glycolysis and oxidative phosphorylation whereas under pathophysiological condition, excessive glucose level can overwhelm the glycolytic process and inhibit glyceraldehyde catabolism, which cause glucose, fructose-1,6-bisphosphate, and glyceraldehyde-3-P to be channelized to other pathways such as enolization and α-ketoaldehyde formation; PKC activation; dicarbonyl formation and glycation; sorbitol metabolism; hexosamine metabolism; and oxidative phosphorylation. All these biochemical pathways generate ROS which have been found to play a major role in the pathogenesis of diabetic nephropathy (Figure 2) and cardiovascular diseases (Figure 3). Diabetic nephropathy is a progressive kidney disease caused by angiopathy of capillaries in the kidney glomeruli. It is characterized by nephrotic syndrome, diffuse glomerulosclerosis and affects approximately 40% of type 1 and type 2 diabetic patients. It increases the risk of death, mainly from cardiovascular causes (Valmadrid *et al.*, 2000), and is defined by increased urinary

albumin excretion (UAE) (Viberti *et al.*, 1982). Diabetic nephropathy is categorized into microalbuminuria (UAE > 20µg/min) and macroalbuminuria (UAE ≥ 200µg/min).

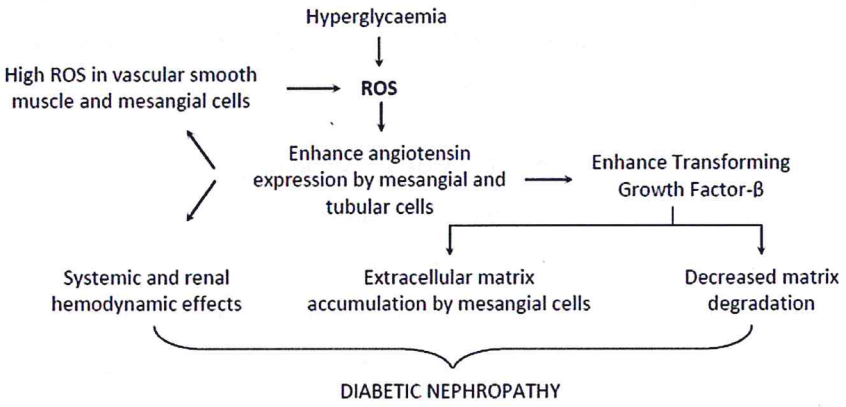


Figure 2. Effects of ROS in the pathogenesis of diabetic nephropathy (Sahib *et al.*, 2009).

Cardiovascular disease (CVD) is a class of diseases that affect the heart and blood vessels. In most cases, this is due to the progressive effects of atherosclerosis in arteries. Common examples of cardiovascular diseases include coronary artery disease, stroke and peripheral vascular disease. Murray and Lopez (1996) predicted that CVD will be the leading cause of death and disability worldwide by 2020. In Mauritius heart diseases and diabetes mellitus were the main causes of mortality in 2008, with 19.6% and 22.8% deaths respectively (Health Statistics Report, 2008).

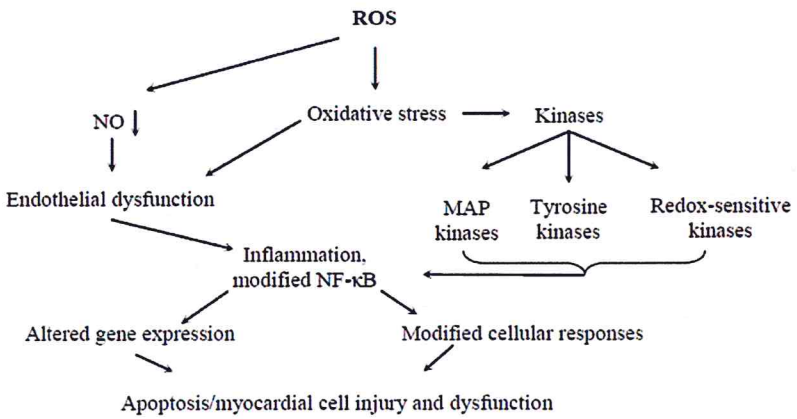


Figure 3. Effects of ROS on signaling systems in cardiovascular disease (Lakshmi *et al.*, 2009).

Lakshmi *et al.* 2009 postulated that ROS reduce nitric oxide bioavailability thereby enhance endothelial dysfunction. Moreover, ROS modulate the expression of redox-sensitive genes, involved in apoptosis and

pathogenesis of CVD, by influencing cellular signaling pathways. Although low fat diets, regular exercise and weight control are evident for the prevention of cardiovascular diseases, it can be anticipated that a large proportion of at-risk individuals will eat non-cautiously and shun exercise. Cellular damage of cardiac tissues as a result of free radical generation is well documented, thus identification of agents able to prevent oxidative modifications to cardiac tissue is a vital pharmacological aim.

Tea is the most widely consumed beverage in the world, second only to water. Green tea contains important phytochemicals such as flavonoids. The main flavonoids present in green tea include catechins (flavan-3-ols) which comprise (-)-epigallocatechin-3-gallate (59% approximately); (-)-epigallocatechin (19% approximately); (-)-epicatechin-3-gallate (13.6% approximately); and (-)-epicatechin (6.4% approximately). Green tea also contains gallic acid and other phenolic acids such as chlorogenic acid and caffeic acid, and flavonols such as kaempferol, myricetin and quercetin (Cabrera *et al.,* 2006). Green tea polyphenols show antioxidant activities *in vitro* by scavenging reactive oxygen and nitrogen species; and chelating redox active transition metal ions such as iron and copper (Kim *et al.,* 2003). They may also function indirectly as antioxidants through inhibition of redox sensitive transcription factors; inhibition of 'pro-oxidant' enzymes, such as inducible nitric oxide synthase, lipoxygenases, cyclooxygenases and xanthine oxidase; and induction of antioxidant enzymes, such as glutathione-S-transferases and superoxide dismutases (Cook *et al.*, 1996 and Tedeschi *et al.*, 2004). Although health benefits have been attributed to green tea consumption since the beginning of its history, biochemical and molecular investigations on this functional beverage have been underway for less than 3 decades and still warrants further research.

Fermented Papaya Preparation (FPP®) is a dietary product developed by OSATO Research Institute, Japan. It is a unique combination of non-genetically modified fermented papaya, dextrose and food yeast, prepared using a novel biotechnological process that strictly adheres to the ISO 9001 and ISO 14001 standardization protocol. The nature of its carbohydrates and possible link to alleviating oxidative stress is the subject of on-going research. Over 40 independent studies on FPP® have examined its antioxidant capacities in lowering blood plasma glucose levels, inhibiting lipid peroxidation (Danese *et al.,* 1996) and protecting plasmid DNA of human T-lymphocytes (Rimbach *et al.,* 2000a). Yoshino *et al.* (2009) suggested that FPP® may up-regulate the redox defense activity in the brain. Further findings of Rimbach *et al.* (2000b) dubbed FPP® as an "immunomodulator" by fact that it can modulate signalling pathways in macrophages for the secretion of nitric oxide and tumor necrosis factor-alpha (TNF-α)-a central regulatory cytokine in macrophage antimicrobial

activity. Marotta *et al.* (2004) successfully demonstrated that FPP® modulated atrophic and metaplastic changes of gastric mucosa in chronic atrophic gastritis patients, a property that is of high relevance in treatment of chronic skin ulcers. Many aspects of the pathology in β-thalassemia and sickle cell anemia are mediated by oxidative stress. Oral administration of FPP® (3 g × 3 times/day for 3 months) resulted in (a) reduced red blood cell sensitivity to hemolysis and phagocytosis by macrophages (b) improved ability to generate oxidative burst – a vital mechanism for bacteriolysis, and (c) reduced platelet tendency to undergo activation, reflected by fewer platelets carrying external phosphatidylserine (Amer *et al.,* 2008).

Epidemiological surveys have brought to light the high prevalence of dental caries, gingivitis and periodontitis amongst patients with diabetes mellitus. In attempt to explain this dilemma multiple pathological mechanisms have been suggested, including: deficient nutritional intake, alterations in host response to oral microflora, compromised neutrophil function and decreased phagocytosis and leukotaxis. Bacteria-triggered secretion of serum pro-inflammatory cytokines in the mouth could induce hyperglycemia and ultimately cause insulin-resistance and contribute to the indirect destruction of pancreatic beta cells (Tsai *et al.,* 2002). A realistic management plan that includes regular oral hygiene practice and basic dental treatment is therefore fundamental for managing diabetes and its associated oral complications.

The human mouth with its diverse niches is an ideal breeding ground for oral microflora. Its conditions favor the development of dental caries, dental plaque, inflammation and infection of the gum surrounding teeth (gingivitis and periodontitis). *In vitro* studies using tooth slabs, saliva or hydroxyapatite-coated glass beads have identified *Streptococcus* species as the principle causative agent of dental caries and biofilm formation. Their affinity to colonize tooth surfaces arises from their ability to synthesize water-insoluble α-linked glucans from sucrose through the action of glycosyltransferases. It is these glucans that provide bacteria with a stable binding site (Islam *et al.,* 2008). In addition, as a by-product of fermentation, *Streptococcoci* release short chain carboxylic acids that demineralize and erode tooth enamel surfaces leading to caries formation. There is presently an escalating demand for compounds that deter the progression of bacterial growth, glycosyltransferase and acid production. Nowadays, active constituents extracted from plants are often included in the preparation of toothpaste, mouth rinses, dental floss and chewing gum to ensure a stronger anti-microbial activity (Bone, 2005). Ongoing studies focusing on the anti-cariogenic properties of polyphenols isolated from green tea (Otake *et al.,* 1991), red chicory (Canesi *et al.,* 2011), cranberry juice (Babu *et al.,* 2012) and shiitake mushrooms (Signoretto *et al.,* 2011) look promising. However despite the numerous studies conducted on such functional foods, only a handful of plants can be clinically used to control dental plaque, caries formation and mouth

infections due to their effectiveness, stability, taste and economic feasibility (Bagramian *et al.,* 2009). Interestingly, dietary agents often lack bactericidal activity after commercial production, but retain their ability to manipulate oral microbiota by exhibiting other important properties such as anti-adhesion, anti-biofilm and anti-inflammatory - **Fermented papaya preparation (FPP, Immun'Age®; Osato Research Institute, Japan) is one** such functional food

However, research must go far beyond the simplistic claims of positive properties in experimental studies. It must be heavily supplemented by accurate scientific findings and toxicity studies. The challenge now is to translate the findings of this study and of other well-resourced studies into effective, feasible preventive strategies that can be implemented in management of diabetes and its secondary complications. The following report describes the antioxidant assay carried out for the period June 2012 to April 2013.

4. METHODOLOGY

4.1 Chemicals

HPLC grade of (+)-catechin, (-)-epigallocatechin, (-)-epigallocatechin gallate, (-)-epicatechin gallate, gallic acid, procyanidin B2, myricetin, kaempferol, hyperoside, cyanidin chloride and quercetin were obtained from Extrasynthèse (Genay, France). HPLC grade of theophylline, theobromine and (-)-epicatechin were purchased from Sigma Co. (St. Louis, MO, USA). Rabbit anti-dinitrophenyl antibody (cat# D9656) and 3,3',5,5'-tetramethylbenzidine liquid substrate system for ELISA were purchased from Sigma Co. (St. Louis, MO, USA). ECL anti-rabbit IgG, horseradish peroxidase-linked species-specific F(ab')2 fragment (from donkey) was purchased from GE Healthcare Ltd. (U.K.). Sterile DMEM, trypsin, fetal bovine serum and L-glutamine were obtained from GIBCO (Grand Island, NY). All other chemicals used were analytical grade from reputable commercial sources.

