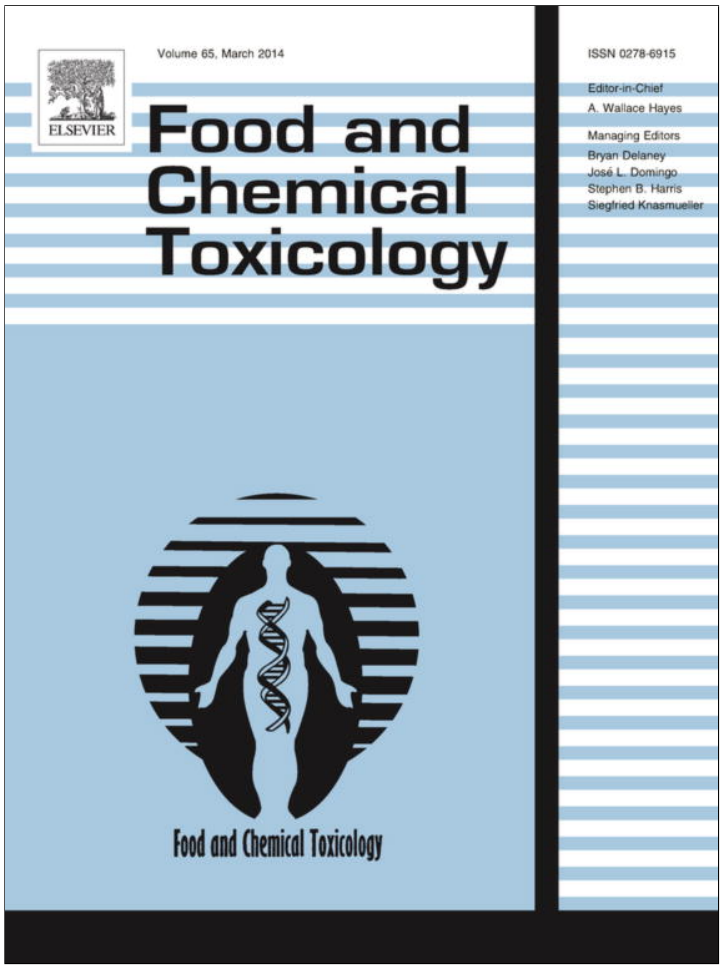

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Relationship between fermented papaya preparation supplementation, erythrocyte integrity and antioxidant status in pre-diabetics ☆

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ABSTRACT

Erythrocytes and their membranes are favorable models to study the relationship between diabetes and susceptibility of erythrocytes to oxidative stress damage. The recommendation for the use of fermented papaya preparation (FPP) as a functional food for dietary management of type 2 diabetes was evaluated by assessing its effect on the human antioxidant status and erythrocyte integrity on a multi-ethnical pre-diabetic population. The *in vivo* effect of FPP was compared with its *in vitro* free radical scavenging potentials. FPP exhibited potent *in vitro* free radical scavenging activities thought to be attributed to residual phenolic or flavonoid compounds. Low doses of FPP significantly reduced the susceptibility of human erythrocytes to undergo free radical-induced hemolysis. The intake of 6 g FPP/day for a period of 14 weeks was observed to significantly reduce the rate of hemolysis and accumulation of protein carbonyls in the blood plasma of pre-diabetics. That FPP consumption on a daily basis can strengthen the antioxidant defense system *in vivo* was clearly demonstrated by the marked increase of total antioxidant status in the FPP-supplemented pre-diabetics. That FPP maintains the integrity of erythrocytes could benefit the strategies to improve the quality of future blood products.

