IJFANS
International Journal of Food And Nutritional Sciences

Volume 4, Issue 5, Oct-Dec 2015, www.ijfans.com e-ISSN: 2320-7876

INTERNATIONAL JOURNAL OF FOOD AND NUTRITIONAL SCIENCES

IMPACT FACTOR ~ 1.021

Official Journal of IIFANS

INTERNATIONAL JOURNAL OF FOOD AND NUTRITIONAL SCIENCES

e-ISSN 2320 –7876 *www.ijfans.com*
Vol.4, Iss.5, Oct-Dec, 2015

Research Paper *Open Access*

CHARACTERIZATION OF FRENCH VICTORIA PINEAPPLE ANTIOXIDANT MICRONUTRIENTS AND POLYPHENOL PROTECTIVE EFFECT ON PREADIPOSE CELLS EXPOSED TO OXIDATIVE STRESS

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Received on: 1st June, 2015 **Accepted on: 23nd December, 2015**

ABSTRACT

Much attention is paid to the health benefits of antioxidant micronutrients such as vitamin C, carotenoids and polyphenols provided by fruits and vegetables. Polyphenols are the most abundant dietary antioxidants and may help to reduce oxidative stress and inflammation in adipose cells during obesity. Thus, the identification of polyphenol-rich sources is of high interest. The present study evaluated the levels of antioxidant micronutrients from Victoria pineapple cultivated in 9 habitats of Réunion Island in France, and the effect of polyphenols on preadipose cells exposed to oxidative stress. We found that micronutrient amounts differed depending on habitats and identified polyphenols as the most abundant antioxidants (40.8±1.7mg) gallic acid equivalent/100g) as compared to vitamin C (29.4±1.1mg/100 g) and carotenoids (57.4±3.5µg) β-carotene equivalent/100g). Polyphenol-rich extracts from pineapple pulp, peel and crown contained gallic, caffeic, ferulic and p-coumaric acids, and exhibited free radical-scavenging capacities. Remarkably, pineapple pulp polyphenols exerted antioxidant and anti-inflammatory effects on preadipose cells exposed to H_2O_2-mediated oxidative stress, by reducing intracellular levels of reactive oxygen species and interleukin-6 pro-inflammatory cytokine production. Altogether, these data led to identify French Victoria pineapple as a relevant source of antioxidants and showed polyphenol benefits on preadipose cells during oxidative stress.

Key words: Victoria pineapple; polyphenols; antioxidants; oxidative stress; inflammation; adipose cells.

INTRODUCTION

Obesity is defined as an abnormal excessive fat accumulation within the white adipose tissue that may alter health and increase mortality. Indeed, excess of fat storage in adipose cells induces oxidative stress with an over-production of metabolic waste products derived from oxygen and called reactive oxygen species (ROS) such as H_2O_2. ROS disrupt adipose cell function and elevate the release of pro-inflammatory cytokines like interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α), leading to the development of insulin resistance and type 2 diabetes (Gregor et.al., 2007 and Houstis et.al., 2006). Epidemiological studies indicate that a fruit and vegetable-rich diet exerts beneficial effects against obesity-related disorders. Although it is important to consider carbohydrate composition of plant products to better manage their impact on blood glucose levels (Miller, 1994 and Parks, 2004), fruits and vegetables are main sources of bioactive micronutrients including vitamins, carotenoids and polyphenols. Vitamin C (ascorbic acid) is considered as a powerful antioxidant (Guorong *et.al.,* 2009) and its daily intake lowers the incidence of several chronic diseases such as diabetes and cardiovascular disorders (Osganian et.al., 2003). Besides their colorant properties, one of the important characteristics of carotenoids like β-carotene is their ability to act as antioxidants, thus protecting cells and tissues from damaging effects caused by ROS (Yahia, 2009). Polyphenols are considered as the most abundant dietary antioxidants (Scalbert, 2005). Several studies have showed the interest of polyphenols such as phenolic acids comprising gallic, caffeic, ferulic or chlorogenic acids, and flavonoids like quercetin or catechin, against oxidative stress and inflammation in adipose cells during obesity (Scalbert, 2005 and Yen, 2011). Our recent data demonstrated that polyphenols exerted antioxidant and anti-inflammatory effects on preadipose cells exposed to H_2O_2-mediated oxidative stress, depending on their structure, dose and time of exposure (Hatia, 2014 and Baret, 2013).

Pineapple (Ananas comosus, Bromelaceae) is one of the most consumed tropical fruits and the total production worldwide is 16 to 18 million tons (Sousa,

2010). Consumption of pineapple has been related to several beneficial properties such as antioxidant (Hossain and Rahman, 2011), anti-inflammatory (Hale, 2005) and anti-diabetic activities (Riya, 2013 and Xie, 2006). Réunion Island is the most recognized site for the production of Victoria pineapple cultivar in France and Europe. To our knowledge, there is a lack of data regarding the levels of antioxidant micronutrients from French Victoria pineapple. Furthermore, there is no data concerning the impact of pineapple polyphenols on preadipose cells despite the fact that these cells play a crucial role in adipose tissue development. Preadipose cells also contribute to the regulation of adipose tissue immune response due to their great plasticity to convert as macrophage-like cells in pro-inflammatory conditions. Such properties of preadipose cells may be profoundly altered during obesity-related oxidative stress and inflammation (Furukawa, 2004, Charrière, 2013, Cousin, 1999).