4.2 Experimental products

Camellia sinensis var. *sinensis* (Chinese Jat) was obtained as homogenous green tea bags (finished product) from Bois Chéri Tea Estate (Bois Chéri, Republic of Mauritius). The tea bag, contained 2 g of green tea, was manufactured on November 2010. A fermented papaya preparation was obtained from Osato Research Institute, Japan.

4.3 Green tea extraction for biochemical and cellular assays

According to conventional tea brewing method, two grams of green tea (equivalent to 1 tea bag) were infused in 200 mL hot water (100 °C) for 6 minutes. The green tea brew was cooled under running tap water and filtered through a 0.2 µm nylon filter. The brew was diluted to generate different concentration of green tea extracts (0.1 mg / mL to 10 mg / mL) for use in biochemical and cellular assays.

4.4 Green tea extraction for HPLC assay

One green tea bag was infused in 100 mL hot water (100 °C) for 6 minutes as described by Wang *et al.* (2000) with minor changes. The green tea brew was cooled under running tap water and centrifuged at 4000 rpm for 15 minutes at 25 °C. For gallic acid, (+)-catechin, (-)-epigallocatechin, (-)-epigallocatechin gallate, (-)-epicatechin gallate, procyanidin B2, theophylline, theobromine and (-)-epicatechin quantification, the supernatant was mixed with HPLC grade methanol in a (2 : 1 v/v, supernatant : HPLC grade methanol) ratio prior to HPLC assay. For myricetin, kaempferol and quercetin quantification, the supernatant was mixed with 6 M HCL in a (8 : 1 v/v,

supernatant : 6 M HCL) ratio and incubated at 85 °C for 2.5 hours. After cooling, the hydrolyse green tea extract was mixed with HPLC grade methanol in a (3 : 1 v/v, hydrolyse green tea extract : HPLC grade methanol) ratio prior to HPLC assay.

4.5 Biochemical assays

4.5.1 Total phenolic content

The Folin-ciocalteu assay, adapted from Singleton and Rossi (1965), was used for the determination of total phenolic present in the green tea extracts. To 0.25 mL of green tea extract, 3.5 mL of distilled water was added followed by 0.25 mL of Folin-ciocalteu reagent (Merck). A blank was prepared using 0.25 mL of water instead of green tea extract. After 3 minutes, 1 mL of 20 % sodium carbonate was added. Tube contents were vortexed before being incubated for 40 minutes in a waterbath set at 40 °C. The absorbance of the blue coloration formed was read at 685 nm against the blank using a spectrophotometer (Thermo Scientific, GENESYS 10S, UV-visible spectrophotometer, USA). Total phenolic was calculated with respect to gallic acid standard graph (concentration range: 0.4 to 1.1 mM). Results were expressed in mg gallic acid equivalent per cup (200 mL). Green tea extracts were analysed in triplicate.

4.5.2 Total flavonoid content

The Aluminium trichloride method (Lamaison and Carnet, 1990) was used to quantify total flavonoid content of green tea extracts. $AlCl_3 \cdot 6H_2O$ (1.5 mL of 2 %) was added to equal volumes of the green tea extract. The mixture was shaken and the absorbance was read at 440 nm against a blank using a spectrophotometer. Total flavonoid was calculated with respect to quercetin standard graph (concentration range: 0.01 to 0.1 mM). Results were expressed in mg quercetin equivalent per cup (200 mL). Green tea extracts were analysed in triplicate.

4.5.3 Total proanthocyanidin content

The HCl/butanol assay adapted from Porter *et al.* (1986) was used to quantify the total amount of proanthocyanidin. To each tube, 0.25 mL of the green tea extract was added, followed by 3 mL of n-butanol/HCl solution (95 : 5 v/v) and 0.1 mL of $NH_4Fe(SO_4)_2 \cdot 12H_2O$ in 2 M HCl. The tubes were capped and incubated for 1 hour at 85 °C. After cooling in the dark, the red coloration was then read spectrophotometrically at 550 nm against a blank. Total proanthocyanidin was calculated with respect to cyanidin chloride standard graph (concentration range: 0.1 to 0.6 mM). Results were expressed in mg cyanidin chloride equivalent per cup (200 mL). Green tea extracts were analysed in triplicate.

4.6 HPLC assay

4.6.1 Chromatographic settings

Green tea phytochemicals HPLC analysis was carried out using a Hewlett Packard 1100 series (Waldbronn, Germany) liquid chromatography system equipped with a vacuum degasser, quaternary pump, auto-sampler, thermostated column compartment and diode array detector. After green tea preparation 60 µL of extract was injected into a Zorbax SB-C18 column (4.6 mm internal diameter x 250 mm length, 3.5 µm pore size) (Agilent Technologies, CA, USA), fitted with a suitable guard column. An elution with a flow rate of 0.7 mL / min at 35 °C was applied as follows: 0-30 min, 0-10 % B in A; 30-50 min, 10-15 % B in A; 50-60 min, 15-25 % B in A; 60-90 min, 25-100 % B in A; 90-100 min, 100-0 % B in A. (Solvent A: acetonitrile/water, 1/9 v/v, pH 2.5; Solvent B: acetonitrile/water, 1/1 v/v, pH 2.5; adjusted with phosphoric acid). The operating pressure range was 115-125 bars. The reference wavelength used was 800 nm ± 10 nm bandwidth. The slit width used was 8 nm.

4.6.2 Phenolic acid, catechins, proanthocyanidin dimer and methylxanthines

Gallic acid, (+)-catechin, (-)-epigallocatechin, (-)-epigallocatechin gallate, (-)-epicatechin gallate, (-)-epicatechin, procyanidin B2, theophylline and theobromine were identified and quantified by comparing their retention times and spectral data with those of their authentic standards at 275 nm ± 15 nm bandwidth. Results were expressed in appropriate standards (mg) per cup (200 mL). Green tea extracts were analysed in triplicate.

4.6.3 Flavonol aglycones

Myricetin, quercetin and kaempferol were identified and quantified by comparing their retention times and spectral data with those of their authentic standards at 365 nm ± 15 nm bandwidth. Results were expressed in appropriate standards (mg) per cup (200 mL). Green tea extracts were analysed in triplicate. Calibration graphs for all authentic standards were prepared by plotting quantity (0.5 - 4.5 µg) versus area. All peaks were plotted and integrated using HP Chemstation software.

4.6.4 Validation of the chromatographic method

A blank injection was run before and after the analysis of green tea samples. No memory effect of green tea phytochemicals was observed.

4.7 Cellular assays

4.7.1 Free radical-induced hemolysis assay

The antioxidant activity of green tea extracts, fermented papaya preparation and human sera (derived from the clinical trial) was evaluated using an assay based on free radical induced hemolysis (Prost, 1992). Blood sample was obtained from the Biochemistry Department of Felix Guyon Hospital, Saint Denis, La Réunion, France. Plasma was removed by centrifugation, at 1700 rpm for 5 minutes at 25 °C, and erythrocytes were washed with an isotonic solution (0.15 M sodium chloride). 100 µL of washed erythrocytes (1/100 dilution in 0.15 M sodium chloride) were disposed, in the absence and presence of green tea extracts, fermented papaya preparation and human sera, in a 96 well-plate. Hemolysis was induced by adding 40 µL of 0.4 M AAPH into each well. Turbidity at 450 nm was recorded every 10 minutes for 840 minutes using a 37 °C thermostated microplate reader FLUOstar OPTIMA (BMG Labtech, Offenburg, Germany). Results were expressed as time taken (minutes) to reach 50 % hemolysis (HT50) which portrayed the antioxidant potential of green tea extracts, fermented papaya preparation and human sera against free radical which might be a causative agent of metabolic syndrome.

4.7.2 HEK-293 MTT assay

Human embryonic kidney cell line (ATCC CRL-1573), obtained from the laboratory of Dr. Lise Bernier (Clinical Research Institute of Montreal, Montreal, Quebec, Canada), was cultivated in DMEM supplemented with 10 % fetal bovine serum, L-glutamine (2 mM), penicillin (100 U / mL) and streptomycin (100 U / mL). For all experiment, cells were seeded in triplicate at 10^4 cells / 200 µL per well in a sterile 96-well plate. The cells were grown in an incubator (5 % CO_2 and 37 °C) for 24 hours. After the first incubation, culture medium was replaced by a solution containing 150 µL of DMEM (1 % fetal bovine serum) and 15 µL of variable concentrations of green tea extract (0.25 mg / mL – 3 mg / mL). The cells were incubated for the second time for 24 hours. After the second incubation, culture medium was further replaced by a solution containing 150 µL of DMEM (1 % fetal bovine serum) and 15 µL of variable concentration of hydrogen peroxide (100 µM - 500 µM). Finally, the cells were maintained in the controlled incubator for the third time for 24 hours before conducting MTT assay.

The effect of variable concentrations of green tea extract on the energy metabolism of HEK-293 cell, post-treated under different oxidative stress conditions, were evaluated by the MTT assay (Mosmann, 1983). The assay is based on the cellular reduction of tetrazolium salt, by NAD(P)H-dependent cytochrome C reductase and succinate dehydrogenase, into insoluble formazan product (Berridge and Tan, 1993). After the final incubation period, 20 µL of MTT dye (5 mg / mL) was added into each well followed by 3 hours incubation. Culture medium

was then carefully removed and 150 µL of dimethyl sulfoxide was added into each well. The 96-well plate was left under gentle agitation in the dark, at room temperature for 30 minutes, to solubilize the formazan crystals. The plate was read using a microplate reader FLUOstar OPTIMA (BMG Labtech, Offenburg, Germany) at 595 nm and 690 nm (for background absorbance). Final absorbance of sample was obtained by subtracting the background absorbance from the optical density at 595 nm. The control samples were cells treated with phosphate saline buffer (1X) (not green tea extracts) and incubated in a DMEM (1 % fetal bovine serum) with variable concentration of hydrogen peroxide. Results were expressed as mean final absorbance of tested samples with respect to mean final absorbance of control samples.

4.8 Anti-caries activity of FPP

A stock solution of final concentration 500 mg / ml was made by dissolving FPP (Fermented Papaya Preparation) in sterile distilled water on an orbital shaker. The mixture was centrifuged at 3000 rpm for 10 minutes to obtain a clear solution from which serial dilutions were made. Aliquots were stored at -4°C until later use. All experiments were carried out in triplicate.

ATCC strains of *Streptococcus mutans* (25175), *S. mitis* (6249) and *Lactobacillus acidophilus* (4356) were grown in sterile brain heart infusion (BHI) or De Man, Rogosa and Sharpe (MRS) broth to stationary culture at 37°C. Standardization of bacterial suspension was made using sterile 0.85% sodium chloride, NaCl with comparison against McFarland standards.

