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African Journal of Biotechnology

Full Length Research Paper

Bioaccumulation of zinc in *Rana tigrina* in different aquatic habitats

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Some creatures are very sensitive to water borne pollutants and can be used as useful bio-indicators of water pollutions. The present study used these bio-indicators to determine the status regarding health of freshwater habitats. The study was carried out to quantify Zn in kidney and liver of *Rana tigrina* from different aquatic habitats (canal, fish pond and sewage water) by atomic absorption spectrometry (AAS). Liver and kidney tissues of *R. tigrina* from sewage habitat showed elevated levels of Zn compared to those collected from canal and fish pond water habitats. Hence, it may be concluded that *R. tigrina* is one of the good bio-indicator species for heavy metals. It can be used to assess the heavy metal load and pollution of aquatic ecosystems.

Key words: Zinc, *Rana tigrina*, atomic absorption spectrometry (AAS), aquatic habitat.

INTRODUCTION

Environmental scientists widely use the term "heavy metals”. Heavy metals have specific gravities range of 4.5 to 22.5. The term heavy metal is used for elements which are toxic. At the same time, many authors (Phipps, 1981; Duffus, 2002) have critically called the term heavy metals as meaningless and objectionable. Heavy metals are natural constituents of freshwater and marine system and are found in very trace amounts. Human activities are responsible for the increment of their amount in the environment and consequently metals pollution is increasing in water resources (Chiarelli et al., 2016). Essential and beneficial metals have cut off values; low and high concentration beyond the cut off values will also be dangerous for life. Some elements have essential role in human being. Physiology of the target species and geochemical behavior are affected due to heavy metals toxicity. The following factors are considered the most important; chemical speciation of metals in aquatic environment, presence of other metals or toxicants, environmental conditions, condition of the organisms tested, adaptation of the organism to the absorption of metals. Bioassays of sediments involving benthic organisms and barytes were carried out at Aberdeen University. Results showed the bioaccumulation of Ba, Zn, Pb and Mn from barytes spiked marine sediments (Ansari et al., 2004).

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Lead is associated with damages to the nervous system (Finkelstein et al., 1998). Hazardous effects of mercury to human include damage to brain and ultimately mercury causes damages to central nervous system (Yoshino et al., 1966). High dose of chromium causes liver and kidney damages, and the circulatory system is also affected (Krishna et al., 2004). The most dangerous effect of selenium exposure to human is breakdown of human circulatory tissues and long term exposure of selenium is believed to damage nervous system (Boadi et al., 1991). Sources of Zn consist of galvanized metal for example wire cages and wire mesh staples. Other sources include cosmetics, skin lotion, shampoos, fertilizers, paints, fungicide and industrial chemicals. In most species, Zinc poisoning is regarded as being rare, but it is the second most common type of heavy metal poisoning in birds (Beyer et al., 2004). Zinc is necessary for standard growth, development and normal functions for all animal species. Death, tumor growth and some chromosomal aberrations is caused by Zinc deficit (Hrabeta et al., 2016; Krebs et al., 2014). It must be mentioned here that lead, cadmium, chromium, mercury etc. are highly toxic heavy metals and do not have any safe limits (Saeed et al., 2005); they have accumulation effects when ingested through food or water and cause various health problems like anemia, kidney diseases, nervous disorder (Gilani et al., 2015), high blood pressure, etc.

Frog belongs to carnivorous group of amphibians. 88% of amphibian species belong to the order *Anura*. These species are around 6645 in number belonging to 33 families. According to an estimate, one third species of frogs have become extinct since the 1980s (Stuart et al., 2004). They are main components of aquatic and terrestrial ecosystems. So they have an important link between human and ecosystem health. Mostly adult amphibians and toads feed on invertebrates and have an energy-efficient trophic link between insects and other vertebrates. So, they are linkers between terrestrial and aquatic habitats and are sensitive to environmental changes both because they have highly semi-permeable skins and different life cycle stages. A little study and information is available on the effects of environmental contamination on frogs and toads.

Kimball (2010) performed a study on the trace element concentrations (P, Ca, Mn, S, Na, Mg, Ba and Zn) in *Rana kl. esculenta* bones and on phalanges. So, concerned studies in this regard will be the best sources to check the pollutants, especially metals but limited studies have been done in this regard. Especially, in developing countries like Pakistan, there is need of time to check these pollutants by using different bio indicators and micro analytical methods like atomic absorption spectrometry etc. Welz and Sperling (1998) analyzed the whole body of *Rana catesbeiana* tadpoles and found the highest concentration of different heavy metals (cadmium, chrome, manganese, arsenic, mercury).

Grillitsch and Chovanec (1995) performed a study on heavy metals in tadpoles (*Rana dalmatina*, *Bufo bufo* and *Rana ridibunda*). Similar study was done by Puky and Oertel (1997) in which they evaluated metals concentration variation depending upon different development stages. These authors found the higher heavy metal concentrations in adults, which may be caused by changes in feeding during development, since they are carnivorous and the detritivorous diet may be richer in metals.

Pavel and Kucera (1986) analysed the accumulation of copper, manganese, zinc and iron in the whole body of *R. esculenta* adults. Zhang et al. (2007) performed a study that the increasing of Cd and Pb concentrations increase the ATP activity in *Bufo raddei* tadpoles. Stolyar et al. (2008) performed a study on heavy metals in the liver of the *R. ridibunda* from a river in Western Ukraine. They have reported concentrations of the metals in the order: Fe>Cu and approximately Zn>Mn>Cd. Simon et al. (2010) analyzed the trace element concentrations (P, Ca, Mn, S, Na, Mg, Ba and Zn) in *R. esculenta* bones and on phalanges. Tao et al. (2012) found that Zn and Cu are the most likely to be accumulated in all aquatic organisms of different tropic levels. Like all these studies, the present study is also a step towards the assessment of extent of metal toxicity in various water habitats.

Atomic absorption spectrometry has wide range application and reliable analytical technique for metal analysis (Welz and Sperling, 2008). For detecting metals and metalloids in ecological samples, flame atomic absorption spectrometry (FAAS) is a very familiar system. It is very consistent and simple to utilize. The aim of study was to evaluate Zn metal concentration in tissues (kidney and liver) of *Rana tigrina* from different habitats (canal water, fish pond and sewage water) and to assess the extent of heavy metal pollution in these habitats using *R. tigrina* as bio-indicator, as limited reports exist from Pakistan point of view.

MATERIALS AND METHODS

Equipment and glassware

The following equipment and glassware were used during this research: Electrical balance (AW220, Shimadzu, Japan), atomic absorption spectrophotometer (A-1800, Hitachi, Japan), heating oven (Gallen Kamp, England), reflux condenser (Quick Fit), personal computer and calibrated pyrex glassware.

Chemicals

Deionized water and analytical grade chemicals were used. Others included the nitric acid (HNO_3), hydrochloric acid (HCl), zinc granules (Zn), Copper metal (Cu) and manganese chloride ($MnCl_2 \cdot 2H_2O$). All these chemicals were of Merck analytical grade.

Sample collection

A total of 75 *R. tigrina* were captured from three different aquatic

Table 1. Statistical data for different parameters of *Rana tigrina* taken from different habitat.

S/N	Location	Parameter	Range	Mean	STDEV
1	Canal water	Total body weight (g)	54.26-98.49	71.266	10.6871
2	Fish pond water	Total body weight (g)	57.20-67.30	60.800	2.6042
3	Sewage water	Total body weight (g)	69.30-89.50	78.556	6.1792
4	Canal water	Kidney weight (g)	0.1324-0.1931	0.1638	0.02080
5	Fish pond water	Kidney weight (g)	0.1158-0.1987	0.1596	0.02580
6	Sewage water	Kidney weight (g)	0.1104-0.1961	0.1614	0.02600
7	Canal water	Zn Conc. (µg/g) in kidney	52.46-179.230	102.6983	3.20470
8	Fish pond water	Zn Conc. (µg/g) in kidney	0.1773-135.34	68.7054	3.25480
9	Sewage water	Zn Conc. (µg/g) in kidney	74.18-277.360	143.1836	4.31720
10	Canal water	Liver weight (g)	0.1246-0.1931	0.1638	0.02080
11	Fish pond water	Liver weight (g)	0.1158-0.1987	0.1594	0.02576
12	Sewage water	Liver weight (g)	0.1291-0.1961	0.1614	0.02600
13	Canal water	Zn Conc. (µg/g) in liver	98.75-232.16	176.2632	3.69134
14	Fish pond water	Zn Conc. (µg/g) in liver	109.64-228.28	164.7224	3.42362
15	Sewage water	Zn Conc. (µg/g) in liver	132.54-231.71	171.2524	3.14737

habitats such as Canal Water, sewage and fish pond during August to September 2012. Among them, 25 frogs were taken from each location. *R. tigrina* from Canal Water were captured from Nawab Pur Road Canal Water, Multan City, Pakistan. Five *R. tigrina* were captured from each of five various sites at equal distance of this Canal Water. *R. tigrina* from sewage water source were captured from sewage water from residence colony of Bahauddin Zakariya University (B.Z.U.), Multan, Pakistan while, *R. tigrina* from fish pond water were captured from a fish pond located in area of Matti Tal Road, Multan City, Punjab, Pakistan. Each was weighed and preserved till sample preparation.

Sample preparation

R. tigrina were dissected and four types of tissues were taken from each: skin, pectoral muscle, liver and kidney. Then weighing, length measurement, preservation in 70% alcoholic solution and storage of each part at -20°C was done. The solution of liver and kidney of each *R. tigrina* was prepared in aqua regia. 0.1 to 0.2 g of each sample was added to 3 ml of aqua regia and then refluxed for 30 min at 150°C. The solution was cooled to 25ºC and diluted with 10 ml of deionized water. The medium was filtered by using Whatman No. 42. After filtration, sample was diluted with 25 ml of deionized water and samples solutions were stored at room temperature.

Standard preparation

Zinc standards were prepared as follows: 1000 ppm stock solution of zinc was prepared by dissolving 0.1000 g of Zn granules in nitric acid and final volume was made up to 100 ml .The different concentration of zinc (100, 10, 2, 1.5, 1, 0.5 and 0.3 ppm) were prepared from stock solution by using dilution formula: $N_1V_1=N_2V_2$.

Zinc analysis

Atomic Absorption Spectrometer (A-1800, Hitachi, Japan) was used to determine metal levels. An instrument was warmed up for about half an hour. A blank was run for each metal to correct measurements. The standards of element of interest were run to check out performance of instrument. Finally, sample solution was aspirated to measure its absorbance. For sets of every ten samples, a sample blank was run to check interference and cross contamination. The concentrations of Zn were calculated using calibration curves in spreadsheets. The following optimized instrumental conditions for an atomic absorption spectrometer for zinc analysis were used: lamp current (mA) 10, slit (nm) 1.3, burner height (mm) 7.5, fuel pressure (Kg/cm^3) 0.20, detection limit (mg/L) 0.01, wavelength (nm) 213.8, calibration range (mg/L) 0.3-3.0 and flame composition C_2H_2/Air.

Data analysis

The samples were analyzed in replicates and statistical data obtained using standard statistical methods. The calibration curves were constructed by plotting absorbance (along ordinate) and concentration (along abscissa) of Zn standards. The linear regression method was used to determine slope and intercept for the Zn calibration data. The values of slope and intercept were used to calculate the concentration of Zn in *R. tigrina* samples. Then, Zn concentrations in mg/l, mg/25 ml, µg/25 ml and µg/g of *R. tigrina* sample from canal water, fish pond and sewage water were calculated.

RESULTS AND DISCUSSION

In this study, Zn analysis was carried out to determine Zn concentrations in different tissues (liver and kidney) of *R. tigrina* from different habitats (canal water, fish pond and sewage water). Zinc concentrations in all Zn standards and *R. tigrina* samples were determined in duplicate. Zn concentrations are summarized in Table 1. It clearly demonstrates that liver tissues of *R. tigrina* taken from all the three habitats (canal water, fish pond and sewage water) contain higher levels of Zn than kidney samples (Table 1).

The range of total body weight of *R. tigrina* found in canal water, fish pond water and sewage water were

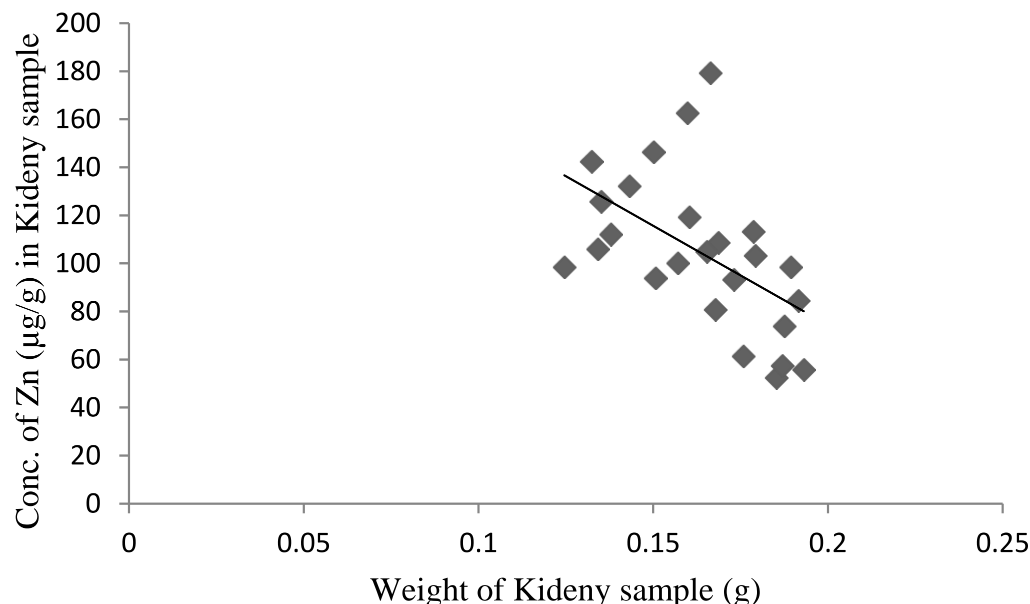

Figure 1. Effect of kidney weight (g) of *Rana tigrina* on the concentration of zinc found in canal water habitat.

recorded as 54.26-98.49, 57.20-67.30 and 69.30-89.5 g, respectively. In case of total body weight, the *R. tigrina* found in canal water has greater standard deviation than *R. tigrina* found in fish pond water and sewage water. The range of total kidney weight of *R. tigrina* found in canal water, fish pond water and sewage water were 0.1324-0.1931, 0.1158-0.1987 and 0.1104-0.1961 g, respectively (Table 1). In case of total kidney weight, the *R. tigrina* found in sewage water has greater standard deviation than *R. tigrina* found in canal water and fish pond water as shown in (Table 1). The range of total weight of liver of *R. tigrina* found in canal water, fish pond water and sewage water were 0.1246-0.1931, 0.1158-0.1987 and 0.1291-0.1961 g, respectively. The *R. tigrina* found in canal water has a bit greater liver average weight of 0.1638 g than that of *R. tigrina* found in fish pond and sewage water.

The range of zinc concentration in kidney of *R. tigrina* found in canal water, fish pond water and sewage water were 52.46-179.230, 0.1773-135.34 and 74.18-277.360 µg/g, respectively. The *R. tigrina* found in sewage water had greater zinc concentration in kidney than *R. tigrina* found in canal and fish pond water as shown in Table 1. It is clear from Figures 1 to 3 that as the weight of the kidney increased the accumulation of zinc decreased. It means that there is inverse relationship between the kidney weight and the zinc concentration. As the total body weight of *R. tigrina* increased the accumulation of zinc decreased in the kidney. It means that there is an inverse relationship between total body weight of *R. tigrina* and the zinc concentration in kidney. It may be concluded that the *R. tigrina* of low weight or small size may have gotten their food from the soil having high concentration of zinc. As the weight or size of the *R. tigrina* increased they got their food from the insects or from dissolved organic or inorganic matter in water.

The range of zinc concentration in liver of *R. tigrina* found in canal water, fish pond water and sewage water was 98.75-232.16, 109.64-228.28 and 132.54-231.71 µg/g, respectively. The *R. tigrina* found in canal water had greater zinc concentration in liver than *R. tigrina* found in fish pond and sewage water. As shown in Figures 4 to 6, as the weight of liver increased the zinc concentration accumulation in liver decreased. It means that there was inverse relationship between liver weight of *R. tigrina* and the zinc concentration. It may be concluded that the *R. tigrina* of low weight or small size may have gotten their food from the soil having high concentration of zinc. Similar trend of metals accumulation in fish was studied by Geffen et al. (1998). They found that tissue concentration of some metals declined exponentially with fish size. Qu et al. (2014) exposed freshwater fish to waterborne zinc under different pH levels and found increased hepatic Zn deposition.

The maximum Zn concentration in kidney of *R. tigrina* that lives in sewage water were found to be 143.1836 ± 4.31720 µg/g and in liver of *R. tigrina* found in canal water was 176.2632 ± 3.69134 µg/g. Shaapera et al. (2013) reported zinc concentration in the intestine of frog 1.81 ± 0.20 mg/kg, Eneji et al. (2011) reported 18.05 mg/kg of Zn in *T. zilli* and 17.76 mg/kg of Zn in *C. gariepinus* from River Benue. Tyokumbur and Okorie (2011) reported 29.84 ppm of Zn in *R. esculentus* and 32.97 ppm in Crabs from Alaro Stream Ecosystem,

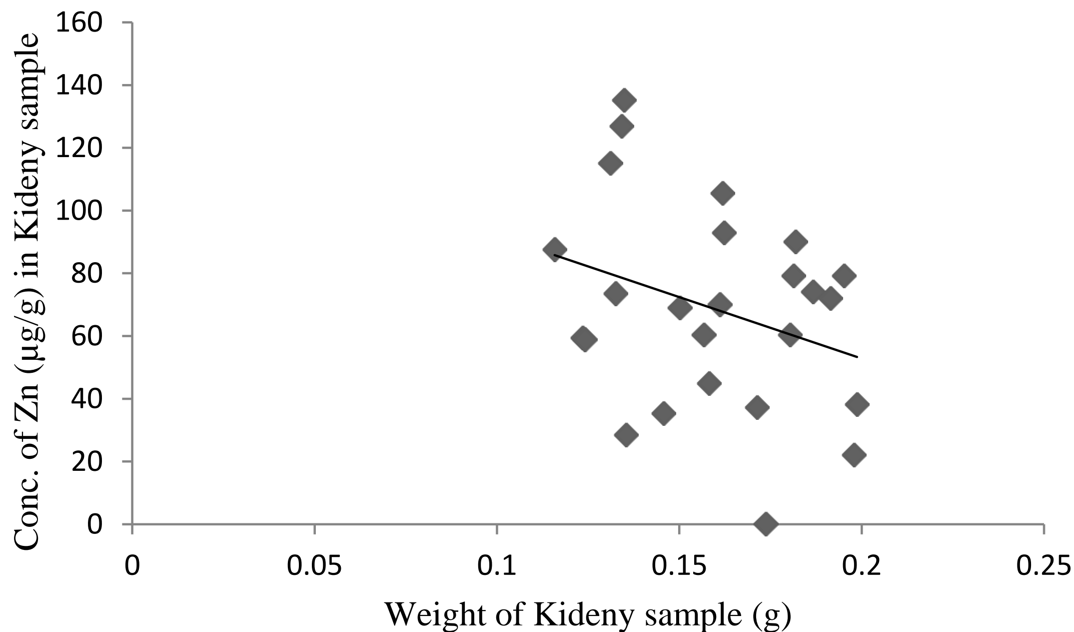

Figure 2. Effect of kidney weight (g) of *R. tigrina* on the concentration of zinc found in fish pond water habitat.

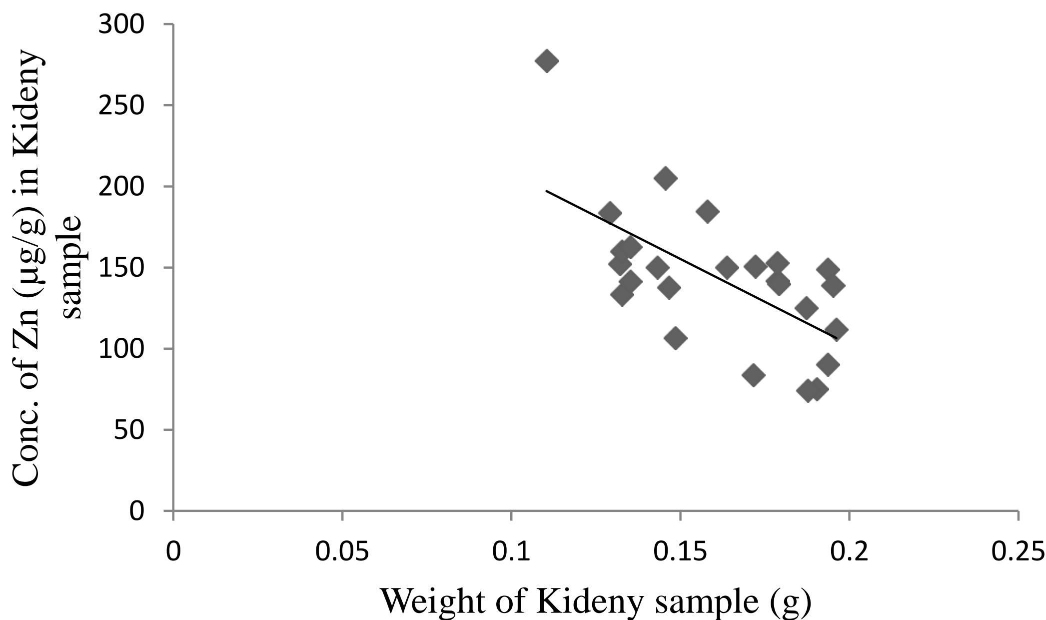

Figure 3. Effect of kidney weight (g) of *R. tigrina* on the concentration of zinc found in sewage water habitat.

Ibadan. Ololade et al. (2008) reported 0.61, 0.23 and 0.10 mg/kg of Zn in crab, fish and periwinkles, respectively. As compared to the reported literature, the present study reported higher concentration of Zinc in various parts of *R. tigrina.* This is because of more heavy metals pollution in various habitats of frogs in Pakistan.

Conclusion

Based on the present study, it can be concluded that liver tissues of *R. tigrina* taken from all the three habitats (canal water, fish pond and sewage water) contain higher level of Zn than kidney. As the weight of the kidney

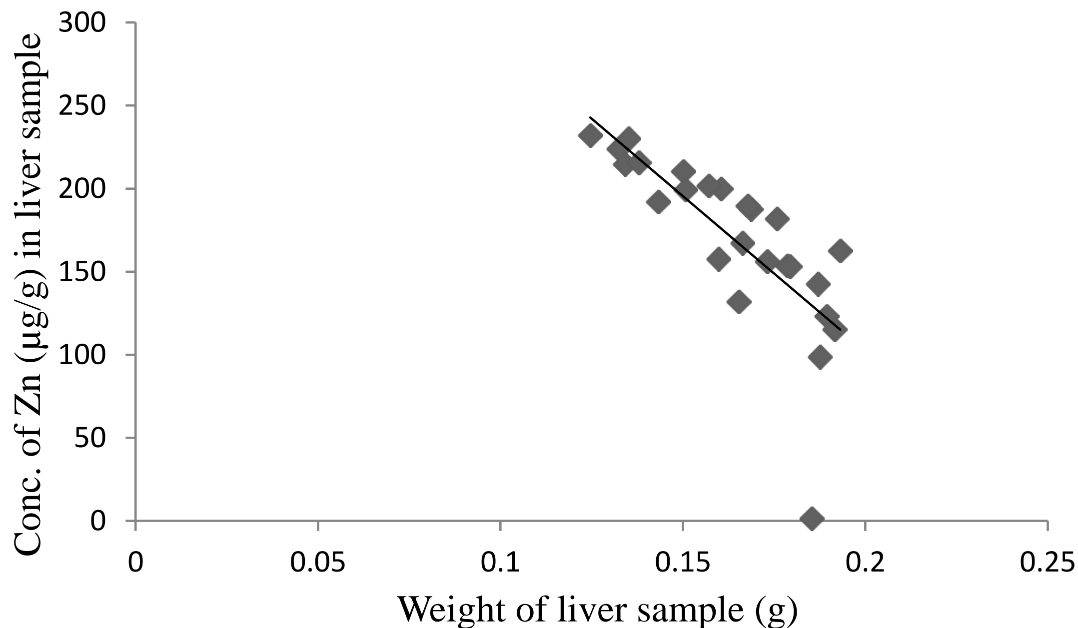

Figure 4. Effect of liver weight (g) of *R. tigrina* on the concentration of zinc found in canal water habitat.

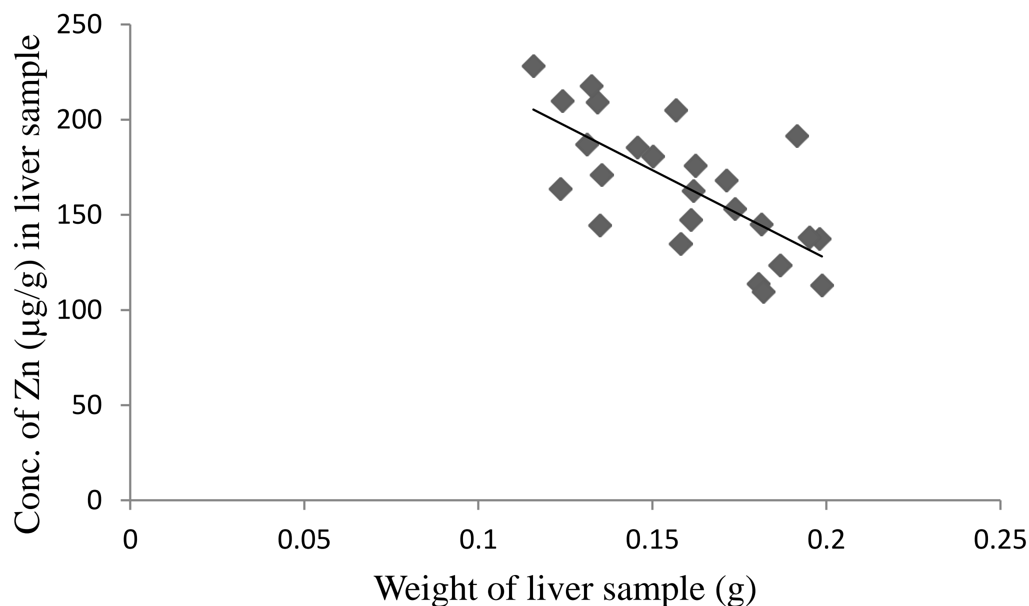

Figure 5. Effect of liver weight (g) of *R. tigrina* on the concentration of zinc found in fish pond water habitat.

increased the accumulation of zinc decreased in the kidney. As the total body weight of *R. tigrina* increased the accumulation of zinc decreased in the kidney. It can also be concluded that as the weight of liver increased the zinc concentration accumulation in liver decreased. As the total body weight or size of the *R. tigrina* increased the concentration of accumulation of zinc in liver increased. *R. tigrina* is a good biological indicator to measure the heavy metals in aquatic environment

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

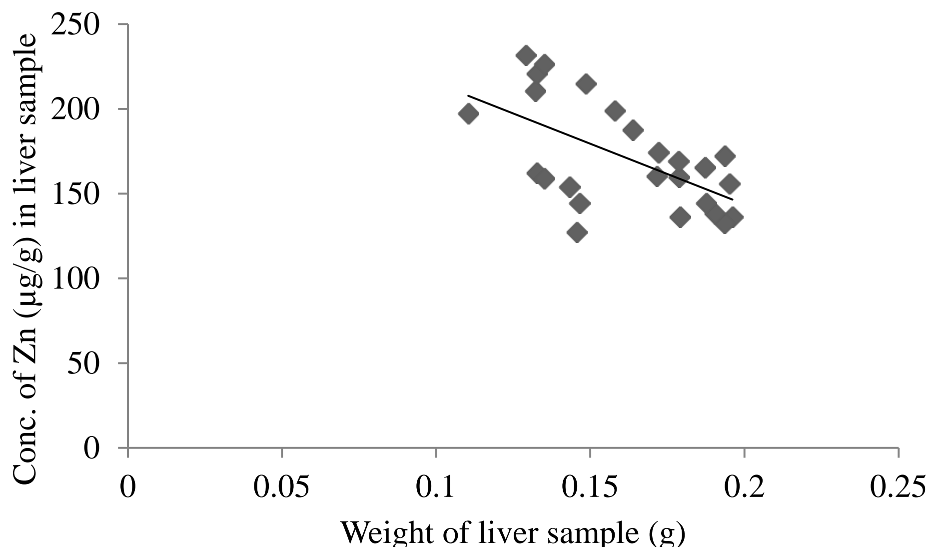

Figure 6. Effect of liver weight (g) of *R. tigrina* on the concentration of zinc found in sewage water habitat.