The present study aimed to evaluate the levels of carbohydrates, vitamin C, carotenoids and polyphenols from the pulp of Victoria pineapple cultivated in 9 habitats of Réunion Island in France. Free radical-scavenging activities of polyphenol-rich extracts from pineapple pulp and by-products (peel and crown) were also explored. Then, we determined the effect of pineapple pulp polyphenols on 3T3-L1 preadipose cells exposed to H_2O_2-mediated oxidative stress, by measuring cell viability, ROS production and IL-6 pro-inflammatory cytokine secretion.

MATERIALS AND METHODS

SAMPLE COLLECTION

Victoria pineapple samples were provided by Vivéa Fruit and Vegetable Farm, and collected from 9 habitats in Réunion Island in France, during the summer period (from November 2013 to February 2014). Habitats were characterized by the Global Positioning System (Table 1) and parameters including pluviometry (Fig.1A), temperature (Fig. 1B) as well as global solar irradiance (Fig.1C) data which were obtained from Meteo France (www.meteofrance.com). For each habitat, 3 fruits were used. The fruits were harvested at optimal eating quality and received at the laboratory one day after harvest. Pulp, peel and crown samples were ground at room temperature (Grindomix GM 200, Germany). Different portions were analyzed immediately or stored at -20°C until analysis.

DETERMINATION OF CARBOHYDRATE, CAROTENOID, VITAMIN C AND POLYPHENOL CONTENTS

Total carbohydrate levels were determined according to the method previously described by Hallmann with slight modifications (Hallmann, 2012). Briefly, 5mL of concentrated HCl were added to pineapple pulp sample (5 g) and the mixture was heated for an acid hydrolysis. Then, 20mL NaOH (2 M) were added and the neutralized solution was mixed with 10mL Fehling solution. A calibration curve was built using a standard solution of glucose. Total carbohydrate contents were expressed as g glucose equivalent/100g of fresh weight.

For the evaluation of carotenoid contents from pineapple pulp, the method published by Khoo et al. (2008) was used and carotenoid levels were measured by UV-Vis spectrophotometry at 450nm (Genesys 10 UV scanning, USA). A calibration curve was built using a standard solution of β-carotene. Total carotenoid contents were expressed as µg β-carotene equivalent/100g of fresh weight.

The 2, 6-dichloro-phenol-indophenol titrimetric method was used to determine the content of reduced ascorbic acid from pineapple pulp (JAOAC, 1984). Briefly, 5g of pineapple pulp sample were diluted with 10mL of metaphosphoric acid-acetic acid solution and 10mL distilled water. The solution was titrated with 0.01% (v/v) of 2, 6-dichloro-phenol-indophenol solution. The final point was considered when the solution had a pink color for 15s. A calibration curve was built using a standard solution of ascorbic acid. Vitamin C contents were expressed as mg ascorbic acid equivalent/100 g of fresh weight.

For polyphenol analysis, 6g of pineapple pulp, peel or crown were added to 30Ml of aqueous acetone (70%, v/v) containing HCl 1.2M. After incubation at 4°C for90min, samples were centrifuged at 3500 rpm for 20min at 4°C. Polyphenol-rich supernatants were collected and stored at -80°C until analysis. Folin-Ciocalteu assay was used to measure the total polyphenol content (Folin *et.al.* 1915). Briefly, 25µL sample, 125µL Folin-Ciocalteu's phenol reagent (Sigma-Aldrich, Germany) and 100 µL sodium carbonate were added in a 96-well micro plate and incubated at 54°C for 5 min, and then at 4°C for 5 min. The absorbance was measured at 765nm (FLUOstar Optima, Bmg Labtech, Germany). A calibration curve was built using a standard solution of gallic acid. Total polyphenol contents were expressed as mg gallic acid equivalent (GAE)/100g fresh weight. Then, polyphenols from pineapple pulp, peel and crown were identified by High-Performance Liquid Chromatography-UV (HPLC-UV) analysis according to a method previously described (Yapo *et.al.*, 2011). Briefly, polyphenol-rich extracts were analyzed through a HPLC unit (Agilent 1200 Series LC, Germany), using a reversed-phase C18 column (ZORBAX Eclipse, 150 x 4.6mm, 5µm, Agilent, Germany) eluted at a flow rate of 0.6mL/min under the solvent system water/formic acid (99.9/0.1,v/v) (A) and acetonitrile/formic acid (99.9/0.1,v/v) (B). The gradient program was 10% B (0-20min), 10 to 50% B (20-40min), 50 to 100% B (40-41min), 100% B (41-50min), 100 to 10% B (50-51 min) and 10% B (51-60min), and the injection volume was 20µL. Spectral data from all peaks were accumulated in the 250-370 nm range and chromatograms were recorded at 280 nm. Polyphenol identification were achieved by analyzing retention times and spectra data related to 19 standard phenolic compounds (Table 2).

EVALUATION OF FREE RADICAL-SCAVENGING ACTIVITIES OF POLYPHENOL-RICH EXTRACTS FROM PINEAPPLE PULP, PEEL AND CROWN

The ability of polyphenol-rich extracts from pineapple pulp, peel or crown to scavenge commercially available and stable free radical 2,2'-diphenyl-1-picrylhydrazyl (DPPH) was measured according to the method published by Hatia *et.al.* (2014). Briefly, 0.25mM DPPH (Sigma-Aldrich, Germany) diluted in methanol was incubated with polyphenol-rich extracts from pineapple pulp, peel or crown, or positive controls including gallic, caffeic, ferulic and p-coumaric acids (25µM, Sigma-Aldrich, Germany). After 25min at 25°C, the optical density (OD) was read at 517nm (FLUO star Optima, Bmg Labtech, Germany). Free radical-scavenging activity was expressed as the inhibition percentage and was calculated using the following formula:

Antioxidant capacity (%) = ((OD control - OD sample) /ODcontrol) ×100.