1. Introduction

The implication of oxidative stress in the etiology of type 2 diabetes mellitus has widely led to the suggestion that incorporation of antioxidant-rich foods into the diet can potentially target oxidative stress pathways and reverse or reduce the progression of type 2 diabetes and development of its related secondary complications. Erythrocytes and their membranes are favorable models to study the relationship between diabetes and susceptibility of erythrocytes to oxidative stress damage. The high proportion of unsaturated lipids, amino acids and DNA nucleotides represent feasible targets for attack by reactive oxygen species. Circulating erythrocytes are continuously burdened by the exposure to high oxygen tension and being naturally abound to iron (hemoglobin)-a major transition metal that promotes radical generation through the Fenton reaction (Puppo and Halliwell, 1988; Prus and Fibach, 2012). Peroxynitrite, superoxide anion and hydroxyl radical related injuries can rupture erythrocyte membranes resulting in critical functional and structural alterations can seriously jeopardize its biological role in the body and interfere with the dynamics of blood flow in the peripheral circulation (Huang et al., 2000; Baskurt et al., 1998). A number of clinical syndromes are associated with marked intravascular hemolysis and circulating free hemoglobin, these include sickle cell disease, paroxysmal nocturnal hemoglobinuria, thalassemias, and hereditary spherocytosis all of which predispose to endothelial dysfunction, thrombosis, and vascular disease (Reiter et al., 2002; Kanias and Acker, 2010; Jandl et al., 1960). Given that chronic inflammation and oxidative stress mechanisms are involved in the pathology of type 2 diabetes, therapeutic interventions using diet- and drug-derived antioxidants to reduce the vulnerability of erythrocytes to oxidative stress continue to be suggested (Aruoma et al., 2007, 2010). Current nutritional recommendations for the prevention of diabetes include modest weight loss, increased fiber intake and the incorporation of functional foods in the diet, such as green tea catechins, pycnogenols, legumes, fruits, vegetables, spices, condiments and beverages that impact insulin action (Aruoma et al., 2007; Rudkowska, 2009; Thielecke and Boschmann, 2009; Zimbadi and Rohdewalde, 2008; Kaushik et al., 2008).

Fermented papaya preparation (FPP) is a dietary supplement that is made from the yeast fermentation of ripe pulp of *Carica papaya* using a biotechnological process that strictly adheres to international quality control regulations. FPP has many prophylactic properties (reviewed in Aruoma et al. (2010)) which also includes the potential to alleviate the oxidative stress burden,

☆ This clinical trial was registered at www.clinicaltrial.gov (NCT01248143).

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especially in pre-diabetics and diabetics (Danese et al., 2006; Somanah et al., 2012). Curbing the incessant rise in type 2 diabetes remains a global health priority which encapsulates the current need for a dietary supplement that can help towards reducing the burden of diabetes on national healthcare systems (Chan et al., 2009). Along this vein, a randomized controlled clinical trial was conducted to determine the effect of a short term supplementation of FPP on the antioxidant status of a multi-ethnical population predisposed to type 2 diabetes mellitus. This study was extended to defining the *in vitro* antioxidant activity of FPP using a battery of free radical scavenging assays namely: 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), 2,2-diphenyl-1-picrylhydrazyl (DPPH), hydroxyl, superoxide anion and nitric oxide scavenging assays. The ability of FPP to modulate 2,2′-azobis(2-methylpropionamidine) dihydrochloride (AAPH)-induced hemolysis and lipid peroxidation was also assessed in attempt to establish a comprehensive antioxidant profile of this functional food.

2. Materials and methods

2.1. Materials

FPP (Osato Research Institute, Gifu, Japan) is made from the yeast fermentation of ripe pulp of *C. papaya* using a specialized biofermentation technique that has received ISO9001:2000 (the international quality standard) and ISO14001:2004 (the international environmental standard) certification and ISO 22000:2005 (the international food safety standard) certifications.

2.2. Estimation of total phenol and flavonoid content in FPP

The methods described in Singleton et al. (2003) and Zhishen et al. (1999) were adapted and used for the quantification of total phenols and total flavonoids in FPP. Briefly a known concentration of FPP was added to 3.5 ml of distilled water, 250 µl of Folin–ciocalteau reagent and 1 ml 20% (w/v) sodium carbonate. The reaction mixture was incubated at 40 °C for 40 min and optical density (OD) read at 685 nm (Genesys™ 10S, Thermo Scientific, USA). For the quantification of total flavonoids: to 500 µl FPP, 150 µl 5% (w/v) sodium nitrite and 2 ml of distilled water were added. After 5 min, 150 µl 10% (w/v) aluminum chloride and 1 ml (1 M) sodium chloride was added and final volume topped up to 5 ml with distilled water. The OD was read at 510 nm (Genesys™ 10S, Thermo Scientific, USA). Estimation of total phenol and flavonoid content was calculated using the formula (adapted from Sharma et al. (2011)):

$$((C * TV)/M)$$

where C is gallic acid or catechin equivalent calculated from standard curve (mg/ml); TV is total volume of reagents used (ml) and M is mass of extract used in assay (g/ml)