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African Journal of Biotechnology

Full Length Research Paper

Changes in phenol metabolism of minimally processed 'baby cassava' under different temperatures: An alternative to commercialization

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This study aimed to evaluate the changes in the phenolic oxidation mediated by polyphenol oxidase (PPO) and peroxidase (POD) in the quality of minimally processed 'baby cassava', during the first hours and days of conservation at different temperatures. Pieces of roots of cassava cv. Recife were minimally processed into 'baby cassava' form and kept at 5 ± 2 and 25 ± 2°C for 12h and/or 10 days. Fresh weight loss (FWL), visual examination (visual appearance), total soluble phenolics (TSP), fluorescence emission at UV-light, PPO and POD activities were evaluated. Non-refrigerated 'Baby cassava' lost overall quality and minimal FWL for 12h. After this period, there was intense darkening, while refrigerated pieces had great quality for 10 days. Non-refrigerated pieces emitted more fluorescence than refrigerated 'baby cassava' at 12h, this possibly resulted in the presence of *Pseudomoas* spp., or fluorescent phenolics compounds. This is associated with an increase of TSP values and PPO and POD activities. On the other hand, the pieces refrigerated at 5 ± 2°C prolong the shelf life until 10 days, suitable for retail market. Moreover, the new form of 'baby cassava' was good for comercialization and consumption, at room temperature, before 12h, an important time for institutional market.

Key words: *Manihot esculenta* Crantz, baby cassava, peroxidase, polyphenolxidase, browning.

INTRODUCTION

Cassava crop (*Manihot esculenta* Crantz) has high economic and nutritional value. According to FAO data, the production in 2013 was 263 thousand tons in a harvested area of approximately 20.5 million hectares (FAO, 2014). Harvest and market are responsible for economic loss of the crop. Cassava roots are sold

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commonly in streets, markets, and *in natura* as a whole or in pieces. They are kept in high temperatures or sold as frozen in supermarkets. In all cases, they undergo few processing and stored for marketing, leading to high risk to consumers' health. This contributes to high perishability after harvest, either in whole or cut form. In addition, improper handling makes them susceptible to rapid postharvest physiological deterioration (PPD), within 48h (Iyer et al., 2010; Sánchez et al., 2013). However, there is no real time for minimally processed form.

PPD is characterized by a series of physiological phenomena that reduce the shelf life of cassava, which also results in an increase of enzymes activity like polyphenol oxidase (PPO) and peroxidase (POD) (Rickard, 1985; Uarrota et al., 2015). As oxidative enzymes, PPO catalyzes two kinds of reactions in the presence of molecular oxygen (co-substrate): first, there is hydrolysis of monophenols to *o*-diphenol and further oxidation of *o*-diphenols to *o*-quinones. These quinones can undergo cyclization or self-polymerization (non-enzymatic reactions with amino acids and proteins) forming melanins, which are variegated colored compounds (red, brown or black pigmentation) (Buckow et al., 2009; Holderbaum et al., 2010).

PODs are oxide-reducing enzymes. They contain iron as a prosthetic group, which catalyzes the reduced reaction of hydrogen peroxide (H_2O_2) using electron donors (phenolic compounds, alkaloids, aromatic amines, auxins) for the formation of water (Almagro et al., 2009). Appropriate handling can minimize these reactions, especially with temperature control, from harvest to processing and marketing.

An alternative that adds value and maintains the organoleptic characteristics of cassava is the application of minimal processing technology. In minimal processing, cassava roots are physically modified by a sequence of steps such as peeling, cutting, slicing, cleaning, centrifuging and packaging, which present the sensory characteristics of a fresh product with high food safety, ready for consumption (Ghidelli and Pérez-Gago, 2016).

However, this handling causes injuries to the tissues which may shorten the shelf life of the product due to PPD. Such tissue damage can be reduced by using cold chain in the processing where temperature is one of the most influencing factors, in the maintenance of quality of minimally processed products (Donegá et al., 2013; Fagundes et al., 2013). The use of cold chain is required in all procedures, since all physical alterations cause a cellular injury by activating oxidative enzymes, generating browning, tissue degradation and sensory changes (Artés, 2004), which vary according to temperature.

The conservation of minimally processed products for marketing is generally done in refrigerated shelves, whose temperatures are between 5 and 10°C. In addition, the minimally processed cassava can be transported for long periods at room temperature (around 25°C) until it is consumed. This can be an extremely strong inducer for anticipating darkening, by an increase of the activities of enzymes cited, increasing the susceptibility of microbial growth. The changes in the biochemical markers associated to phenolic compounds can be an important tool to coordinate browning and shelf life of minimally processed cassava.

In this work, the proposed 'baby cassava' used is recently developed (Freire et al., 2015); it has potential market due to its added value; it makes consumers' life easier by making cooking fast and does not require pressure cooker and has attractive shapes. Thus, understanding the changes in the PPD and biochemical markers in the first hours after minimal processing simulates transport and market. Therefore, this study aimed to evaluate the changes in the phenolic oxidation mediated for PPO and POD in the quality of minimally processed 'baby cassava', during the first hours of conservation at different temperatures

MATERIALS AND METHODS

Plant material and minimal processing

Cassava cv. Recife harvested was acquired in a street market, in the city of Serra Talhada – PE 12 months prior to the study. The roots were transported to the experimental kitchen of the Unidade Acadêmica de Serra Talhada of the Universidade Federal Rural de Pernambuco. They were selected, and washed in running water with the aid of a brush; they were kept in a refrigerator at 8 ± 2°C for 24 h.

Minimal processing was performed according to the method described by Brito et al. (2013). Roots were cross-cut into pieces of 3.0 cm and peeled. They were longitudinally cut into pieces called 'baby cassava' with a stainless knife. The 'baby cassava' was initially rinsed by immersion for 10 s in water at 5 ± 2°C; it was sanitized in chlorinated water solution at a temperature of 5 ± 2°C and 200 mg L^{-1} of active chlorine (sodium dichloroisocyanurate dihydrate) for 10 min. Then, it was finally rinsed in 5 mg L^{-1} chlorinated solution for 10 min.

The pieces were centrifuged in a centrifuge (Model C2A05BBBNA Consul) for 30 s. Approximately 150 g of 'baby cassava' was packed in polypropylene bags (PP - 15 cm x 10 cm x 0.6 µm thick) at a temperature of 5 ± 2°C, 75 ± 5% relative humidity (RH), 25 ± 2°C and 69 ± 5% RH for 10 days.

Average temperatures were recorded with a digital thermo-hygrometer (Model 7663.02.0.00). During the first hours (0, 2, 4, 6, 8, 10 and 12 h) in every two days (0, 2, 4, 6, 8 and 10 days), analyses on the most superficial tissue were carried out (± 3 mm from the equatorial side of the 'baby cassava'). The evaluations are as follows.

Fresh weight loss (FWL)

The fresh weight loss of the 'baby cassavas' was determined gravimetrically according to the following equation: % FWL = ([FWi-FWf] *100) / FWi. Where: FWL = fresh weight loss; FWi = initial fresh weight; FWf = final fresh weight.

Visual assessment (general appearance)

The 'baby cassava' was evaluated based on its overall appearance,

by a trained panel of ten. A subjective scale of grades ranging from 5 (best grade) to 1 (worst grade) was used. Score 3 was set as an acceptance limit and the overall score corresponds to the average of the scores for each 'baby cassava' (Freire et al., 2015).

Total soluble phenolics (TSP)

TSP was determined as described by Reyes et al. (2007), and as used for cassava by Freire et al. (2015). One gram of the surface portion (± 3 mm from the equatorial region of the 'baby cassava') was collected and macerated in a mortar containing 10 mL of pure methanol. The extract was kept in the dark at a temperature of 5°C for 24 h. They were centrifuged at 7690 g, for 21 min and 2°C (Hettich centrifuge, Model Universal 320 R).

For determination of the phenolic compounds, reaction with 150 µL of methanolic extract was performed and diluted in 2400 µL of distilled water with 150 µL of Folin Ciocalteu reagent (0.25 N) added. After 3 min of homogenization of the mixture, 300 µL of sodium carbonate (1N) was added and stirred for 1 min. It was kept in the dark at 20 ± 2°C for 2h. Readings were taken with a spectrophotometer (Biochrom, Model British S8) at a wavelength of 725 nm. The TSP content was quantified from the gallic acid standard curve.

Fluorescence emission

Fluorescence emission was determined with two representative slices of each treatment and was photographed using a semi-professional digital camera (Nykon; D3100 14.2 megapixels); it was coupled in a dark room (CN-6; Vilber Lourmat), under ultraviolet light at 365 nm with 1 x 6 Watts filter 1 x 6 Watts and 220 V 50/60 Hz (VL-6.l; Vilber Lourmat) (Simões et al., 2016).

Extraction and assay of polyphenol oxidase (PPO – EC 1.14.18.1) and peroxidase (POD – EC 1.11.1.7) isozymes

PPO and POD isozymes were extracted using the methodology of Freire et al. (2015) for cassava. It was collected at 0.25 g of superficial tissue (± 3 mm from the equatorial side of the 'baby cassava'). This material was macerated in a mortar containing sodium phosphate buffer (0.2 M, pH 6.0), and was centrifuged at 7690 g and 4°C for 23 min (Hettich centrifuge, Model Universal 320 R).

The PPO assay was performed according to Freire et al. (2015), wherein a mixture was made by adding 100 µL of the enzymatic extract (supernatant) and 1.5 mL of sodium phosphate buffer (0.1 M, pH 6). This was added to catechol (89.65 mM), and diluted in phosphate buffer (0.1 M, pH 6.0). The reaction was observed in a spectrophotometer (Biochrom, Model Libra S8) with change in absorbance at 425 nm, for a period of 2 min. Readings were recorded for 10 s, at 20 ± 2°C. For blank sample, 100 µL of sodium phosphate buffer (0.2 M, pH 6) was used replacing the enzymatic extract.

The POD assay was performed following the method of Freire et al. (2015), with some modifications. A mixture containing 300 µL of an enzymatic extract (supernatant) was made with 1 mL of sodium phosphate buffer (0.2 M, pH 6). 100 µL of hydrogen peroxide (0.08%) and guaiacol (0.5%) were added (both diluted in 0.2 M phosphate buffer) and the reaction was monitored in a spectrophotometer with a change in absorbance 470 nm, for a period of 2 min. For white sample, 300 µL of sodium phosphate buffer (0.2 M, pH 6) was used to replace the enzymatic extract. The results of PPO and POD were expressed in Enzyme Unit (EU - 0.001absorbance per minute) per gram of fresh weight per minute.

Experimental design and statistical analysis

This design was completely randomized (CRD); it was divided into two factorial schemes with four replications, each with 5 pieces in package: the first factorial scheme was 2x8: two temperatures (refrigerated at 5 ± 2°C and 25 ± 2°C) and seven storage periods (0, 2, 4, 6, 8, 10 and 12 h after minimal processing) used for evaluations of FWL, appearance, TSP and PPO and POD activity.

The second factorial scheme was 2x6: two temperatures (refrigerated at 5 ± 2°C and 25 ± 2°C) and six storage periods (0, 2, 4, 6, 8 and 10 days) used only for FWL and appearance assessments; due to the deterioration stage of the 'baby cassava' kept at room temperature, biochemical analyses (TSP, PPO and POD activities) were not performed from the fourth day.

RESULTS AND DISCUSSION

Effect of temperature on the weight loss and visual appearance in minimally processed cassava

The 'Baby cassava' kept at room temperature had greater weight loss than those kept under refrigeration (Figure 1A). It was found that, at 12 h after minimal processing, the fresh weight loss was approximately 0.035 and 0.002% for the 'baby cassava' maintained at 25 ± 2 and at 5 ± 2°C, respectively (Figure 1A to i). Nevertheless, this dehydration was not enough to generate losses in the visual integrity of the pieces (Figure 2).

Furthermore, the pieces kept at room temperature for long had severe dehydration reaching 3.14% at 10 days (Figure 1A). Unlike the refrigerated pieces, values were within 0.05% in the same period (Figure 1A). The refrigerated 'baby cassava', during the first 12 h after minimal processing, maintained maximum values according to the visual scale (Figure 1B). The pieces were kept at room temperature, and a decrease was noticed in the appearance values (Figure 1B to i) at 12 h. Even with lower values, these were still above 3 (acceptance limit) (Figure 1B and 2), reaching its value in two days (Figure 1B and 2). From the second day until the end of the experiment, the most observed features were: pronounced browning of the surface; exudation of liquid appearance of a totally contaminated product and loss of firmness.

The severe dehydration of the 'baby cassavas' kept at room temperature, in relation to the refrigerated ones, can be explained partly by the relative humidity, which was around 75 ± 5% for the refrigerated cassavas and 69 ± 5% for those kept at room temperature. In the latter, the atmosphere was drier, increased water loss and respiration as observed for minimally processed radish (Aguila et al., 2006) and pumpkin (Sasaki et al., 2014).

Browning rates in cassava roots may vary according to the various conditions relating to plant material and minimal processing. "Baby cassava" has smaller signs of browning, when conserved for more than 8 days (Brito et al., 2013; Freire et al., 2015). This is due to the removal of the first layers of the periderm in which it presents

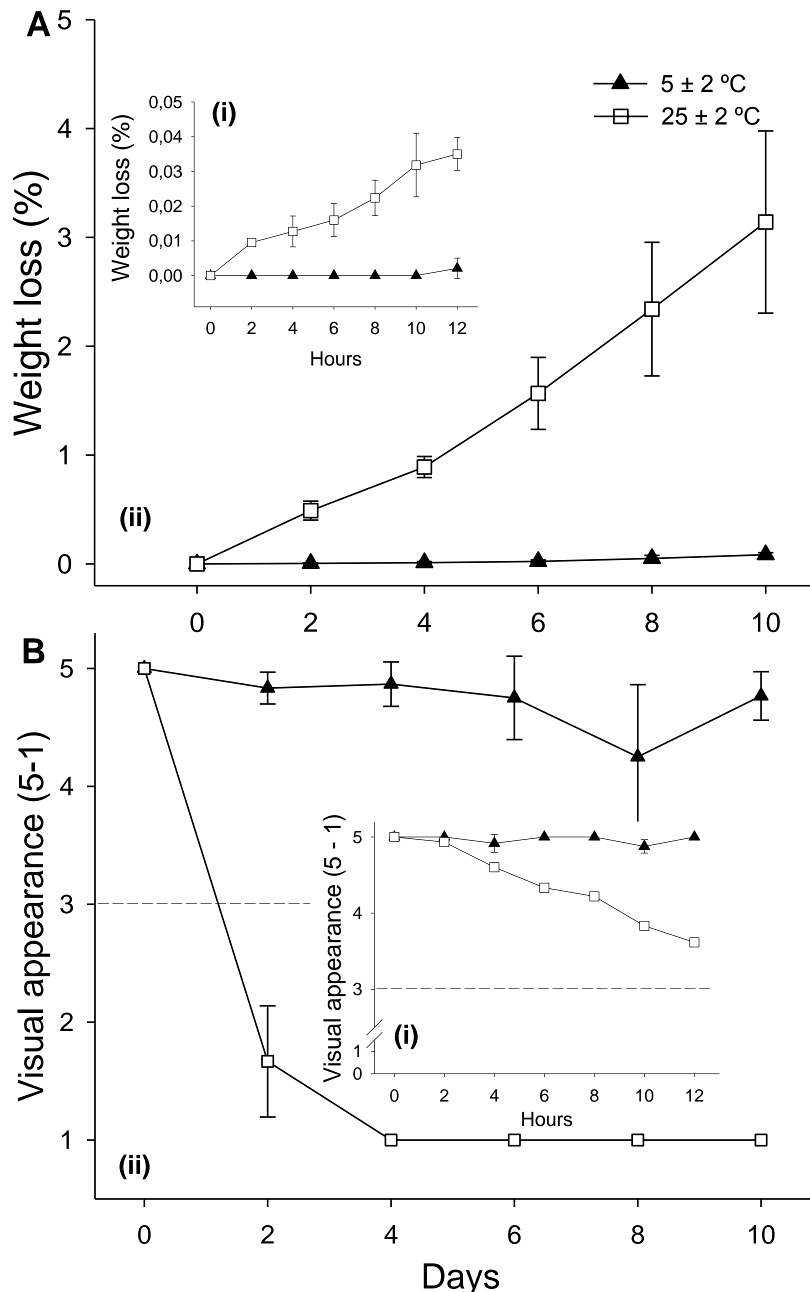

Figure 1. Weight loss (A) and visual appearance (B) in minimally processed cassava in the 'baby cassava' format for 12 h (i) or for 10 days (ii) at 5 ± 2°C (refrigerated) and 25 ± 2°C (ambient) after processing. The dotted line has in visual appearance (B) which represent the acceptance limit (note 3).

secondary phloem, being therefore more metabolically active, as seen in 'cassava sticks' during 12 days of storage (Freire et al., 2015).

These results indicate that, the minimally processed cassava can be marketed without the use of refrigeration, provided that the consumption is fast, less than 12 h (Figures 1 and 2). This is important as in the case of the institutional market, that is, industrial kitchens, schools, companies, among others, in which consumption takes place in a few hours or even after a short period of transportation at room temperature. Although, these pieces had lower grades and good characteristics, with light to medium severe symptoms of browning are observed (Figure 1B, and Figure 2), provided the product is correctly sanitized.

Thus, these results suggest that dehydration of non-refrigerated 'baby cassava', after 12 h of storage slightly reduced its visual quality. The type with minimal

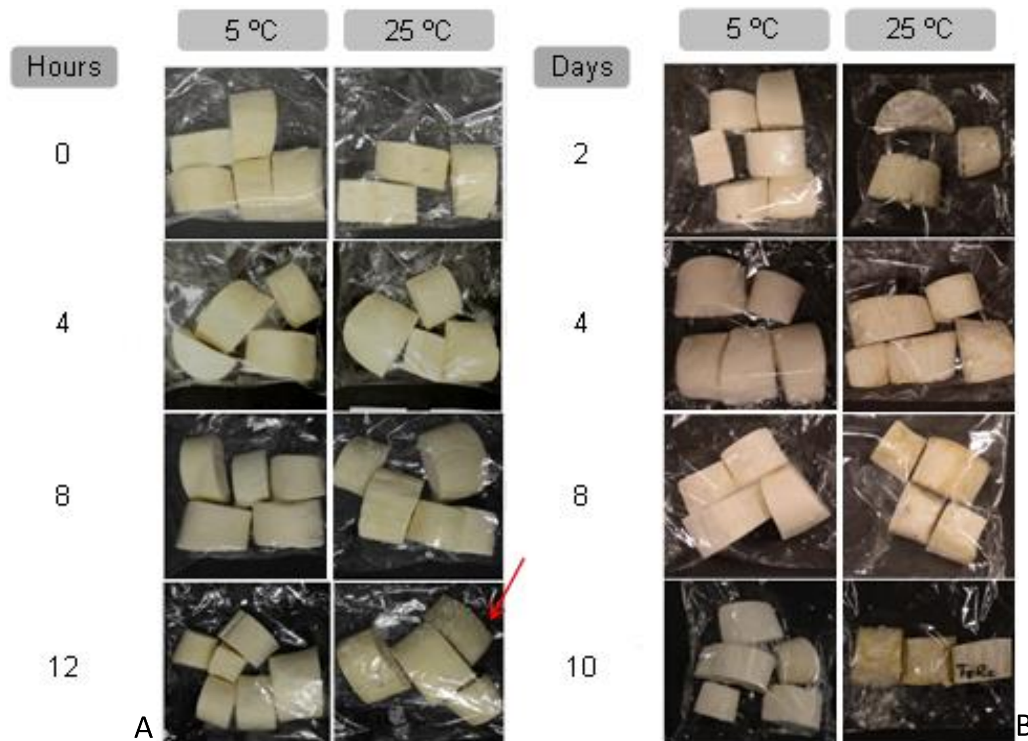

Figure 2. Fresh cut of 'baby cassava', maintained under refrigeration (5 ± 2°C) and room temperature (25 ± 2°C) for A (12 h) and (B) 10 days.

dehydration can be accepted in the market. On the other hand, in retail market in which time is needed, the 'baby cassava' needs to be refrigerated.

Effect of temperature on the total soluble phenolics, oxidative enzymes activity and fluorescence emission on 'baby cassava'

It was observed that, for both temperatures there was an increase in TSP content expressed in gallic acid content in the first hours after minimal processing, with a peak of 1.7 times at 6 h at 25°C, and of 1.33 times for 8 h at 5°C (Figure 3A). When storage was extended, the 'baby cassava' kept at room temperature was damaged, not being made by TSP quantification in these materials after 48 h. On the other hand, in the refrigerated ones, the TSP continued to rise (Figure 3A).

These authors reported that, the rapid increase in TSP biosynthesis is as a result of the mechanism of response to an injury caused in plant tissues (Xu et al., 2013). This increase was anticipated to the synthesis or activation of phenylalanine ammonia-lyase (PAL) enzyme (Beeching et al., 2002; Sanchéz et al., 2013). In this work, the room temperature resulted in larger TSP content, browning and loss of visual quality of 'baby cassava' at room temperature after 48 h (Figure 2 and 3A).

When the 'baby cassava' was refrigerated it could be seen that, the phenolic compounds content were increased by 1.6 times in the sixth day of storage (Figure 3C to ii) as compared to the initial day of processing, next to 1.7 times after 6 h at room temperature (Figure 3C to i). This shows the importance of refrigeration in the delay of peak TSP for 'baby cassava' to be less brown. The same trend was observed in cassava roots, over a few days of storage at room temperature (Uarrota and Maraschin, 2015) and also 'baby cassava' (Freire et al, 2015), as well as some varieties of potato (Cantos et al., 2002).

The short and long storage in different temperatures of the 'baby cassavas' influenced the PPO and POD activities (Figure 3B and C). Enzymatic activities increased for the minimally processed cassava for 10 days under refrigerated storage (5 ± 2°C) (Figure 3B to ii, C to ii). The PPO activity in the 'baby cassavas' kept at room temperature slightly increased more than 3 times in its original value in relation to those stored at 5 ± 2°C at the end of 12 h (Figure 3B). Moreover, the POD activity after 12 h at 5 ± 2°C remained stable, while at room temperature, it increased at about 2 times (Figure 3C). In this context, the browning observed for 'baby cassava' occurred after explosion in the enzymatic activities studied, especially those kept at room temperature. Vitti et al. (2011) also reported these parameters, where the initial symptoms of senescence in minimally processed potatoes increase in enzyme activity.

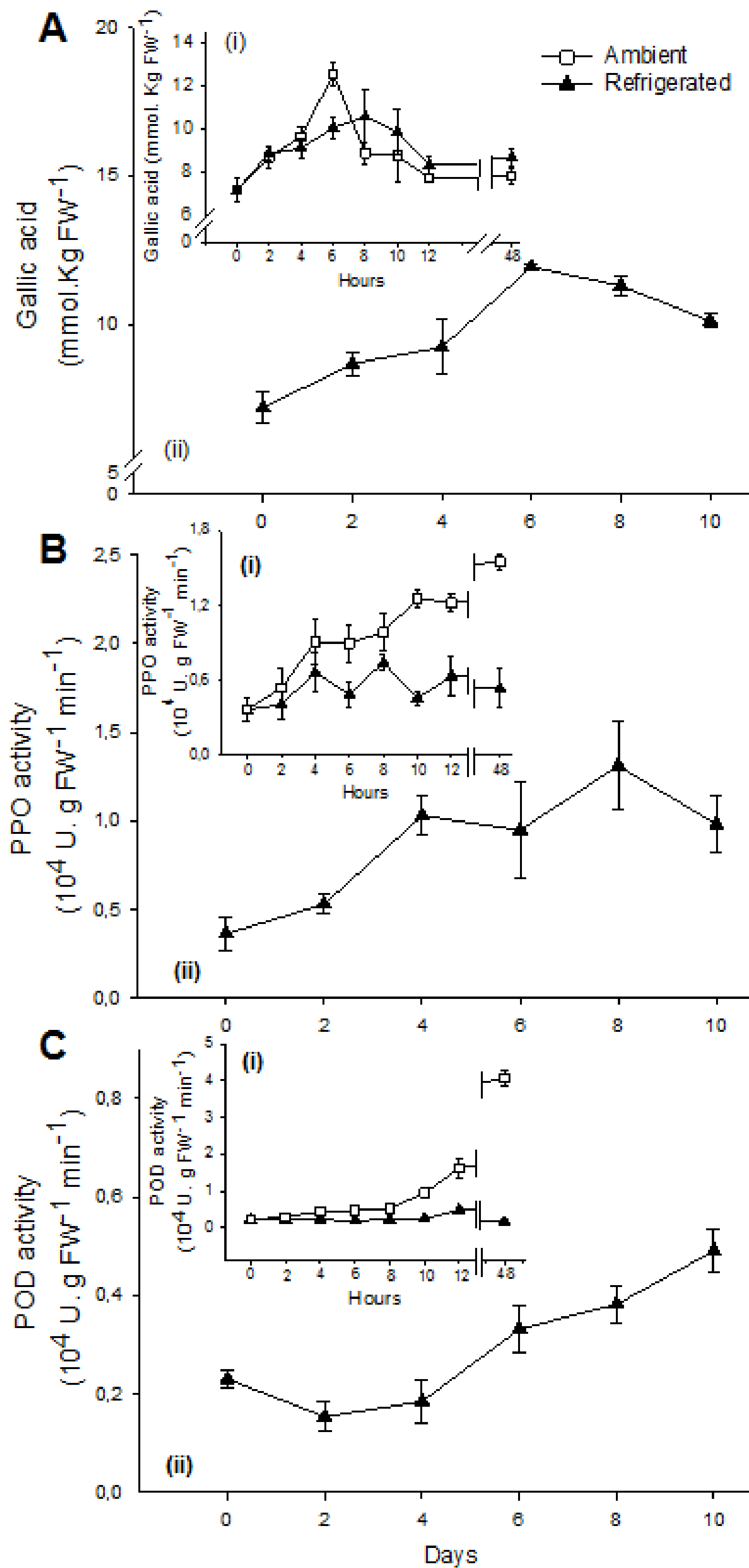

Figure 3. Content of total soluble phenols (A); polyphenoloxidase (B) and peroxidase (C) activity in hours(i) or days (ii) after processing for 'baby cassava' format, and stored at 5±2°C and 25±2°C for 10 days.