Free radical-scavenging activities of polyphenol-rich extracts from pineapple pulp, peel or crown were also evaluated by Oxygen Radical Absorbance Capacity (ORAC) test, according to the method previously described (Hatia, 2014). Analyses were conducted in phosphate buffer saline (PBS, pH 7.4). Peroxyl radical was generated using 2, 2'-azobis [2-methyl-propionamidin] dihydrochloride (AAPH) (Sigma-Aldrich, Germany) which was prepared fresh for each run. Fluorescein (Sigma-Aldrich, Germany) was used as the substrate. Briefly, 25 µL samples diluted 40 times with PBS and 150 µL of 8.4×10^{-5}mM fluorescein were placed in a 96-well black plate, and after 15 min at 37°C, 25µL of 153mM AAPH radical were added to each well. Then, the fluorescence was measured for 1 h 40 min at an excitation wavelength of 485nm and an emission wavelength of 530 nm (Infinite 200, Tecan). Positive controls including gallic, caffeic, ferulic and p-coumaric acids were also used (25µM). Values were calculated based on net area under the curve (AUC) obtained by subtracting the AUC of the blank from that of a sample and compared to Trolox standard curve.

EVALUATION OF THE EFFECT OF POLYPHENOL-RICH EXTRACTS FROM PINEAPPLE PULP ON 3T3-L1 PREADIPOSE CELLS EXPOSED TO H_2O_2

3T3-L1 cells were obtained from American Type Culture Collection (USA) and grown in Dulbecco's Modified Eagle's Medium supplemented with 25mM glucose, 10% heat-inactivated fetal bovine serum, L-glutamin (5mM), streptomycin (2µg/mL) and penicillin (50µU/mL). Cells were maintained at 37°C with 5% CO_2.

For cell viability measurement, MTT (3-(4-5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was performed according to the method previously described by Hatia *et al*. (2014). Cells were plated in 96-well plate at a density of 5×10^3cells/well. After 24h, the culture medium was removed and cells were exposed to polyphenol-rich extracts from pineapple pulp (25µM GAE) in the presence or not of H_2O_2 (200µM) (Sigma-Aldrich, Germany) for 24h. Five hours before the end of the experiment, 20 µL of sterile filtered MTT solution (5 mg/mL) (Sigma-Aldrich, Germany) prepared in PBS, were added to each well and the plate was incubated at 37°C. Then, the unreacted dye was removed by centrifugation, the insoluble for mazan crystals were dissolved in 200 µL/well dimethyl sulfoxide and the absorbance was measured at 560nm (FLUO star Optima, Bmg Labtech, Germany).

The level of intracellular ROS was assessed by measuring the oxidation of 2', 7'-dichlorofluorescein-diacetate (DCFH-DA) according to the method previously published by Hatia *et al.* (2014) and Baret, 2013. Briefly, cells were cultured in 96-well black plate (5×10^3 cells/well) for 24h. Then, the medium was removed and replaced by PBS containing 10 µM of DCFH-DA (Sigma-Aldrich, Germany), and cells were kept in a humidified atmosphere (5% CO_2, 37°C) for 45 min. Next, cells were co-exposed to polyphenol-rich extracts from pineapple pulp (25µM GAE) in the presence or not of H_2O_2 (200µM) for 1h. Fluorescence was measured at an excitation wavelength of 492 nm and an emission wavelength of 520nm (FLUO star Optima, Bmg Labtech, Germany).

For the evaluation of the cellular inflammatory response, culture media collected from preadipose cells exposed to polyphenol-rich extracts from pineapple pulp (25µM GAE) in the presence or not of H_2O_2 (200µM) during 24h, were analyzed using Mouse IL-6 ELISA kit (eBioscience, UK). Absolute values were normalized according to total cellular protein contents assessed by Bradford test (Bradford, 1976).

STATISTICAL ANALYSIS

Data were expressed as means ± SEM of three independent experiments with triplicate analysis. Statistical analysis was performed using Prism software (USA). Significant differences ($p<0.05$) between the means were determined by either the Bonferonni or Dunnett tests.

RESULTS AND DISCUSSION

First, considering that different levels of dietary carbohydrates give rise to different glycemic responses and may also have considerable effects on the sensory properties of foods (Miller, 1994, Parks, 2004 and Olsson, 2004), it was important to determine the carbohydrate pattern of Victoria pineapple pulp. Results showed that carbohydrate levels ranged from 14.4±0.2-16.5±0.1g glucose equivalent/100g (Fig. 2A). Two statistically different groups of fruits were identified and originated from habitats 1 and 2 on one hand, and from habitats 3-9 on the other hand ($p<0.05$). Whereas pineapple pulp from both habitats 1 and 2 exhibited a similar carbohydrate content to that found in literature data (Martinez, 2008), samples from other habitats were characterized by a carbohydrate level significantly higher ($p<0.01$). Carbohydrate contents measured from Victoria pineapple pulp were close to those reported for orange, apple or cherry pulps and other tropical fruits such as mango and litchi (12.0-14.0g/100g). Noticeably, Victoria pineapple

pulp appears as a less abundant source of carbohydrates as compared to banana (22.8 g/100g) which is recognized as another tropical fruit largely consumed worldwide (USDA, 2010). Here, results also indicated that there was a low degree of variability in carbohydrate contents between fruits from 9 habitats. This is in agreement with literature data reporting that carbohydrate levels are not considerably modified by environmental stimuli such as the light, temperature, irrigation or fertilization systems when fruits originated from the same cultivar and were harvested at the same ripeness stage and at the same season, as described in the present study. However, such environmental stimuli are known to regulate the biosynthesis pathways of plant secondary metabolites including carotenoids, vitamin C and polyphenols (Melino, 2011).