2.3. In vitro antioxidant activities of a fermented papaya preparation

The methods of Campos and Lissi (1997), Duan et al. (2007) and Halliwell et al. (1987) were used for the ABTS, DPPH, hydroxyl radical scavenging assays respectively. The methods of De Andrade et al. (2010), Kumar et al. (2011) and Sunil et al. (2012) were used to assess the ability of FPP to inhibit lipid peroxidation and scavenge superoxide anions and nitric oxide. The scavenging activity of FPP was measured using the formula (Kumar et al., 2011):

$$([(A_0 - A_1)/A_0] * 100)$$

where A_0 is absorbance of reaction mixture only and A_1 is absorbance of reaction mixture with FPP.

2.4. Protective effect of FPP on the antioxidant defenses of pre-diabetics

2.4.1. Study design of clinical trial

127 Pre-diabetic Mauritians were recruited based on the following 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; and (6) not receiving glucose-lowering, cholesterol-lowering or anti-hypertension treatment. The study consisted of a randomized, controlled clinical trial with treatment and control groups running in parallel. The treatment group received 6 g FPP dissolved in 200 ml (1 cup) water per day before mealtimes for a period of 14 weeks. This supplementation period was then followed by a 2 weeks wash out. 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).

2.4.2. Blood collection and analysis

Blood was collected at the Cardiac Center, SSRN hospital, Pamplemousses, at baseline, week 14 and after wash out following a 10 h fast. Samples were centrifuged and analyzed for total antioxidant status (TAS) using an automated clinical chemistry analyzer (Olympus AU480, Beckman Coulter® Inc.) at the Apollo Bramwell Hospital, Mauritius. Commercial kits (NX 2332) were purchased from Randox® laboratories (Crumlin, UK). Random samples were sent to an independent laboratory for cross-checking (Biohealth Ltd., Mauritius). Serum was stored at −20 °C for further analysis.

2.4.3. Inhibition of hemolysis in human erythrocytes

Human blood samples were obtained from the Biochemistry Department of Félix Guyon Hospital (Saint Denis, Réunion, France). Using the modified method of Prost (1992), erythrocytes were isolated, washed and diluted in 0.15 M NaCl. To 100 µl (2×10^7 erythrocytes/µl) of diluted red blood cell solution, 100 µl serum extract obtained from the clinical trial (serially diluted in 0.15 M NaCl) or FPP (200–1000 µg/ml) was added. Lysis of erythrocytes was initiated by adding 40 µl 2,2′-azobis(2-methylpropionamidine) dihydrochloride (AAPH, 0.5 M). Turbidity at 450 nm was recorded at intervals of 10 min using a thermostated microplate reader.

2.4.4. Quantification of protein carbonyls

A serum sample (5 µl) was denatured by 5 µl 12% (w/v) sodium dodecyl sulfate (SDS). Protein carbonyls present in samples were converted to 2,4 dinitrophenylhydrazine (DNP) hydrazone by DNP in 2 M HCl for 20 min at room temperature. Samples were diluted in coating buffer, adsorbed to wells of an ELISA plate and blocked with PBS/0.1% tween/1% bovine serum albumin, then probed with an anti-DNP antibody (Sigma D9656) and a horseradish peroxidase (HRP) conjugated secondary antibody (ECL anti-rabbit IgG, HRP-linked whole antiobody from donkey) for 3 h and 1 h respectively at room temperature. A tetramethylbenzidine (TMB) substrate was then added and allowed to oxidize for 15 min to a sapphire-blue complex at room temperature. The reaction was stopped by addition of 2 M HCl and OD measured at 450 nm.

2.5. Statistical analysis

Data derived from antioxidant assays were fitted into an appropriate regression model that allowed us to determine AA_{50} of FPP (mg/ml). Whereas data obtained from the supplementation study was entered into Medcalc (version 11.4.2, Mariakerke, Belgium) and Prism™ (GraphPad, version 3.0) for statistical analysis. After omission of outliers, statistical differences within groups over time were determined using Student's paired samples t-test, where data was non-normal, the non-parametric alternative Wilcoxon test was used. Statistical differences between groups over time were determined using Student's independent samples t-test, where Mann–Whitney test was used for non-normal data. A two-tailed P value less than 0.05 was considered to be significant. Significance was set at 95% CI.