4.8.1 Effect of FPP on the growth of oral microbes

An aliquot of 50 µl standardized microbial suspension (2 x 10^8 CFU/ml was dispensed into sterile test tubes (10 mm x 90 mm) containing 3 ml of fresh BHI/MRS broth, 300 µl of FPP (0.05-50 mg/ml) and 100 µl Tween 80 (0.001% v/v). Ciprofloxacin (0.02 mg/ml) was used as positive control. Cultures were incubated at 37°C for 24 hours. At the end of the incubation period turbidometric analysis was performed at 600 nm against a blank of broth.

4.8.2 Effect of FPP on the adhesion of oral microbes to a hydrocarbon

Cell surface hydrophobicity of *S. mutans*, *S. mitis* and *L. acidophilus* was investigated in the presence of FPP using the hydrocarbon hexadecane. Bacterial strains were grown in 50 ml BHI/MRS broth to stationary phase for 18 hours at 37°C and cells harvested by centrifugation (2500 rpm, 15 min) and washed phosphate-urea-magnesium (PUM) buffer. Cells were re suspended in PUM buffer (pH 7.1) so that their optical density was 0.5 -

0.6 at 600 nm. 1 ml of bacterial suspension was added to sterile test tubes in triplicates, followed by an equal amount of FPP (0.05 – 50 mg/ml). After standing at room temperature for 20-30 minutes, 300 µl of *n*-hexadecane was added and agitated uniformly on a vortex for 1 minute. After allowing complete separation of the aqueous phases - the upper phase was discarded and the cell density of the lower aqueous phase determined at 600 nm. The percentage of cells partitioned to the hexadecane was calculated as the loss in absorbance relative to that of the initial absorbance using the following formula:

$$\text{Hydrophobicity (\%)} = \left(\frac{\text{Abs}_{600\text{nm}} \text{ before adsorption} - \text{Abs}_{600\text{nm}} \text{ after adsorption}}{\text{Abs}_{600\text{nm}} \text{ before adsorption}} \right) * 100$$

4.8.3 Effect of FPP on the adherence of oral microbes to a glass surface

Assessment of the adherence of growing cells of *S. mutans*, *S. mitis* and *L. acidophilus* to a glass surface was preformed according to the original method of Hamada *et al.,* 1981 with modifications. Briefly, bacterial strains were grown in BHI/MRS broth to stationary culture for 24 hours at 37°C. To sterile test tubes (10 x 90 mm) containing 3 ml BHI/MRS broth, 50 µl standardized microbial suspension (2 x 10^8 CFU/ml), 300 µl FPP (0.05-50 mg/ml), 300 µl sucrose/glucose (1%) and 100 µl tween 80 (0.001%) were dispensed in triplicates. Ciprofloxacin was used as positive control. Tubes were inclined at an angle of 30° and incubated for 18/48 hours at 37°C. After incubation period, the supernatant was decanted into a clean tube and adhered cells were removed by addition of NaOH (0.5 mol). Both test tubes were centrifuged (3000 rpm, 15 minutes) and the aqueous supernatant discarded. Bacterial cells were re-suspended in 3ml NaOH (0.5 mol) and the percentage of adherence quantified by reading absorbance at 600 nm and applying the following formula:

$$\text{Adherence (\%)} = \left(\frac{\text{Abs}_{600\text{nm}} \text{ adhered cells} - \text{Abs}_{600\text{nm}} \text{ non-adhered cells}}{\text{Abs}_{600\text{nm}} \text{ adhered cells}} \right) * 100$$

4.8.4 Effect of FPP on the acid production of oral microbes

To examine the effect of FPP on acid production of *S. mutans*, *S. mitis* and *L. acidophilus,* bacterial strains were grown in BHI/MRS broth to stationary culture for 24/48 hours at 37°C. In sterile culture tubes, 20 ml phenol red broth containing 3% glucose (w/v) was inoculated with 300 µl standardized microbial suspension (2 x 10^8 CFU/ml) followed by addition of 1 ml FPP (0.05-50 mg/ml). Using a calibrated pH meter, the pH of the culture was recorded before (T_0) and after incubation at 37°C for 24/48 hours ($T_{24/48}$).

4.9 Effectiveness of FPP on humans predisposed to type 2 diabetes

4.9.1 Effect of a short term supplementation of FPP on AAPH-induced hemolysis

For this study, 127 pre-diabetic Mauritians were recruited based on inclusion criteria: (1) fasting blood glucose range 5.1 – 5.9 mM/L (2) age 25-60 years (3) non-smoker or stopped for more than 6 months (4) alcoholic consumption less than 2 standard drinks/day (5) post-menopausal women not receiving hormone replacement treatment (6) not receiving glucose-lowering, cholesterol-lowering or anti-hypertension treatment. The study consisted of a randomized, controlled clinical trial (clinicaltrial.gov identifier NCT01248143) with treatment and control groups running in parallel. The treatment group received 3 grams FPP dissolved in 200 ml water twice daily before meals for 14 weeks followed by a 2 week wash out period. The control group consumed an equivalent amount of water. Written consent was obtained from all subjects. This study was conducted in accordance to guidelines set by the National Ethics Committee of the Ministry of Health and Quality of Life (Republic of Mauritius). Blood was collected at the Cardiac Center, SSRN hospital, Pamplemousses, at baseline, week 14 and after wash out following a 10 hour fast. Samples were centrifuged and serum was stored at -20°C for further analysis.

4.9.2 Effect of a short term supplementation of FPP on carbonylated protein content

Proteins frequently exposed to free radicals might undergo carbonylation reaction to generate carbonylated proteins which constitute a sensitive biomarker of oxidative stress. 3 µL of diluted human serum (contained 3 - 5 µg of protein) was denatured by 7 µL of 10 % sodium dodecyl sulfate for 10 minutes at room temperature. Human serum was treated with 5 mM 2,4-dinitrophenylhydrazine in 2 M HCL for 20 minutes at 37 °C to derivatize protein carbonyl group into a stable dinitrophenyl hydrazone product. Excess acidity of sample mixture was then neutralised by sodium carbonate buffer (0.1 M, pH 9.6). Proteins from the sample mixture are, non-specifically adsorbed to an ELISA plate (NUNC Maxisorp) for 3 hours at 37 °C and blocked with phosphate saline buffer/tween (0.1 %)/bovine serum albumin (1 %) overnight at 4 °C. These proteins were then probed with a rabbit anti-dinitrophenyl antibody at room temperature for 3 hours, followed by a secondary antibody (ECL anti-rabbit IgG horseradish peroxidase-linked species-specific F(ab')2 fragment from donkey) for 1 hour at room temperature. Tetramethylbenzidine substrate was then added and allowed to oxidize for 5 minutes at room temperature, leading to the formation of a blue complex. The reaction was stopped by the addition of 2 M HCL and absorbance was measured at 490 nm. Results are expressed as mean absorbance of tested human sera which illustrate the effect of FPP and water regimen on the formation of carbonylated proteins *in vivo*.

4.10 STATISTICAL ANALYSIS

Results are expressed as mean values ± standard deviation, where values in brackets ( ) represent percentage change and error bars represent standard deviation. Both descriptive and inferential statistical analyses were carried out using Microsoft Excel®, MedCalc® (version 11.5.1) and Prism® (Graphpad Software, version 3.0). Shapiro-Wilk's test was used to define the normality of data. A box-and-whisker plot was carried out on data to identify any outliers, which were then excluded from statistical analysis. After data cleaning, significant testing was performed by Student's paired *t*-test comparing the mean values of two samples. For data which followed a non-normal distribution, the non-parametric alternative Wilcoxon test was used. Differences were considered significant where value of $P < 0.05$ (two-tailed). Further significance testing of observed mean differences was carried out using the Student's independent *t*-test. For data that followed a non-normal distribution, Mann-Whitney *U*- test was used as a non-parametric alternate. Differences were considered significant where value of $P < 0.05$ (two-tailed).

5. RESULTS

Polyphenolic and biochemical data on Mauritian Green Tea

Total phenolic, total flavonoid and total proanthocyanidin contents of Mauritian green tea (var. Chinese Jat) were calculated and the data were set out in Table 1.

Table 1. Polyphenolic contents of Mauritian green tea.

Biochemical assays	Polyphenolic contents - mg per cup (200 mL)
Total phenol content	235.13 ± 30.89 gallic acid equivalent [a]
Total flavonoid content	7.52 ± 0.97 quercetin equivalent [b]
Total proanthocyanidin content	7.89 ± 0.63 cyanidin chloride equivalent [b]

Data are expressed as mean ± standard deviation (n = 3). Statistical analyses were performed, using independent samples t-test, for multiple comparisons. Different alphabetical superscripts between rows represent significant differences between mean polyphenolic contents ($P < 0.05$).

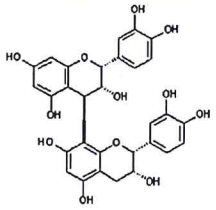
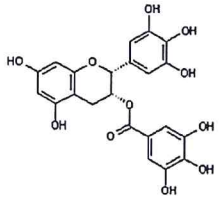
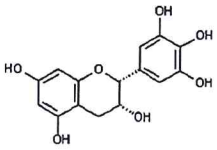
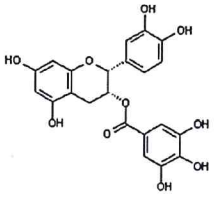
Mauritian green tea showed to be an important source of phenol, flavonoid and proanthocyanidin phytochemicals. One cup (200 mL) of Mauritian green tea contained 235.13, 7.52 and 7.89 mg of gallic acid, quercetin and cyanidin chloride equivalent respectively.

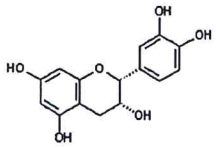
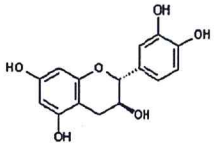
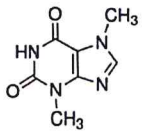
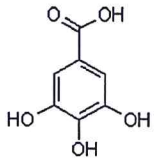
- **Mauritian green tea phytochemicals HPLC data**

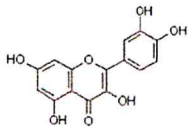
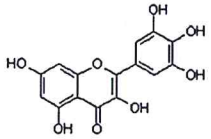
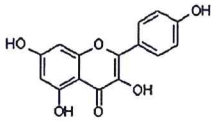
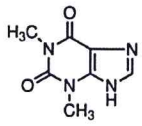
HPLC analytical method has been used to determine 12 green tea phytochemicals. This method of choice was the reversed phase liquid chromatography with UV absorbance detection. The experiment elucidated the presence of 5 catechins [(-)-epigallocatechin gallate, (-)-epigallocatechin, (-)-epicatechin gallate, (-)-epicatechin, (+)-catechin], 1 proanthocyanidin dimer (procyanidin B2), 3 flavonol aglycones (myricetin, kaempferol, quercetin), 2 methylxanthines (theobromine, theophylline) and 1 phenolic acid (gallic acid). The HPLC analytical method represents a benchmark for the comprehensive separation of tea constituents. The Mauritian green tea phytochemicals HPLC data are shown in Table 2. The 12 green tea phytochemicals were ranked, according to their quantity per cup (200 mL), in the following decreasing order: procyanidin B2 > (-)-epigallocatechin gallate > (-)-epigallocatechin > (-)-epicatechin gallate > (-)-epicatechin > (+)-catechin >

theobromine > gallic acid > quercetin > myricetin > kaempferol > theophylline. Procyanidin B2 has been found to be the most predominating bioactive molecule with 496.92 mg per cup (200 mL) and theophylline was the least bioactive molecule with 0.10 mg per cup. No significant difference ($P > 0.05$) has been found between quercetin and myricetin phytochemical content. According to figures 4 and 5, the HPLC chromatograms also showed that each identified phytochemicals had a good resolution peak with a significant ($P < 0.0001$) retention time (Table 2). The 12 green tea phytochemicals were ranked, according to their retention time (minutes), in the following increasing order: gallic acid > theobromine > theophylline > (-)-epigallocatechin > (+)-catechin > procyanidin B2 > (-)-epicatechin > (-)-epigallocatechin gallate > (-)-epicatechin gallate > myricetin > quercetin > kaempferol. Gallic acid has been found to have the lowest retention time of 5.22 minutes while kaempferol has been found to have the highest retention time of 82.47 minutes.