Carotenoids constitute natural pigments present in fruits and vegetables and are important vitamin A precursors. Results reported in Fig.2B showed a marked variability in carotenoid contents ranging from 40.1±0.7-84.9±0.2µg β-carotene equivalent/100g of fresh weight. Five statistically different groups of fruits could be identified depending on habitats. Pineapple pulp from habitats 7 and 9 presented the lowest carotenoid contents which were close to those published for pineapple from Brazil by Silva *et al.* (2013). Pineapple pulp samples from habitats 1, 2, 6 and 5 exhibited carotenoid levels significantly higher than those of habitats 7 and 9 ($p<0.05$) but lower than those depicted for habitats 8 and 3 ($p<0.05$). Interestingly, pineapple pulp from habitat 4 identified as the most abundant source of carotenoids, as compared to all other habitats ($p<0.05$), was characterized by an amount of carotenoids (84.9±0.2µg β-carotene equivalent/100g of fresh weight) similar to that reported for orange (USDA, 2010), guava and mango fruits which are known to be rich in carotenoids (Setiawan, 2001). Such an abundance of carotenoids in plants such as tropical fruits has been correlated with changes in the expression of genes involved in carotenoid biosynthesis pathways, in response to environmental stimuli integrating photo-oxidative stress and light-signaling cascades. Indeed, carotenoids play important roles in functioning, survival and expansion of plants. They are found in all photosynthetic organs where they act as light-collecting pigments and photo-protectors in photosynthesis. Carotenoids such as β-carotene exert a beneficial photo-protective action by quenching excited chlorophyll molecules as well as an antioxidant effect in stabilizing membrane lipids against photo-damage (Yahia, 2009). Our data showed a pronounced variability in carotenoid levels depending on habitats. This could be explained by the complex interaction between environmental stimuli and agricultural practices, such as soil irrigation frequency or cultivation mode on open/shade sites, as nicely reported for β-carotene content in cloudberry fruits from 10 habitats in northern Finland (Jaakkola, 2011).

Vitamin C is also established as one of the most abundant antioxidants provided by fruits and vegetables (Guorong, 2009). We found that ascorbic acid contents ranged from 15.0±0.2-36.5±0.1 mg/100g of fresh weight and distinguished four statistically different groups of samples depending on habitats (Fig.2C). Pineapple pulp from habitat 1 contained the lowest amount of vitamin C, followed by samples from habitats 6 and 2 ($p<0.05$) for which the concentrations of ascorbic acid were in accordance with values reported for pineapple from Brazil (Hassimotto *et.al.,* 2005). Samples from habitats 3, 4 and 8 exhibited higher levels than those found for the previous habitats ($p<0.05$) but lower than those from habitats 7, 9 and 5 ($p<0.05$) which were identified as the most abundant sources of ascorbic acid with levels close to those described in literature (USDA, 2010). Such vitamin C amounts were two-fold lower than that reported for fresh orange (71mg/100g) which is considered as one of the most consumed vitamin C sources (USDA, 2010). Here, the variability in vitamin C contents depending on habitats could has been influenced by the availability of light to the crop and to individual fruits (Wall, 2006). Indeed, pineapple fruit samples which were identified as the most abundant sources of vitamin C were harvested from habitats 5, 7 and 9 particularly exposed to a high global solar irradiance (Fig. 1C). Interestingly, habitats 5, 7 and 9 were also characterized by a high index of pluviometry (Fig. 1A). Accordingly, soil irrigation is established as a key factor controlling vitamin C contents in plant products (Melino, 2011). Moreover, several literature data have reported that nitrogen fertilization affects plant growth and vitamin C content (Gómez-López, 2013). In the present study, data concerning the application of an organic fertilizer were not collected but it should be of interest to consider such an important parameter to better understand the variability in vitamin C contents from Victoria pineapple pulp depending on habitats.

Similarly to carotenoids and vitamin C, polyphenols are also referred as major dietary antioxidants. They are mainly involved in fruit quality (role in the aroma, flavor) and their consumption has been associated with several health benefits due to their antioxidant and anti-inflammatory properties (Scalbert, 2005). Folin-Ciocalteu assay was performed to evaluate total polyphenol contents from Victoria pineapple pulp. As shown in Fig.2D, three statistically different groups of samples could be identified depending on habitats. Indeed, samples from habitats 9 and 2 exhibited the highest polyphenol levels (44.9±2.9 and 49.5±2.0 mg GAE/100 g, respectively), followed by samples from habitats 3, 4, 8, 7 and 1 (from 35.5±2.5 to 41.5±2.0 mg GAE/100 g, $p<0.05$) and from habitats 5 and 6 (32.2±1.2 and 34.1±1.7 mg GAE/100 g, respectively, $p<0.05$). Interestingly, total polyphenol contents measured for samples from habitats 9, 2, 3, 4, 8, 7 and 1 were in agreement with values published by other authors, whereas polyphenols amounts depicted for habitats 5 and 6 were lower than those from literature data (Almeida, 2011). Several factors may affect polyphenol levels in plants, such as cultivar, agronomic, environmental, handling and storage parameters (Tomas *et.al.,* 2001).