3. Results

3.1. Total phenol and flavonoid content in FPP

Average gallic acid equivalent was estimated to be 5.81 ± 0.69 mg g^{-1} FPP. Whereas, the catechin equivalent was found to be approximately 2.26 ± 0.15 mg g^{-1} FPP.

3.2. Assessment of the in vitro antioxidant activities of FPP

The antioxidant profile of FPP was established using 7 independent assay methods using a concentration range varying between 1 and 200 mg/ml. A ranking order indicating the concentration of FPP required to attain an antioxidant activity of 50% (AA_{50}) for each assay was established as follows (Table 1): hydroxyl scavenging (4.13 ± 0.76 mg/ml) > ABTS scavenging (14.56 ± 1.65 mg/ml) > DPPH scavenging (55.69 ± 6.46 mg/ml) > inhibition of AAPH-induced lipid peroxidation (68.06 ± 22.79 mg/ml) > superoxide scavenging (88.70 ± 22.48 mg/ml) > nitric oxide scavenging (116.55 ± 40.59 mg/ml) > inhibition of iron (III)/ascorbate-induced lipid peroxidation. The activity of FPP was observed to be dose dependant in all assays (data not shown).

Table 1
In vitro antioxidant activities of a fermented papaya preparation (FPP).

In vitro antioxidant activity	AA_{50} of FPP (mg/ml)
$OH^{\cdot}$ scavenging	4.13 ± 0.76^{a}
$ABTS^{+}$ scavenging	14.56 ± 1.65^{a}
DPPH scavenging	55.69 ± 6.46^{b}
Inhibition of AAPH-induced lipid oxidation	68.06 ± 22.79^{c}
$O_2^{\cdot -}$ scavenging	$88.70 \pm 22.48^{b,c,d,f}$
$NO^{\cdot}$ scavenging	$116.55 \pm 40.59^{b,e,f}$
Inhibition of iron (III)/ascorbate induced lipid oxidation	$130.20 \pm 14.96^{b,c,f}$

Data for AA_{50} is expressed as mean ± standard deviation ($n = 4$). Statistical analyzes were performed, using independent samples *t*-test, for multiple comparisons different alphabetical superscripts between rows represent significant differences between mean AA_{50} ($P < 0.01$). AA_{50} is defined as the concentration of FPP (in mg/ml) that attained an antioxidant activity of 50%. Abbreviations: $OH^{\cdot}$: hydroxyl radical; ABTS: 2,2'-azino-bis (3-ethybenzthiazoline-6-sulfonic acid) radical; DPPH: 2,2-diphenyl-1-picrylhydrazyl radical; APPH: 2,2′-azobis(2-amidinopropane) hydrochloride radical; $O_2^{\cdot -}$: superoxide radical; $NO^{\cdot}$: nitric oxide.

3.3. Effect of FPP supplementation on free radical induced hemolysis

FPP increased the hemolysis HT_{50} of red blood cells in a non-dose-dependent manner. A minimum dose of 200 μg/ml significantly increased HT_{50} by 144.6 ± 7.5% compared to a control ($P < 0.01$). The capacity of FPP to delay red blood cell hemolysis gradually reduced as from 400 μg/ml upwards ($P < 0.05$) (Table 2). The measurement of HT_{50} is very reproducible with intra- and inter-assay coefficients of variation, 1.32% and 3.85%, respectively (Bourdon et al., 1999). Indeed in humans and animal models where oxidative stress has been well documented, HT_{50} have been shown to be representative of the total defense against free radicals.

Basal total antioxidant status (TAS) values varied between 1.48–1.88 mM/l in males and 1.35–1.75 mM/l in females of the treatment group (Table 3). After 14 weeks of consuming 6 g FPP/day, the TAS of males and females of the treatment group were observed to increase by 4.9% ($P < 0.05$) and 5.7% ($P > 0.05$) respectively. Post wash out analysis showed a continuous increase ($P < 0.001$) of mean TAS values in both genders of the treatment (Fig. 1). These results demonstrate the prolonged prophylactic effects of FPP on the general functioning of the immune system.