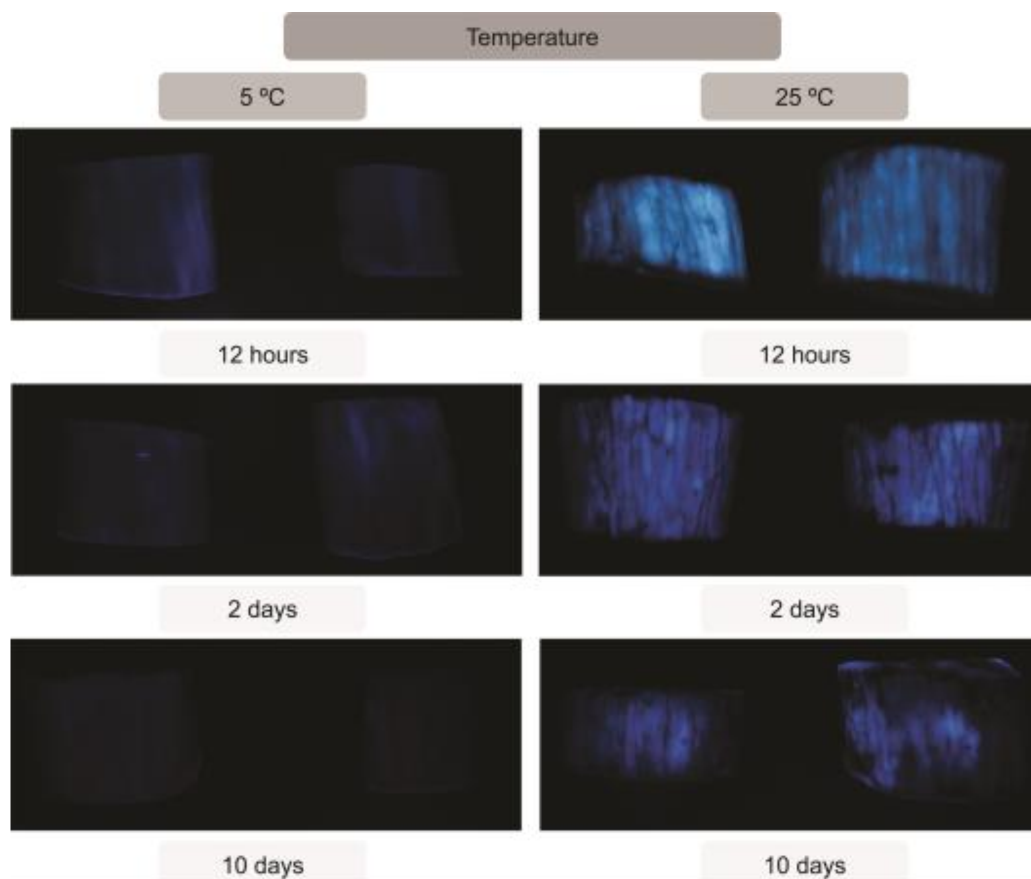

Figure 4. Fluorescence emission of 'baby cassava' maintained under refrigeration (5 ± 2°C) and room temperature (25 ± 2°C) for 12 h at 5 ± 2°C and 25 ± 2°C for 10 days.

The increase of enzymes activities accompanied an increase in the phenols oxidation to 6 h, under room temperature until 6 days at 5°C (Figure 3A). This shows a close association between PPO, POD activities, TSP and darkening in cassava as described by Yingsanga et al. (2008). Increased PPO activity and browning levels in vegetables, which darken are seen in some studies on minimally processed jicama roots (*Pachyrhizus erosus*) (Aquino-Bolaños and Mercado-Silva, 2004).

Notwithstanding, Vitti et al. (2011) and Cantos et al. (2002) found no direct relationship between the PPO activity and browning of some cultivars of minimally processed potatoes, even with high levels of the enzyme and high susceptibility to darkening in some varieties. The same was reported by Cantos et al. (2001), for minimally processed lettuce leaves. This is not fully elucidated and influenced by plant and its structures.

In another approach, the increase of POD activity in 'baby cassava' may be related to the defense mechanism of abiotic stress (Liu et al., 2010). In this work, it was high temperature and cut. Thus, it plays an important role in the lignin synthesis of cassava (Beeching et al., 2002; Canto et al., 2013), and to a lesser extent comparable to other tuberous roots such as taro (*Colocasia antiquorum*), sweet potato (*Ipomoea batatas* L. Lam) and jicama (*Pachyrhizus erosus* L. Urban) (Aquino-Bolaños and Mercado-Silva, 2004).

In this work, it is possible to see the simultaneous action of the enzymes POD and PPO, for both forms of temporary storage. This behavior can be related to the synergistic action of these two enzymes (Cantos et al., 2001). In the reaction catalyzed by PPO, oxidizing the phenolic compounds resulted in the release of hydrogen peroxide (H_2O_2), inducing POD activity. Another factor may also be associated with the *de novo* synthesis of POD, as observed by Cantos et al. (2001) who obtained a linear increase in POD activity and synthesis of new isoenzymes, attributing tissue repair to this factor (Yingsanga et al., 2008).

It was observed that at 12 h and at the end of 10 days, there was no fluorescence emission on 'baby cassava' at 5°C (Figure 4). On the other hand, at 25 °C, the pieces emitted fluorescence in the ultraviolet light, as was also evident, in later stages: stickiness on the surface, after the second day. Simões et al. (2016) observed *Pseudomonas* ssp., using fluorescence emission under ultraviolet light, in which it has aerobic metabolism (Sillankorva et al., 2004). The packaging used evidenced

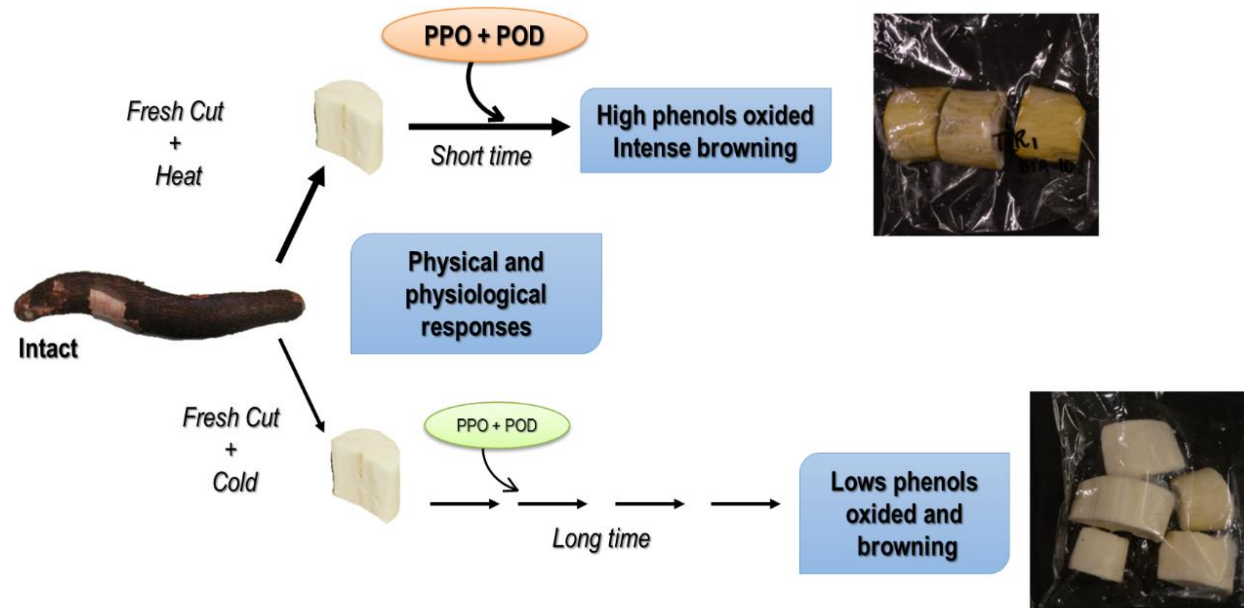

Figure 5. Model illustrative of the handling and responses of minimally processed cassava kept at high and low temperatures. Changes in PPO and POD activities with phenolics compound, response to shelf life of 'baby cassava'. The bold letters represent larger enzymatics activities.

a certain aerobic environment, due to high PPO activity (Figure 3) in which it uses O_2 in the catalysis of the reaction. Moreover, some phenolic compounds can emit fluorescence under ultraviolet light (Wulf et al., 2005). In this work, PPO and POD activities and phenolic compounds increased during conservation (Figure 3); perhaps the fluorescence emitted is related to the increase of phenols.

Thus, in the present work, the fluorescent spot observed is associated to surface stickiness, which may show microbiological contamination in the 'baby cassava' at 25°C, after two days. However, no quantitative analysis was done. This evidence is important for quick handling and consumption, not more than 12 h of 'baby cassava' at room temperature.

Thus, high POD and PPO activities and accumulation of phenols compounds were biochemical markers that preceded the browning in 'baby cassava'. This shows that maintenance of minimally processed cassava at low temperatures delayed the metabolism studied, reduced the effects caused by injuries of processing, probably retarding microbial growth and consequently prolonging shelf life of 'baby cassava' (Figure 5). Moreover, the new form of 'baby cassava' made it possible for commercialization and consumption, at room temperature before 12 h.

Conclusion

The minimally processed 'baby cassavas' from the roots of cassava cv. Recife remained with high visual quality for 12 days at 5 ± 2°C, and not more than 12 h at 25 ± 2°C. The accumulation of phenolic compounds and the increases in the PPO and POD activities in early hours were more pronounced in the 'baby cassavas' kept at 25 ± 2°C, as compared to those kept at 5 ± 2°C in all cases, with browning.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interest.

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Full Length Research Paper

High-performance liquid chromatography-diode-array detector (HPLC-DAD) analysis and evaluation of antioxidant and photoprotective activities of extracts from seeds of *Simira gardneriana* M. R. V. Barbosa and Peixoto (Rubiaceae)

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Simira gardneriana **is a Rubiaceae species commonly found in the Brazilian Northeast region, presented several therapeutic and biotechnological applications. In this paper, the antioxidant and photoprotective properties of extracts from the seeds of *S. gardneriana* were highlighted. The antioxidant activity of ethanol and methanol extracts (Si-EtOH and Si-MeOH, respectively) was determined, using 1,1-Diphenyl-2-picryl-hydrazyl (DPPH) free radical scavenging assay. The photoprotective activity of the extracts was evaluated using a spectrophotometric method. Total phenolic and flavonoid content was determined. In addition, a qualitative analysis of phytochemical markers and a high-performance liquid chromatography-diode-array detector (HPLC-DAD) analysis were also performed for both extracts. Concerning the antioxidant activity *in vitro*, Si-MeOH (EC_{50} = 70.94±2.17 µg/ml) showed greater activity in comparison to Si-EtOH (EC_{50} = 138.60±7.39 µg/ml). Once the sun protection factor spectrophotometric ($SPF_{spectrophotometric}$) of samples was calculated, it was demonstrated that the extracts show a similar photoprotective effect on all concentrations tested. Si-EtOH and Si-MeOH tested on a concentration of 100 mg/l, exhibited SPF values equal to 3.37±0.006 and 3.36±0.007, respectively. HPLC fingerprints was recorded and showed the presence of characteristic peaks for phenolic compounds. The extracts showed significant phenols and flavonoids content according to the quantification methods used. According to the results, it was concluded that Si-EtOH and Si-MeOH have significant antioxidant and photoprotective activities. These activities are probably related to the profile of flavonoids and phenolic compounds found in this species.**

Key words: Phenolic and flavonoids compounds, photoprotective, oxidative stress, plant, Rubiaceae.

INTRODUCTION

Exposure to UV radiation promotes a range of damaging effects on the body which include production of reactive

oxygen species (ROS) in skin. In particular, ultraviolet B (UVB) (290 to 320 nm) can reach the skin and cause erythema, burns, local inflammation, DNA damage and early aging. In addition, ultraviolet A (UVA) radiation (320 to 400 nm) penetrates deeper into the epidermis and dermis and stimulates ROS production ($O_2^{\cdot}$ and $OH^{\cdot}$ for example), which can modify proteins, lipids and DNA structure (Stevanato et al., 2014; Surget et al., 2015). An alternative that has been used to combat the damage caused by solar exposition is the use of natural products.

Rubiaceae family comprises 637 genera and about 13,000 species mainly distributed in tropical and subtropical regions (Rogers, 2005). In America, this family represents 229 genera and 5,200 species (Delprete, 1999), while in Brazil, Rubiaceae is represented by about 1,500 species, making this one of the main families of Brazilian vegetation (Souza and Lorenzi, 2005). Some Rubiaceae species have described biological properties, such as the species *Uncaria tomentosa* (Willd) D.C., popularly known as "unha-de-gato", being widely used in folk medicine for various indications: arthritis, asthma, cancer, gastric ulcer, inflammation and bleeding (Heitzman et al., 2005). Moreover, pharmacological studies also demonstrated that other species of this family have anti-inflammatory (Zhu et al., 2012), antinociceptive (Déciga-Campos et al., 2006), antibacterial (Comini et al., 2011), antitumor and antioxidant activities (Dreifuss et al., 2010).

In relation to family phytochemistry, some alkaloids were mentioned as important chemical markers (Moraes et al., 2009). In addition to these compounds, the presence of flavonoids, benzenoid derivatives, anthraquinones, coumarins, saponins, lignoids, terpenoids, cucurbitacines, amides and pheophytins has also been reported in Rubiaceae species (Rudrapaul et al., 2014; Mongrand et al., 2005; Moreno et al., 2014; Ferreira-Júnior et al., 2012).

The *Simira* genus is an important genera of Rubiaceae and comprises about 45 species, predominantly found in neotropical regions, of which 16 occur in Brazil (Sampaio et al., 2002). Some of these species are used in folk medicine as natural remedies. In fact, studies describe their phototoxic activity, justified by the presence of bioactive secondary metabolites (Araújo et al., 2012; Arnason et al., 1983). In Caatinga, a biome is located in the Northeast region of Brazil. There are six *Simira* species, among which *S. gardneriana* M. R. V. Barbosa and Peixoto is the only one endemic (Sampaio et al., 2002).

S. gardneriana is known as "pereiro-de-tinta" or "pereiro-vermelho" and is used as forage during the dry season (Sampaio et al., 2002). However, there have been no reports on the phytochemical profile and biological properties of this species until now. Thus, considering that several Rubiaceae species are promising sources of bioactive molecules, this study aimed to evaluate the antioxidant and photoprotective activities of extracts from the seeds of *S. gardneriana* and to investigate its phytochemical profile through high-performance liquid chromatography-diode-array detector (HPLC-DAD) analysis.

MATERIALS AND METHODS

Plant material

The seeds of *S. gardneriana* were collected in the city of Afrânio, State of Pernambuco, Brazil, in February 2012 (coordinates 08°28'40.60" S and 40°56'10.60" W). A voucher specimen of the plant (13949) was deposited in the Herbarium Vale do São Francisco (HVASF) of the Universidade Federal do Vale do São Francisco.

Preparation of extracts

Initially, the dried and pulverized plant material (1.938 g) was subjected to maceration with 95% ethanol. Five extractions were performed and the solvent was replaced every 72 h. The extraction solution obtained was filtered and concentrated in a rotary evaporator apparatus oven at 50°C, providing 115 g of ethanol extract (Si-EtOH, 5.93%).

Subsequently, the maceration was continued with absolute methanol. Three extractions were carried out and the solvent was replaced every 72 h. The extraction solution obtained was concentrated under the same conditions as Si-EtOH, resulting in 162 g of methanol extract (Si-MeOH, 8.36%).

Qualitative analysis of phytochemicals

Extracts solutions (1 mg/ml) were evaluated on thin layer chromatographic plates of silica gel 60 F_{254} aluminum supports, applied with a micropipette and eluted in different solvent systems as previously described (Wagner and Bladt, 1996), seeking to highlight the main groups of secondary metabolism (Table 1).

HPLC-DAD analysis

Solutions of Si-EtOH and Si-MeOH extracts (1 mg/ml, in methanol) were individually analyzed by High Performance Liquid Chromatography (HPLC), following parameters which is previously described (Cai et al., 2003). The solvents used were of analytical grade from Merck®. A Milli-Q System® (SMART, China) was used to purify the water. Analysis was performed on a liquid chromatograph Shimadzu® equipped with a quaternary pump system (LC-20ADVP), a SPD-20AVP Diode-Array Detector (DAD), and an SIL-20ADVP auto sampler.

The data was acquired and processed using Shimadzu® LC solution 1.0 software. The extracts were analyzed using a reverse-phase HPLC column: Ascentis® C18 (250 x 4, 6 mm, 5 μm) column

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Table 1. Elution systems and revelators are used to characterize the main secondary metabolites from the extracts of seeds of *Simira gardneriana* by thin layer chromatography.

Phytochemicals	Elution systems	Revelators
Alkaloids	Toluene:ethyl acetate: diethylamine (70:20:10, v/v)	Dragendorff reagente
Anthracene derivatives	Ethyl acetate:methanol: water (100:13.5:10, v/v)	10% ethanolic KOH reagente
Coumarins	Toluene:ethyl ether: (1:1 saturated with acetic acid 10%, v/v)	10% ethanolic KOH reagente
Flavonoids and tannins	Ethyl acetate:formic acid: glacial acetic acid: water (100:11:11:26, v/v)	NEU reagente
Lignans	Chloroform:methanol: water (70:30:4, v/v)	Vanillin sulfuric reagente
Mono and diterpenes	Toluene:ethyl acetate (93:7, v/v)	Vanillin sulfuric reagente
Naphthoquinones	Toluene:formic acid (99:1, v/v)	10% ethanolic KOH reagente
Triterpenes and steroids	Toluene:chloroform: ethanol (40:40:10, v/v)	Liebermann-Burchard reagente

(Supelco®). The mobile phase was composed of solvent (A) H_2O/trifluoroacetic acid 0.1% and solvent (B) MeOH. The solvent gradient was composed of A (100 to 90%) and B (0 to 10%) for 0 to 7 min, A (90 to 60%) and B (10 to 40%) for 7 to 20 min, A (60 to 100%) and B (40 to 0%) for 20 to 25 min, and finally A (100 to 90%) and B (0 to 10%) for 25 to 40 min. A flow rate of 1.0 ml/min was used in an oven at 37°C, and 20 µl of each sample was injected. The procedure was repeated three times for each sample. Samples and mobile phases were filtered through a 0.22 µm Millipore filter prior to HPLC injection. Spectra data were recorded from 200 to 400 nm during the entire run and the chromatograms of extracts obtained at a wavelength of 254 nm were selected for analysis of its components.

Total phenolic content

Total phenolic contents were performed using the Folin-Ciocalteu reagent, based on a method previously reported, in which only the volumes were reduced (Slinkard and Singleton, 1977). Si-EtOH and Si-MeOH were diluted (1000 mg/l), an aliquot (40 µl) was added to 3.16 ml of distilled water and 200 µl of the Folin-Ciocalteu reagent. The mixture was shaken and allowed to stand for 6 min. Then, 600 µl of sodium carbonate solution was added and shaking to mix. The solutions remained at room temperature for 2 h and the absorbance of each sample was determined at 765 nm against the blank (Spectrophotometer Quimis, Brazil).

Total phenolic contents were expressed as mg gallic acid equivalents per gram (mg GAE/g) through the calibration curve with gallic acid (50 to 1000 mg/l, R^2 = 0.997). All samples were performed in triplicates.

Total flavonoid content

Total flavonoid content was determined according to a colorimetric method described previously (Santos and Blatt, 1998; Marques et al., 2012). Si-EtOH and Si-MEOH extracts were diluted (1000 mg/l) and 0.20 ml of extracts or quercetin standard solution were mixed with 3.80 ml of distilled water, in a test tube followed by the addition of 200 µl of a 2.5% $AlCl_3$ solution.

After 30 min of reaction at room temperature, the absorbance was measured against the blank at 408 nm using a spectrophotometer (QUIMIS, Brazil), in comparison with the standards prepared similarly with known quercetin concentrations. The results were expressed as mg of quercetin equivalents per gram of extracts (mg QE/g) through the calibration curve with quercetin (1 to 20 mg/l, R^2 = 0.995). All assays were performed in triplicate.

Antioxidant activity *in vitro* - DPPH free radical scavenging assay

The free radical scavenging activity was measured using the 2, 2-diphenyl-1-picrylhydrazyl radical (DPPH) assay (Falcão et al., 2006). Sample stock solutions (1.0 mg/ml) of extracts were diluted to final concentrations of 243, 81, 27, 9, 3 and 1 µg/ml, in ethanol. One millilitre (1 ml) of 50 µg/ml DPPH ethanol solution was added to 2.5 ml of sample solutions of different concentrations, and allowed to react at room temperature. After 30 min of reaction, the absorbance values were measured at 518 nm and converted into the percentage antioxidant activity (AA) using the following formula: AA % = [(absorbance of the control – absorbance of the sample) / absorbance of the control] × 100. Ethanol (1.0 ml) plus plant extracts solutions (2.5 ml) were used as blank and DPPH solution (1.0 ml) plus ethanol (2.5 ml) was used as negative control. Ascorbic acid, BHA (butylated hydroxyanisole) and BHT (butylated hydroxytoluene) were used as positive controls. Assays were carried out in triplicate.

Photoprotective activity *in vitro* – determination of the maximum absorption wavelength and sun protection factor spectrophotometric (SPF)

For determining the maximum absorption wavelength (λ_{max}), the extracts were diluted in absolute ethanol, obtaining concentrations of 5, 25, 50 and 100 mg/l. Subsequently, spectrophotometric scanning was performed at wavelengths between 260 to 400 nm, with intervals of 5 nm. The readings were performed using 1 cm quartz cell, and ethanol used as blank (Mansur et al., 1986). Calculation of SPF was obtained according to the equation:

$$\mathbf{SPF_{spectrophotometric} = CF \times \sum_{290}^{320} EE\ (\lambda) \times I\ (\lambda) \times Abs\ (\lambda)}$$

Where: EE (λ) = erythemal effect spectrum; I (λ) = solar intensity spectrum; Abs (λ) = absorbance of sunscreen product; CF = correction factor (=10). The values of EE x I are constants. They were previously determined (Sayre et al., 1979). Benzophenone-3 (10 mg/l) was used as a positive control.

Statistical analysis

The data obtained were analyzed using the GraphPad Prism® version 5.0 and expressed as mean ± S.D. The EC_{50} values were obtained by interpolation from non-linear regression analysis with 95% confidence level.

Table 2. Phytochemical characterization of extracts from the flowers of *S. gardneriana***.**

Phytochemicals	Si-EtOH	Si-MeOH
Alkaloids	-	-
Anthracene derivatives	-	+
Coumarins	+	++
Flavonoids and tannins	+++	+++
Lignans	++	+
Mono and diterpenes	+++	+++
Naphthoquinones	-	-
Triterpenes and steroids	++	++

-, Not detected; +, low presence; ++, moderate presence; +++, strong presence.

EC_{50} is defined as the concentration sufficient to give 50% of maximumeffect estimated at 100%. Statistically significant differences were calculated by the application of Student's *t*-test. Values were considered significantly different at $p < 0.05$.

RESULTS

Qualitative analysis of phytochemicals

The phytochemical profile of the extracts was characterized by thin layer chromatography. In general, the extracts showed positive results for the presence of several classes of secondary metabolites, especially flavonoids, mono and diterpenes, coumarins, lignans, triterpenes and steroids, as shown in Table 2. The intensity scale of the identified phytochemicals was defined by comparison with standard samples whenever possible.

HPLC-DAD analysis

HPLC fingerprint for Si-EtOH and Si-MeOH are presented in Figure 1. The chromatogram shows the presence of six majority peaks for both extracts with different retention times. Furthermore, the λ_{max} values observed for compounds 1 to 6 are characteristic of phenolic constituents for the analyzed wavelength (254 nm). Based on their UV-Vis spectral data and their retention time, the compounds have UV band characteristic for phenolic acids and flavonoid derivatives (Table 3). These compounds are under investigation.

Total phenolic and flavonoid content

The total phenols and flavonoids contents for extracts were determined using different methods. Total phenol content was determined by the method of Folin-Ciocalteu reagent, where Si-EtOH and Si-MeOH showed 183.70±9.87 and 166.50±1.67 mgGAE/g, respectively. In relation to determination of total flavonoids, a colorimetric assay using quercetin was conducted as a standard.

In this method, Si-EtOH and Si-MeOH showed 4.55±0.73 and 4.05±1.60 mgEQ/g, respectively. However, the extracts showed no significant differences in total phenolic and flavonoids contents found (Figure 2). The results are expressed in mg of gallic acid equivalents per gram of sample (mg GAE/g) and in mg of quercetin equivalents per gram of sample (mg QE/g), respectively. The Student's *t*-test was used for analysis of the results.

Antioxidant activity *in vitro*

Concerning the antioxidant activity *in vitro*, Si-MeOH (EC_{50} = 70.94±2.17 µg/ml) showed better activity in comparison to Si-EtOH (EC_{50} = 138.60±7.39 µg/ml) in DPPH free radical scavenging assay. However, ascorbic acid, BHA and BHT proved more effective than both extracts, presenting EC_{50} of 3.65±0.04, 3.76±0.09 and 6.10±0.31 µg/ml, respectively (Figure 3).

Photoprotective activity *in vitro*

To evaluate the photoprotective effect of the extracts, the spectrophotometric method was adopted. This test is based on spectrophotometric absorption capacity of the sample in order to evaluate the ultraviolet region of the spectrum (100 to 400 nm) at which the sample shows a higher absorbance value. Accordingly, it was found that both extracts (100 mg/l) showed absorption bands in UVA (320 to 400 nm) and UVB (290 to 320 nm) regions, possibly suggesting photoprotective activity (Figure 4).