Taken together, our data led to identify polyphenols as the most abundant antioxidant micronutrients detected in Victoria pineapple pulp (average level of 40.8±1.7 mg GAE/100 g) as compared to

vitamin C (average level of 29.4±1.1 mg ascorbic acid equivalent/100 g) and carotenoids (average level of 57.4±3.5 µg β-carotene equivalent/100 g). As polyphenols may be used in medicine and food industries as antioxidants and conservatives, it appeared relevant to assess their presence in pineapple by-products, namely peel and crown as well as their potential free radical-scavenging activities. For a better understanding, data obtained were expressed as means ± SEM from 9 habitats. As reported in Fig.3A, polyphenol-rich extracts from pineapple pulp and peel exhibited polyphenol contents (49.5±2.0 and 48.4±1.9 mg GAE/100 g, respectively) significantly higher ($p<0.001$) than those measured for pineapple crown (34.8±1.7mg GAE/100 g). Whereas here there was no statistically significant difference between pulp and peel polyphenol amounts, other studies have reported that total polyphenol contents from the peel were higher than those from the pulp (Contreras, 2011). This was attributed to the accumulation of polyphenols in the dermal tissues of fruit body due to their potential role in protection against ultraviolet radiations, acting as attractants in fruit dispersal, and as defense chemicals against pathogens and predators (Toorand Savage, 2005).

To complete this study, a HPLC-UV analysis of pineapple polyphenols was performed and the identification of compounds was achieved by comparing their retention times and spectra to those of 19 standard phenolic compounds (Table 2). Four phenolic acids were detected and quantified, namely gallic, caffeic, ferulic and p-coumaric acids (Table 3). Whereas polyphenol-rich extracts from pineapple pulp were characterized by the presence of gallic, caffeic and ferulic acids, those from pineapple peel contained only caffeic and ferulic acids. Ferulic acid was also depicted in polyphenol-rich extracts from pineapple crown which presented p-coumaric acid. Accordingly, such compounds have been commonly found in several fruits and vegetables (Yapo *et.al.*, 2011).

Then, to assess free radical-scavenging activities of polyphenol-rich extracts from Victoria pineapple pulp, peel or crown, both DPPH and ORAC assays were performed. The results obtained with DPPH method were presented inFig.3B. Antioxidant capacities of polyphenol-rich extracts from pulp, peel and crown were compared to those of standard phenolic acids including compounds identified by HPLC-UV assay. Polyphenol-rich extracts from pineapple peel and pulp exhibited the strongest antioxidant activities (63.1±2.0 and 58.9±1.3% of DPPH reduced, respectively) as compared to pineapple crown (31.8±1.9% of DPPH reduced) ($p<0.001$). Such data were close to that measured for ferulic acid which was identified in pineapple pulp and peel extracts, but lower than those determined for caffeic and gallic acids ($p<0.001$).Comparatively, the antioxidant capacity measured here for polyphenol-rich extracts from pineapple pulp was lower than the value (87.5%) reported by Alothman *et.al.* (2009). In the present study, antioxidant capacities determined for polyphenol-rich extracts from pineapple pulp, peel and crown were similar to those found through a DPPH assay for strawberry (34%), guava (40%), banana (46%) and plum (54%) but were lower than those published for mango (84%) and passion fruit (94%) (Vasco *et.al.*, 2008).

Table 1: Characterization of 9 habitats according to the Global Positioning System

Habitats	1	2	3	4	5	6	7	8	9
South latitude	21°22'09"	21°18'54"	21°19'59"	21°15'01"	21°03'29"	21°20'34"	21°07'34"	21°12'32"	21°16'02"
East longitude	55°35'54"	55°28'59"	55°29'39"	55°22'17"	55°42'23"	55°34'15"	55°46'05"	55°24'01"	55°24'22"

Table 2: Retention times of standard phenolic compounds analyzed by HPLC-UV (280 nm)

Standard phenolic compounds	Retention times (min)
gallic acid	03.92±0.01
4-hydroxybenzylalcohol	06.39±0.01
protocatechuic acid	07.01±0.01
chlorogenic acid	10.59±0.07
catechin	12.80±0.04
vanillic acid	15.98±0.06
caffeic acid	17.72±0.04
syringic acid	18.23±0.03
4-hydroxybenzaldehyde	20.05±0.04
epicatechin	24.60±0.11
vanillin	26.83±0.04
p-coumaric acid	28.95±0.04
ferulic acid	30.76±0.03
myricetin	34.26±0.06
resveratrol	35.77±0.02
quercetin	37.32±0.03
t-cinnamic acid	38.21±0.01
apigenin	39.54±0.02
kaempferol	40.18±0.01

Table 3: Phenolic acids identified in polyphenol-rich extracts from pineapple pulp, peel and crown from the habitat 2

	Phenolic acids (mg/100 g of fresh weight)			
	Gallic acid	Caffeic acid	Ferulic acid	p-Coumaric acid
Pulp	1.18± 0.11	1.73±0.13	0.37±0.05	ND
Peel	ND	0.89±0.15	0.41±0.04	ND
Crown	ND	ND	0.09±0.01	0.07±0.02

Values are means ± SEM of three independent experiments with triplicate analysis. ND: not detected.