Fig. 2 illustrates the effect of hemolysis on human erythrocytes exposed to plasma from 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 (Table 3). FPP consumption induced a slight increase ($P < 0.05$) of 3.3% in males, whereas HT_{50} dropped by 1.5% in females at week 14. Post wash out analysis showed a continuous non-significant increase of 1.3% ($P > 0.05$) and 4% ($P > 0.05$) in males and females of the treatment group respectively, providing further evidence of the prophylactic effects of FPP. The mean carbonyl protein content in pre-diabetics at baseline ranged between 156–840.8% and 224.2–947.7% in males and females of the treatment group (Table 3). The effect of a short term intake of FPP on the level of protein carbonyls in the blood plasma is illustrated in Fig. 3. After 14 weeks of FPP supplementation there was a decrease of 1.9% in males and 9.7% females of the treatment group (non-significant). A continued non-significant reduction of 5.8% and 11.9% in males and females respectively was observed after the wash out period. The extent of which FPP influences the accumulation of protein carbonyls in the blood plasma even after the wash out period reflects the *in vivo* capacity of FPP to markedly lower oxidative stress in pre-diabetics.

4. Discussion

It is of intriguing importance that hemoglobin oxidation can largely dictates the ability to effectively preserve red blood cells. That FPP may have a potential in preserving erythrocyte integrity was the subject of the investigation here reported. Indeed, factors affecting the rate of hemoglobin oxidation during red blood cell *ex vivo* storage include compromised antioxidant activity, high concentrations of glucose in the storage media and the presence of molecular oxygen. That FPP intake could positively alter diabetes-related risk factors, including a significant decrease in systemic inflammation, an improvement of lipid profile, liver and kidney functionality and mean arterial blood pressure, is seminal (Somanah et al., 2012).

Table 2
The effect of fermented papaya preparation (FPP) on the rate of free radical-induced hemolysis of human erythrocytes.

Hemolysis (HT_{50})						
Concentration of FPP (μg/ml)	Control	200	400	600	800	1000
HT_{50} (%)	100	144.61 ± 7.49**	135.78 ± 6.38*	121.17 ± 20.12	112.67 ± 18.80	111.39 ± 8.71

HT_{50} denotes the time taken (in min) for 50% hemolysis to occur compared to a control (1% NaCl). Values are expressed as mean percentage ± standard deviation ($n = 3$).
* $P < 0.05$ vs. control.
** $P < 0.01$ vs. control.

Table 3
Baseline data for participants enrolled in the clinical study. Data is expressed as mean baseline value ± standard deviation.

Mean baseline value										
		Age (years)	MABP (mmHg)	BMI	Waist–hip ratio	Fasting blood glucose (mmol/l)	HbA1c (%)	TAS (mmol/l)	Hemolysis half-time (%)	Carbonyl protein accumulation (%)
FPP group	Male ($n = 24$)	49.7	98.47 ± 7.92	26.75 ± 2.64	0.92 ± 0.05	5.26 ± 8.22	6.03 ± 0.53	1.53 ± 0.14	102.05 ± 29.33	567.88 ± 181.54
	Female ($n = 20$)	49.5	92.40 ± 10.68	26.90 ± 3.50	0.82 ± 0.05	5.39 ± 6.66	6.04 ± 0.45	1.67 ± 0.18	101.01 ± 28.22	579.38 ± 211.91
Control group	Male ($n = 29$)	46.9	95.33 ± 6.10	26.31 ± 4.77	0.92 ± 0.06	5.05 ± 7.66	5.95 ± 0.36	1.62 ± 0.13	115.84 ± 29.91	553.0 ± 130.92
	Female ($n = 27$)	47.2	89.14 ± 8.59	26.55 ± 3.35	0.83 ± 0.05	5.08 ± 9.70	5.93 ± 0.24	1.48 ± 0.11	83.70 ± 23.47	552.83 ± 173.22

MABP: mean arterial blood pressure; BMI: body mass index; HbA1c: glycated hemoglobin; TAS: total antioxidant status.