When calculating $SPF_{spectrophotometric}$ of samples, it was found that the extracts show a similar effect at all concentrations tested. Si-EtOH and Si-MeOH tested at a concentration of 100 mg/l, for example, SPF exhibit values equal to 3.37±0.006 and 3.36±0.007, respectively (Figure 5). Furthermore, it was found that the photoprotective activity of the extracts is directly proportional to the concentration used suggesting an effect of the concentration dependent type as described in previous studies, to extracts fractions of plants with photoprotective activity (Sônia et al., 2015; Serafini et al., 2014). Benzophenone-3 exhibited $SPF_{spectrophotometric}$ value, which is equal to 5.09 ± 0.147.

DISCUSSION

In this study, it was indicated that Si-EtOH and Si-MeOH have phenolic compounds, which are possibly responsible for their antioxidant and photoprotective properties. A HPLC fingerprint of phenolic compounds was developed and showed the presence of characteristic peaks for these compounds. The extracts showed significant

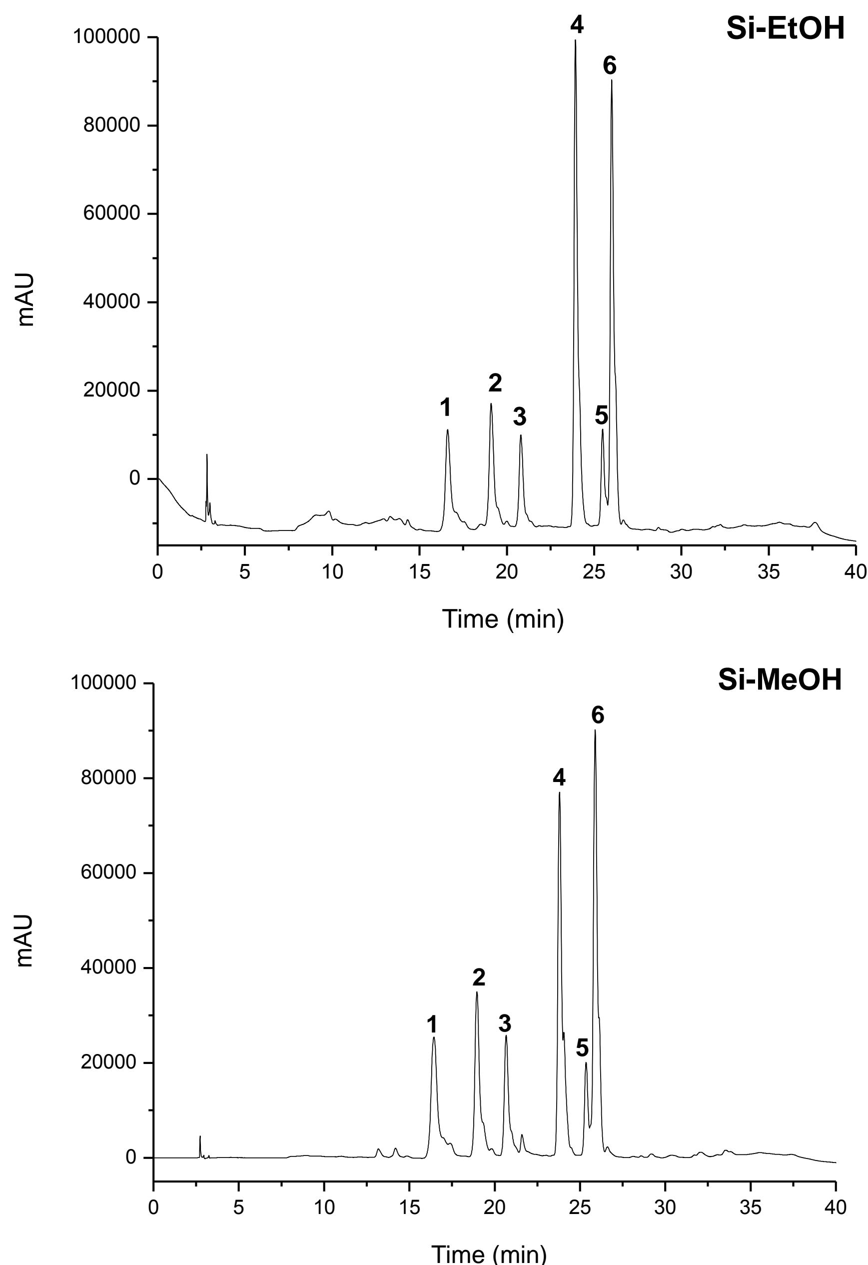

Figure 1. High performance liquid chromatography profile (HPLC fingerprint) of Si-EtOH and Si-MeOH extracts recorded at 254 nm.

Table 3. Retention time (RT) and wavelength for maximum absorbance λ_{max} of the major components (1-6) identified for Si-EtOH and Si-MeOH by HPLC-DAD (254 nm).

Peak	Si-EtOH		Si-MeOH	
	RT (min)	λ_{max} (nm)	RT (min)	λ_{max} (nm)
1	16.61	326	16.45	242 and 324
2	19.10	263. 311 and 374	18.97	255. 311 and 375
3	20.81	263. 311 and 363	20.60	255. 311 and 374
4	23.93	257. 303 and 369	23.80	246. 303 and 369
5	25.50	268 and 269	25.36	223
6	26.01	268	25.90	220

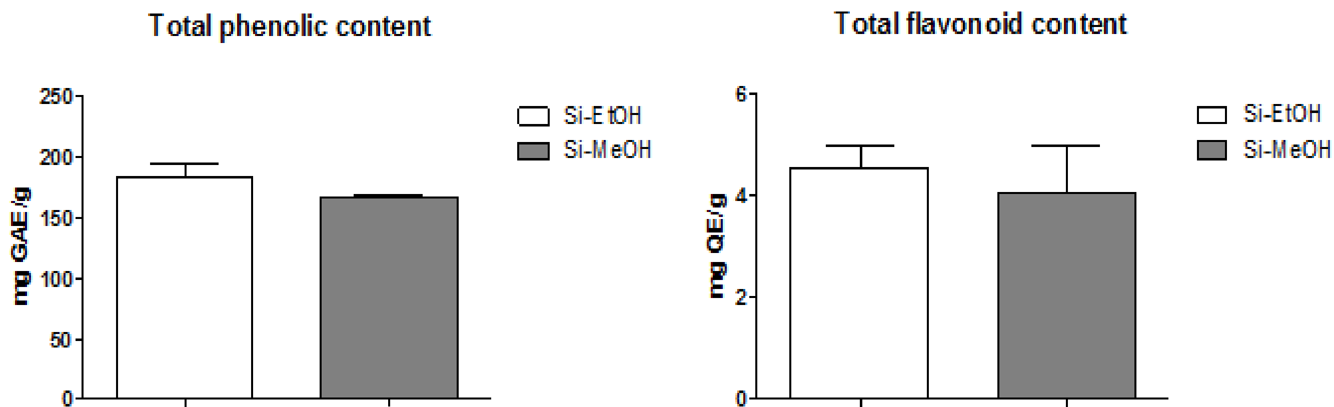

Figure 2. Determination of total phenols flavonoids for Si-EtOH and Si-MeOH.

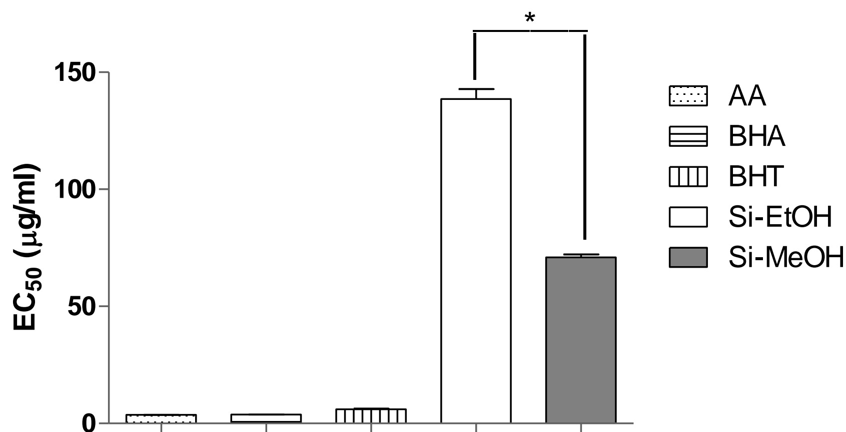

Figure 3. Antioxidant activity *in vitro* of Si-EtOH and Si-MeOH. AA: ascorbic acid. BHA: butylhydroxy anisole. BHT: butylhydroxy toluene. The Student's *t*-test was used for analysis of the results, where * ($P < 0.05$) indicates significant difference (Si-EtOH vs Si-MeOH).

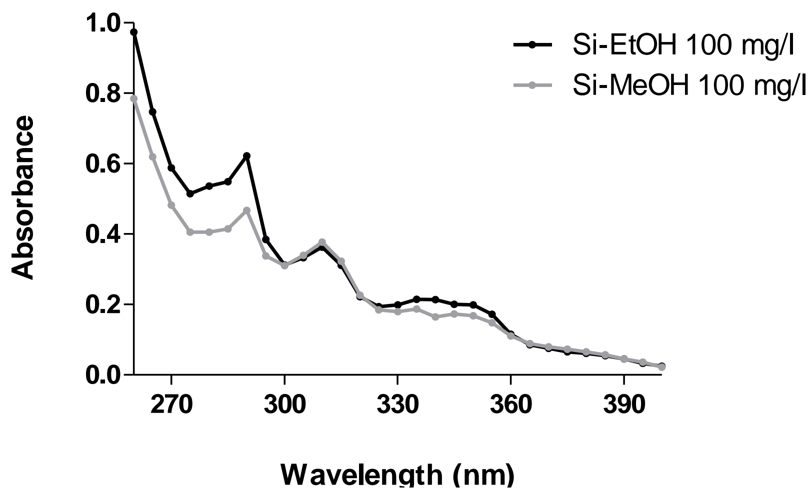

Figure 4. Spectrophotometric absorption profile of Si-EtOH and Si-MeOH extracts (260-400 nm).

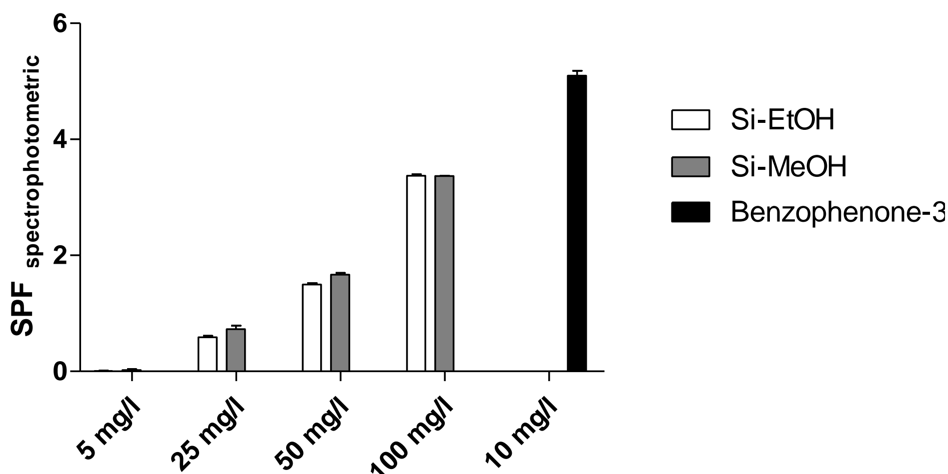

Figure 5. Determination of sun protection factor spectrophotometric ($SPF_{spectrophotometric}$) of Si-EtOH, Si-MeOH and benzophenone-3. The Student's *t*-test was used for analysis of the results.

phenols and flavonoids content through the quantification methods used. However, there was no statistically significant difference between them.

Flavonoids represent an important class of secondary metabolites that possesses photoprotective and antioxidant efficacy and tolerability greater than currently used synthetic filters. In general, flavonoids and other phenolic compounds have the ability to reduce the oxidative damage caused by short solar wavelengths and reduce the risk of generation of ROS (Stevanato et al., 2014).

The antioxidant ability of the *S. gardneriana* extracts was investigated through DPPH method, commonly used for screening antioxidants from plant extracts. DPPH is a stable free radical that reacts with compounds which can donate a hydrogen atom. This assay is based on the scavenging of DPPH through the addition of an antioxidant that decolorizes the DPPH solution (Lima-Saraiva et al., 2012).

In this model, Si-MeOH was more effective than Si-EtOH, with a minor EC_{50} value. Several publications with plant extracts have demonstrated linear correlations between the profile of phenolic compounds and antioxidant activity. However, it is possible that other compounds present in Si-EtOH and Si-MeOH act as antioxidants since the flavonoid and phenolic content was similar in both extracts.

The photoprotective activity was determined by the spectrophotometric method developed by Mansur et al. (1986) using UVB region, which is considered to be the region of greatest incidence during the day. Although this test has been performed *in vitro*, there is a relevant correlation with *in vivo* tests because it relates the absorbance of the samples with its photoprotective potential in combating an erythematogenic effect, caused by radiation at specific wavelengths between 290 and 320 nm (UVB region) (Violante et al., 2009).

Si-EtOH and Si-MeOH showed characteristic absorption bands in UVB and UVA regions, suggesting a possible photoprotective potential. The maximum absorption wavelength (λ_{max}) for extracts was 225 (UVC), 290 (UVB), 310 (UVB) and 335 nm (UVA). Concerning the SPF values, the extracts showed an interesting photoprotective activity in a concentration dependent manner. These results can be justified by the presence of flavonoids in the extracts. Some reports correlate the concentration of flavonoids in plant extracts and fractions with their photoprotective activity. In fact, flavonoids have the ability to reduce the oxidative damage caused by short solar wavelengths and reduce the risk of generation of ROS by absorption and stabilization of the energy, emitted by UVB radiation on the skin (Stevanato et al., 2014).

Conclusion

According to the results shown, it was concluded that Si-EtOH and Si-MeOH have significant antioxidant and photoprotective activities. These activities are probably related to the profile of flavonoids and phenolic compounds found in this species.

This study provides the use of extracts of *S. gardneriana* in pharmaceutical preparations as sunscreens. However, other studies are needed to reach the isolation of the compounds responsible for the properties of the extracts.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Histochemical GUS expression of beta tubulin promoter in transgenic tobacco

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Agrobacterium mediated plant transformation is a useful technique for stable transformation of plants. This study aimed at stable transformation of the beta tubulin (β) gene promoter and its expression analysis by histochemical GUS assay. The beta tubulin promoter sequence was cloned in plant expression vector (pGA482) and transformed stably in tobacco through _Agrobacterium_ mediated transformation. The stable GUS expression assays for this promoter in various tissues of _Nicotiana tabacum_ indicated its functional importance in regulating gene expression in a constitutive manner. It was concluded that the β tubulin promoter is constitutively expressed with a strength equivalent to CaMV 2X35S promoter. The isolated promoter can be used in plant genetic engineering for crop improvement in future.

Key words: Constitutive, gene expression, *GUS,* transformation.

INTRODUCTION

Transgenic plants contain genes which are genetically modified by using genetic engineering techniques. The mode of insertion of transgene is completely artificial. The modified genes can be obtained from completely different species, for example transgenic Bt corn contains a gene from a bacterium and produces its own insecticide. The aim is to introduce a new trait to the plant and this trait does not occur naturally in the species. The production of transgenic plants is a useful process. Transgenic plants can express foreign proteins with industrial and pharmaceutical value. This will help to increase shelf life, quality and resistance against biotic and abiotic stresses. Several new methods of gene transformation has been used for improvement of certain crops. A very common technique known as “Particle Bombardment” is used for transformation of foreign genes in plants. It uses biolistic approach for shooting a piece of DNA into the recipient plant tissue. Gene gun is used in which gold particles are coated with the plasmid vector having gene of interest. These coated particles are accelerated by helium gas and bombarded on particular plant tissues. The gold particles pass through external barrier, that is, the cell

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wall of plant and most of them may enter the nucleus under suitable conditions. The major drawback of this technique is tissue damage, copy number and low transformation efficiency. This method can be used on both monocotyledonous and dicotyledonous species.

Dicotyledonous plants develop from two cotyledons in the seed. They can be recognized by the branching veins in their leaves. Dicots of commercial value include many horticultural plants such as petunias, and crops such as tobacco, tomatoes, cotton, soybean and potatoes. The other method of great importance for gene transformation is electroporation. It works by using electric field that generates holes in the plasma membrane allowing DNA to be taken up by the cell. According to some studies, the reported mortality rate of cells is much high, almost 25 to 50% survival. This technique can be used for variety of species and tissue types. The related drawback is that it needs an established protoplast regeneration system for the plant species being manipulated with foreign DNA.

Agrobacterium-mediated gene transfer method has also gained much importance for production of transgenic plants. *Agrobacterium* is a Gram negative soil pathogen used to transfer DNA to plant cells for the purposes of plant genetic engineering. A number of different reports related to gene transfer through *Agrobacterium* mediated transformation have been reported. Examples in monocotyledonous plant species include rice, barley, maize and wheat (Cheng et al., 1997; Hiei et al., 1994; Rashid et al., 1996; Tingay et al., 1997; Ishida et al., 1996). *Agrobacterium tumefaciens* is a Gram-negative soil pathogen which can naturally infects the wounded sites in plants. The infection results in the formation of crown gall tumors under natural conditions (Smith and Townsend, 1907). The bacterium transfers mobile DNA segment also called T-DNA into the nucleus of infected cells along with DNA coded by some virulence proteins. The transferred DNA is then stably integrated into the host genome and transcribed (Nester et al., 1984; Binns and Thomashaw, 1988). The bacterium is involved in transformation of several dicots (DeCleene and DeLey, 1976). It may even transform various fungal species and human cells (Bundock et al., 1995; De Groot et al., 1998; Gouka et al., 1999; Kunik et al., 2001).

Microtubules occur as a result of cell division and cell elongation. They are mainly composed of α- and β-tubulin having some evolutionary conserved genes (Little and Seehaus, 1988). Simply, tubulin are globular proteins that make up microtubules. Many plant tubulin genes are found as a part of multigene family. However, almost six tubulin genes exist in Arabidopsis and about seven in maize. All tubulin genes works differently in a different manner. Some of them work in a constitutive manner and few are tissue specific genes (Ludwig et al., 1988; Kim and An, 1992; Kopczak et al., 1992). More generally, the expression of tubulin genes in plants can be best studied in dividing tissues.

Studies reported the cloning of tubulin which was achieved from chicken cDNA during 1980s (Cleveland et al., 1980). However, a number of different tubulin genes from different organisms have been isolated and characterized. Tubulin was long thought to be specific to eukaryotes. The basic unit of microtubules is a heterodimer protein composed of α and β-tubulin polypeptides. It is surprising why a large number of tubulin genes in eukaryotes and a single type of α and β-tubulin would be sufficient to fulfill the polymerization of microtubules. In the higher plants, both alpha and β-tubulin genes form multigene families. Some of these tubulin genes are expressed constitutively, while many others exhibit tissue, organ, or cell specific expression patterns. The isotype-specific expression of tubulins suggests some functional distinction among these proteins. Studies have indicated that the β-tubulin promoter contains a GC-rich region between the TATA box and the transcription initiation site, with 7 copies of 10 bp sequence motifs called tub box. These tub box motifs are involved in the induction of transcription. Indeed, removing 4 or 5 tub box motifs prevents transcriptional increase but it does not significantly affect the transcription level (Davies and Grossman, 1994).

In order to develop transgenic plants with specialized characteristics and properties, it is important to study and understand the functions of dicot plant promoters for gene expression studies. The present study was planned to characterize the dicot promoter from tubulin family. The β-tubulin promoter, therefore, was selected in this study to figure out, if it really represents a constitutive promoter and can substitute the equivalent promoters for construction of multiple gene expression cassettes.

MATERIALS AND METHODS

A variety of different tobacco leaves was selected to perform a tissue culture experiment. All reagents and chemicals used in this protocol were of high purity and analytical grade, meeting the standards of plant cell tissue culture applications. The antibiotic solutions were filter sterilized, while the equipment's were sterilized by autoclaving.

Transformation in *Agrobacterium*

The β tubulin construct was transformed in tobacco through *Agrobacterium* mediated transformation method. For this, the *Agrobacterium* strain LBA4404 was used as a carrier strain. The plasmid DNA of the pGA482 clone having beta tubulin promoter cassette was isolated from overnight culture. The isolated plasmids were further transformed in electrocompetant cells of *Agrobacterium* strain LBA4404 by electroporation. This method works by applying electric shock. The agar plates containing antibiotics were used to spread the transformed cells. These plates were prepared by adding Rifampicin (25 µg/ml) and Kanamycin (50 µg/ml) and were incubated at 28°C for 48 h. Moreover, for *Agrobacterium* growth, single colonies were cultured in LB broth containing antibiotics with relative concentrations. Finally, plasmid were isolated after 48 h by using classical alkaline lysis technique and amplified by PCR with repeated cycles of denaturation,

Table 1. PCR profile for amplification of promoter fragment.

1	Initial denaturing temperature (one cycle)	94°C	4 min
2	Denaturing temperature	94°C	1 min
3	Annealing temperature	46-52°C	1 min
4	Extension temperature	72°C	2 min
	40 cycles from step 2 to step 4		
5	Final extension temperature (one cycle)	74°C	10 min

annealing and extension. The amplified plasmids was further verified by agarose gel electrophoresis for clear visualization of bands. The confirmed clones were then preserved as glycerol stock prepared with 30% concentration for future use.

The seeds of tobacco (*Nicotiana tabaccum* L.) were germinated and grown *in vitro*. For this purpose, surface sterilized tobacco seeds were used. About 1 g of tobacco seeds were soaked in 10 ml bleach prepared with 15% concentration for about 15 min, rinsed in deionized water five times and dried on sterile Whatman filter paper. Ten seeds were planted on Petri dish containing the MS medium. Seeds were germinated and grown in growth room under 16 h constant light at 25°C. The stems with single node and two leaves were cut out from grown plantlets and then transplanted to fresh MS medium.

Regeneration of transformed callus

The prepared glycerol stock was streaked on LB agar plates containing Rifampicin and Kanamycin antibiotics to get true recombinant colonies. The single colonies were cultured in LB media. The cultures were incubated at 28°C with 130 rpm for 48 h. The overall objective of tobacco leaf discs preparation was to maximize wounded surface area for *Agrobacterium* attachment and maintaining tissue health for efficient regeneration of cells. Sterile tobacco leaves were cut from plantlets and soaked in MS liquid medium in a sterile Petri dish to avoid dehydration. After two days, 10 to 20 ml *Agrobacterium* inoculum of each of the four clones was poured in separate petri dishes. About 20 to 30 leaf disks were added per Petri plate, covered and placed at room temperature to allow physical attachment of *Agrobacterium* to plant tissues for about half an hour. After co-cultivation, 5 to 7 leaf disks were placed per petri dish on the co-cultivation media to physical transfer of genetic material by *Agrobacterium* causing virulence. Petri dishes were covered and sealed well with PVC cling film and incubated at 26°C for almost 36 to 42 h.

The leaf disks co-cultivated with *A. tumefaciens* were collected, washed 4 to 5 times with MS liquid medium containing Cefotaxime (250 µg/ml) to remove extra growth of *Agrobacterium* and blotted to remove excessive water. About 4 to 5 leaf disks were placed per Petri dish on the shoot selection medium for induction of callus formation. Regenerated plants were carefully transferred to the pots containing mixture of loamy soil and sand (1:1), covered with water soaked polythene bags and placed at 25°C in the green house. Plants were lightly irrigated after every 2 days interval. When plants attained a height of about 5-inches, they were shifted to soil pots till maturity and collection of seeds. The putative transgenic and control tobacco plants were routinely observed and noted for their morphological appearance during developmental stages.

Molecular analysis of transgenic plants

Young leaves of putative transgenic plants and negative control tobacco plant were selected for DNA isolation by CTAB method. The isolated DNA was re-suspended in 50 µl of ultrapure sterile H_2O and stored at -20°C. Positive control plasmid DNA for each construct was isolated by using miniprep plasmid isolation kit Fermantas. The primers for transgene analysis were designed at specific sites inside the promoter and gene for amplification of junction regions near promoter. The PCR products for transgene analysis were analyzed by electrophoresis on agarose gel prepared with 1% concentration containing 0.05% concentrated EtBr along with standard 1 kb DNA ladder. Leaves of young plants containing transgenes was selected for DNA isolation by CTAB method with a negative control. The isolated DNA was re-suspended in 30 µl of ultrapure sterile H_2O and stored at -20°C. Gene specific primers for transgene analysis were designed for amplification of junction regions near gene. Finally, the presence of transgenes was confirmed by PCR reaction. The PCR profile is shown in Table 1.

β-Glucuronidase expression

The expression of transgenes was studied by using histochemical *GUS* assay. The tissues of selected plants were covered with 20 µl of 0.1 M X-gluc staining solution. The samples were incubated at 37°C in dark overnight and vacuum infiltrated. At the completion of incubation time, the leaves, stems and roots were treated with different serial dilutions of ethanol. The expression of *GUS* in all the tissues was recurrently monitored. The detailed observation was carried out with light microscope and photographed using a camera.

RESULTS

Agrobacterium-mediated tobacco transformation

The construct for β tubulin was transformed into the *Agrobacterium* LBA4404 strain by electroporation. The clones were confirmed by PCR using reverse and forward promoter specific primers to amplify the selected gene promoter. The results are shown in Figure 1.

Tobacco transformation

A number of different leaf discs were cut and co-cultivated with the cultured *Agrobacteroium* containing plant expression vector. This gives a fair chance to the *Agrobacterium* to transform the gene of interest into the plant causing virulence. Leaf disc were placed on solidified MS0 medium till shooting and rooting for proper growth. Cultivated tobacco plants at different stages are shown in Figure 2.

Molecular characterization of transgenic plants

The transgenic plants were grown in a growth chamber to

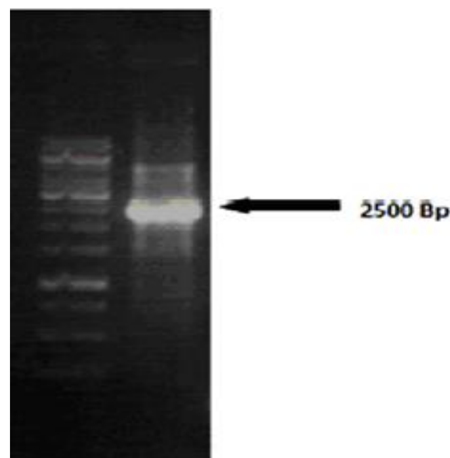

Figure 1. Confirmation of clones in *Agrobacterium*. Lane M: 1kb DNA ladder, PCR amplification of beta tubulin gene promoter of *Agrobacterium* clone.