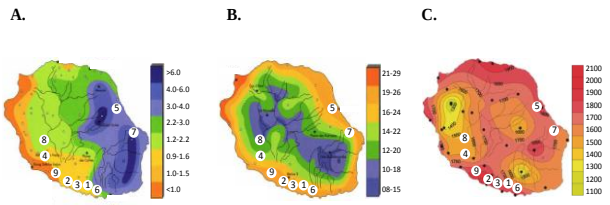

Figure 1

Characterization of 9 habitats according to environmental parameters. Fruit samples were collected from 9 habitats in Réunion Island in France which were characterized by environmental parameters including (A) pluviometry (m/year), (B) temperature (°C) and (C) global solar irradiance (kWh/m^2/year) data.

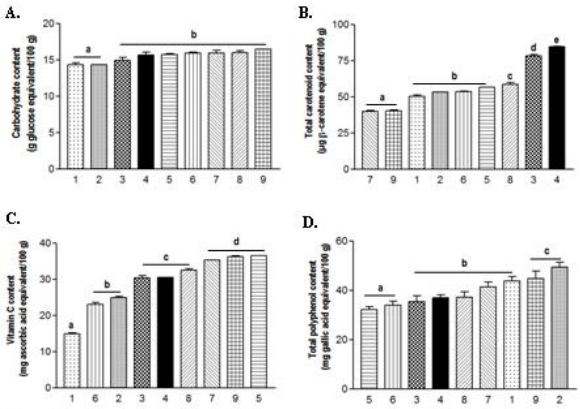

Figure 2

Carbohydrate, carotenoid, vitamin C and polyphenol contents from Victoria pineapple pulp.

Levels of carbohydrates and antioxidant micronutrients were determined in pineapple pulp from 9 habitats in Réunion Island. (A) Carbohydrate content was determined by using a redox method and expressed as g glucose equivalent/100g of fresh weight. (B) Total carotenoid content was determined by using a colorimetric method and expressed as µg β-carotene equivalent/100 g of fresh weight. (C) Vitamin C amount was determined by using a titrimetric method and expressed as mg ascorbic acid equivalent/100 g of fresh weight (D). Total polyphenol content was determined by using a colorimetric assay and expressed as mg gallic acid equivalent/100 g of fresh weight. Results are means ± SEM of three independent experiments with triplicate analysis. Means with different letters (a–e) are significantly different ($p<0.05$).

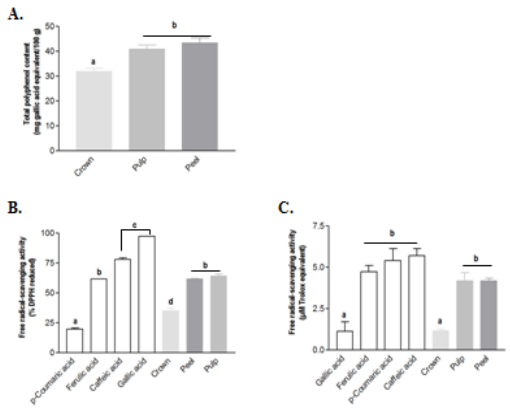

Figure 3: Total polyphenol contents and free radical-scavenging activities of polyphenol-rich extracts from Victoria pineapple pulp, peel and crown. (A) Polyphenol contents from pineapple pulp, peel and crown were evaluated by a colorimetric assay and expressed asmg gallic acid equivalent/100g of fresh weight. Then, free radical-scavenging activities of polyphenol-rich extracts from pineapple pulp, peel, crown and standard polyphenols (25µM) were measured (B) through DPPH method and expressed as % DPPH reduced, or (C) by ORAC assay and expressed as µM Trolox equivalent. Results are means ± SEM of three independent experiments with triplicate analysis. Means with different letters (a–d) are significantly different ($p<0.05$).

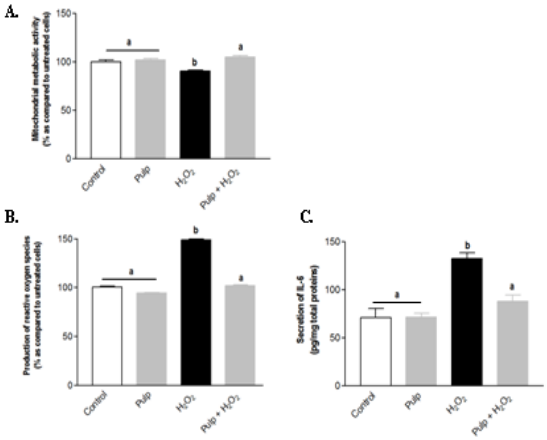

Figure 4: Effect of polyphenol-rich extracts from Victoria pineapple pulp on the mitochondrial metabolic activity, ROS production and IL-6 secretion of 3T3-L1 preadipose cells exposed to H_2O_2. (A) Cells were exposed to polyphenol-rich extracts from pineapple pulp (25µM GAE) in the presence or not of H_2O_2 (200µM) for 24 h. Then, the mitochondrial metabolic activity was determined by MTT assay. (B) Cells were exposed to DCFH-DA (10µM) for 45 min. Then, they were treated with polyphenol-rich extracts from pineapple pulp (25 µM GAE) in the presence or not of H_2O_2 (200µM) for 1 h. ROS production was measured by DCFH-DA assay. (C)

Cells were exposed to polyphenol-rich extracts from pineapple pulp (25µM GAE) in the presence or not of H_2O_2 (200µM) for 24h. Then, culture media were collected and IL-6 levels evaluated by ELISA kit. Results are means ± SEM of three independent experiments with triplicate analysis. Means with different letters (a–b) are significantly different ($p<0.05$).