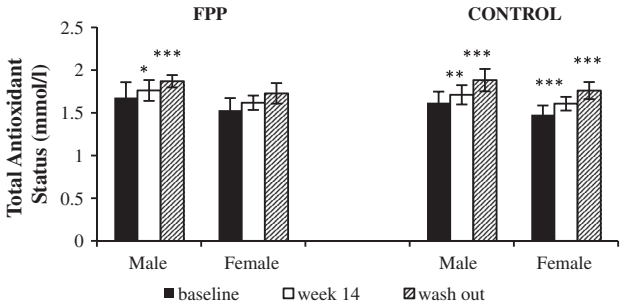

Fig. 1. Effect of FPP on the total antioxidant status (TAS) in a pre-diabetic population under the FPP (male, $n = 20$; female, $n = 16$) and control regimes (male, $n = 28$; female, $n = 25$). Data is expressed as mean TAS value (mmol/l) where error bars represent standard deviation. Significance: $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ vs. baseline value.

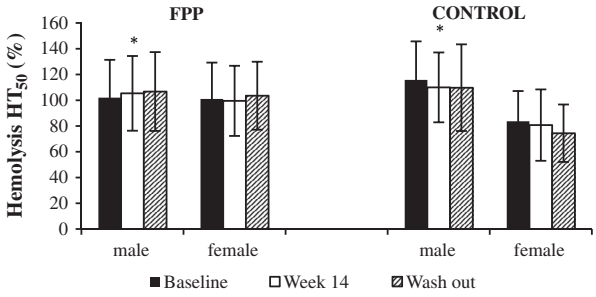

Fig. 2. The effect of FPP on hemolysis half-time (HT_{50}) in a pre-diabetic population under the FPP (male, $n = 22$; female, $n = 15$) and control regimes (male, $n = 25$; female, $n = 26$). Data is expressed as mean percentage, where error bars represent standard deviation. Significance: $^{*}P < 0.05$ vs. baseline value.

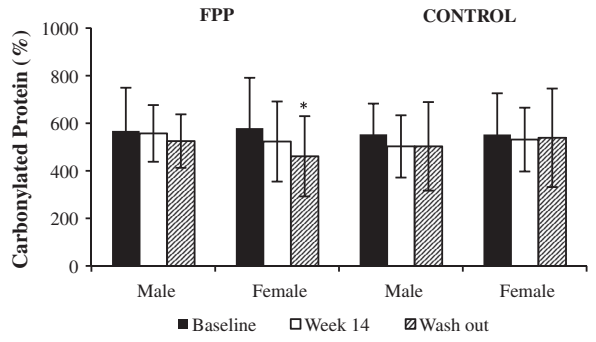

Fig. 3. The effect of FPP on the accumulation of carbonylated proteins in a pre-diabetic population under the FPP (male, $n = 23$; female, $n = 18$) and control regimes (male, $n = 30$; female, $n = 28$). Data is expressed as mean percentage where error bars represent standard deviation. Significance: $^{*}P < 0.05$ vs. baseline value.