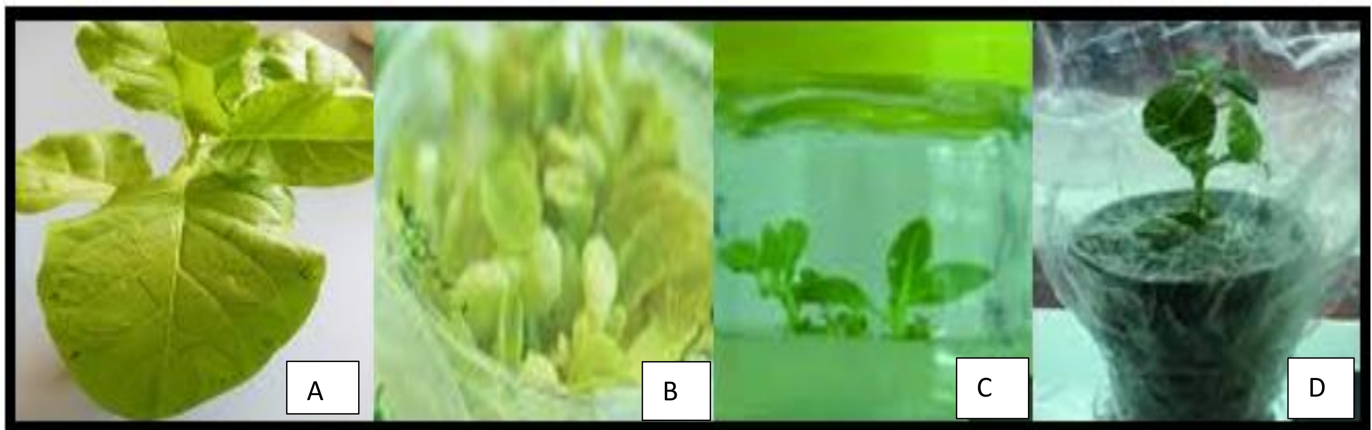

Figure 2. Stages of *Agrobacterium*-mediated tobacco transformation. (A) Tobacco leaf discs for co-cultivation with *Agrobacterium* inoculums. (B) Tiny plantlets of tobacco leaf discs co-cultivation with *Agrobacterium* inoculum. (C) Excised 7 days old plantlets which survived on Kanamycin selection media shifted to jar. (D) Mature plantlets shifted to soil.

study their morphology. More than 100 events were established in the soil. The maximum number of transformed plants was fertile and produced many seeds. All the transgenic plants were analyzed by polymerase chain reaction (PCR) to check the presence of tubulin gene. The analysis showed positive incorporation of tubulin gene in various examined tobacco plants. Out of 5 experiments, the average transformation efficiency of callus producing plants was found to be highest in experiment no. 2 with approximately 79.5% average. The transformation efficiency was calculated by dividing total number of explants showing transformation with number of explants inoculated. The results obtained are shown in Table 1 and Figure 3.

Evaluation of transgenic plants

The insecticidal activity in all 5 experiments was performed to measure the resistance levels. Results showed different resistance levels including high and low levels with variation in all experiments. Screening of plants with bioassays was useful to measure the different resistance levels in the selected plants. The obtained results are presented in Table 2 and Figure 4.

Analysis of transgene and *GUS* expression

The genomic DNA was isolated by using CTAB method

Table 2. Different resistance levels in the selected transgenic tobacco plants.

Experiment	Number of Plants used	Resistance Level
1.	5	Highly resistant
2.	4	Less resistant
3.	6	Moderately resistant
4.	3	Less resistant
5.	4	Highly resistance

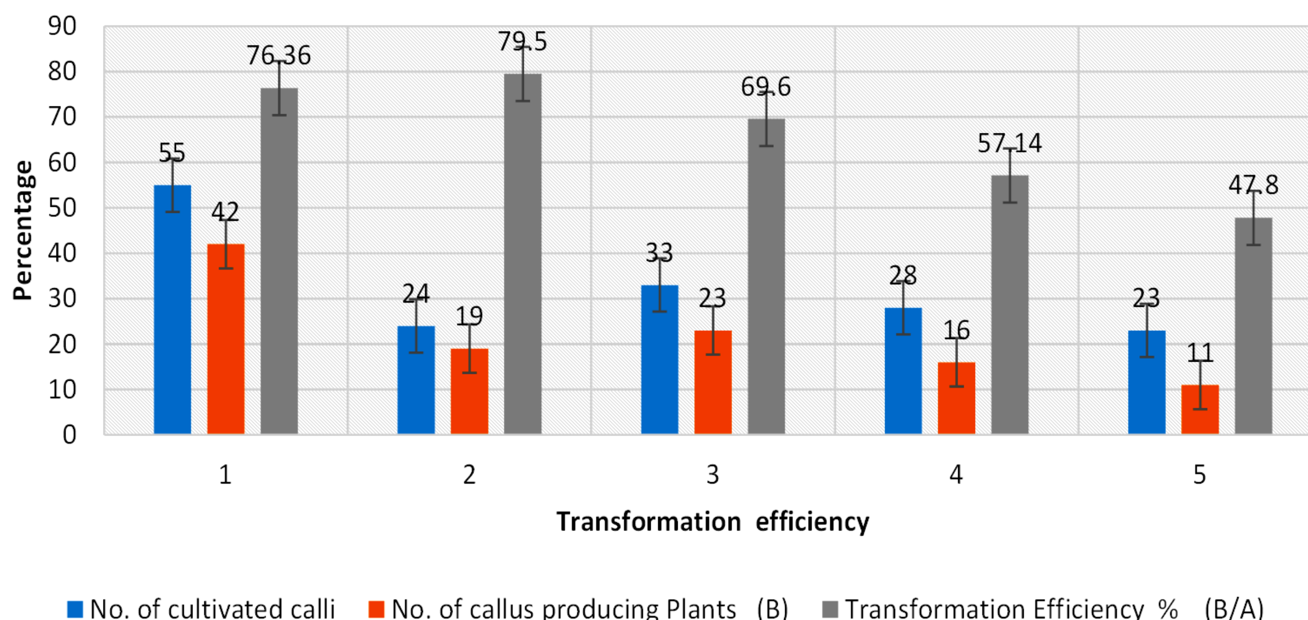

Figure 3. The average transformation efficiency of transgenic plants.

from the putative transgenic and non-transformed negative control plants. The transgenic plants were confirmed by PCR using promoter specific primers. Results of PCR analysis are shown in Figure 5. The results show amplification of expected fragments from the transgenics for β tubulin construct. The amplifications in the transgenic yielded identical size of amplified DNA fragment that could also be seen in the positive control. However, no amplification was observed in the genomic DNA of control plant. Further, the selected leave tissues of beta tubulin transgenic plants were stained for *GUS* activity. Staining patterns of representative leaf tissues are shown in Figure 6. All the leaf tissues were dipped in staining solution to monitor color intensity of expression levels in different transgenic events. The plants tissues expressing *GUS* using *2X35S* and beta tubulin promoter was stained for 24 h. The staining reaction showed that beta tubulin promoter was constitutively expressed in leaves. The staining of leaves from non-transgenic plants did not reveal any expression.

DISCUSSION

Transformation of plants by *Agrobacterium*-mediated gene transfer is the most commonly used method for transferring gene of interest in plants. According to some recent reports, the method of *Agrobacterium*-mediated transformation has been applied in a number of monocotyledonous plant species (Hiei et al., 1994; Rashid et al., 1996), barley (Tingay et al., 1997), maize (Ishida et al., 1996) and wheat (Cheng et al., 1997). The integration of T-DNA into host plant can infer resistance against disease.

Stress tolerance in plants can be improved by using several enzymes of biosynthetic pathways (Lata et al., 2011). The *Agrobacterium* mediated plant transformation can be used for genetic improvements. This is due to small copy number of T-DNA and variable expression of transferred genes (Murai, 2013). Gene regulation plays a vital role in controlling genetic modification of plants. Cellular homeostasis can be maintained with proper

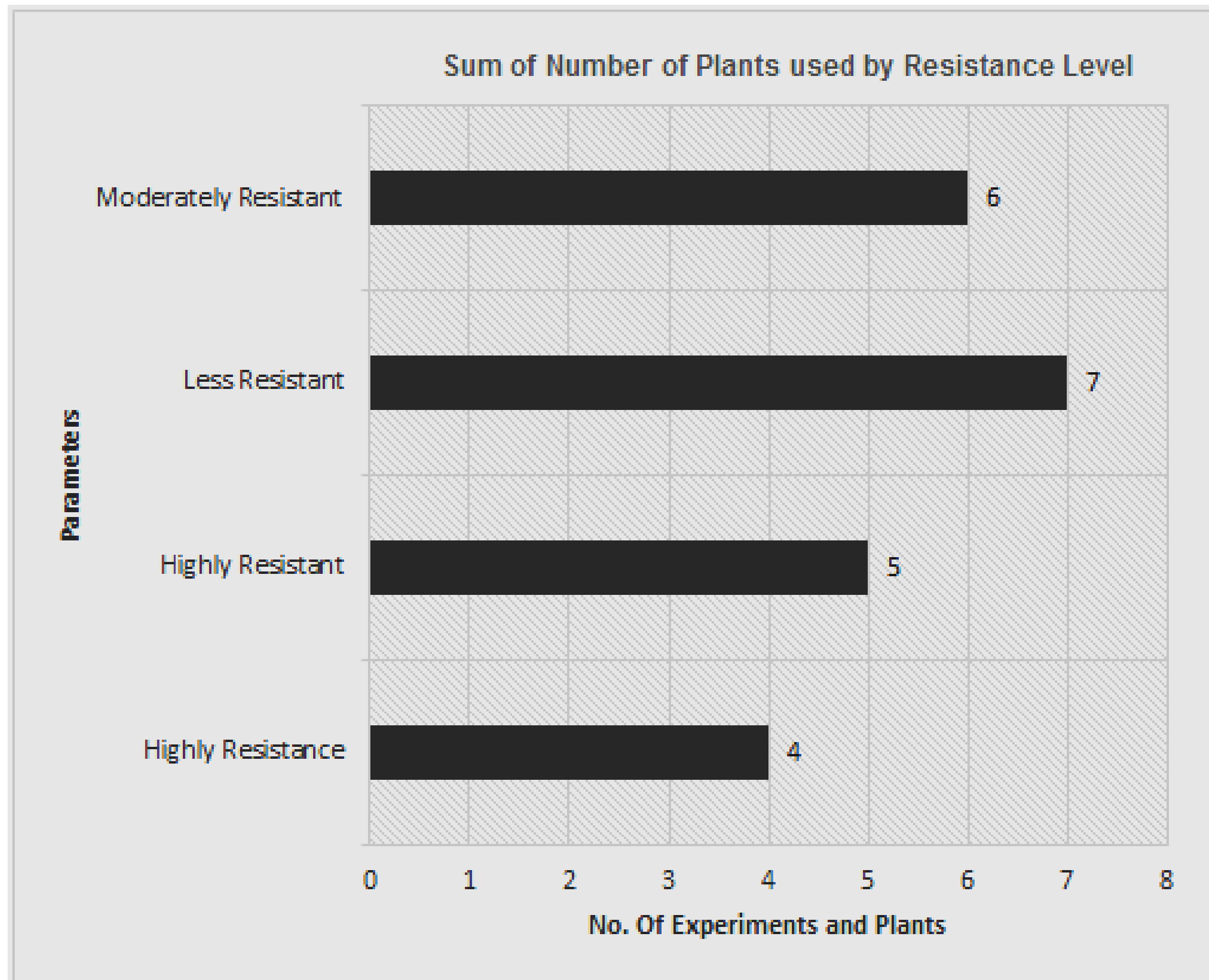

Figure 4. Resistance levels in different plants with variation of resistance.

regulation and growth (Arie, 2002; Mizoi and Yamaguchi-Shinozaki, 2013). Transcriptional regulation involves regulation of all genes involved in regulatory pathways (Julieta, 2014). Transcription factors are key factors in transcription regulatory mechanism acting as gene switches.

A number of different factors exists and can limit this transformation efficiency in both monocots and dicot plants. This can also affect plant regeneration process. Mostly, the regeneration rates are poor with monocotyledonous plants. *Gus* is a very useful reporter gene expression system used in plant transformation. The histochemical GUS assay has been used for subcellular localization of *GUS* associated fusion proteins. However, efficient methods of *Agrobacterium* mediated gene transfer have been established mainly for dicotyledoneous plants. The optimization of *Agrobacterium tumefaciens* for plant transformation is possibly the most important aspect to be considered. Expression of transgenes in tobacco leaves and callus was determined using histochemical GUS assay. Some of the relevant findings about *Gus*-reporter gene have been studied in tobacco plant (Nakashima et al., 2014).

To evaluate the accuracy of tubulin promoter that has been transformed through stable transformation, it is preferred to generate its large number of transformants. Out of which few was screened for gene expression analysis. Therefore, important elements that control the tubulin transcriptional expression may lie in both the 5′- and 3′-flanking regions of the genes (Stotz and Long, 1999; Doyle and Han, 2001). Moreover, it was found that 5′-flanking regions are more commonly involved in this process. Some of the previous studies predicted, to increase the level of GUS expression of rice tubulin genes, the first intron of *tubulin-α1* was sufficient (Jeon et al., 2000). This was observed in many transformation experiments performed with the use of *GUS* as the reporter gene. The expression of the reporter gene was controlled by the tubulin sequences 5′-upstream of the

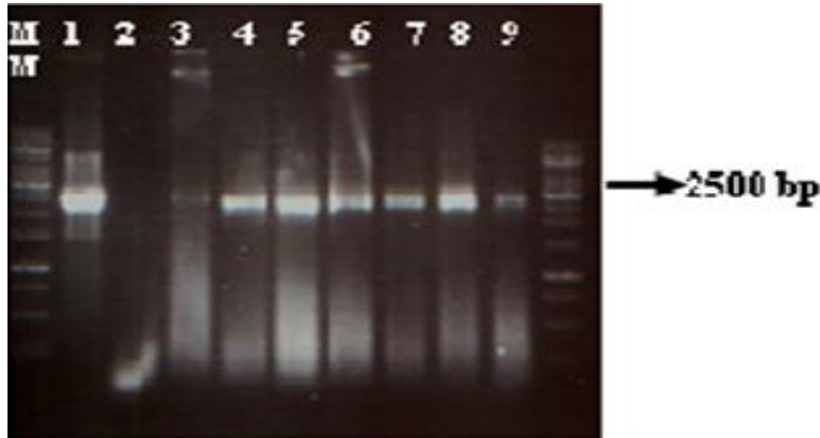

Figure 5. PCR analysis of putative transgenic tobacco plants for β tubulin promoter. M: 1 kb DNA ladder, Lane 1: PCR of positive control using plasmid DNA as a template, Lane 2: Negative control of PCR master mix, Lane 3: Negative control of tobacco Lanes 4 to 8: PCR analysis of 5 randomly selected putative transgenic plants using promoter specific primers, showing expected amplification product of 2500 bp.

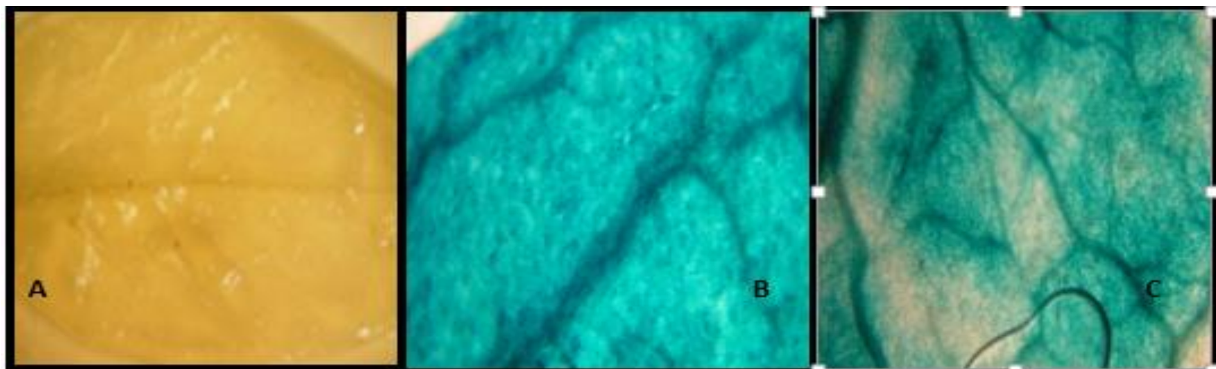

Figure 6. Histochemical assay for *GUS* activity in stably transformed tobacco plants. A: negative control of tobacco, B: *GUS* activity in leaves controlled by 2X35S, C: Close view of leaf tissue stained for *GUS* activity controlled by β tubulin promoter.

ATG in construct being used (Carpenter et al., 1992). Therefore, *GUS* expression will be high in these experiments (Uribe et al., 1998; Stotz and Long, 1999; Cheng et al., 2001). The study was designed to characterize and analyze the expression of the tubulin promoter in tobacco (*Nicotiana tabacum* L.) by using *Agrobacterium*-mediated transformation. The tubulin gene from beta family was selected and cloned in plant expression vector (pGA482) for stable transformation. Beta tubulin promoter was retrieved from high throughput genomic sequences and its sequence was analyzed through databases. The transgenic plants were obtained and *GUS* activity in each of the transgenic was determined through *GUS* staining. The selected tubulin promoter showed GUS expression in various plant tissues of tobacco and cotton. Also, it was found to highly constitutive dicot promoter for expression of transgenes in various dicot plants. The *GUS* expression studies revealed that the selected beta tubulin promoter actively control *GUS* expression in different transgenic events of the stably transformed tobacco plants. *In situ GUS* activity on leaf tissues of transgenic plants showed, the β tubulin promoter was active with different levels of expression.

Conclusions

It is concluded that β tubulin promoter can be utilized to confer a constitutive gene expression in all plant tissues. The *2X35S* promoter has been reported to exhibit strong constitutive activity in different plant species and the

same was observed in the current experiments. It is proposed that the promoter identified through this study may be utilized in future to overcome the major issues related to gene silencing and to ravel novel mechanisms of different plants. Moreover, highly expressed constitutive dicot promoters can be utilized for the transgene expression in economically important agricultural crops in future.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interest.

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Full Length Research Paper

Phenotypic diversity in physic nut (*Jatropha curcas* L.) *in vivo* germplasm bank for superior parent selection

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***Jatropha curcas* is an interesting alternative for biodiesel production due to the high oil content in its seeds, its ability to grow in a wide range of climate and soil conditions as well as low cost of production. However, the species is considered to be in domestication and there are no defined cultivars. Therefore, it is extremely important to understand the genetic diversity of the species for selection and characterization of promising genotypes to initiate breeding programs. The objective of this study is to evaluate the phenotypic diversity of physic nut in order to select the most divergent and superior genotypes to compose future breeding programs, using multivariate analysis. Eleven agronomic characters were evaluated in 165 *J. curcas* genotypes from the *in vivo* germplasm bank, which were: Plant height, stem diameter, number of primary branches, fruit length, width, weight and shape, seed length, width and weight plus the oil content. The data were analyzed by principal component analysis (PCA), cluster analysis by Ward and k-means methods. The character fruit shape was removed from the multivariate analysis as the only one with qualitative character. The PCA resulted in 4 main components (PC), which explained 71.62% of total variance. The characters selected in PC1 were seed weight, fruit width, fruit length and fruit weight. There were 22 promising genotypes highlighted, with potential to be exploited in breeding programs. Cluster analysis by Ward and k-means methods generated 9 groups influenced by all analyzed characters, of which five groups of genotypes had advantageous characters. Regarding fruit shape, 13 genotypes had an ellipsoid lanceolate shape and the others had an ellipsoid spherical shape. Multivariate analyses allowed genotype characterization, indicating good strategies used for the selection in genetic breeding programs.**

Key words: Agronomic characters, *Jatropha curcas*, oleaginous, multivariate analysis.

INTRODUCTION

Jatropha curcas, also known as physic nut, is a shrub, perennial and monoecious species that belongs to the Euphorbiaceae family. It is in the same family with castor bean, cassava and rubber tree. It is believed that this

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species originated from Central America; however, it may have adequate development in many tropical and subtropical countries (Laviola and Dias, 2008). *Jatropha* genus is entomophilous (Saturnino et al., 2005), which increases the genetic variability probability within species. The height of physic nut plant ranges from 2 to 5 m; it can live up to 35 years, and produces fruit with three seeds of 20 mm long, 11 mm wide and 9 mm thick. They are oblong and black. Its young leaves are reddish, and become dark-green during maturity; they have three to five lobes, petiolate, alternate. Its flowers are small and greenish-yellow (Drummond et al., 1984).

Due to the global energy crisis from environmental and climatic impacts caused by the high fossil energy consumption, the demand for renewable fuels that produce lower pollutants is increasing. In this context, physic nut is an alternative source because of some of its advantages: the high oil content of its seed, between 22 and 42% (Sunil et al., 2008; Achten et al., 2008), its low production cost, it is an edible crop and meets environmental demands, since its oil contains sulfur in insignificant amounts. The physic nut can reach between 500 and 3000 kg ha^{-1} depending on genotype and environment (Wani et al., 2016), producing about 2000 kg ha^{-1} of oil in the fourth year of cultivation, depending on the spacing (Laviola and Dias, 2008). However, the cultivation depends on its domestication in order to achieve higher productivity and production uniformity (Fairless, 2007). This potential can be surpassed with breeding programs along with production system improvement.

Since there are no defined cultivars and descriptors for physic nut, its genetic diversity exploration and characterization is of great importance in the species genetic breeding programs (Achten et al., 2010). Therefore, studies concerning genetic diversity among the genotypes around the world have been done at the molecular level (Pazeto et al., 2015; Pecina-Quintero et al., 2014; Sinha et al., 2016) as well as the phenotypic level (Freitas et al., 2015; Oliveira et al., 2016; Priyanka et al., 2015) stating that there is a large genetic variability, making physic nut a prosperous species for domestication and breeding.

Multivariate techniques are important tools in predicting genetic diversity, germplasm classification, accession variability ordering and analysis of genetic relationships between characteristics and existing genetic material (Iqbal et al., 2008). Among these techniques, it can be highlighted the principal component analysis (PCA) and cluster analysis (Cruz et al., 2004; Gonçalves et al., 2008). The principal component analysis is a useful tool used to identify characters containing more information for the germplasm characterization, even as to inform which characters contribute less to the total variation available (Cruz et al., 2004). Cluster analysis aims to gather, by some classification criteria, the sample units in groups, for there to homogeneity within the group and heterogeneity among them (Neto and Moita, 1998). The PCA technique was applied for physic nut by Singh et al. (2016) to distinguish parental accessions for plant improvement, Nietsche et al. (2015) to evaluate the variability in reproductive traits and by Tripathi et al. (2015) to study the genetic diversity of Indian accessions. Different methods of cluster analysis have also been widely used for the species aiming to study its genetic diversity (Noor Camellia et al., 2012; Silva Junqueira et al., 2016; Reddi et al., 2016). Due to the importance of physic nut genotypes characterization for domestication and the need to obtain superior genotypes for future use in breeding programs, the present study aimed to evaluate phenotypic diversity to select the most divergent and superior genotypes from a physic nut germplasm bank using multivariate analysis strategies.

MATERIALS AND METHODS

We evaluated 165 physic nut plants from 50 accessions from four Brazilian states: Paraíba, Pernambuco, Tocantins and São Paulo (Table 1). The genotypes belong to the *in vivo* Germplasm Bank of the College of Agricultural and Veterinary Sciences of the Universidade Estadual Paulista - UNESP, Jaboticabal, SP, located in the Plant Production Department experimental area. Harvesting and agronomic characters were evaluated from December 2014 to May 2015.

To define the characters evaluated, some descriptors used for castor bean (*Ricinus communis* L.) were used (MILANI, 2008), since the two species are from the same family. Eleven agronomic traits were evaluated: Plant height - measured from the plant collar to the apex (m); stem diameter - measured using a digital caliper (ZAAS brand) at 10 cm ground level interval (cm); number of primary branches - the primary branches of each genotype were observed; fruit length - length of 10 fruits per genotype were measured using a digital caliper (cm); fruit width - widths of 10 fruits per genotype were measured using a digital caliper (cm); fruit weight - 10 fruits per genotype were weighed using a digital scale (g); fruit shape - 10 fruits per plant were evaluated, classified as ellipsoid spherical or ellipsoid lanceolate, according to Laviola et al. (2011); seed length - 10 seeds per genotype were measured using a digital caliper (cm); seed width - 10 seeds per genotype were measured using a digital caliper (cm); seed weight - 10 seeds per genotype were weighed using a digital scales (g); oil content - extraction was performed with a soxhlet extractor, the method includes leaching of the oil in the material via contact with a particular solvent in a series of cycles, according to the AOCS official method (AOCS, 2003) (%). The process was performed in duplicate for each genotype and subsequently the average was calculated for each plant, with results presented in g/100 g.

All fruits were harvested when their color was brown and at random from each plant over the previously mentioned period by multiple harvests. To perform the agronomic traits analyses, seeds and fruits evaluated were randomly chosen from each genotype. For fruit length, width and weight characters, as well as seed oil content, the mean values for each genotype were considered.

Principal Components Analysis and Cluster Analysis were performed using Statistica software, version 10 (STATSOFT, 2010) for all agronomic traits except fruit shape since it has a qualitative characteristic. Data were standardized resulting in mean zero and variance one for all the variables analyzed. Hierarchical clustering was performed by the Ward method and dissimilarity estimates were generated using the Euclidean distance procedure.

Table 1. Physic nut origin, identification and number of plants used in this study.