ORAC is also used as one of the most reliable and standard methods to assess the antioxidant capacity of food products. In agreement with previous data obtained by DPPH assay, polyphenol-rich extracts from pineapple crown exerted the lowest anti-radical activity yielding 1.2±0.1 µM Trolox equivalent, similarly to standard gallic acid (Fig.3C). Polyphenol-rich extracts from pineapple pulp and peel presented higher antioxidant activities (5.4±0.2 and 4.3±0.4 µM Trolox equivalent, respectively) than pineapple crown ($p<0.001$) which were close to those measured for standard ferulic, p-coumaric and caffeic acids. Accordingly, both ferulic and caffeic acids were identified by HPLC-UV analysis in the present study. Data obtained for pineapple pulp, peel and crown agree with those published for some fruits and vegetables including white grape and onion (4.5 µM Trolox equivalent),but are lower than those concerning red grape (7.4 µM Trolox equivalent), orange (7.5 µM Trolox equivalent) and plum (9.5 µM Trolox equivalent) (Lachnicht, 2002).

Few studies have reported antioxidant activities of by-products derived from fruits. Peels of apples, peaches and star fruits have been found to contain higher amounts of polyphenols than those measured from the edible fresh parts. Thus, a scientific processing of under-utilized fruit waste could be relevant in pharmaceutical and food industries with opportunities of developing new nutraceutical and/or pharmaceutical products, reducing industrial waste and cost, and providing a positive economic and environmental impact (Gorinstein, 2002). Interestingly, our data led to establish a correlation between total polyphenol contents and antioxidant activities from pineapple samples. More precisely, a correlation was observed between total polyphenol contents and free radical-scavenging activities measured by DPPH assay ($R^2=0.9159$). Similarly, a high correlation was found between total polyphenol contents and antioxidant capacities explored by ORAC method ($R^2=0.9513$). Finally, data obtained from both DPPH and ORAC assays were strongly correlated ($R^2=0.9949$).

The adipose tissue development is governed by preadipose cell proliferation and differentiation capacities, and is profoundly altered by obesity-related oxidative stress (Furukawa, 2004). To assess the ability of polyphenol-rich extracts from Victoria pineapple to protect preadipose cells against oxidative stress, 3T3-L1 murine preadipose cell line was selected as a reliable model for adipose cell growth and metabolism. For this study, only polyphenol-rich extracts from pineapple pulp were tested as the pulp constitutes the most commonly consumed part of the fruit. The effect of polyphenol-rich extracts from pineapple pulp on 3T3-L1 preadipose cell viability was determined by MTT assay measuring the mitochondrial metabolic activity. Cells were treated with polyphenol-rich extracts from pineapple pulp (25µM GAE) in the presence or not of H_2O_2 (200 µM) during 24 h. For this experiment, we selected polyphenol-rich extracts from pineapple pulp originated from the habitat 2 based on its identification as the most significantly abundant source of polyphenols. Here, the selection of polyphenol dose tested was in accordance with our recent published data showing the absence of cytotoxic effect of major dietary polyphenols used at this concentration (Hatia, 2014 and Baret, 2013). The choice of H_2O_2 concentration was also based on our previous works and in agreement with literature data. Indeed, it has been reported that H_2O_2 exerted a dose-dependent cytotoxic effect for doses more than 200 µM whereas until 200 µMa transient proliferation arrest was observed due to the ability of cells to undergo detoxification or repair, and reinitiate cell cycle progression (Hatia, 2014 and Baret, 2013). As shown in Fig.4A, polyphenol-rich extracts from pineapple pulp did not affect the basal mitochondrial metabolic activity of cells, leading to suggest an absence of cytotoxic effect. Importantly, H_2O_2 significantly decreased it (from 100.0±1.0 to 90.9±1.0%, $p<0.001$), by inducing a cell proliferation arrest according to our published data (Hatia, 2014 and Baret, 2013). The mitochondrial metabolic activity of cells exposed to H_2O_2 in the presence of polyphenol-rich extracts from pineapple pulp did not significantly differ from that of cells treated with pineapple pulp extract or control cells. This result indicates that polyphenol-rich extracts from pineapple pulp totally inhibited H_2O_2-mediated anti-proliferative action. Such a protective effect of polyphenols from pineapple pulp could be attributed to their antioxidant activity discussed above. The presence of phenolic acids such as caffeic acid derivatives detected in polyphenol-rich extract from pineapple pulp could contribute to this protective action. This is in accordance with our recent data showing the ability of both caffeic and ferulic acids to protect 3T3-L1 preadipose cells from H_2O_2 (Hatia, 2014).

In order to emphasize the protective antioxidant effect of polyphenol-rich extracts from Victoria pineapple pulp on preadipose cells exposed to H_2O_2, their impact on the cellular production of ROS was evaluated. Cells were treated with polyphenol-rich extracts from pineapple pulp (25µM GAE) in the presence or not of H_2O_2 (200µM) for 1h. ROS generation was measured through the DCFH-DA fluorescent probe assay. As described in Fig.4B, polyphenol-rich extracts from pineapple pulp did not affect the basal production of ROS, whereas H_2O_2 significantly increased it (from 100.7±0.8 to 149.9±0.5%, $p<0.001$). Remarkably, polyphenol-rich extracts from pineapple pulp exerted an antioxidant effect by preventing the elevation of ROS production induced by H_2O_2. This effect could be partly mediated through their free radical-scavenging activity as demonstrated above with DPPH and ORAC data. Polyphenols such as those identified in pineapple pulp extracts by HPLC-UV assay, namely gallic, caffeic and ferulic acids, have been found to be strong antioxidants that can neutralize free radicals by donating an electron or hydrogen atom. They suppress the generation of free radicals, thus reducing the rate of oxidation by inhibiting the formation or deactivating the active species and precursors of free radicals. More