The methods used for estimation of polyphenols were selected based on their simplicity, reproducibility and integration of non-hazardous reagents. Our results show that FPP contained relatively low equivalents of gallic acid and catechin. Identification and quantification of the secondary metabolites present in fresh *C. papaya* has been focus of many studies. GC–MS data of Canini et al. (2007) revealed the presence of caffeic acid (0.25 mg/g fresh weight (FW)), protocatechuic acid (0.11 mg/g FW), *p*-coumaric (0.33 mg/g FW), and trace amounts of kaempferol and quercetin (≈0.04 mg/g FW) in its leaves; while its fruit pulp contained cysteine endopeptidase, glutaminyl cyclase and linalool (Azarkan et al., 2006). Luximon-Ramma et al. (2005) reported moderate total phenol levels in methanolic extracts of papaya pulp (variety: exotica) (576 ± 41 μg gallic acid/g FW; 376 ± 15 μg quercetin/g FW; 208 ± 21 μg cyaniding chloride/g FW); whereas important quantities of ascorbic acid were detected (929 ± 19 μg/g FW). Ethyl acetate fractions of papaya seeds, on the other hand, were found to contain much lower quantities: 1.945 ± 45.55 μg gallic acid/100 g DW and 0.117 ± 15.54 μg rutin/100 g DW (Zhou et al., 2011). Whilst the polyphenolic profile of fresh papaya is firmly established, the exact phytochemical composition of FPP remains an area of on-going research. Processing steps (*in vacuo* concentration, prolonged storage and pasteurization) that are commonly involved in large scale commercialization of fruit-based products (juice, puree, jam and wine) are largely suspected to contribute to the reduced quality and nutrient loss of the final product. Findings of Kloptek et al. (2005) demonstrated that mashing and heating could indeed lead to an extensive loss of vitamin C and phenolic compounds as reflected by the reduced ABTS scavenging activity (−20%) of strawberry puree. But, upon fermentation of strawberry puree with yeast, the antioxidant/anti radical effect of strawberry wine was found to have been significantly boosted by 69%. Interestingly Qin et al. (2010) also found that the use of yeast (*Issatchenkia orentalis*) and *Bacillus* spp. for the fermentation of black soybeans (*Glycine max*) could in fact boost both its polyphenolic content and DPPH scavenging activity threefold in comparison to a non-fermented control sample. Microorganisms have the ability to modify a plant's nutritive composition upon fermentation. For example, a resulting enhancement of vitamins, essential amino acids and proteins not only improves its nutritional quality and digestibility, but also greatly enhances its biochemical properties. Eom et al. (2010, 2011) reported that fermentation of an edible Korean brown alga *Eisenia bicyclis* and *Laminaria japonica* by yeast (*Candida utilis*) resulted in a superior total phenol content and amino acid profile compared to non-fermented controls. This suggests that the process of fermentation can greatly modify the ratio of free amino acids which may contribute to the functional qualities of FPP. Besides the abundance of amino acids, oligosaccharides and vitamin B6 in FPP, papaya pulp also contains a variable amount of glucose, saccharose, citric acid, malic acid all known to possess hydroxyl groups that may greatly influence its free radical scavenging (Lo scalzo and Venezian, 2010). It also contains β-carotene a known oxidative stress suppressant in diabetic rats (Maritime et al., 2002). Hydroxyl radicals produced during aerobic metabolism by the Fenton reaction between iron or copper ions and hydrogen peroxide, are the most predominant and reactive of oxygen species that are responsible for base mutations in DNA (Aruoma et al., 1989). We report the potential of FPP to scavenge hydroxyl radicals (AA_{50} 4.13 ± 0.76 mg/ml, Table 1). Comparable observations were equally made by Imao et al. (1998) and Santiago et al. (1991) where electron spin resonance spectrometry data revealed low AA_{50} values for the hydroxyl scavenging activity of FPP in iron-induced epileptic foci of rats. In line with these findings, Antioxidant Biofactor (AOB®) another commercial dietary supplement made from the *Aspergilus oryzae* fermentation of soybean, wheat, rice bran, sesame, citron and malted rice was also reported to significantly counteract ferric-nitriloacetate-induced DNA damage (Mizote et al., 2008). The possibility of such fermented health products to deviate hydroxyl radicals away from π bonds of C_5 and C_6 pyrimidines and C_4 and C_8 of purines, thus protecting DNA from any structural modification has been proposed (Marotta et al., 2007). The DPPH assay is a preferred model for determining the anti-radical power with respect to the decay of the stable radical DPPH. Osato et al. (1995) reported the remarkable DPPH scavenging activity of Runn-Runn® a Japanese skin care product made from a mixture FPP and fermented rice bran (99% at 100 mg/ml), whereas the anti-radical activity of Bio-Catalyzer No. 11®, a fermented preparation of unripe papaya pulp exhibited minimum DPPH scavenging activity (11% at 25 mg/ml). These findings suggest that the scavenging activity of FPP may be attributed to its

strong hydrogen donating ability and that it has an improved potency when in synergy with other antioxidant rich products. The presence of polyunsaturated fatty acids in membranes makes it a susceptible target to free radical attack and propagation. Since membranes are fundamental to the normal functioning of cellular organelles (e.g., mitochondria, lysosomes, endoplasmic reticulum and peroxidases) the damage caused by lipid peroxidation is highly detrimental to the cell's structure and functioning. In the present study, we report the AA_{50} of FPP to be 68.06 ± 22.79 mg/ml and 130.20 ± 14.96 mg/ml in the AAPH- and iron (III)/ascorbate-induced lipid peroxidation system assays respectively. A similar membrane lipid protective effect was demonstrated in the earlier work of Marcocci et al. (1996) and Haramaki et al. (1995), where thiobarbituric acid reactive substance levels (an index of peroxyl radical induced lipid peroxidation) were observed to significantly drop in kidney, brain and heart homogenates of FPP supplemented mice (0.1%/day/8 weeks). Manda®, a Japanese health product produced by the yeast fermentation of fruits, seaweed and black sugar, demonstrated a similar protective effect on brain homogenates of senescent rats orally administrated Manda® (200 g/day/8 days) (Kawai et al., 1998). In the present study, the relatively high AA_{50} values of FPP denote the moderate scavenging of both nitric oxide and superoxide radicals (Table 1). Besides being a scavenger, FPP can also present itself as a ROS modulator protecting SH-SY5Y neuronal cells from cytotoxic-induced apoptosis inflicted by over accumulation nitric oxide and superoxide radicals within cells (Zhang et al., 2006).