Origin	Coordinates	Accession identification	Number of plants
Tocantinópolis – TO	06°19'S 47°24'W	1	3
Garanhuns - PE	08°56'S 36°30'W	2 / 3 / 4 / 5 / 6 / 7 / 8 / 9 / 10 / 11 / 12 / 13 / 14	2 / 2 / 5 / 2 / 2 / 3 / 4 / 5 / 1 / 2 / 3 / 2 / 3
Mundo Novo – PE	07°35'S 37°11'W	15 / 16 / 17 / 18 / 19 / 20 / 21 / 22	3 / 6 / 5 / 3 / 2 / 8 / 5 / 7
Pugmil - TO	06°24'S 37°48'W	23 / 24 / 25 / 26 / 27	2 / 2 / 3 / 1 / 3
Alagoinha - PB	06°57'S 35°32'W	28 / 29 / 30 / 31	9 / 6 / 6 / 6
Oliveira de Fátima - TO	10°42'S 48°54'W	32 / 33 / 34 / 35 / 36 / 37 / 38	2 / 3 / 3 / 2 / 2 / 3 / 1
Marizópolis - PB	09°47'S 49°39'W	39 / 40 / 41 / 42	5 / 1 / 6 / 3
Ituverava - SP	20°20'S 47°46'W	43 / 44 / 45 / 46 / 47 / 48 / 49 / 50	3 / 2 / 3 / 2 / 2 / 2 / 2 / 2

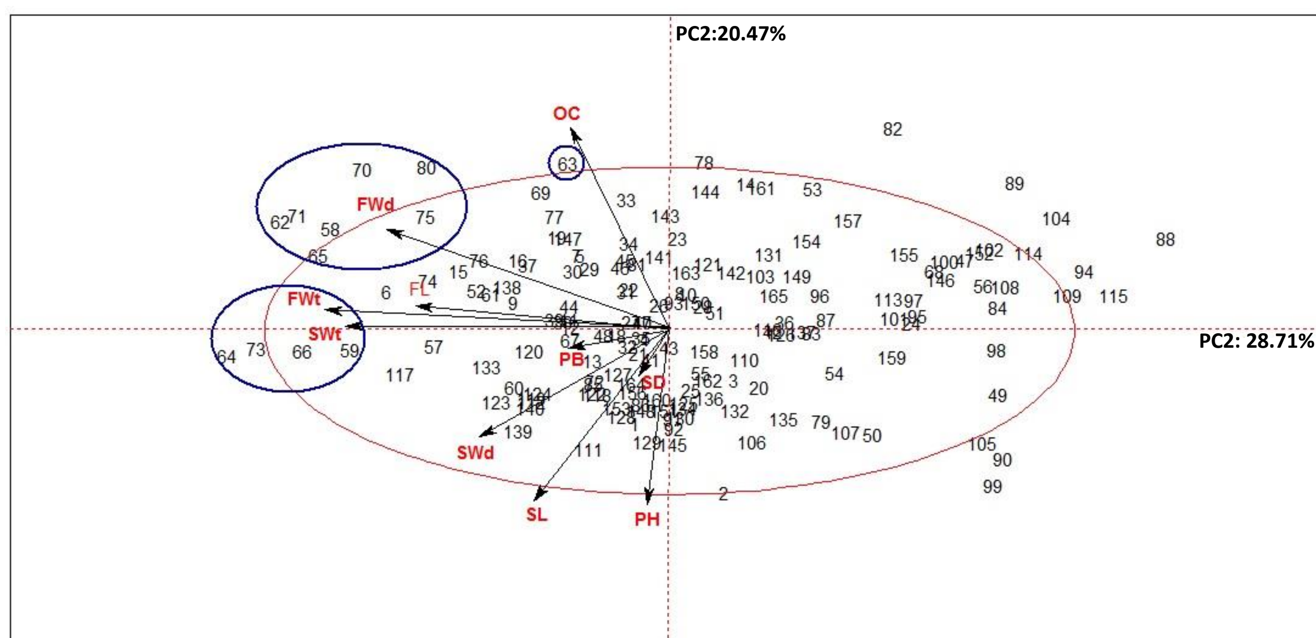

Figure 1. Biplot graph with dispersion of 165 physic nut genotypes according to the principal components PC1 x PC2 and vectors projection of the agronomic traits: plant height (PH), stem diameter (SD), number of primary branches (PB), seed weight (SWt), seed width (SWd), seed length (SL), fruit width (FWd), fruit length (FL), fruit weight (FWt) and oil content (OC).

RESULTS AND DISCUSSION

The first four principal components explained 71.62% of the variance contained in the original ten variables, and the first principal component (PC1) retained 28.71% of the original variance (Figure 1). The principal characters that explained this variance retention (PC1) were the production components: seed weight, fruit weight, width and length (Table 2). The second main component (PC2) retained 20.47% of the variance, explained by different characters of the plants such as plant height, seed length and oil content (Table 2). The third principal component (PC3) retained 12.00% of the variance, which was explained by the plant proportions: stem diameter and number of primary branches (Table 2). The fourth principal component (PC4) retained 10.42% of the variance and was contributed by fruit length and seed width; loads presenting absolute value greater than 0.5 were considered relevant (Table 2).

The 165 genotypes were distributed along the axis of the principal components. That means the closer a genotype is to the other; the more similar they will be, while the genotypes that are further away from the axis of the principal components are the most discrepant. The two-dimensional plane formed by PC1 (28.71%) and PC2 (20.47%) components retained altogether 49.18% of the original variance. It can be observed in Figure 1, that 58, 59, 62, 63, 64, 65, 66, 70, 71, 73, 75 and 80 genotypes are located to the left of PC2, indicating negative correlations, differentiated by the seed weight, fruit width,

Table 2. Correlation between the variables and the principal component (PC) of *J. curcas* genotypes for agronomic traits: Plant height (PH), stem diameter (SD), number of primary branches (PB), seed weight (SWt), seed width (SWd), seed length (SL), fruit width (FWd), fruit length (FL), fruit weight (FWt) and oil content (OC).

Parameter	PC1	PC2	PC3	PC4
PH	-0.061	-0.733	0.081	-0.229
SD	-0.079	-0.216	-0.793	-0.169
PB	-0.236	-0.084	-0.680	0.465
SWt	-0.824	-0.031	0.070	0.029
SWd	-0.489	-0.437	0.219	0.592
SL	-0.341	-0.725	0.188	0.042
FWd	-0.717	0.370	0.039	-0.013
FL	-0.638	0.078	-0.072	-0.567
FWt	-0.886	0.072	-0.013	-0.111
OC	-0.246	0.767	0.075	0.239

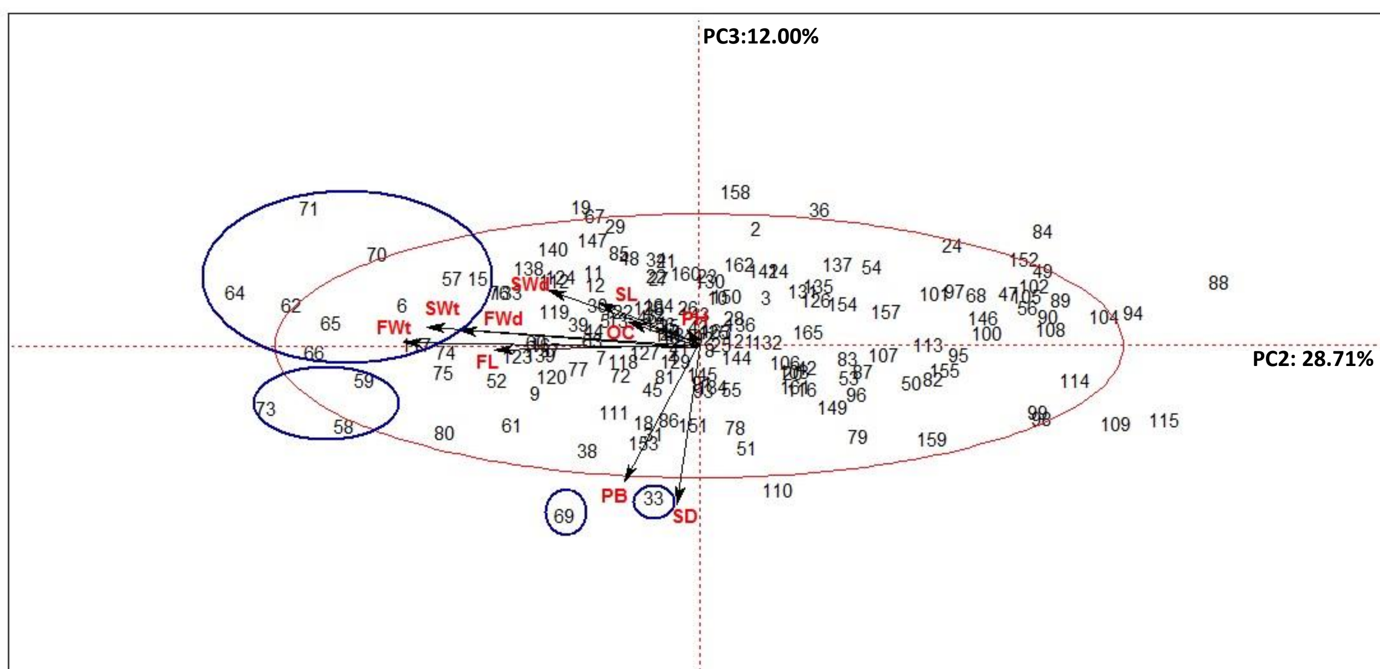

Figure 2. Biplot graph with dispersion of 165 physic nut genotypes according to the principal components PC1 x PC3 and vectors projection of the agronomic traits: Plant height (PH), stem diameter (SD), number of primary branches (PB), seed weight (SWt), seed width (SWd), seed length (SL), fruit width (FWd), fruit length (FL), fruit weight (FWt) and oil content (OC).

length, and weight, plant height and oil content variables. The two-dimensional plane formed by PC1 and PC3 (12.00%) components retained 40.71% of the variance and was characterized by seed weight, fruit width, length and weight, stem diameter and number of primary branches variables, differentiating genotypes 6, 15, 33, 57, 58, 62, 64, 65, 66, 69, 70, 71, 73 and 117, located to the left of PC3 (Figure 2).

The two-dimensional plane formed by PC1 and PC4 (10.42%) components retained 39.13% of the variance, and was characterized by the seed weight, fruit width, length and weight, plus seed width variables, differentiating genotypes 13, 17, 19, 52, 58, 59, 62, 64, 65, 66, 69, 70, 71, 73, 74, 80 and 128, located to the left of CP4 (Figure 3).

The seed weight and oil content characters also had great importance in the principal components analysis of Malaysian physic nut (Shabanimofrad et al., 2013) and castor accessions (Anjani, 2010). According to the report of Reis et al. (2015) in their study on physic nut

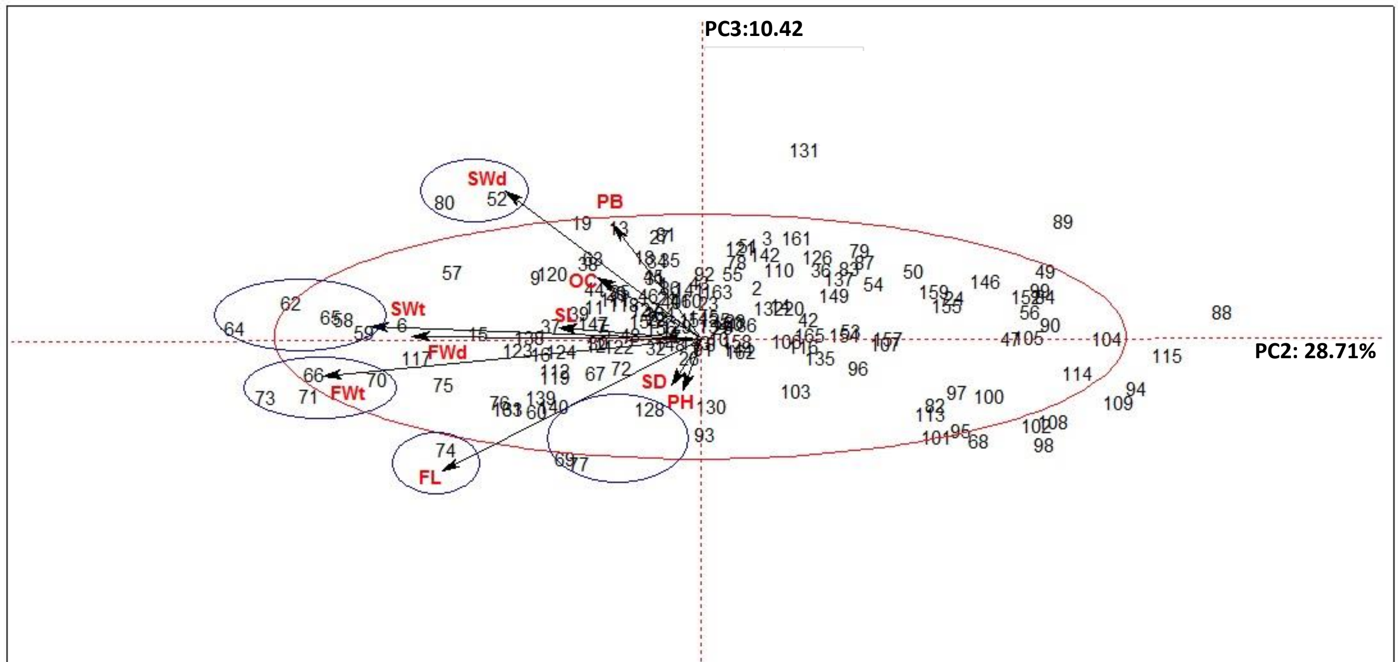

Figure 3. Biplot graph with dispersion of 165 physic nut genotypes according to the principal components PC1 x PC4 and vectors projection of the agronomic traits: plant height (PH), stem diameter (SD), number of primary branches (PB), seed weight (SWt), seed width (SWd), seed length (SL), fruit width (FWth), fruit length (FL), fruit weight (FWt) and oil content (OC).

accessions, oil production per plant showed coefficient of variation of 60.00%, indicating that this is a character with high phenotypic variability. On the other hand, characteristics related to the seed presented the lowest coefficients of variation, with values below 10%. Seed morphological characters of the wild accessions are considered to be the first step in ascertaining genetic variability of the population. Large seeds, for example, may be favoured because they generate larger and more vigorous seedlings with better chances of survival than small seeds, on the other hand, the small seeds may have a selection advantage thanks to its wider and more effective dispersal (Eriksson, 1999).

The variables that have the same sign act in the same direction; that is, when there is an increase in one variable, it also occurs in the other, and those with opposite signs act in opposite directions; when the value of one increases, the other decreases. Thus, in accordance with the correlations indicated in Table 2, in PC1 and PC3, variables considered with higher discriminatory power act directly. In PC2, plant height and seed length characters act directly, but indirectly to the oil content. In turn, in PC4, seed width and fruit length characters act indirectly.

Carvalho (2010) reported that the number of primary branches was a character that had high contribution in the third principal component in physic nut, and may be the variable discarded according to Cruz et al. (2004) and Pereira et al. (2003)'s criteria, showing similar results presented in our study. Thus, seed width variable could also be discarded, since it showed a correlation only in the fourth principal component. However, considering the economic potential of the understory crop in the initial years of establishment, the characters plant height and number of branches are considered important for major selection indices when the objective is to incorporate physic nut in an agroforestry system wherein balanced trade off can be made on yield (Rao et al., 2008).

By observing the agronomic traits values for each genotype selected by PCA (Table 3), genotypes 63 and 53 were selected only due to their high seed-oil content, genotypes 75 and 15 for their high fruit width values, genotype 69 for the greatest number of primary branches and high stem diameter value, genotypes 64, 66 and 59 for their high seed and fruit weights and genotype 73 for higher seed weight and fruit length and weight. Genotypes 71, 65, 62 and 117 had higher seed weight, fruit width and weight, 57 had high seed weight, genotype 6 had higher seed weight and fruit weight, 58 had higher fruit width, length and weight values. Genotype 33 was selected due to its high stem diameter value. Genotypes 17 and 128 were selected due to the high plant height and larger stem diameter, genotype 52 had high seed width value and genotype 74 had the longest fruit length.

Laviola et al. (2011) found that the stem diameter and plant height traits contributed 12 and 11%, respectively, to the genetic diversity of physic nut accessions. Considering that physic nut is a bushy plant that can

Table 3. Physic nut genotypes averages selected by principal component analysis for 10 agronomic traits: Plant height (PH), stem diameter (SD), number of primary branches (PB), seed weight (SWt), seed width (SWd), seed length (SL), fruit width (FWd), fruit length (FL), fruit weight (FWt) and oil content (OC).

Accession	Genotype	PH (m)	SD (cm)	PB	SWt (g)	SWd (mm)	SL (mm)	FWdh (mm)	FL (mm)	FWt (g)	OC (%)
21	75	3.00	21.00	4.00	0.79	10.64	17.83	23.06	30.93	3.62	60.89
20	69	2.50	28.00	5.00	0.75	10.51	16.98	21.67	34.54	3.48	57.37
19	64	3.30	18.00	4.00	0.82	11.23	19.24	23.83	30.72	3.65	58.60
21	73	3.00	28.00	4.00	0.82	11.07	18.75	23.10	32.07	3.84	56.00
19	66	3.00	27.00	3.00	0.86	11.17	18.86	23.17	30.97	3.45	56.80
19	59	3.00	26.00	4.00	0.86	11.08	18.74	22.81	29.68	3.53	56.87
20	71	2.50	12.00	2.00	0.83	10.99	18.75	23.06	33.08	3.61	60.56
20	70	2.60	16.00	2.00	0.80	10.96	17.92	23.13	31.55	3.74	62.09
19	65	3.00	18.00	4.00	0.84	11.14	17.90	23.70	30.17	3.57	58.70
19	62	2.70	16.00	4.00	0.85	11.16	18.04	23.97	30.54	3.54	59.17
6	15	2.55	16.00	3.00	0.69	11.07	18.73	23.34	29.79	3.84	56.62
18	57	3.00	15.00	4.00	0.78	11.42	18.77	22.23	29.75	3.55	58.20
3	6	3.00	15.00	4.00	0.78	11.12	18.04	23.10	29.78	3.93	56.24
22	80	2.15	26.00	5.00	0.83	11.25	17.59	22.71	28.19	3.53	62.28
18	58	2.50	30.00	4.00	0.80	11.16	18.38	22.76	31.25	3.65	62.61
11	33	2.60	31.00	5.00	0.68	10.66	17.83	22.77	29.10	3.26	61.94
19	63	2.15	12.00	5.00	0.76	11.00	17.36	22.87	29.95	3.22	57.50
32	117	3.20	21.00	4.00	0.75	11.06	18.77	23.49	29.92	3.65	54.01
7	17	3.20	22.00	3.00	0.71	11.19	17.82	22.40	29.08	3.24	57.17
21	74	2.95	18.00	4.00	0.78	11.48	18.58	22.66	33.13	3.52	57.38
16	52	2.65	18.00	6.00	0.76	11.23	18.62	22.94	28.28	3.30	58.82
16	53	2.60	19.00	4.00	0.68	10.45	17.49	22.49	28.41	2.90	59.00
36	128	3.20	24.00	2.00	0.75	11.10	18.76	22.20	30.26	3.02	52.98

reach up to 5 m in height (Saturnino et al., 2005) and its harvest is mainly performed manually, selecting smaller genotypes will improve the harvesting process. In addition, for commercial purposes, those genotypes that have taller trees, low oil content, and low productivity are not feasible.

Genotypes 70 and 80 were considered the most promising and with potential to be used in genetic plant breeding programs, as they presented higher seed weight, fruit weight and width, oil content and also lower plant height. In a study of physic nut seeds using genotypes from Suriname, Ethiopia, Nigeria, Brazil and China, Vaknim et al. (2011) found that the oil content varied between 39 and 62%, Aguilera-Cauich et al. (2015) verified an average of 50.52% oil content in American physic nut accessions, whereas in the present work values observed vary between 50.81 and 62.89%.

Cluster analysis was performed using the Ward method, which generated the dendrogram shown in Figure 4. The dendrogram allowed the formation of nine groups from a cutoff level where abrupt changes were observed, as recommended by Cruz et al. (2004). Cluster analysis by the non-hierarchical k-means method (Figure 5) allowed the characterization of the nine groups formed, according to the generated dendrogram. It is possible to observe that five within nine groups had oil content above average, and these same groups presented below average for plant height; that is, they were considered groups with genotypes that presented good results to be considered in genetic breeding programs. Group 1 was considered the best group, especially for presenting the highest values for seed weight, fruit width, length and weight and oil content, plus the low plant height. Group 2 presented the largest stem diameter and higher number of primary branches, besides high oil content and low plant height. Groups 3, 5 and 6 were classified as having the worst performance, as they had low oil content and high plant height values, therefore not considered relevant. Group 4 is composed of genotypes with high oil content, but low number of primary branches. Groups 5 and 6 were characterized as having high plant height values and low oil content. Group 7, despite presenting low values for all traits related to seed and fruit, had high oil content. Group 8 was also considered good due to the low plant height and high oil content. In contrast, Group 9 was not considered interesting since it was characterized by having low values for traits related to fruit and seed, and low oil content.

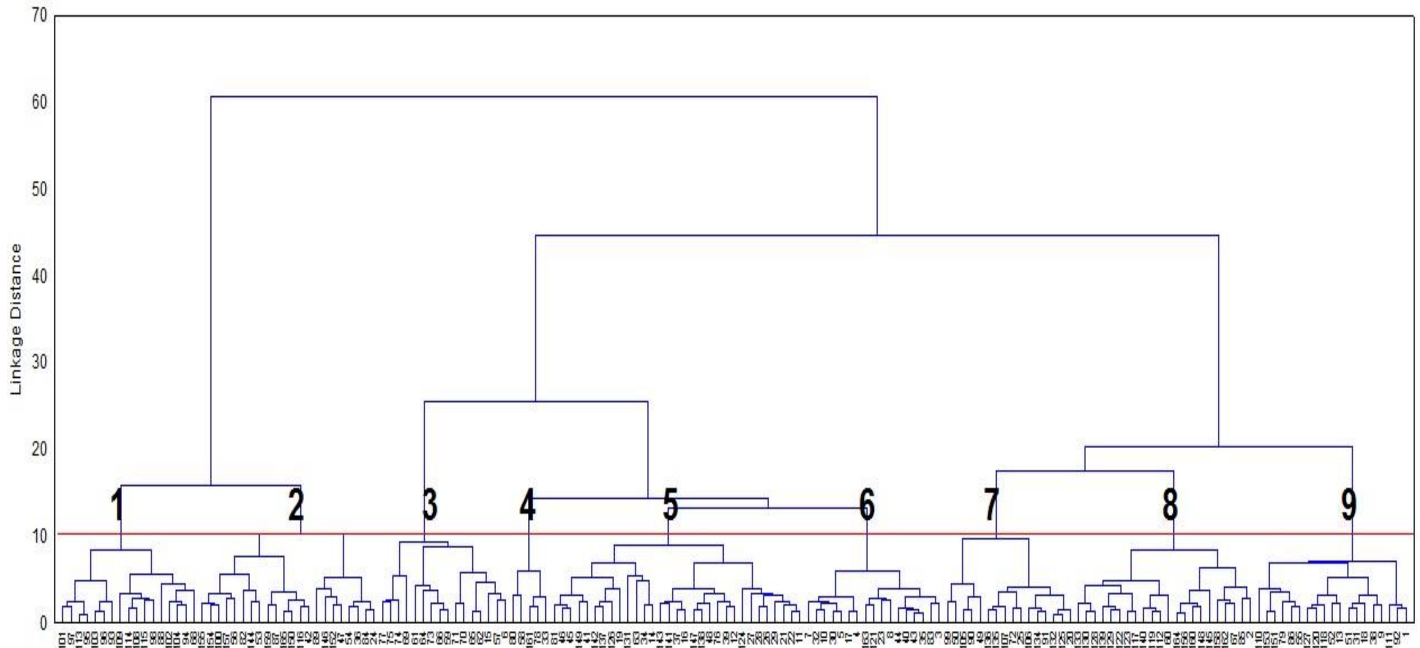

Figure 4. Hierarchical cluster analysis dendrogram using the Euclidean distance and the link between the groups by Ward method for agronomic traits: Plant height, stem diameter, number of primary branches, seed weight, seed width, seed length, fruit width, fruit length, fruit weight and oil content.

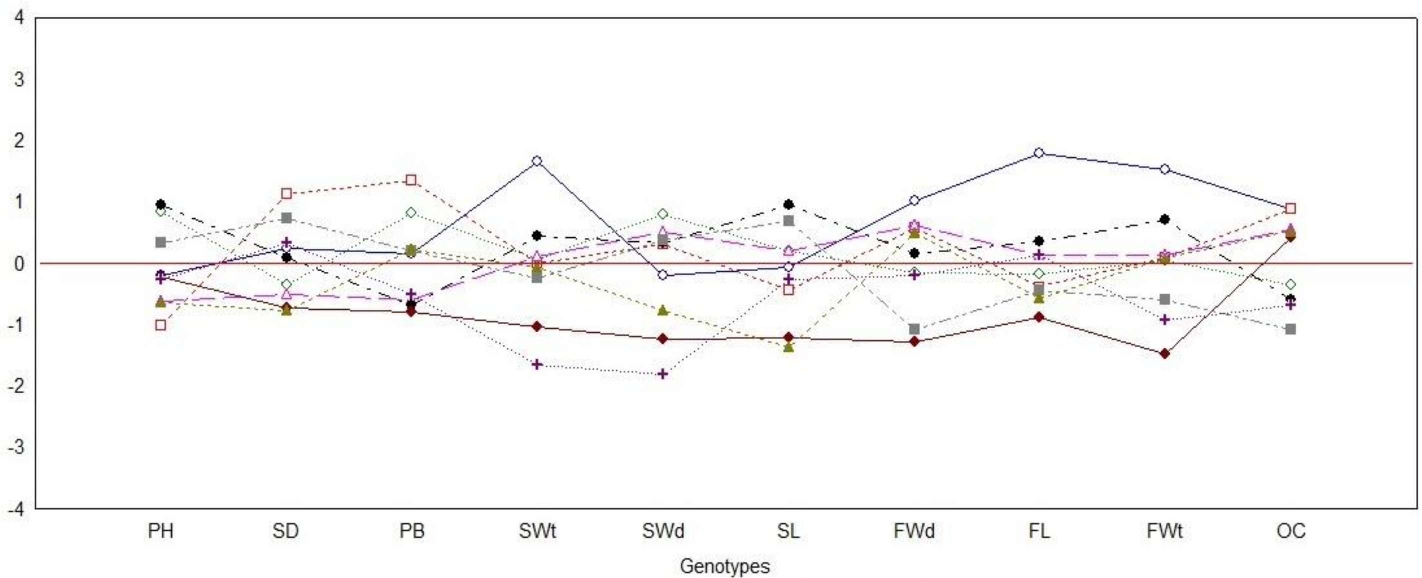

Figure 5. Profile graph of group centroid distributions in the cluster analysis by k-means, formed by the variables**:** Plant height (PH), stem diameter (SD), number of primary branches (PB), seed weight (SWt), seed width (SWd), seed length (SL), fruit width (FWd), fruit length (FL), fruit weight (FWt) and oil content (OC).

According to groups formed by Ward's dendrogram, hybridizations between groups 1 x 4, 1 x 7, 1 x 8, 2 x 4, 2 x 7, 2 × 8, 4 x 7 and 4 x 8 can be recommended, due to the distance between the groups, thus having

heterogeneity between them. Also they have genotypes with attractive characteristics for the physic nut production system, such as a low plant height and high oil content. It is noteworthy that, within groups, the genotypes belonging to different accessions had similar values in some characters. Rao et al. (2008) and Spinelli et al. (2010) observed positive correlation between the number of branches and plant height, and productivity character as well. This is a very important information that can be used to facilitate the selection of promising genotypes for this crop which still has a lot of genetic variability to be exploited.