Table 2. Mauritian green tea phytochemicals HPLC data.

Phytochemical	Structural formula	MW	Number of OH group	mg per cup (200 mL)	Phytochemical RT in green tea medium (min)
Procyanidin B2		578.53	10	496.92 ± 20.08 [a]	15.37 ± 0.035 [l]
(-)-Epigallocatechin gallate		458.37	8	234.68 ± 12.08 [b]	20.69 ± 0.073 [m]
(-)-Epigallocatechin		306.28	6	205.12 ± 25.42 [c]	9.69 ± 0.020 [n]
(-)-Epicatechin gallate		442.37	7	28.46 ± 1.80 [d]	41.77 ± 0.115 [o]

(-)-Epicatechin		290.28	5	24.42 ± 2.08 [e]	19.44 ± 0.065 [p]
(+)-Catechin		290.28	5	13.90 ± 2.20 [f]	12.83 ± 0.039 [q]
Theobromine		180.16	0	3.16 ± 0.76 [g]	6.64 ± 0.008 [r]
Gallic acid		170.13	4	1.66 ± 0.16 [h]	5.22 ± 0.003 [s]

Quercetin		302.27	5	1.50 ± 0.16 [i]	75.77 ± 0.024 [t]
Myricetin		318.25	6	1.48 ± 0.14 [i]	63.42 ± 0.080 [u]
Kaempferol		286.25	4	0.90 ± 0.08 [j]	82.47 ± 0.016 [v]
Theophylline		180.16	0	0.10 ± 0.06 [k]	8.99 ± 0.006 [w]

Data are expressed as mean ± standard deviation (n = 3). MW = molecular weight, OH = hydroxyl, RT = retention time. Statistical analyses were performed, using independent samples t-test, for multiple comparisons. Different alphabetical superscripts, between rows within same column, represent significant differences between mean phytochemical contents (P < 0.05) or between mean phytochemical retention time in green tea medium (P < 0.0001).

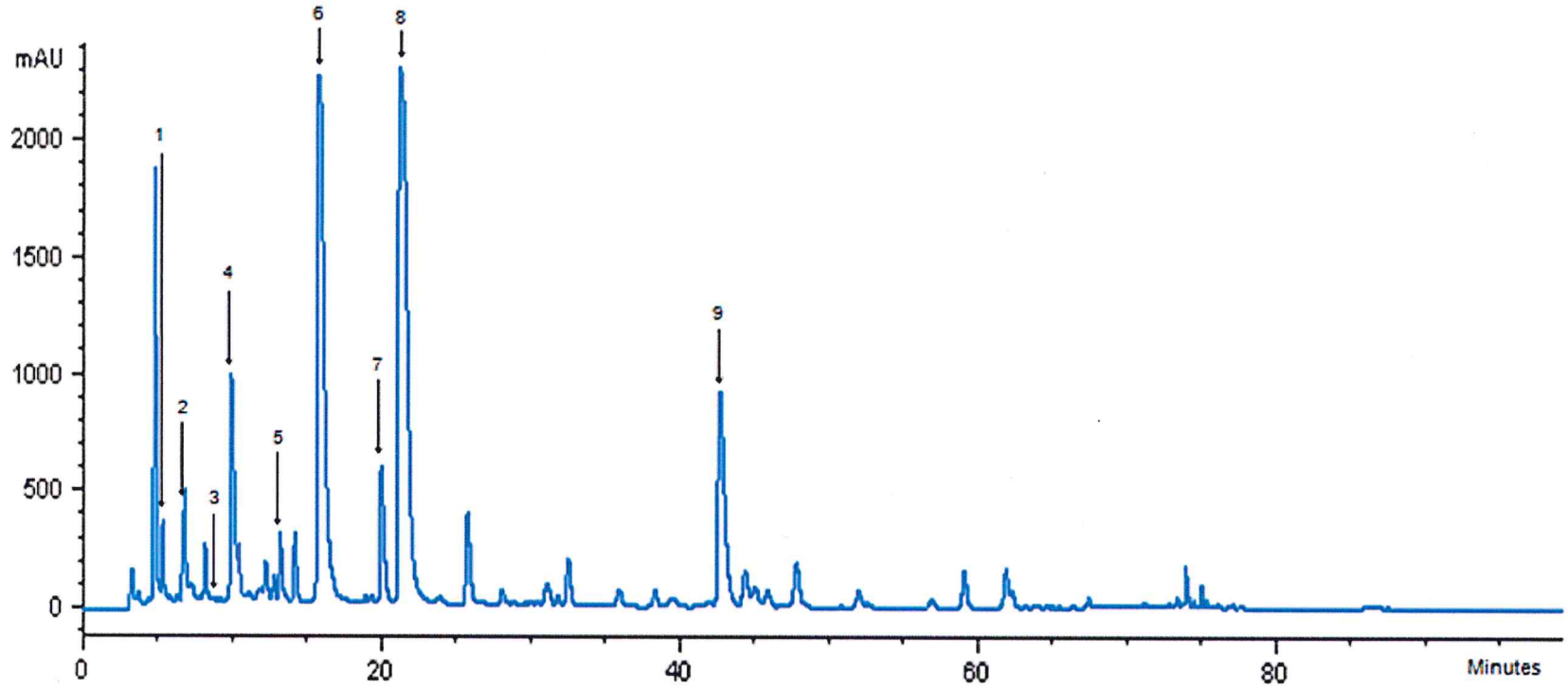


Figure 4. HPLC chromatogram of phenolic acid, catechins, proanthocyanidin dimer and methylxanthines in unhydrolysed green tea extract at 275 ± 15 nm. 1 = gallic acid, 2 = theobromine, 3 = theophylline, 4 = (-)-epigallocatechin, 5 = (+)-catechin, 6 = procyanidin B2, 7 = (-)-epicatechin, 8 = (-)-epigallocatechin gallate, 9 = (-)-epicatechin gallate. mAU = milli absorbance unit.

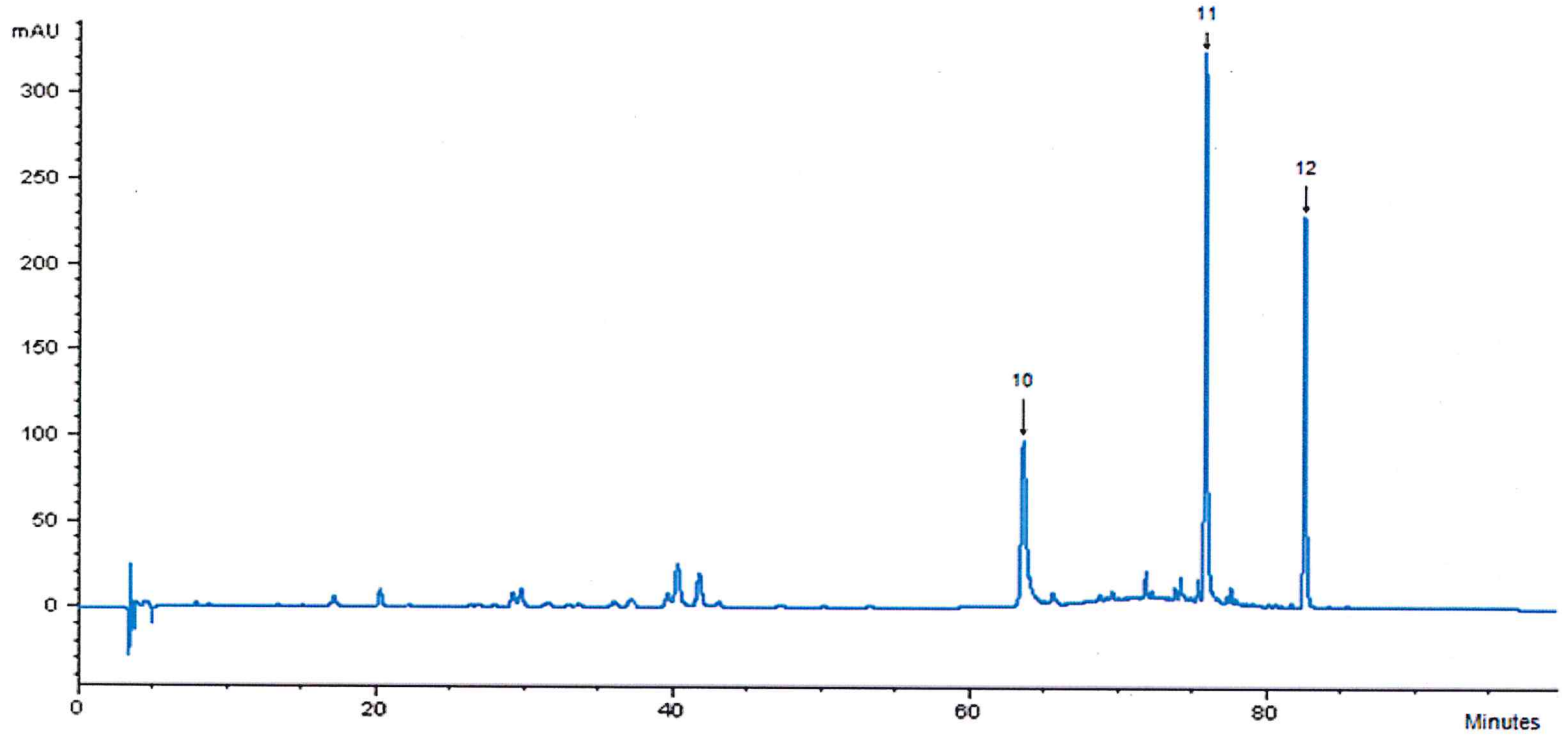


Figure 5. HPLC chromatogram of flavonol aglycones in hydrolysed green tea extract at 365 ± 15 nm. 10 = myricetin, 11 = quercetin, 12 = kaempferol. mAU = milli absorbance unit.