frequently, they act as direct radical scavengers of the lipid peroxidation chain reactions (chain breakers). Chain-breakers donate an electron to the free radical, neutralizing the radical and themselves becoming stable (less reactive) radicals, thus stopping the chain reactions (Hatia, 2014). Additionally, other mechanisms such as inhibition of xanthine oxidase and elevation of endogenous antioxidants should be considered. Indeed, dietary polyphenols can induce antioxidant enzymes such as glutathione peroxidase, catalase and superoxide dismutase that decompose hydro-peroxides, hydrogen peroxide and superoxide anions (Du, 2007). Interestingly, our published studies led to demonstrate that dietary polyphenols protected against H_2O_2-mediated elevation of ROS production from 3T3-L1 preadipose cells which were either preconditioned with polyphenols during 24h, then rinsed and exposed to H_2O_2 for 24 h, or co-treated with polyphenols and H_2O_2 for 24 h (Hatia, 2014 and Baret, 2013). The protective effect observed in pretreatment condition could be explained by the interaction of polyphenols with the cell membranes and reduction of lipid peroxidation. Indeed, it has been reported that the constitution of polyphenols in terms of hydrophilic and hydrophobic domains can determine their interaction with lipid bilayers (Du, 2007). The protective action of polyphenols may also is explained by their uptake into the cells as it has been reported that they are able to interact with proteins such as enzymes or transcription factors. These interactions have different biological effects, including the modification of enzymatic activities, receptor-lig and binding and transcription factors binding to their specific sites in DNA (Fraga, 2010 and Middleton *et.al.,* 2000). Inside the cells, polyphenols could also exert their protective effect against mitochondrial alterations induced by H_2O_2 according to our previous results found on 3T3-L1 cells preconditioned with polyphenols and then exposed to H_2O_2 (Baret, 2013). Thus, this raises the possibility that here pineapple polyphenols could exert their antioxidant action through different extracellular and/or intracellular mechanisms.

Then, it appeared relevant to evaluate the impact of polyphenol-rich extracts from pineapple pulpon the production of IL-6, known as a major pro-inflammatory cytokine involved in adipose tissue insulin resistance during obesity (Gregor, 2007). Moreover, several literature data have established a link between adipose tissue and immune-competent cells. This link is illustrated by the great cellular plasticity exhibited by preadipocytes to be very efficiently and rapidly converted into macrophages in an inflammatory environment. Thus, the ability of preadipocytes to function as macrophage-like cells may play an important role in the adipose tissue inflammatory response (Charrière, 2003 and Cousin, 1999). As previously described, 3T3-L1 cells were treated with polyphenol-rich extracts from pineapple pulp (25 µM GAE) in the presence or not of H_2O_2 (200 µM). After 24 h, cell culture media were collected, and levels of IL-6 measured by ELISA kit. Polyphenol-rich extracts from pineapple pulp did not affect IL-6 basal secretion level, whereas H_2O_2 significantly elevated it (from 71.7±9.4 to 132.9±5.8pg/mg total proteins, $p<0.001$) (Fig.4C). Such a pro-inflammatory effect of H_2O_2 was counteracted by polyphenol-rich extracts from pineapple pulp which decreased IL-6 secretion to 88.1±6.9pg/mg total proteins. Mechanistically, it has been shown that H_2O_2 pro-inflammatory effect could be mediated through the activation of MAPK and NF-κB signaling pathways (Mc Cubrey *et.al.,* 2006). Concerning the anti-inflammatory action of polyphenol-rich extracts from pineapple pulp, it could be attributed to the presence of compounds such as gallic, caffeic and ferulic acids identified here by HPLC-UV assay, and known to down-regulate both major pro-inflammatory signaling pathways cited above (Yen, 2011).

CONCLUSION

The present study evaluated for the first time the levels of carbohydrates and antioxidant micronutrients from Victoria pineapple cultivated in France. We found that micronutrient amounts differed depending on habitats and identified polyphenols as the most abundant antioxidants as compared to vitamin C and carotenoids. Four phenolic acids, namely gallic, caffeic, ferulic and p-coumaric acids were detected from pineapple pulp and its by-products (peel and crown). Moreover, polyphenol-rich extracts from pineapple pulp, peel and crown exhibited free radical-scavenging activities. Importantly, polyphenol-rich extracts from pineapple pulp protected 3T3-L1 preadipose cells against H_2O_2-mediated oxidative stress by reducing ROS production, and exerted an anti-inflammatory action by decreasing the release of IL-6 pro-inflammatory cytokine. Thus, this study led to identify Victoria pineapple as a relevant source of antioxidants and showed that pineapple polyphenols could exert beneficial effects against oxidative stress and inflammation occurring in adipose cells during obesity. Altogether, our new findings should contribute to the growing literature data reporting the interest of dietary polyphenols against obesity-related disorders. Further work will be needed to evaluate the effect of polyphenols from Victoria pineapple on human adipose cells and more particularly on mature adipocytes responsible for fat storage. In vivo studies in animal models and then in obese subjects will also be crucial to better consider polyphenol absorption, metabolic fate and ability to reach target cells.

ACKNOWLEDGEMENTS

We gratefully thank all colleagues from Vivéa Fruit and Vegetable Farm who provided Victoria pineapple fruit samples from 9 habitats of Réunion Island. This work was supported by the European Union, the French Ministry of Education and Research and the Federative Structure for Environment, Biodiversity and Health from the University of Réunion Island. ASM is a recipient of a Région Réunion fellowship.

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