The antioxidant propensities demonstrated through the multitude of assays described in this paper are not only consistent with the functional bioefficacy that have been ascribed to FPP, but also supports the emerging view that FPP may modulate the signaling mechanisms involved in redox regulation (Marotta et al., 2010; Aruoma et al., 2010). Data obtained from the supplementation study provides substantial evidence that a daily intake of 6 g FPP can certainly contribute to an improved functioning of the antioxidant defense system, as evidenced by the elevated TAS levels in FPP-supplemented group. This notion is further supported by the decrease in protein carbonyl levels, an index of oxidative stress observed here and in Haramaki et al. (1995). Chronic oxidative stress can increase the susceptibility of erythrocytes to undergo hemolysis, which is a likely result of free radical attack to membrane proteins and lipids (Shinar and Rachmilewitz, 1990). The ability of FPP to counteract oxidative stress in human erythrocytes has recently been reported in the work of Prus and Fibach (2012). In the present paper, we confirm earlier studies that a short term intake of FPP can indeed influence the susceptibility of human erythrocytes to under hemolysis.

A minimal dose of FPP was found to be sufficient to significantly reduce the rate of hemolysis, suggesting that specific membrane stabilizing compounds may be more active at this dose (200 μg/ml in fact reflects a realistic concentration of FPP in the body after its bio-assimilation has taken place in the intestinal tract and liver). The membrane protecting effects of FPP have been attested by Ghoti et al. (2010) and Amer et al. (2008) who describe the ability of FPP to reduce the tendency of thalassemic erythrocytes to undergo hemolysis and phagocytotic uptake by macrophages. The latter is an expected outcome of free radical attack on membrane proteins bands 3, 4.1 and spectrin and may explain the high incidence of thromboembolic complications encountered by diabetics and the aged (Shinar and Rachmilewitz, 1990; Beneke et al., 2005). Elevated quantities of glutanic acid, glycine and methionine naturally present in FPP are casually suggested to contribute to glutathione synthesis – an important building block of glutathione peroxidase – a major endogenous antioxidant enzyme and deactivation of erythocyte membrane glycoproteins (Fibach et al., 2010; Amer et al., 2008). The up-regulation of several other endogenous antioxidant enzymes notably, superoxide dismutase, catalase and xanthine oxidase by FPP has been widely reported in literature (Marotta et al., 2004, 2010). The ability of FPP to permeate the bloodstream after oral ingestion and act in synergy with other locally present dietary antioxidants such as vitamin C and α-tocophenol may equally contribute to the overall decrease of oxidative stress in the treatment group as well. Globally our findings warrant a complete chemical characterization of both enzymatic and non-enzymatic constituents of FPP in order to understand the nutrageonomic mechanisms reported herein.

5. Conclusion

FPP is a potential therapeutic functional food that can not only contribute to the antioxidant status of pre-diabetics but also provide a significant level of protection to human erythrocytes against oxidative stress-induced hemolysis. Given the complex mechanisms involved in the human body burdened by oxidative stress, there is urgent need to define the significance of this in diabetes and in other pathophysiological conditions characterized by this phenomenon (oxidative stress and overt inflammation). It is intriguing to suggest that an understanding of the mechanisms with which dietary factors maintain erythrocyte integrity could add to strategies to improve the quality of future blood products.

Contributor statements

Jhoti Somanah researched data. Jhoti Somanah, Emmanuel Bourdon, Philippe Rondeau, Okezie I. Aruoma and Theeshan Bahorun/reviewed/edited the manuscript.

Conflict of Interest

Okezie I. Aruoma is actively involved in biomedical research involving fermented papaya preparation with the Osato Research Institute, Gifu, Japan. All authors declare that there are no conflicts of interest.

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