- **Cellular Assays**

Effect of green tea on AAPH-induced hemolysis

The present free radical induced hemolysis assay relates to a novel way of determining the antioxidant capacity of human serum and green tea extract by means of AAPH. The free radical - generating azo compound is gaining prominence, as a model oxidant, for its ability to initiate oxidative reactions via both nucleophilic and free radical mechanisms. According to figure 6, the human sera of male and female experimental group increased significantly the time taken to reach 50 % hemolysis by 2.7 % ($P < 0.1$) and 5.1 % ($P < 0.01$) respectively on week 14. On the contrary, the human sera of male and female control group decreased insignificantly the time taken to reach 50 % hemolysis by 2.9 % and 0.9 % respectively on week 14. Furthermore, the antioxidant capacity of green tea extract at different concentrations is shown in figure 7. Relative to control, the green tea extracts (0.50 mg / mL, 1.00 mg / mL and 1.50 mg / mL) increased significantly ($P < 0.01$) the time taken to reach 50 % hemolysis by 100 %, 186 % and 278 % respectively. It is important to note that the 0.25 mg / mL green tea extract did not differ significantly from the control in its ability to delay hemolysis.

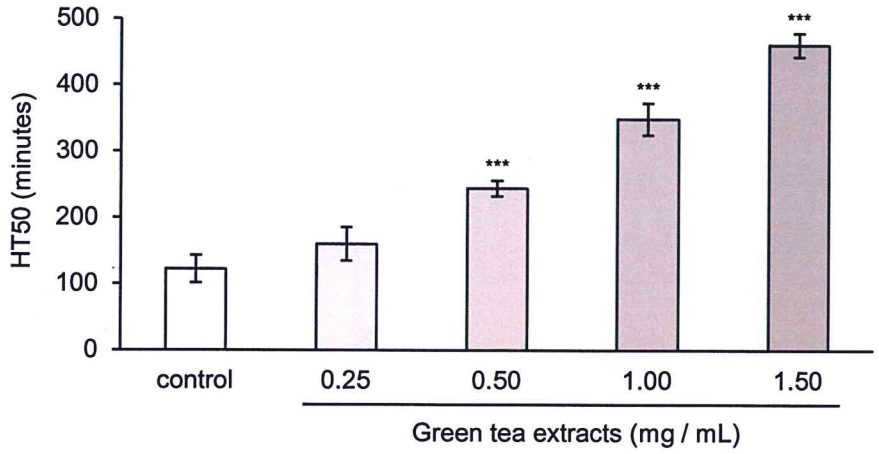


Figure 6. HT50 level of green tea extract at different concentrations. Main and error bars represent mean values and standard deviations respectively, for control (n = 3) and green tea extracts (each group has a sample size of 3). Statistical analyses were performed, using independent samples t-test, for multiple comparisons. * Time taken to reach 50 % hemolysis was significantly different from that of control ($P < 0.01$).**

Effect of FPP on AAPH-induced hemolysis

The inhibition of free radical-induced oxidative hemolysis by the dietary supplement fermented papaya preparation (FPP) Immun'Age® is illustrated in Figure 7. In this assay, the intrinsic effect of FPP to protect AAPH-treated human erythrocytes from hemolysis was evidenced by a higher hemolysis halftime (HT_{50}) in comparison to the control. This antioxidant activity was observed to be non-dose dependant. A minimum concentration of 200 µg/ml could significantly increase HT_{50} by 144.61 ± 7.50% compared to control of 1% NaCl ($P<0.01$). Interestingly, concentrations above 400 mg/ml were found to decrease hemolysis HT_{50} values strongly suggesting that low doses of FPP can more effectively scavenge peroxyl radicals in biological systems.

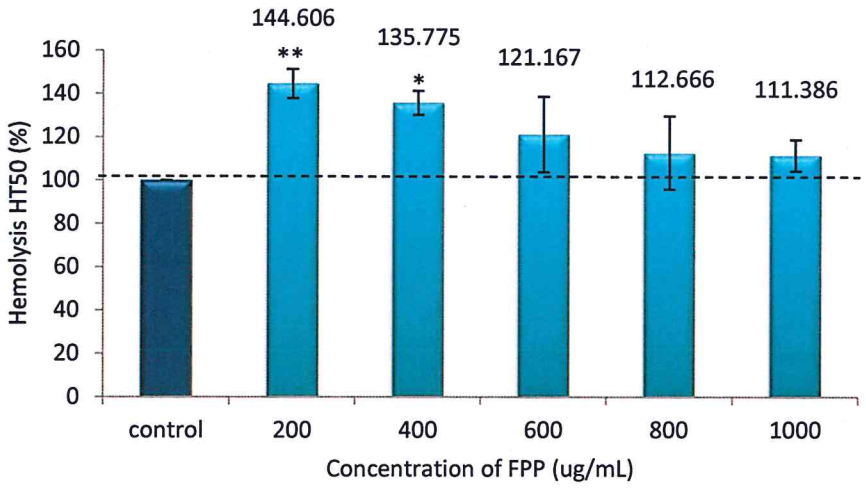


Figure 7. The antioxidant effect of various FPP concentrations on hemolysis half-time (HT_{50}) as a percentage compared to control (1% NaCl) where error bars represent standard deviation (*P<0.05, **P<0.01, ***P<0.001).

Energy metabolism of HEK-293 cell

The present interest in MTT assay is elicited as cellular reduction of tetrazolium salt, more related to NADH production through glycolysis than to respiration, involved mainly NAD(P)H-dependent enzymes of the endoplasmic reticulum and succinate dehydrogenase of the mitochondrial complex II. The energy metabolism of HEK-293 cell, pretreated with variable concentrations of green tea extract, under different oxidative stress conditions are shown in figure 8. Under normal condition, the green tea extracts were inversely proportional to the energy metabolism of HEK-293 cell. Green tea extracts at 0.25 mg / mL and 0.50 mg / mL increased significantly the energy metabolism of HEK-293 cell by 81.5 % ($P < 0.01$) and 56.1 % ($P < 0.05$) respectively when compared to the control. Under 100 µM H_2O_2-induced oxidative stress, the green tea extracts did not significantly influence the energy metabolism of HEK-293 cell relative to the control. Under 200 µM and 500 µM H_2O_2-induced oxidative stress, green tea extract at 3.00 mg / mL significantly reduced ($P < 0.05$) the energy metabolism of HEK-293 cell by 36.6 % and 20.7 % respectively when compared to the control.

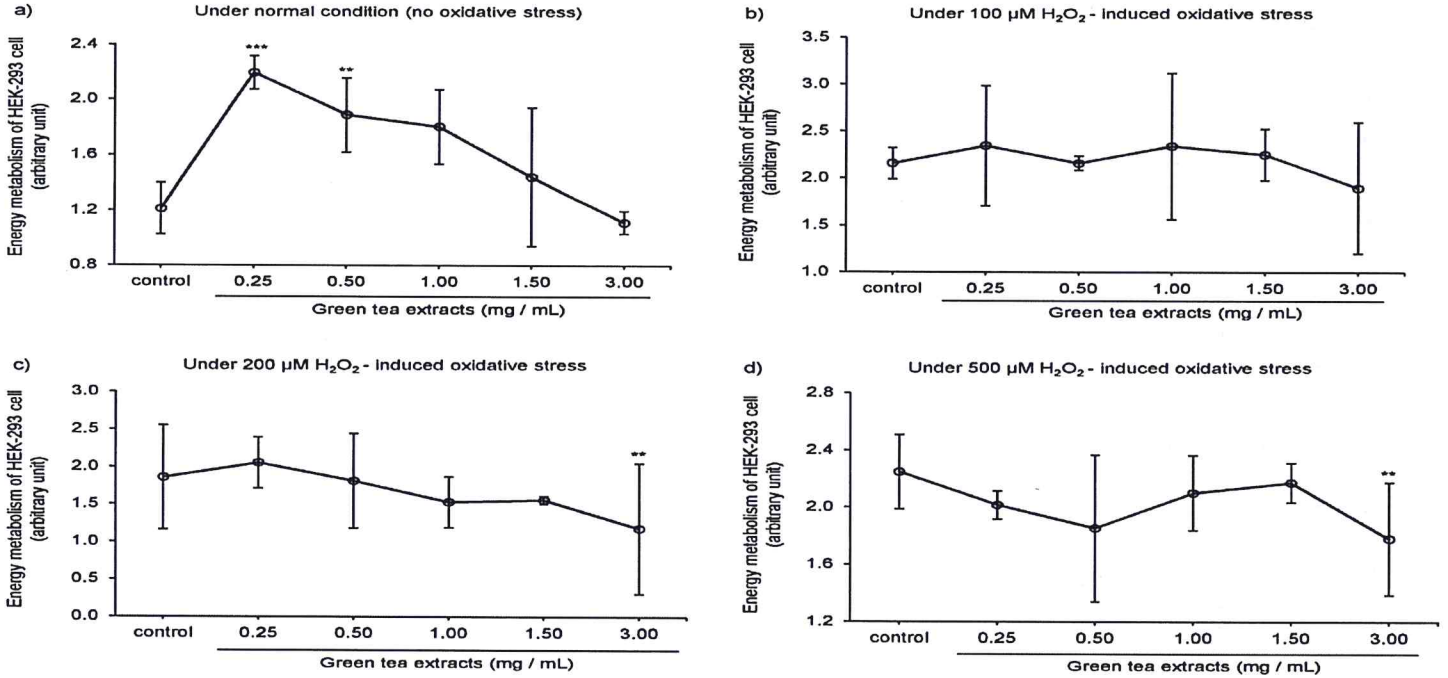


Figure 8. The energy metabolism of HEK-293 cell, pretreated with variable concentrations of green tea extract, under different oxidative stress conditions. Markers and error bars represent, respectively, mean values and standard deviations of a triplicate experiment. Statistical analyses were performed, using paired Student's t-test, for multiple comparisons. ** Mean value is significantly different from that of control ($P < 0.05$) (compared within same oxidative stress condition). * Mean value is significantly different from that of control ($P < 0.01$) (compared within same oxidative stress condition).**

- **Anti-caries activity of FPP**

Effect of a fermented papaya preparation on microbial growth

Figure 9 illustrates the effect of a fermented papaya preparation Immun'Age® on the growth of *Streptococcus* after a 24 hour incubation period. For *S. mutans*, the optical densities varied between 1.45 ± 0.05 and 1.63 ± 0.02. The growth of *S. mutans* was found to be generally inhibited in a dose-dependent manner within the concentration range 0.05-5 mg/ml where a significant reduction of 6.9% ($P<0.05$) was noted at 5 mg/ml. But, a slight but insignificant increase in growth (2.9%, $P>0.05$) was observed at 50 mg/ml.