Cluster analysis indicated that there is variability within the accessions, as the genotypes belonging to the same accession are in different groups and their characteristics do not always resemble each other. In a study with phenotypic diversity of physic nut, Aguilera-Cauich et al. (2015) found variability within the accessions and concluded in their study that are greater diversity among the American physic nut accessions evaluated in comparison with reports on diversity for India and Malaysia. Likewise, Trebbi et al. (2015) verified an increased genetic variability and heterozygosity in physic nut accessions of Mexico and Guatemala. These results can be explained by the fact that Central America is the center of origin of the species. Genetic variability in physic nut population was also found by Brasileiro et al. (2013), as well as higher estimates of heritability, in which it was possible to obtain genetic gains for growth and production traits. Reinforcing this information, Abreu et al. (2009), in physic nut accessions, obtained high heritability coefficients for plant height, first leaf height, stem diameter and number of leaves, due to the wide genetic variability among accessions. The higher the heritability of a characteristic, the better is the prediction of genetic value by individual performance and the faster the response to selection for this trait (Oliveira et al., 2007).

In a study with multivariate analysis for resistant peanut genotypes selection, Pitta et al. (2010) concluded that Ward and K-means clustering methods were efficient and complementary to the principal components analysis, also presented in this study. Due to the presented variability within the accessions, the generated dendrogram did not reveal a pattern with similar geographic regions, and it is explained by the fact that each group brings together different accessions within it. Similar results with physic nut were obtained by Tripathi et al. (2015) that used the k-means method to group accessions from different parts of India, Jun-ling et al. (2010) that studied by UPGMA method 38 accessions from different regions of China and Indonesia, and Kaushik et al. (2007) that analyzed accessions from India by non-hierarchical Euclidian cluster analysis, concluding that geographical diversity need not obligatorily be related to genetic diversity.

For fruit shape, genotypes 29, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75 and 77 presented an ellipsoid lanceolate, and most of them were in Group 3. All the other 152 genotypes had an ellipsoid spherical fruit shape. These results corroborate with that of Laviola et al. (2011)'s study where among 195 physic nut accessions evaluated, 190 had fruit in an ellipsoid spherical shape, four had ellipsoid lanceolate shape and one had ellipsoid ovoid fruit shape. It can be concluded, then, that fruit shape is a qualitative trait that contributes little to the variance among accessions.

Conclusions

The study showed that there is genetic variability for the physic nut accessions evaluated for the traits assessed and the results are very important information to be exploited in a genetic breeding program. Multivariate analyses allowed genotype characterization and also indicated those that are different from each other, allowing the targeting of crossings. All agronomic traits allowed genotype discrimination and characterization.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Chemical composition and antioxidant activity of *Lippia alba* essential oil obtained by supercritical CO_2 and hydrodistillation

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***Lippia alba* essential oils were obtained by hydrodistillation (HD) and supercritical fluid (SFE) extraction methods. These were analyzed by gas chromatography-mass spectrometry-flame ionization detector (GC-FID-MS). Antioxidant activity was tested by DPPH and ABTS methods, and total soluble phenolics (TSP) were also determined. While in the SFE extract 14 compounds were identified (mostly: myrcenone 3.4%, α-terpineol 1.0% and β-caryophyllene 2.3%), in the HD extract 17 compounds were identified (mostly: eucalyptol 15.6%, myrcenone 9.3% and Z-ocimenone 5.8%). The results showed that *L. alba* essential oils obtained by SFE was IC_{50}=17.35 mg/mL and by HD was IC_{50} = 12.45 mg/mL in DPPH assay. *L. alba* has an excellent eucalyptol content.**

Key words: Antioxidants, bioactive compounds, supercritical fluid, green technologies.

INTRODUCTION

The Verbenaceae family consists of 76 genera with over 2000 species widely distributed across almost all the planet. The genus *Lippia* includes about 200 species that are abundantly present in Central America, South America and Africa. *L. alba* produces aromatic leaves and flowers and aerial portions of the plant are routinely used in native medicines. Some of the medicinal properties of *L. alba* or other *Lippia* species have been attributed to the presence of biologically active volatile components found in the essential oil of the plant (Hennebelle et al., 2006). The composition of the essential oil is variable and depends on the characteristics of the geographic location, climate and soil. Based on the identity of the major constituents found in essential oil samples from around the world, it is proposed that at least seven chemotypes exist (Table 1) (Hennebelle et al., 2006). Although the essential oil composition of *L. alba* samples from many regions has

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Table 1. Characteristics of the seven chemotypes of *Lippia alba* essential oil.

Chemotype	Major compounds
I	Citral, linalool, and β-Caryophyllene
II	Ocimenone and myrcenone
III	Limonene, carvone, dihydrocarvone, piperitone, piperitenone and lippione.
IV	Myrcene
V	ɣ-Terpinene
VI	Camphor and Eucalyptol
VII	Estragole

been reported, there are presently no reports for the analysis of plants harvested in Mexico (García-Abarrio et al., 2014; Linde et al., 2016; Braga et al., 2005). In Mexico, *L. alba* is an aromatic shrub or tree, up to 2 m tall; this plant is popularly known by the name of mints leaves (Willman et al., 2000). The leaves and flowers of this plant are commonly prepared as an infusion or decoction that is employed as folk remedies for the treatment of diarrhea and stomach ache (Pascual et al., 2001).

Aromatic and medicinal plants have been extensively studied for their antioxidant activity among other functional properties. This is mainly due to an obvious consumer preference for natural ingredients combined with concerns about toxic effects of synthetic antioxidants (Agnaniet et al., 2005; Wojdylo et al., 2007). Essential oils are composed of many chemical compounds; therefore some of them might be regarded as valuable components and might exhibit antioxidant potency (Kamaliroosta et al., 2012).

With the exception of the work by Stashenko et al. (2004), *L. alba* essential oil extracts have been principally obtained by a singular method, either hydrodistillation or microwave radiation-assisted hydrodistillation (Bahl et al., 2000; Lorenzo et al., 2001; Fischer et al., 2004; Mesa-Arango et al., 2009; Escobar et al., 2010). The Supercritical Fluid Extraction (SFE) systems extract chemical compounds normally using supercritical carbon dioxide instead of an organic solvent, as compared to the hydrodistillation method. The supercritical fluid state occurs when a fluid is above its critical temperature (Tc) and critical pressure (Pc), when it is between the typical gas and liquid state (Oliveira et al., 2016; Zermane et al., 2014; Bagheri et al., 2014).

Stashenko et al. (2004) compared different methods of extraction in terms of composition. Extracts were obtained through hydrodistillation, microwave radiation-assisted hydrodistillation, distillation solvent extraction and supercritical fluid extraction and their results showed that the method of extraction affected quantitatively the essential oil composition. In other work, Duran et al. (2007) found that extraction time directly impacted the composition of *L. alba* essential oil extracts prepared by microwave radiation-assisted hydrodistillation. The influence of extraction method on the resulting essential oil composition has also been reported for clove buds, patchouli, and *Mirtus communis* L. (Wenqiang et al., 2007; Donelian et al., 2009; Ghasem et al., 2011). Thus, it appears essential to use more than a single extraction technique when characterizing a plant's volatile secondary metabolite composition (Stashenko et al., 2004).

Given the above, this work aimed to obtain fractions of essential oil of *L. alba* by supercritical CO_2 and hydrodistillation, and to evaluate their chemical composition and antioxidant activity.

MATERIALS AND METHODS

Plant material

L. alba leaves were collected in Sinaloa, Mexico (24°45´56.8´´N, 107°39´03.6´´W), and plants were confirmed and authenticated by the botanical researcher Rito Vega-Aviña from the herbarium "Jesus Gonzalez-Ortega" located at the Agricultural School from the Sinaloa State University [Lippia alba (Mill.) N. E. Br. ex Britton & Wilson, ACJA 85 (UAS), AEJA 43 (UAS); PTJL 86 (UAS); VAR 7248 (UAS)]. The leaves (old and young) were separated from the plant manually and combined into a single lot. *L. alba* leaves were dried in an oven at 40°C for 12 h. The dried leaves were ground with a grinder to produce a fine powder that was stored at room temperature in polyethylene bags.

Chemicals

Cinnamon essential oil, butylated hydroxytoluene (BHT), carvacrol, limonene, carvone, geraniol, camphor, citral, p-cymene, thymol eucalyptol, β-caryophyllene, cinnamaldehyde, benzyl alcohol, estragole, myrcene, α-terpineol, caryophyllene oxide, linalool, eugenol and ɣ-terpinene were purchased from Sigma Aldrich (St Louis, Mo, USA). Origanum oil was purchased from LKT Laboratories. Oregano, lemongrass and chinese cinnamon oils were purchased from Lhasa Karnak Herb company (Berkeley, Ca, USA). Piperitone was purchased from TCI America (Portland, Or, USA). Dichloromethane (HPLC grade) was purchased from Acros (New Jersey, USA) and methanol (HPLC grade) was purchased from Fisher Scientific (New Jersey, USA). Kovats standard (C7-C30) was purchased from Supelco (Bellefonte, PA, USA). 2,2-diphenyl-1-picrylhydrazyl (DPPH) was purchased from MP Biomedicals (Aurora, Ohio,USA).

Hydrodistillation

Dried leaves (10 g of powder) were submitted to hydrodistillation for 2 h, using a distilling flask, a vigrex column, a condenser and a receiving vessel. The essential oil layer and aqueous layers were separated and the oil collected. The oil was weighed using an analytical balance (Mettler-Toledo, Columbus, OH, USA) and stored in a glass vial at 4°C protected from light.

Supercritical fluid extraction

Extraction of *L. alba* essential oil was prepared using a supercritical fluid equipment (Model SFT-150, Supercritical Fluid Technologies, Inc., Newark, DE, USA). The flow rate of supercritical CO_2 was 5 mL/min. The extraction vessel (100 mL) was charged with 10 g of plant powder. The conditions of extraction were 47°C and 3800 Psi. The extract was collected in a glass vial. In order to improve the collection efficiency, the collected vial was placed in an ice bath during the dynamic extraction. The extract weight was measured using an analytical balance and stored in a glass vial at 4°C protected from the light.

GC and GC/MS analyses

GC analyses were performed using a Hewlett-Packard 6890 series gas chromatograph (Wilmington, DE, USA) equipped with a flame ionization detector (FID) and a DB-1 column (60 m × 0.2 mm i.d. x 0.25 µm of film thickness) (Agilent Technologies, Wilmington, DE, USA). Oven temperature was programmed at 45°C (2 min), and then increased to 270°C (20 min) at a rate of 4.5°C/min. Injector temperature was 250°C and detector temperature was 270°C. Helium was used as carrier gas with a linear velocity of 32 mL/min. The samples (1 µL) were injected using the splitless mode.

The GC/MS analyses were carried out on two separate systems. The first system consisted of a HP-589066 with a DB-Wax column (60 mx 0.25 mm i.d. × 0.25 µm of film thickness) coupled to a HP-5972 MSD. The GC oven temperature was programmed from 35°C hold for 5 min to 230°C hold for 40 min at 4.5°C/min. Injector temperature was 250°C. The temperature of the ionization chamber was 285°C. The second system was a HP-6890 series (Wilmington, DE, USA) with a DB-1 column (60 m × 0.2 mm i.d. x0.25 µm of film thickness) coupled to a HP-5972 MSD (Agilent Technologies, Wilmington, DE, USA). The GC oven temperature was programmed from 45°C hold for 5 min to 270°C hold for 20 min at 4.5°C/min. Injector temperature was 250°C. The temperature of the ionization chamber was 285°C. The samples were diluted in dichloromethane and 1 µL of the solution were injected using the splitless mode. The compounds of the extracts were identified by Kovats retention indices and comparing their mass spectra with those in the NIST 2011 mass spectra library and with authentic standards.

Antioxidant activity

DPPH radical assay

The radical scavenging capacity was determined using 2,2-diphenyl-1-picrylhydrazyl (DPPH) following the Agnaniet et al. (2005) method with some modifications. Cinnamon, oregano, origanum, Chinese cinnamon and *L. alba* (HD) extracts and standard samples were dissolved in methanol at different concentrations. Supercritical fluid extraction of *L. alba* extracts was dissolved in methanol, centrifugated (1300 rpm, 5 min at 5°C) and then filter (13 mm and 0.22 µm porosity, PVDF. Millex-GV, Millipore). DPPH was dissolved in methanol to give a 100 µM solution. To 1.2 mL of the methanolic solution of DPPH were added 100 µL of a methanolic solution of the antioxidant compounds (standards and essential oils) at different concentrations. The control is represented by the DPPH methanolic solution containing 100 µL of methanol. After a 30 min incubation period at room temperature the decrease in absorption at 517 nm was measured with a SpectraMax Plus 384 spectrophotometer (Molecular Device, USA). Antioxidant activity was reported as IC_{50} (the amount of antioxidant necessary to decrease the initial DPPH concentration by 50%) and was calculated using SoftMax® Pro software Version 5.0.1 (Molecular Devices Corp., USA). BHT was used as a positive control. The assay was carried out in triplicate and results are reported as averages.

ABTS radical cation decolorization assay (TEAC)

The antioxidant activities of essential oils were determined by the method of Re et al. (1999) with some modifications. Cinnamon, oregano, origanum, Chinese cinnamon and *L. alba* (HD) oils were dissolved in methanol. Supercritical fluid extraction of *L. alba* essential oil was dissolved in methanol, centrifuged (1300 rpm, 5 min at 5°C) and then filtered (13 mm and 0.22 µm porosity, PVDF. Millex-GV, Millipore). ABTS was dissolved in water (HPLC grade) to a 7 mM concentration. ABTS radical cation was produced by reacting ABTS stock solution with 2.45 mM potassium persulfate and allowing the mixture to stand in the dark at room temperature for 16 h before use. For the determination of antioxidant activity the ABTS radical solution was diluted with methanol to an absorbance of 0.70 ± 0.01 at 734 nm. To 20 µL of the sample was added 400 µL of diluted ABTS radical solution. After incubation for 6 min at 30°C, the absorbance was measured with a SpectraMax Plus 384 spectrophotometer at 734 nm. Ascorbic acid (0.05 mg/mL) and BHT (0.125 mg/mL) were used as a positive control and cinnamic acid (1 mg/mL) as a negative control. The activities of essential oils were estimated within the range of Trolox calibration curve and results were expressed as µmol Trolox equivalent per gram (Tr eq µmol/g). The assay was carried out in triplicate and results are reported as averages.

Total soluble phenolics

The Folin-Ciocalteau method for the colorimetric estimation of total polyphenols was adapted to a 96-well plate format according to Breksa et al. (2010). Cinnamon, oregano, origanum, chinese cinnamon and *L. alba* (HD) oils were dissolved in methanol. Supercritical fluid extraction of *L. alba* essential oil was dissolved in methanol, centrifuged (1300 rpm, 5 min at 5°C) and then filtered (13 mm and 0.22 µm porosity, PVDF. Millex-GV, Millipore). Standards (100 µL) were mixed with water (1500 µL) in a 2 mL polypropylene plate. Trolox (250 µg/mL) and BHT (250 µg/mL) were used as positive controls and cinnamic acid (1.0 mg/mL) was used as a negative control. Samples were mixed with water 100:1500 (sample: H_2O). Controls were diluted with water in the same way as samples. All dilutions were mixed with Folin Ciocalteu's phenol reagent (1 N, 100 µL). After a brief incubation at room temperature (5 min), saturated sodium carbonate (300 µL, 75 g/L) was added. Solutions were mixed and incubation continued at room temperature. After 2 h, 300 µL were transferred to a well of a 96-well plate and the absorbance measured at 765 nm with a molecular Devices Spectromax 384-Plus plate reader (Sunnyvale, CA). Quantification was based on the standard curve generated with 50, 100, 200, 300, 400 and 500 mg/L of gallic acid. Samples with absorbance values greater than the 500 mg/L standard were diluted and reanalyzed. Values were reported as µmol gallic acid equivalents (GAE) per gram material ± SD and represent the average of three independent analyses.

Table 2. Chemical composition of *L. alba* essential oil obtained by hydrodistillation and SFE.

Number of peak	Compound	RI (DB-1)	HD	SFE
1	Octen-3-ol	968	1.6	n.d.
2	Myrcene	980	n.d.	n.d.
3	Benzyl alcohol	1010	n.d.	n.d.
4	p-Cymene	1017	n.d.	n.d
5	Limonene	1023	n.d.	n.d.
6	Eucalyptol	1024	15.6	n.d.
7	ϒ-Terpinene	1053	n.d.	n.d.
8	Trans-Linalool oxide	1060	Tr	n.d.
9	Linalool	1086	6.2	0.4
10	Myrcenone	1123	9.3	3.4
11	Camphor	1124	n.d.	n.d.
12	Terpine-4-ol	1164	0.5	0.1
13	α-Terpineol	1175	7.0	1.0
14	Estragole	1177	n.d.	n.d.
15	(E)-Tagetone	1130	0.5	Tr
16	(Z)-Tagetone	1151	0.7	Tr
17	(Z)-Ocimenone	1206	5.8	0.8
18	Neral	1217	n.d	n.d.
19	Carvone	1218	n.d	n.d.
20	Piperitone	1229	n.d	n.d.
21	Cinnamaldehyde	1232	n.d	n.d.
22	Geraniol	1235	n.d	n.d.
23	Geranial	1245	n.d	n.d.
24	Thymol	1268	n.d	n.d.
25	Bornyl acetate	1274	n.d	n.d.
26	Carvacrol	1278	n.d	n.d.
27	Eugenol	1333	n.d	n.d.
28	α-Copaene	1382	0.9	0.4
29	Geranyl acetate	1362	n.d	n.d.
32	β-Caryophyllene	1426	0.9	2.3
33	α-Humulene	1458	Tr	0.4
34	Allo-Aromadendrene	1463	1.1	0.5
35	Germacrene D	1487	0.7	8.2
36	Spathulenol	1576	1.7	0.9
37	Caryophyllene oxide	1584	8.0	1.5

RI, Retention index obtained using a DB-1 column. HD, Hydrodistillation; SFE, supercritical fluid extraction; n.d., no detected; Tr, Traces.

RESULTS AND DISCUSSION

Chemical composition

Table 2 shows the relative proportion of the compounds identified in both samples (SFE and HD) using a DB-1 column. Also in order to confirm the identity of the compounds a DB-wax column was used (data not show) but it is important to highlight that the retention index value for the ketone: myrcenone was 1588, based on the fact that no previous reports were found using this type of column.

L. alba is probably the most studied species in the *Lippia* genus however, in spite of the diversity and medicinal properties of this plant, in Mexico no previous reports have reported about the chemical compositions of *L. alba* essential oil (Hennebelle et al., 2008). As show in Table 2 the major compounds in *L. alba* essential oil obtained by SFE were myrcenone (3.4%), α-terpineol (1.0%), β-caryophyllene (2.3%), germacrene-D (8.2%) and caryophyllene oxide (1.5%), while by hydrodistillation the major compounds were eucalyptol (15.6%), myrcenone (9.3%) and (Z)-ocimenone (5.8%) (Table 2). Based on these results and in accordance with Henebelle et al. (2006) chemotypes classification (Table 1), *L. alba* essential oil could correspond to chemotype II (ocimenone

Table 3. Main compounds in essential oils.

Sample	Main compounds
L. alba oil (HD)	Eucalyptol (15.6%), myrcenone (9.3%), caryophyllene oxide (8.0%), α-terpineol (7.0%), linalool (6.2%) and (Z)-ocimenone (5.8%),
L. alba oil (SFE)	Myrcenone (3.4%), α-terpineol (1.0%), β-caryophyllene (2.3%), germacreno-D (8.2%) and caryophyllene oxide (1.5%)
Cinnamon oil	Cinnamaldehyde (71.63%), eugenol (7.40%), limonene (3.82%), benzyl alcohol (3.30%), 3-phenylpropanol (2.56%) and linalool (1.25%)
Chinese cinnamon oil	Cinnamaldehyde (81.74%), cinnamaldehyde,o-methoxy (7.58%), cinnamyl ester (2.71%), coumarin (2.10%) and benzaldehyde (1.28%)
Lemongrass oil	Geranial (46.52%), neral (31.35%), geraniol (5.14%), β-caryophyllene (2.91%), limonene (2.42%), geranil acetate (1.79%), caryophyllene oxide (1.47%) and linalool (1.15%)
Oregano oil	Carvacrol (66.68%), p-cymene (13.87%), ɤ-terpinene (6.63%), thymol (3.99%), α-pinene (1.33%), linalool (2.86%) and β-myrcene (1.20%)
Origanum oil	Carvacrol (63.52%), linalool (16.91%), p-cymene (6.45%), ɤ-terpinene (3.68%), β-myrcene (1.46%) and thymol (0.56%)

% identified by GC-FID (DB-1 column) and GC-MS (DB-1 column).

and myrcenone) since both compounds are present in the sample composition of *L. alba* extract obtained by hydrodistillation.

In the SFE prepared sample the sesquiterpenes including β-caryophyllene (2.3%), α-humulene (0.4%) and germacrene-D (8.2%), were at a greater proportion than found in the hydrodistillation sample. A similar result was observed by Stashenko et al. (2004) who found more sesquiterpenes in the supercritical CO_2 extract of *L. alba* than in extracts obtained with other extraction techniques. Eucalyptol was reported in *Lippia alba* essential oil as the main compound in samples obtained from Uruguay while was differed from that reported from Colombia where the main compounds were carvone (51%) and limonene (32.60%) carvone (31.8-52.6%) and geraniol (15-21.5%) (Mesa-Arango et al., 2009; Dellacassa et al., 1990). In Brazil *L. alba* essential oil was rich in geranial (12.9%) and myrcene (15%) (Oliveira et al., 2006). While in Guatemala, Senatore and Rigano (2001) reported as the main compounds limonene (44%) and piperitone (31%). Fischer et al. (2004) analyzed 16 *L. alba* populations from all over Guatemala. They reported myrcenone (37.8-58%) as the main compound in 14 samples and neral (17.6-18.9%) and geranial (24.7-27%) as the main compounds in two samples collected from two different geographic locations. The variability in the chemical composition of *L. alba* essential oil was attributed to different factors such as season of harvest, collection site, soil composition, water hydric stress, extreme temperatures and sunlight (Nogueira et al., 2007; Olivero-Verbel et al., 2010; Blanco et al., 2007). However, other works have suggested that the composition of the essential oil of *L. alba* may not only be due to climatic or environmental conditions. Pandelo et al. (2012) analyzed three chemotypes of *L. alba* cultivated at the same conditions and were collected at the same time. No changes were observed in the main compounds of the samples. The authors concluded that these results are a consequence of genetic variation among the chemotypes. Also, it was observed that developmental stage such as flowering and vegetative growth affect the total oil production but not the quality of oil in leaves of *L. alba*, reinforcing the suggestion of genetic control.

In order to compare the chemical composition of *L. alba,* essential oil commercial oils were tested in this work. As show in Table 3 six main compounds were identified in cinnamon oil, representing 89.96% of the total oil. The most abundant components were cinnamaldehyde (71.63%) and eugenol (7.4%). Cinnamaldehyde is one of the main aroma-active compounds in cinnamon essential oil (Schmidt et al., 2006). The results found in this work are in accordance with those of Tomaino et al. (2005) who reported cinnamaldehyde (45.84%) and eugenol (49.09%) as the main compounds in cinnamon oil. The major compounds in Chinese cinnamon oil were cinnamaldehyde (81.74%) and cinnamaldehyde,o-methoxy (7.58%) but eugenol was not present in the sample analyzed. The composition of the essential oil of Chinese cinnamon oil (*Cinnamomum cassia*) have been reported that trans-cinnamaldehyde and cinnamaldehyde,o-methoxy as the main compounds and eugenol content was no present in the sample evaluated (Geng et al., 2011). The major compounds in lemongrass were geranial (46.52%) and neral (31.35%), representing already of 80% of the total oil. These results are similar with previous reports. Sacchetti et al. (2005) reported geranial (41.30%), neral (32.30%) and geraniol (3.35%) as the main compounds. Katsukawa et al. (2010)

Table 4. Table 4. Antioxidant activities (DPPH and ABTS) and soluble phenolics (TSP) of essential oils and essential oil of Lippia alba obtained by hydrodistillation and CO2 supercritical extraction.

Sample	DPPH (IC50 mg/mL)	TSP (GA Eq µmol/g)	ABTS (Tr eq µmol/g)
BHT (control, 0.125 mg/mL)	0.21 ± 0.01	2718.3 ± 210.8	1042.0 ±75.4
Ascorbic acid (control, 0.050 mg/mL)	NA	NA	5803.4 ± 92.4
Cinnamic acid (control, 1.0 mg/mL)	NA	NA	No activity
Trolox (control, 0.250 mg/mL)	NA	1220.0 ± 27.6	NA
Lippia alba oil (HD)	12.45 ± 0.57	137.5 ± 1.6	15.6 ± 0.1
Lippia alba oil (SFE)	17.35 ± 0.92	84.0 ± 4.7	55.2 ± 1.6
Cinnamon oil	0.62 ± 0.02	448.8 ± 16.1	6322.1 ± 143.5
Chinese cinnamon oil	No activity	141.8 ± 7.9	74.5 ± 1.7
Lemongrass oil	33.68 ± 1.99	16.9 ± 0.1	48.3 ± 1.1
Oregano oil	3.06 ± 0.21	1501.0 ± 39.4	33204.0 ± 810.4
Origanum oil	3.72 ± 0.21	1368.4 ± 47.5	28158.7 ± 684.7

HD, Hydrodistillation; SFE, supercritical fluid extraction; NA = not analyzed.

reported citral (mixture of geranial (31.43%) and neral (26.03%) as the main compound in lemongrass essential oil. The most abundant components in oregano were carvacrol (66.68%) and p-cymene (13.87%) and for origanum, carvacrol (63.52%) and linalool (16.91%) (Table 1). In both essential oils, carvacrol was the main compound identified. It is well known that in *Origanum* species carvacrol is the main constituents (Hussain et al., 2011). However, thymol concentrations found in this work were lower than that reported previously (Puertas-Mejia et al., 2002).

Antioxidant activity

The chemical complexity of essential oils, often a mixture of dozens of compounds with different functional groups, polarity and chemical behaviour, could lead to scattered results depending on the test employed (Sacchetti et al., 2005). DPPH and ABTS (TEAC) assays are useful for determining the activity of both hydrophilic and lipophilic species (Sacchetti et al., 2005; Katsukawa et al., 2010; Hussain et al., 2011; Puertas-Mejia et al., 2002). The radical scavenging activity of essential oils, assessed by the antioxidant concentration required for 50% reduction in DPPH radical concentration in 30 min (IC_{50}), decreased in the following order: cinnamon oil>oregano oil>origanum oil>*L. alba* oil (obtained by hydrodistillation)>*L. alba* (obtained by SFE)>lemongrass oil; chinese cinnamon oil showed no antioxidant activity (Table 1). It was found that the essential oils analyzed showed very different antioxidant activity (Table 4). The radical scavenging activity (IC_{50}) of standard compounds decreased in the following order: eugenol> carvacrol> thymol. The other standards tested showed low radical scavenging activity (IC_{50} = >20 mg/mL) (Table 5). Table 4 also shows the antioxidant activities of essential oils tested by ABTS assay. The highest antioxidant activity decreased in the following order: oregano > origanum > cinnamon > Chinese cinnamon > *L. alba* (SFE) > lemongrass > *L. alba* (HD). The antioxidant activity tested was different between both methods. Antioxidant activities of essential oils from aromatic plants are mainly attributed to their chemical structure (Saleh et al., 2010). Politeo et al. (2006) suggested that the antioxidant activity may be attributed either to high percentage of the main constituents but also to the presence of other constituents in small quantities or to synergy among

Table 5. Antioxidant activities of references compounds.