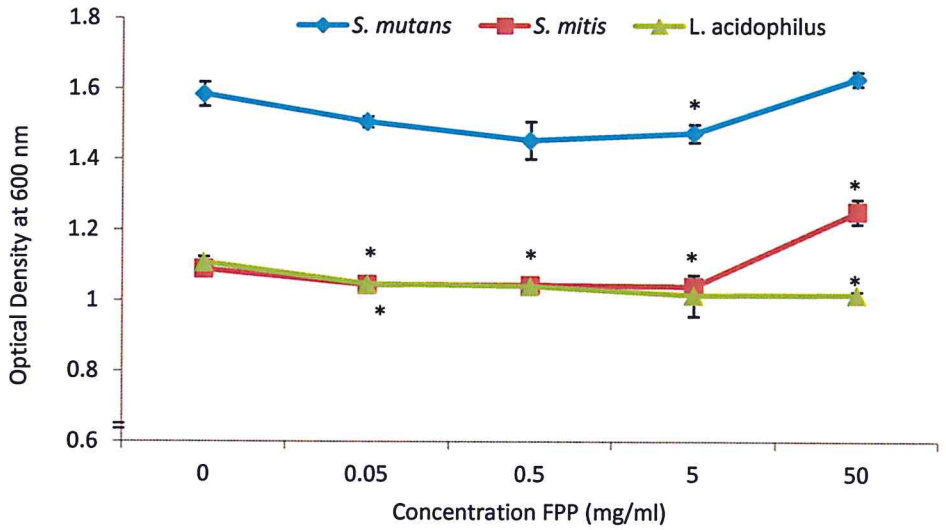


Figure 9. Effect of a fermented papaya preparation (0.05-50 mg/ml) on the growth of *Streptococcus mutans*, *Streptococcus mitis* and *Lactobacillus acidophilus*. Values are expressed as mean optical density at 600 nm of triplicate determinations (n=3), where error bars represent ± standard deviation. Significance: $P<0.05$ versus control

For *S. mitis,* the optical densities ranged from 1.04 ± 0.01 to 1.25 ± 0.04. In contrast to *S. mutans*, *S. mitis* was found to be significantly more responsive to FPP treatment. Growth was observed to drop significantly by 4.07%, 4.16% and 4.47% ($P<0.05$) at 0.05, 0.5 and 5 mg/ml respectively upon comparison to control (without extract). Similar to the trend observed with *S. mutans*, at a concentration of 50 mg/ml growth of *S. mitis* was found to significantly rise by 15.12% ($P<0.05$).

The effect of FPP on the growth of *Lactobacillus acidophilus* was observed to be inhibitory in a concentration dependant manner. Optical densities ranged from 1.05 ± 0.004 to 1.02 ± 0.01 where a significant decrease of 5.51% and 8.19% (P<0.05) was noted at concentrations 0.05 and 50 mg/ml.

Effect of a fermented papaya preparation on the microbial adherence to a hydrocarbon

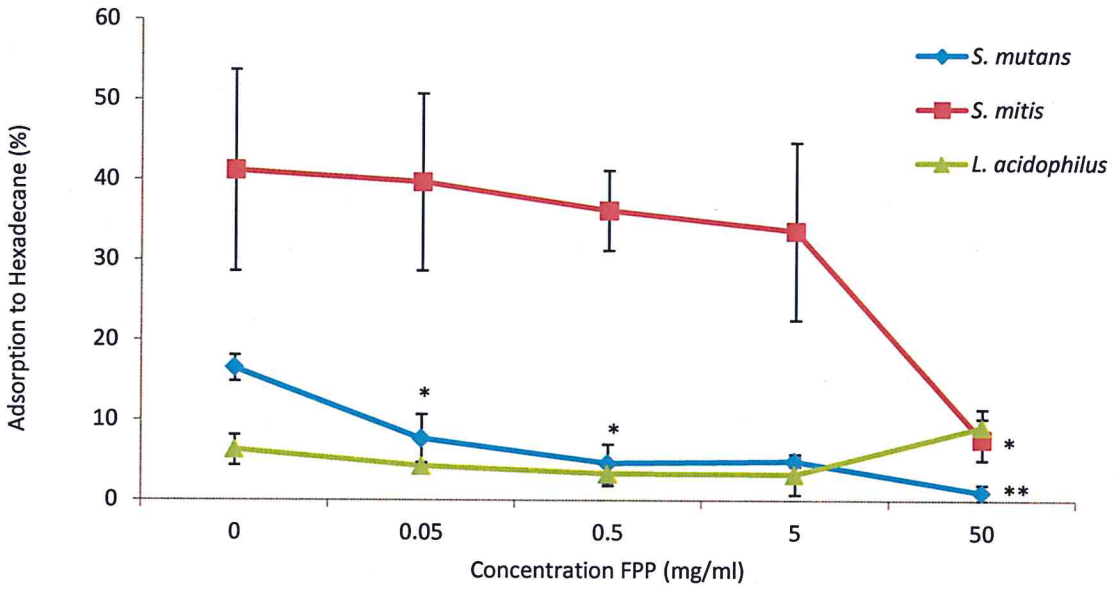


Figure 10. The effect of a fermented papaya preparation on cell surface hydrophobicities of *Streptococcus mutans, S. mitis* and *Lactobacillus acidophilus*. Values are expressed as mean (%) of triplicate determinations (n=3), where error bars represent standard deviation. Significance: * P<0.05, **P<0.01 versus control.

Amongst the three strains tested in the MATH assay (Figure 10), *Streptococcus mitis* demonstrated itself as being the most hydrophobic. The percentage of adsorption to the hydrocarbon hexadecane for the latter was 7.66 ± 2.62 % at 50 mg/ml, whereas the binding affinity to hexadecane of *S.mutans* was comparatively lower at this same concentration.

It was shown that the cell surface hydrophobicity of *Streptococcus* was affected by treatment with FPP. Exposure of *S. mutans* to 0.05 and 0.5 mg/ml of FPP significantly reduced adsorption to 39.66 ± 11.038

and 36.16 ± 5.01 % respectively (P<0.05) compared to control; whereas non-significant reductions were observed for *S. mitis.* Interestingly, a significant loss in hydrophobicity was noted in *S. mitis* at a concentration of 50 mg/ml (P<0.05). At this same concentration of FPP, the reduction was less drastic in *S. mutans* but significantly lower than its respective control (7.66 ± 2.62 %, P<0.01).

Lactobacillus acidophilus was also observed to be slightly affected by FPP treatment. Adsorption values were found to vary from 4.22 ± 0.51 to 3.23 ± 2.52 % within the concentration range 0.05-5 mg/ml. In comparison to its control the gradual loss of bacterial hydrophobicity- although insignificant (P>0.05)- was observed to be concentration dependent. At 50 mg/ml increase of 9.19 ± 2.23 % in bacterial adhesion to hexadecane was noted; this rise was non-significant.

Effect of a fermented papaya preparation on microbial adherence to a glass surface

The inhibitory effect of different concentrations of a fermented papaya preparation on adherence of *Streptococcus mutans* to a glass surface is shown in Figure 11 a. In the presence of 1% sucrose, the adherence of bacterial cells was found to reach a minimum of 43.79 ±4.44 % at a concentration of 0.05 mg FPP /ml. The percentage of adhered cells was found to gradually increase in a dose-dependent manner to reach a maximum value of 53.85 ± 0.49 % at 50 mg/ml. Statistical analysis of data showed this trend to be non-significant when compared to the control (without extract). Interestingly, for the sucrose non-dependant adherence assay, bacterial adherence was found to reduce significantly from 47.21 ± 2.24 to 32.32 ± 4.48 % at 0.5 mg/ml when compared to the control (without extract) (P<0.05). A further non-significant increase in bacterial cell adherence was noted at 50 mg/ml.

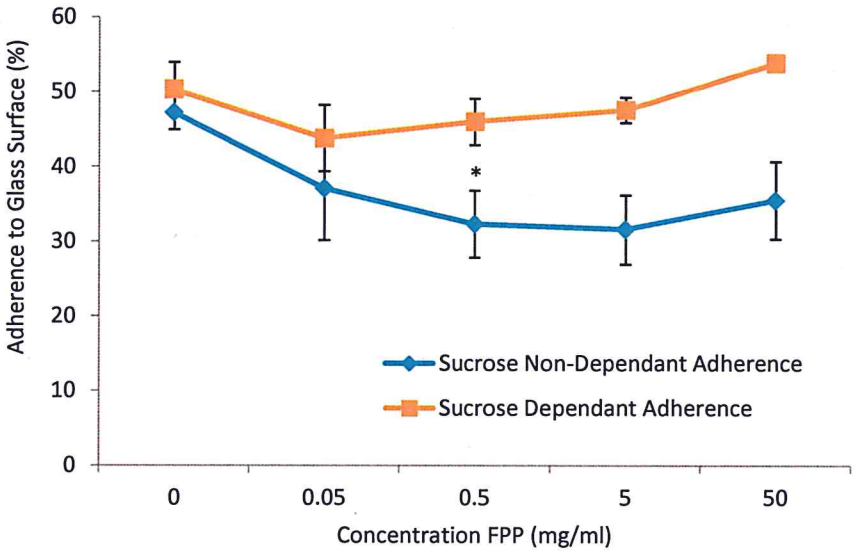


Figure 11 a. The effect of a fermented papaya preparation on adherence of *Streptococcus mutans* to a glass surface in presence or absence of 1% sucrose. Values are expressed as mean (%) of triplicate determinations (n=3), where error bars represent standard deviation. Significance: * P<0.05 versus control

In general, regardless of the presence or absence of sucrose/glucose in the basal media, the adherence of *Streptococcus mitis* to a glass surface was observed to be much greater than that of *S. mutans.* The inhibitory effect of varying concentrations of a fermented papaya preparation on adherence of *Streptococcus mitis* to a glass surface is illustrated in Figure 11 b. In the presence of 1% sucrose (sucrose dependant assay), minimum adherence of bacterial cells was observed at a concentration of 0.05 mg FPP /ml (70.08 ± 7.12 %). The percentage of adhered cells was found to gradually, but non-significantly, increase in a dose-dependent manner to reach a maximum value of 77.16 ± 2.61 % at 50 mg/ml (P>0.05). A similar trend was also observed for the sucrose non-dependant assay, where bacterial adherence was found to slightly drop from 62.52 ± 6.07 to 59.65 ± 1.97 % at 0.05 mg/ml (P>0.05). Despite consequent increases in bacterial cell adherence within the range 0.5 to 50 mg/ml, these values were considered to be insignificant in comparison to the control (without extract).

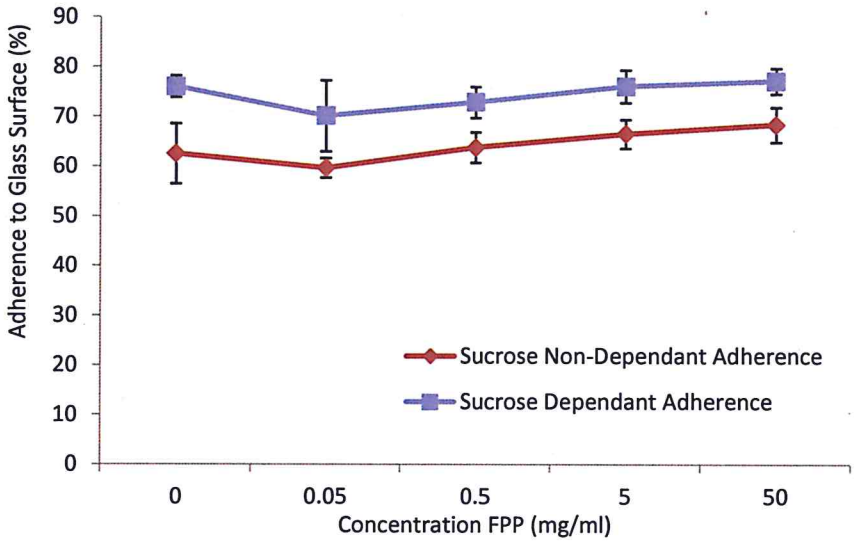


Figure 11 b. The effect of a fermented papaya preparation on adherence of *Streptococcus mitis* to a glass surface in presence or absence of 1% sucrose. Values are expressed as mean (%) of triplicate determinations (n=3), where error bars represent standard deviation

For *Lactobacillus acidophilus* (Figure 11 c), a trend similar to that observed with *S. mitis* was obtained. In the absence of glucose (i.e. glucose non-dependant) bacterial cell adherence ranged from 84.057 ± 2.780 % to reach a maximum of 92.399 ± 2.082 % at 50 mg/ml- this slight increase (+7%) was found to be non-significant when compared to the control (without extract, P>0.05). In the same experiment carried out with the addition of 3% glucose to the growth medium, exposure of *L.acidophilus* to low concentrations of FPP (0.05 mg/ml) resulted in a drop of 21.7% in cell hydrophobicity. Although this change was substantial in comparison to the control, it was non-significant (P>0.05). In general, the percentage of bacterial cell hydrophobicity to the smooth surface of the glass wall was found to increase in accordance to FPP concentration. A maximum adherence of 93.57 ± 3.98 % was reached at 50 mg/ml.

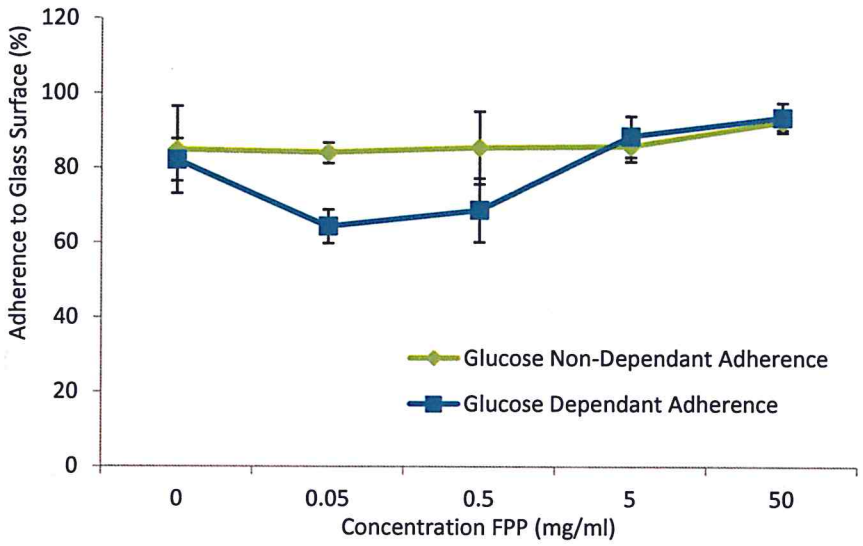


Figure 11 c. The effect of a fermented papaya preparation on adherence of *Lactobacillus acidophilus* to a glass surface in presence or absence of 1% glucose. Values are expressed as mean (%) of triplicate determinations (n=3), where error bars represent standard deviation

Effect of a fermented papaya preparation on the acid production

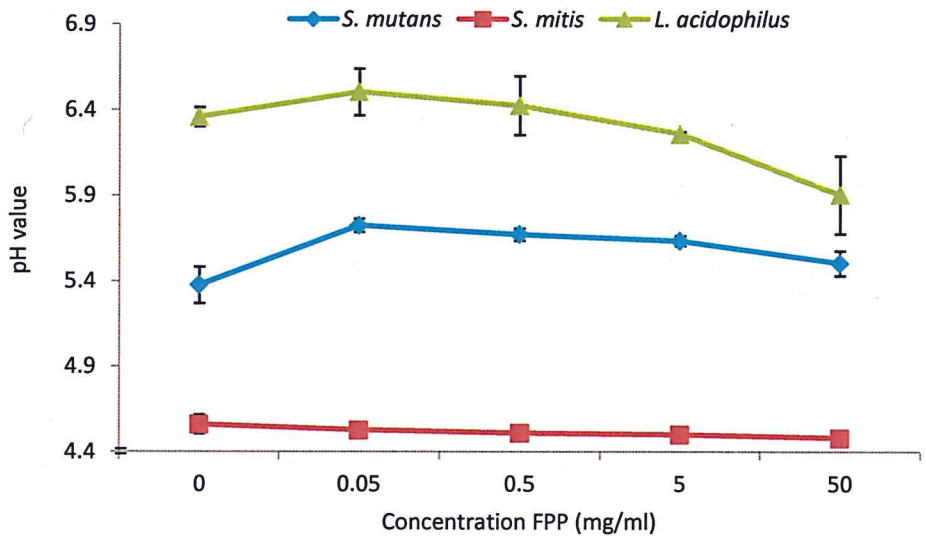


Figure 12. Effect of a fermented papaya preparation (0-50 mg/ml) on the acid production of *Streptococcus mutans, S. mitis* and *Lactobacillus acidophilus.* Values are expressed as mean pH values recorded after a 24/48 hour incubation of triplicate determinations (n=3), where error bars represent ± standard deviation.

Figure 12 illustrates the pH profiles obtained when differing concentrations of FPP were incubated with *S. mutans, S. mitis* and *L. acidophilus* for a period of 24 hours. For all bacterial strains, the pH of the basal medium was found to gradually acidify in a dose-dependent manner however, amongst the three strains of oral bacteria tested, *S. mutans* was found to be the most responsive to the presence of FPP. A slight non-significant reduction in the acidic response of *S. mutans* was noted at the lowest concentration of FPP tested (0.05 mg/ml), where the pH value was found to rise from 5.38 ± 0.11 to 5.72 ± 0.04. Gradual non-significant acidification was observed within the concentration range 0.5-50 mg/ml, where after a 24 hour incubation period the final pH reached was 5.50 ± 0.07 at 50 mg/ml.

On the other hand, FPP had no evident effect on the acid production of *Streptococcus mitis* within the concentration range tested. The final pH of the latter's medium was found to drop from 4.53 ± 0.06 to 4.48 ± 0.01 after 24 hours. In general, the slight, but non-significant, acidification of the medium was noted to be dose-dependent, where the effect of 0.05 mg FPP/ml on acid production was less pronounced than that of 50 mg/ml.

For *L. acidophilus,* the final pH levels ranged from 6.50 ± 0.06 to 5.90 ± 0.23, in which non-significant increases (P>0.05) in acidity were noted within the concentration range of FPP tested. From Figure 12 it can be noted that treatment of bacterial cells to 0.05 mg/ml FPP was observed to reduce the acidity of the medium by 2.2% (P>0.05) compared to the control (without extract).

- **Effectiveness of FPP on humans predisposed to type 2 diabetes**

Effect of a short term supplementation of FPP on AAPH-induced hemolysis

Figure 13 demonstrates the effect of hemolysis on human erythrocytes exposed to peroxyl radicals in a pre-diabetic population supplemented with 6 g FPP/day for 14 weeks. Mean basal HT_{50} values ranged between 65.62 – 164.1% in males and 47.54 – 164.4% in females of the treatment group. FPP consumption was observed to influence a slight increase (P<0.05) of 3.3% in males, whereas HT_{50} dropped by 1.5% in females at week 14. Wash out treatment caused continued non-significant increases to reach 106.78 ± 30.63% (+1.3%, P>0.05) and 103.53 ± 26.40% (+4%, P>0.05) in males and females respectively. In contrast, an opposing trend was observed in the control group. A t-test for independent samples indicated an overall statistically significant difference between females of the pre and post supplementation groups when compared to control (P<0.05).

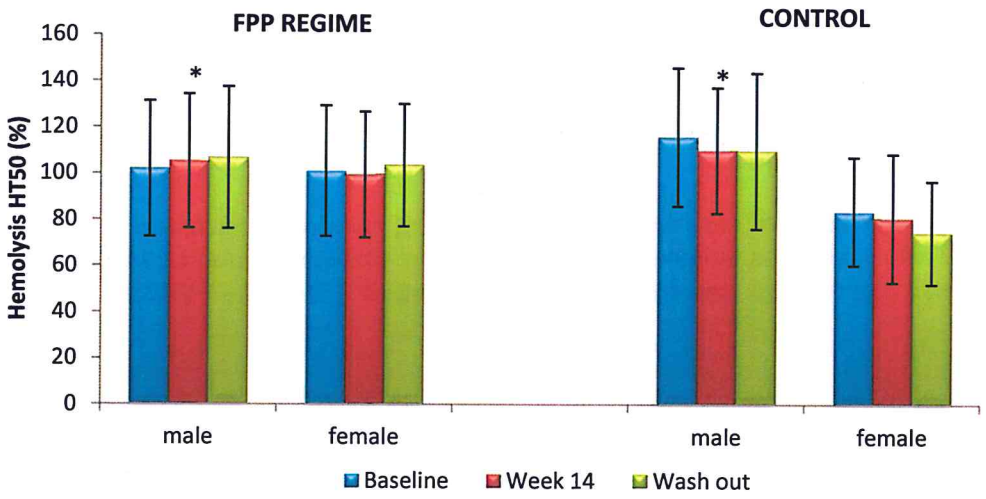


Figure 13. The effect of a FPP supplementation on hemolysis half-time (HT_{50}) as a percentage compared to control (1% NaCl) in males and females of a pre-diabetic population under the FPP and control regimes; error bars represent standard deviation (*P<0.05, **P<0.01, ***P<0.001)

Effect of a short term supplementation of FPP on protein carbonyl accumulation

The mean carbonylated protein content in the pre-diabetic population at baseline ranged between 159.97 – 840.78% and 224.18 – 947.67% in males and females of the treatment group in that order. The protective effect of a short term intake of 6g FPP per day influenced a general reduction in protein carbonyl accumulation. At week 14, a non-significant decrease of 1.9% and 9.7% could be noted in males and females of the treatment group. A continued non-significant reduction of 5.8% and 11.9% was observed after the wash out period. A similar non-significant trend was followed in both genders of the control at week 14. However, post wash out analysis showed mean carbonylated protein content to remain relatively unchanged in both genders (Figure 14).

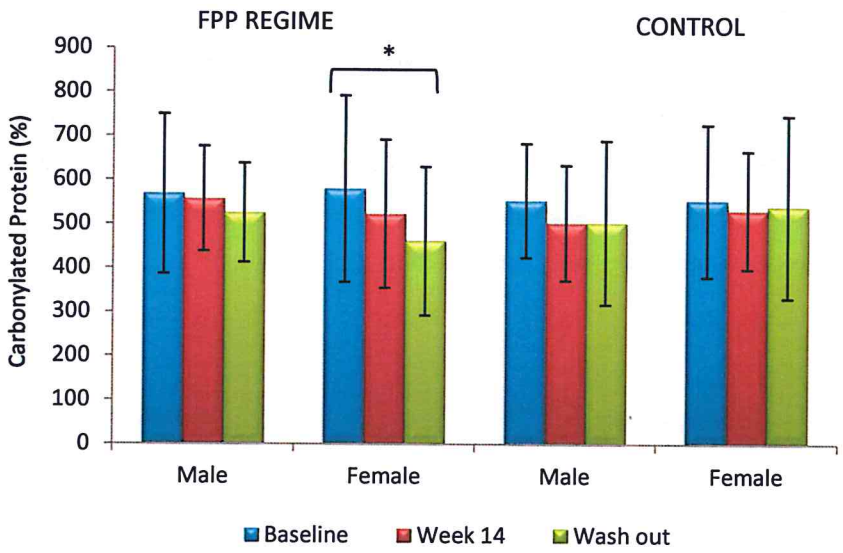


Figure 14. The effect of a FPP supplementation on carbonyl protein accumulation as a percentage compared to control (1% NaCl) in males and females of a pre-diabetic population under the FPP and control regimes; error bars represent standard deviation (*P<0.05, **P<0.01, ***P<0.001)

REFERENCES

AMER J, GOLDFARB A, RACHMILEWITZ EA, FIBACH E. Fermented papaya preparation as a redo regulator in blood cells of ß-thalassemic mice and patients. *Phytother Res.* 2008; 22: 820-828.

ARAKI E, NISHIKOWA T. Oxidative stress: A cause and therapeutic target of diabetes complications. *J Diab Res.* 2010; 3: 90-96.

ARUOMA, O.I. AND HALLIWELL, B., 1987. Action of hypochlorous acid on the antioxidant protective enzymes superoxide dismutase, catalase and glutathione peroxidase. *Biochem J,* 248, 973-6.

BABU, J., BLAIR, C., JACOB, S., ITZHAK, O. 2012. Inhibition of Streptococcus gordonii metabolic activity in biofilm by cranberry juice high-molecular-weight component. *J Biomed Biotech*, doi: 10.1155/2012/590384

BAGRAMIAN RA, GARCIA-GODOY F, VOLPE AR. 2009. The global increase in dental caries, a pending public health crisis. *Am J Dent*, 22:3-80

BENZIE, I.F. AND STRAIN, J.J., 1996. The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": the FRAP assay. *Anal Biochem,* 239, 70-6.

BERRIDGE, M.V. AND TAN, A.S., 1993. Characterization of the cellular reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT): subcellular localization, substrate dependence, and involvement of mitochondrial electron transport in MTT reduction. *Arch Biochem Biophys,* 303, 474-82.

BONE K. 2005. Phytotherapy for periodontal disease and improved oral hygiene. *Townsend Lett Dr Patients*, 263:38

CABRERA, C., ARTACHO, R. AND GIMÉNEZ, R., 2006. Beneficial effects of green tea – A review. *Journal of the American College of Nutrition*, 25(2), 79-99.

CANESI, L., BORGHI, C., STAUDER, M., LINGSTRÖM, P. 2011. Effects of fruit and vegetable low molecular mass fractions on gene expression in gingival cells challenged with Prevotella intermedia and Actinomyces naeslundii. *J Biomed Biotech,* doi: 10.1155/2011/230630

COOK, N.C. AND SAMMAN, S., 1996. Flavonoids – Chemistry, metabolism, cardioprotective effects, and dietary sources. *The Journal of Nutritional Biochemistry*, 7, 66-76.

DAMESE C, ESPOSITO D. D'ALFONSO V, CIRENE M, AMBROSINO M, COLLOTTO M. Plasma glucose level decreases as collateral effect of fermented papaya preparation use. *Clin Ter.* 2006; 157: 195-198.

GARRATT, D.C., 1964. *The quantitative analysis of drugs*. Volume 3. Japan: Chapman and Hall Ltd.

GLEISSNER CA, GALKINA E, NADLER JL, KEY K. Mechanisms by which diabetes increases cardiovascular diseases. Drug Discovery. 2007; 4: 131-140.

GUPTA A, GUPTA R, LALL B. Effect of *Trigonella foenum graecum* (fenugreek seeds) on glycaemic control and insulin resistance in type 2 diabetes mellitus: a double-blind placebo controlled study. *J Assoc Physicans India.* 2001; 49: 1057-1061.

HEALTH STATISTICS REPORT, 2008. Island of Mauritius and Island of Rodrigues, Ministry of Health and Quality of life.

HEALTH STATISTICS REPORT 2007: Island of Mauritius and Island of Rodrigues, Ministry of Health and Quality of life.

HENRIQUEZ, C., ALIAGA, C. AND LISSI, E., 2002. Formation and decay of the ABTS derived radical cation: A comparison of different preparation procedures. *International Journal of Chemical Kinetics,* 34, 659-665.

KIM, J.H., KANG, B.H. AND JEONG, J.M., 2003. Antioxidant antimutagenic and chemopreventive activities of a phyto-extract mixture derived from various vegetables, fruits and oriental herbs. *Food Science and Biotechnology*, 12, 631–638.

LAKSHMI, S.V.V., PADMAJA, G., KUPPUSAMY, P. AND KUTALA, V.K., 2009. Oxidative stress in cardiovascular disease. *Indian Journal of Biochemistry and Biophysics*, 46, 421-440.

MARKESBERRY WR. Oxidative stress hypothesis in Alzhimer's disease. *Free Rad Bio Med.* 1997; 23: 134-147.

MAROTTA F, BARRETO R, TAJIRI H, BERTUCCELLI J, SAFRAN P, YOSHIDA C, FESCE E. The aging/precancerous gastric mucosa. A pilot nutraceutical trial. *Ann N.Y Acad Sci.* 2004; 1019: 195-199.

MURRAY, C. J. AND LOPEZ. A.D., 1996. *Global Burden of Disease and Injury Series, Vols. I and II, Global Health Statistics*. Boston: Harvard School of Public Health.

NISHIKIMI, M., APPAJI, N. AND YAGI, K., 1972. The occurrence of superoxide anion in the reaction of reduced phenazine methosulfate and molecular oxygen. *Biochem Biophys Res Commun,* 46, 849-54.

RIMBACH G, GUO Q, AKIYAMA T, MATSUGO S, MOINI H, VIRGILI F, PACKER L. Ferric nitrilotriacetate induced DNA and protein damage; inhibitory effect of a fermented papaya preparation. *Anticancer.* 2000a; 20: 2907-2914.

RIMBACH G, PARK YC, GUO Q, MOINI H, QURESH N, SALIOU C, TAKYAMA K, VIRGILI F AND PARKER L. Nitric oxide synthesis and TNF-alpha secretion in RAW 264.7 macrophages. Mode of action of a fermented papaya preparation. *Life Sci.* 2000b; 67: 679-94.

ROBERTSON, R.P., 2004. Chronic oxidative stress as a central mechanism for glucose toxicity in pancreatic islet beta cells in diabetes. *The Journal of Biological Chemistry*, 279, 42351-42354.

SAHIB, M.N., ABDULAMEER, S.A., AZIZ, N.A. AND HASSAN, Y., 2009. Pathogenesis of diabetic kidney disease: review of cellular aspects of renal lesions. *African Journal of Pharmacy and Pharmacology*, 3(11), 507-514.

SHARMA, O.P. AND BHAT, T.K., 2009. DPPH antioxidant assay revisited. *Food Chemistry,* 113, 1202-1205.

STOOKEY, L.L., 1970. Ferrozine - a new spectrophotometric reagent for iron. *Analytical Chemistry,* 42, 779-781.

GLEISSNER, C.A., GALKINA, E., NADLER, J.L., KEY, K., 2007. Mechanisms by which diabetes increases cardiovascular diseases. Drug Discov. 4: 131–140.

ISLAM, B., KHAN, S.N., HAQUE, I., ALAM, M., MUSHFIQ, M., KHAN, A.U. 2008. Novel anti-adherence activity of mulberry leaves: inhibition of *Streptococcus mutans* biofilm by 1-deoxynojirimycin isolated from *Morus alba. J Antimicrob Chemother,* 64: 751-757

LAMAISON, J.L.C. AND CARNET, A., 1990. Teneurs en principaux flavonoids des fleurs de *Crataegus monogyna* Jacq et de *Crataegus laevigata* (Poiret, D.C.) en fonction de la vegetation. *Plant medicinales et phytotherapie*, XXV, 12-16.

MOSMANN, T., 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J Immunol Methods,* 65, 55-63.

OTAKE S, MAKIMURA M, KUROKI T, NISHIHARA Y, HIRASAWA M. 1991. Anti caries effects of polyphenolic compounds from Japanese green tea. *Caries Res*, 25:438-443

PORTER, L.J., HRSTICH, L.N. AND CHAN, B.G., 1985. The conversion of procyanidins and prodelphinidins to cyanidin and delphinidin. *Phytochemistry,* 25, 223-230.

PROST, M., 1992. Process for the determination by means of free radicals of the antioxidant properties of a living organism or a potentially aggressive age. *U.S. patent application 5135850*.

SIGNORETTO, C., BURLACCHINI, G., MARCHI, A., GRILLENZONI M. 2011. Testing a low molecular mass fraction of a mushroom (Lentinus edodes) extract formulated as an oral rinse in a cohort of volunteers. *J Biomed Biotech*, doi: 10.1155/2011/857987

SINGLETON, V.L. AND ROSSI, J.A., 1965. Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. *American Journal of Enology and Viticulture,* 16, 144-158.

TEDESCHI, E., MENEGAZZI, M., YAO, Y., SUZUKI, H., FÖRSTERMANN, U. AND KLEINERT, H., 2004. Green tea inhibits human inducible nitric oxide synthase expression by down-regulating signal transducer and activator of transcription-1α activation. *Molecular Pharmacology*, 65, 111-120.

TSAI, C., HAYER, C., TAYLOR, G.W. 2002. Glycemic control of type 2 diabetes and severe periodontal disease in the US adult population. *Community Dentistry and Oral Epidemiology.* 30: 182-192

VALMADRID, C.T., KLEIN, R., MOSS, S.E. AND KLEIN, B.E., 2000. The risk of cardiovascular disease mortality associated with microalbuminuria and gross proteinuria in persons with older-onset diabetes mellitus. *Archives of Internal Medicine*, 160, 1093-1100.

VIBERTI, G.C., HILL, R.D., JARRETT, R.J., ARGYROPOULOS, A., MAHMUD, U. AND KEEN, H., 1982. Microalbuminuria as a predictor of clinical nephropathy in insulin-dependent diabetes mellitus. *Lancet*, 1, 1430-1432.

VISCONTI D, GRIECO D. New insights on oxidative stress in cancer. *Curr Opin Drug Discov Develop.* 2009; 12: 240-245.

WANG, H., HELLIWELL, K. AND YOU, X., 2000. Isocratic elution system for the determination of catechins, caffeine and gallic acid in green tea using HPLC. *Food Chemistry,* 68, 115-121.

WANG, L., BASSIRI, M., NAJAFI, R., NAJAFI, K., YANG, J., KHOSROVI, B., HWONG, W., BARATI, E., BELISLE, B., CELERI, C. AND ROBSON, M.C., 2007. Hypochlorous acid as a potential wound care agent: part I. stabilized hypochlorous acid: a component of the inorganic armamentarium of innate immunity. *J Burns Wounds*, 6, 65-79.

YOSHINO F, LEE M-C-I KOBAYASHI K, HAYASHI Y, ARUOMA OI. Assessment of the effect of fermented papaya preparation on oxidative damage in spontaneously hypertensive rat brain using electron spin resonance (ESR) imaging and L-band ESR spectroscopy. *J Func Foods.* 2009 *in press*