Standards compounds	DPPH (IC_{50} mg/mL)
BHT (control)	0.21 ±0.01
Carvacrol	1.85 ±0.14
Eugenol	0.041 ±0.001
Thymol	2.10 ±0.11
α-Terpineol	> 20
Benzyl alcohol	> 20
β-Caryophyllene	> 20
Camphor	> 20
Carvone	> 20
Caryophyllene oxide	> 20
Cinnamaldehyde	> 20
Citral	> 20
Eucalyptol	> 20
Geraniol	> 20
Estragole	> 20
ɤ-Terpinene	> 20
Limonene	> 20
Linalool	> 20
Myrcene	> 20
p-Cymene	> 20
Piperitone	> 20

them. Respectively the antiradical scavenging activity of *L. alba* (HD) was modest in comparison to the others oils with an IC_{50}= 12.45 mg/mL and 15.6 Trolox eq µmol/g in DPPH and TEAC assay. In this case it was difficult to attributed the antioxidant activity to the main compounds since eucalyptol showed a low antiradical activity, whereas can be attributed to high percentage of the others main constituents but also to the presence of other compounds in small quantities or to synergy among them (Politeo et al., 2006). Puertas-Mejia et al. (2002) reported that *L. alba* essential oil samples from Colombia showed a low IC_{50}=0.28 kg oil/mmol DPPH and also displayed lower activity against the ABTS radical (14.4 mmol Trolox/kg oil).

Cinnamon essential oil demonstrated the highest inhibitory activity (IC_{50}=0.62 mg/mL) compared to other essential oils tested and this is attributed to the hydrogen-donating capacity of the phenolic component eugenol and this compound was present in significant concentration in the essential oil tested and also presented the highest inhibitory activity with an IC_{50}=0.041 mg/mL (Schmidt et al., 2006; Tomaino et al., 2005) while in ABTS assay, cinnamon oil showed lower activity than oregano and origanum oils. It is of note that Chinese cinnamon oil showed no antioxidant activity by DPPH method and this can be attributed to the absence of eugenol in its composition but showed antioxidant activity by ABTS assay. Mantle et al. (1998) reported that cinnamon oil showed the highest antioxidant activity against ABTS assay but showed no significant evidence of antioxidant activity against hydroxyl and superoxide radicals. Suggesting that the variation in antioxidant capacity of oils extracts depends on the particularly assay method employed to determine antioxidant status. This result can be attributed to the fact that ABTS radicals involve electron transfers process while DPPH radical involve H atoms transfers (Kaviarasan et al., 2007). On the other hand, oregano and origanum essential oil exhibited DPPH radical scavenging activity and this can be related to their chemical compositions since both samples are rich in carvacrol and this compound showed radical scavenging activity with an IC_{50}=1.85 mg/mL. Also, thymol was present in the samples, this compound showed a radical scavenging activity similar than carvacrol with an IC_{50}=2.10 mg/mL. Sahin et al. (2004) reported that DPPH radical scavenging activity of *Origanum vulgare* spp. essential oil was low and this was related to its chemical composition because the percentage of carvacrol were low (0.057%) in the composition. Oregano and origanum essential oils showed better activity by ABTS assay; 33204 and 28158.7 Trolox eq µmol/g, respectively, than positive control (Table 4). Puertas-Mejia et al. (2002) found that total antioxidant activity of *O. vulgare* L. essential oil against ABTS radical was 25.1 Trolox eq mmol/kg oil while Karakaya et al. (2011) reported that oregano essential oil had a strong inhibitory effect on ABTS radical cation oxidation with a value of 2.69 Trolox eq µmol/ µL of oil.

The radical scavenging of lemongrass essential oil was lower than the other essential oils. The low antioxidant activity can be attributed to the main compounds composition since the standard compounds present in the essential oil were tested and also these compounds showed low activity (Table 5). Also, the antioxidant activity tested by ABTS was low (48.3 Trolox eq µmol/g). Sacchetti et al. (2005) reported an intermediate inhibition with 60% of radical scavenging activity percentage. These results demonstrate the difficulties in comparing data on antioxidant effectiveness obtained by different assays. Therefore, an approach with multiple assays in screening work is highly advisable (Sacchetti et al., 2005).

Total soluble phenolic

The presence of phenolic compounds in essential oil has been investigated in terms of antioxidant activity. This activity is mainly due to their redox potential, which can play an important role in adsorbing and neutralizing free radicals and quenching reactive oxygen species (Kähkönen et al., 1999). The total soluble phenolic (TSP) were measured by Folin-Ciocalteu reagents in terms of the acid gallic equivalent. TSP content of essential oils decreased in the following order: oregano> origanum> cinnamon>chinese cinnamon>*L. alba* (HD)>*L. alba* (SFE)>lemongrass (Table 4). Generally, a positive correlation between the phenolic content and antioxidant capacity is reported. It has been shown that the antioxidant activity of extracts is roughly connected to their phenolic composition and strongly depends upon their phenolic structures (Chaillou and Nazareno, 2006). It is of note that oregano and origanum had the highest TSP content; 1501 and 1368 GA Eqv µmol/g, respectively. In this case both essential oils were rich in carvacrol, a phenolic compound. On the other hand, Chinese cinnamon (141.8 GA eq µmol/g) showed less activity but greater than lemongrass. Lemongrass showed lower phenolic content (16.9 GA eq µmol/g); these results are in contradiction with previous reports. Mirghani et al. (2012) reported a high phenolic concentration of 2100.7 mg/L GA eq. No data was found concerning the total soluble phenolic content of *L. alba* (SFE and HD) essential oil in previous reports. It does appear that this is the first report. It is of note that the essential oil tested did not seem to depend on TSP since antioxidant activity by DPPH and ABTS assay in all samples were very different between both methods.

Conclusions

The chemical composition and antioxidant activity of the

extracts obtained by SFE and hydrodistillation methods were different. SFE extracts was characterized by the presence of sesquiterpenes compounds. *L. alba* plants from Mexico can be classified in chemotype II (ocimenone and myrcenone). Results provided here show that *L. alba* essential oil has a good eucalyptol content, which makes it an important natural source for the nutraceutical industry.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Modelling the rheological properties of gruels produced from selected food products from Cameroon

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This study investigated the flow behaviour of some food gruels obtained from pre-treated banana, sorghum or sesame flours. The Herschel-Bulkley and the power law models were used to evaluate the consistency and flow indices while an Arrhenius-type equation was used to analyse the effect of temperature on viscosity. The Ostwald de Waele model gave a good fit with experimental data, with p-values less than 0.01 and R^2 values greater than 0.96 for most of the experiments. The results revealed a pseudoplastic, dilatant and time independent character for the sorghum gruels while the sesame and banana gruels revealed a pseudoplastic and time-dependent character. The effect of temperature on viscosity led to an activation energy and second Arrhenius parameter varying from 964 to 21,070 $J.mol^{-1}$ and from 81 to 34,484 Pa.s respectively. The concentration dependence of the consistency index was modelled using an exponential equation and showed a decrease with temperature.

Key words: Sorghum gruel, sesame gruel, banana gruel, power law, Arrhenius law, time dependency.

INTRODUCTION

In Africa and particularly in Cameroon, several foods are traditionally used in the preparation of infant gruels, especially during weaning. Amongst these foods, sorghum (Matalanis et al., 2009; Onyango et al., 2010; Onyango et al., 2011; Sanoussi et al., 2013; Okoye and Ojobor, 2016; Wanjala et al., 2016), sesame (Arslan et al., 2005; Razavi et al., 2007; Elleuch et al., 2007; Çiftçi et al., 2008; Onabanjo et al., 2009; Ikujenlola, 2014) and banana (Guerrero and Alzamora, 1997; Forster et al., 2003; Abbas et al., 2009; Honfo et al., 2011) are mostly preferred. The three foods are of potential sources of nutrients; carbohydrates for sorghum, proteins and lipids for sesame, minerals and vitamins for bananas. Sorghum and sesame, in addition to being available, are commonly used in the preparation of food supplements for infants. Banana is well appreciated by children and is used by mothers as baby desserts. Moreover, Banana in the form of flour and incorporated into the porridge allows easy storage. Cameroon's annual production of these foods is growing and has reached about 1,187,531 tons for millet/sorghum, 43,963 tons for sesame and 3,182,184 tons for banana in 2010 (Minader, 2012).

Conventionally, before being fed to infants, foods are generally transformed into flour for purée (also called porridge or gruel depending on the consistency) production, whose flow properties are not often mastered. Taking into account the reduced nature and low activity of infant gut (Sanogo, 1994; Giamarchi and Trèche, 1995;

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Laurent, 1998; Mouquet et al., 1998), it is important to master the rheological properties of gruels as they significantly affect the mechanism of absorption and digestion (Sanogo, 1994). Several rheological and textural studies on different flour-based products have been presented in the literature. Matalanis et al. (2009) studied the textural and thermal properties of sorghum starch pastes; Onyango et al. (2011) presented the effect of cassava starch on the rheological and crumb properties of sorghum-based batter and bread respectively. Abdelghafor et al. (2015) studied the effects of sorghum flour addition on rheological pasting properties of hard white winter wheat; Mahajan and Gupta (2015) compared the elasticity behaviors of sorghum and wheat flour doughs. Other authors studied the rheological behaviour of banana purée (Guerrero and Alzamora, 1997), suspensions made of banana and wheat flours (Mohamed et al., 2010), semi-solid sesame paste (Abu-Jdayil, 2003; Çiftçi et al., 2008) and sesame-based products (Razavi et al., 2007; Akbulut et al., 2012). However, the results obtained cannot accurately predict the rheological behaviour of infant gruels, due to the difference in consistencies between infant gruels and other flour-based products. Although some rheological studies have been carried out on infant gruels (Mouquet and Trèche, 2001; Trèche and Mouquet, 2008; Gnahé Dago et al., 2009; Nwakego Ayo-Omogie and Ogunsakin, 2013), they involved specific operating conditions with neither models presented to predict the effect of temperature and concentration on the rheological parameters, nor curves to study the time dependency of the gruels.

This study was therefore undertaken to evaluate the flow properties of sorghum, sesame and banana gruels, present models to describe the effects of concentration and temperature on the rheological parameters and study the effect of time on the flow properties.

MATERIALS AND METHODS

Raw materials for gruel production

The raw materials considered for gruel production were sorghum (*S. bicolor* cv. *Safrari*), white sesame (*S. indicum*), both obtained from the Institute of Agricultural Research for Development (IRAD) at Maroua (Far-North Region, Cameroon) and ripe banana (*Musa acuminata*, *Cavendish*) obtained from a local market in Ngaoundere (Adamawa Region, Cameroon).

Sample preparation

The sorghum and sesame were separately treated as described in the literature (Elmaki et al., 1999; Elkhalifa and Bernhardt, 2010; Tizazu et al., 2010) sorted, winnowed, and washed twice with distilled water. The cleaned grains were soaked in distilled water for 24 h at 22 ± 1°C with the soaking water renewed at the twelfth hour. After soaking, the seeds were then drained using a sieve, spread on a wet tissue that was made to imbibe water every 24 h, and allowed to germinate in the dark over a period of 72 h at 22 ± 1°C (Hahm et al., 2009). The germinated seeds were dried at 40 ± 1°C for 48 h. After cutting-off and discarding their cyanide-containing radicles (Traoré et al., 2003), the dried seeds were ground and sieved to obtain small particle-sized flour (< 1 mm).

The bananas were peeled, cut into slices of about 5.0 ± 0.5 mm thickness and subjected to a combined dewatering-impregnation-soaking process/blanching as described by Jiokap Nono et al. (2002). They were then dried (40 ± 1°C for 72 h) to reduce water activity, limit protein denaturation/browning reactions and then ground to small particle sizes (< 1 mm).

The flours were mixed for 5 min with water at 45°C (quantity depending on the required concentration) and the mixture (1 litre) was placed in a stainless steel pot (2 litres capacity) and cooked with gentle heat using a two burner gas stove for 10 min at atmospheric pressure, after reaching 95°C. The mixture was slowly stirred during cooking using a stainless steel spoon. As mentioned by Trèche (1995), this procedure leads to the production of low viscosity purées and as such most appropriate for infants.

Physicochemical analysis

The waste content (*Wc*) of cereals was calculated as presented in equation 1, where M (10 g) is the mass of sample and M_g, the mass of good grains in the sample. The cleanliness of the grain was evaluated according to the CODEX (1989) with maximum admissible value for sorghum equal to 8%.

$$Wc=[(M-M_g)/M].100 \quad (1)$$

The rate of germination (*Gr*) was determined through a germination test using 100 good grains initially soaked in distilled water. The soaked grains were then spread on a wet filter paper, put in a petri dish maintained at 22±1°C and the filter paper was watered every 12 h. The germinated grains were counted each day till stabilization, corresponding to the time of germination. In Equation 2, N_g and N_0 are respectively the number of germinated grains at the end of germination and the number of initial good grains.

$$Gr = (N_g/N_0).100 \quad (2)$$

The mass of 1000 grains (*Mm*) which gives an idea on the quantity of matter and especially starch available in the grains was calculated as shown in Equation 3. M_g is the mass of good grains in the sample, N_0 the number of good grains in the sample and *MS* the mass of dry matter in 100 grams of good grains.

$$Mm= [(M_g.1000.MS)/(N_0 .100] \quad (3)$$

The length of the bananas was measured using a tape while the diameter was obtained using a Mitutoyo digital caliper. The classification of banana ripening was done using a colour index (Aurore et al., 2009).

The water content was determined by the AOAC (1990) method, the ash content by AFNOR (1981) method and the total nitrogen by the Kjeldahl method (AFNOR, 1984); the nitrogen content was multiplied by 6.25 to obtain the protein content; the colorimetric technic of Devani et al. (1989) was used for the chemical dosing and the protein content was determined using the conventional conversion coefficient of 6.25 (AOAC, 1975). The determination of reducing sugars was done by the DNS (3,5 dinitro salicylic acid) colorimetric method of Fisher and Stein (1961) and the total available sugars were determined in the same way after hydrolysis of the sugars by hydrogen sulfate (H_2SO_4, 1.5 N).

Measurement of rheological properties

Experimental procedures

Rheological analyses were conducted using a Brookfield DV-III

Ultra rheometer (model HBDV-III Ultra, 8534447, Brookfield Engineering Lab., Massachusetts, USA). The disk-shaped spindle HA/HB-2 of 133 mm height; 47.12 mm diameter and 1.65 mm thickness was used.

Three gruels were prepared at different flour concentrations (dry matter): 15, 25 and 35% w/w. After cooking, 500 ml of each was put in a graduated beaker and gently stirred while cooling in a temperature-controlled bath at different temperatures (30, 40, 50 or 60°C). Analyses for each experiment were conducted in triplicate with a scanning speed ranging from 0.01 to 250 rpm. The concentrations of 15, 25 and 35% were chosen in view of the fact that the average dry matter concentration of infant gruel is around 25%. A study of the effect of the concentration shows the cases of dilutions (15%) where swallowing is easy and nutrients are insufficient; and cases of very viscous porridge (35%) where nutrients would be sufficient and swallowing difficult. The usual consumption temperature is around 45°C. While feeding the child, this temperature may drop and reach room temperature. A study of the effect of the temperature up to 60°C allows us to have at least three points which will be used to determine the activation energy.

Determination of rheological parameters

The apparent viscosity was calculated as described by Anonymous (1998) with a dimensionless factor of the spindle equals to 3200/N, where N (rpm) is the rotation speed. For the disk-shaped spindle N°2, the shear rate $\dot{\gamma}$ (s^{-1}) was determined as presented in Equation 4 (Mitschka, 1982):

$$\dot{\gamma} = (0.119 \,.\, \mathrm{Tw}) / \mu \quad (4)$$

Where T_w (%) and μ (Pa.s) are respectively the torsion torque and the apparent viscosity for each value of the rotation speed.

The threshold shear stress ($\boldsymbol{\tau_c}$), the flow index (*n*) and the consistency index (k) were determined by adjustment, either using the Herschel-Bulkley model (Equation 5) or using the power law model (Equation 6):

$$\tau = k \cdot (\dot{\gamma})^n + \tau_c \quad (5)$$

$$\tau = k \cdot (\dot{\gamma})^n \quad (6)$$

The model with a better coefficient of determination and p-values less than 0.05 was chosen.

Evaluation of the effect of concentration and temperature

In literature, there is limited information regarding models presenting a correlation between rheological parameters and substrate concentration for the case of gruels. However, for other types of food pastes, an exponential model (Equation 7) has been presented to describe the consistency index behaviour in function of the concentration (Arslan et al., 2005).

$$k = k_0 \cdot exp(a \cdot C) \quad (7)$$

For each operating temperature, the relationship between consistency index and substrate concentration was studied using Equation 7.

The dependency of apparent viscosity on temperature was evaluated using an Arrhenius-type equation (Equation 8):

$$\mu = A \cdot exp(E_a/(R \cdot T)) \quad (8)$$

Where T is the absolute temperature in kelvin, in the range 30 - 60°C; A (Pa.s) is the Arrhenius constant; E_a ($J.mol^{-1}$) is the activation energy and R ($J.K^{-1}.mol^{-1}$) is the perfect gas constant. Measurements were conducted at a constant shear rate of 100 rpm (3.8 s^{-1}).

Study of the time effect

The effect of time on each gruel was studied at 30°C by monitoring the evolution of gruel viscosity (30% gruel) with time at a constant shear rate of 100 rpm (3.8 s^{-1}). The hysteresis curves were obtained by increasing, directly followed by reducing, the rotating speeds. This procedure for the forward and backward curves was done without interruption.

Model fitting and statistical analysis

The fitting of the models was done using the Sigmaplot © Software Version 11 (wpcubed, GmbH, Germany) while the mean comparison was carried out with Duncan's multiple range test ($P < 0.05$) using IBM SPSS Statistics software version 20.0.0.

RESULTS AND DISCUSSION

Raw material characterization

The average length and diameter of the bananas were respectively 18±1 cm and 3.9±0.2 cm while the colour index according to the commercial peel colour scale was located between 6 and 7. This classification of banana ripening was done using a colour index as presented by Aurore et al. (2009). Table 1 presents the physical characteristics and germination rates of sorghum and sesame. According to the CODEX (1989), the percentage of waste obtained for sorghum and sesame are low (1.62 and 0.41% respectively), reflecting the good quality of the grains. The weight of 1000 grains shows that sorghum grains are on average twice as heavy as, and more uniform than sesame grains. The weight of 1000 grains gives an indication of the quantity of matter (mainly starch in the case of cereals) that can be extracted from the different seeds. The observed difference in grain weight could be essentially due to variations in grain dimensions (Purseglove, 1972), growing conditions of the plant or storage conditions after harvest (FAO, 1989). Under the tested experimental conditions (22±1°C and saturated atmosphere), the stabilization phase during germination occurred at the third day with a respective rate of 95 and 99% for sorghum and sesame. It was also observed that, the germination rate of sesame was higher than that of sorghum throughout the germination period. The observed low percentage germination of sesame compared to that of sorghum can be due to the fact that, radicals grow during germination mainly by using carbohydrate reserves and sorghum has more carbohydrates than sesame. The results were different from that of Hahm et al. (2009) who obtained a sesame germination rate greater than 99% after four days of germination at 35°C and in a saturated atmosphere. This

Table 1. Percentage of waste, weight of 1000 grains and germination rate of sorghum and sesame.

Grains	Percent of waste	Weight of 1000 grains (g)	Germination rate (%)
Sorghum	1.62 ± 0.36	50.71 ± 1.73	95.33 ± 1.53
Sesame	0.41 ± 0.13	23.80 ± 6.05	99.67 ± 0.58

Table 2. Proximate analysis of the raw materials and corresponding flours.

Substrate	Humidity (g/100g w-b)	Total sugars (g/100g d-b)	Soluble sugars (g/100g d-b)	Lipids (g/100g d-b)	Total proteins (g/100g d-b)	Ash (g/100g d-b)
SON	11.47±0.91^{d}	78.04±10.47^{bc}	1.29±0.10^{b}	1.88±0.86^{a}	6.14±0.27^{a}	0.98±0.02^{bc}
SOG	11.33±1.15^{d}	72.49±2.53^{b}	5.43±0.32^{d}	1.09±0.56^{a}	6.02±0.36^{a}	0.99±0.01^{c}
SEN	3.33±1.15^{a}	2.59±0.64^{a}	0.57±0.11^{a}	57.24±2.39^{c}	21.65±0.52^{b}	0.93±0.04^{a}
SEG	6.00±0.00^{b}	2.40±0.40^{a}	1.02±0.52^{b}	50.71±2.52^{b}	18.91±0.16^{b}	0.95±0.01^{ab}
BF	76.67±1.15^{e}	77.31±11.25^{bc}	3.53±0.76^{c}	0.36±0.13^{a}	3.87±0.98^{a}	0.96±0.01^{ab}
BD	8.44±0.51^{c}	85.37±2.02^{c}	5.75±2.36^{d}	0.46±0.09^{a}	3.06±0.86^{a}	1.00±0.01^{c}

On the same column. data followed by the same superscript letter are not significantly different at the 5% level. SON: non-germinated sorghum ; SOG: germinated sorghum; SEN: non-germinated Sesame; SEG: germinated sesame; BF: fresh banana; BD: dried banana.

observed difference in the germination time could be attributed to differences in germination temperatures and absence of an initial soaking step (24 h soaking at 22°C in our case). In addition, several authors have shown the importance of the soaking step in the efficiency of the germination (Elmaki et al., 1999; Eneje et al., 2004).

Table 2 presents the physico-chemical characteristics of the raw materials and the derived flours. The results show that for sorghum and banana, the carbohydrates occupy more than 77% of the dry matter, followed by proteins (more than 3%), while for sesame, lipids come first (57%) followed by proteins (22%). These results are similar to those reported by Onyango et al. (2011) for sorghum; Forster et al. (2003) and Abbas (2009) for banana and Elleuch et al. (2007), Ciftçi et al. (2008) and Hahm et al. (2009) for sesame. The germination presents a significant effect ($P<0.05$) on the carbohydrate content of sorghum and on the lipid content of sesame, as also reported by Hahm et al. (2009). Compared to total sugars, soluble sugar contents are much lower for all the biological materials. However, the soluble sugar content was observed to be higher after germination, due to the increase in α-amylases activity (Elkhalifa and Bernhardt, 2010) resulting in a corresponding increase in starch hydrolysis. The osmotic dehydration applied to banana explains the higher soluble sugar content in the dried fruits compared to the fresh fruits (Jiokap Nono et al., 2002). This is advantageous as the presence of soluble sugars in flour destined for infant gruel increases the energy intake of the child (Gerbouin, 1996; Joshi and Verma, 2015). All the treatments applied do not have significant effect on the ash and protein contents; this is important for weaning foods where proteins have an important role (Elkhalifa and Bernhardt, 2010).

Effects of temperature and concentration on the rheological behaviour of the gruels

Sorghum purée at 15 and 25% w/w presented a two-phase behaviour, the first at shear rates less than 1.2 s^{-1} and the second at shear rates greater than 1.2 s^{-1}, while that of 35% showed a single phase behaviour (Figure 1). Similar to the first phase of 15 and 25% w/w concentrations, the 35% concentration showed a pseudoplastic behaviour (decrease of the viscosity with the spindle's rotation speed) throughout the range of the shear rate. The observed pseudoplastic behaviour could be due to the progressive breakdown of inter-molecular forces resulting from the breakdown of hydrogen bonds that maintains the main structural component of sorghum (Steffe, 1996; Guerrero and Alzamora, 1997). Concerning the dilatant behaviour observed at lower concentrations, it could be accounted for reformation of already broken bonds at high shear rates. These behaviours were observed for the four tested temperatures (30°C, 40°C, 50°C and 60°C). Very few studies have been carried out on the rheological properties of sorghum gruels and those presented in the literature relate to the rheological properties of sorghum starch during gelatinization (Vallons et al., 2009; Matalanis et al., 2009; Onyango et al., 2010; Onyango et al., 2011) but not after cooking as it is the case in the present work.

Unlike sorghum gruels, all the three concentrations of sesame gruels presented a single phase pseudoplastic behaviour throughout the tested range of shear rates (Figure 2). The decrease in resistance to flow could be due to structural deformation, bursting of lipid droplets (main component of sesame) and breakdown of primary and secondary bonds by shear-induced hydrodynamic

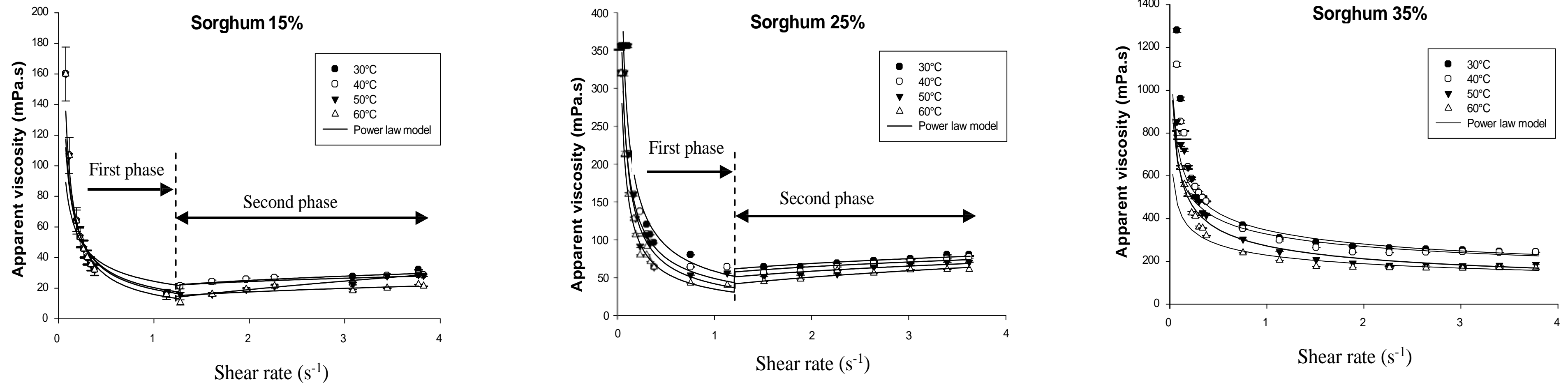

Figure 1. Effects of the rotation speed on viscosity of sorghum gruels at different concentrations and temperatures.

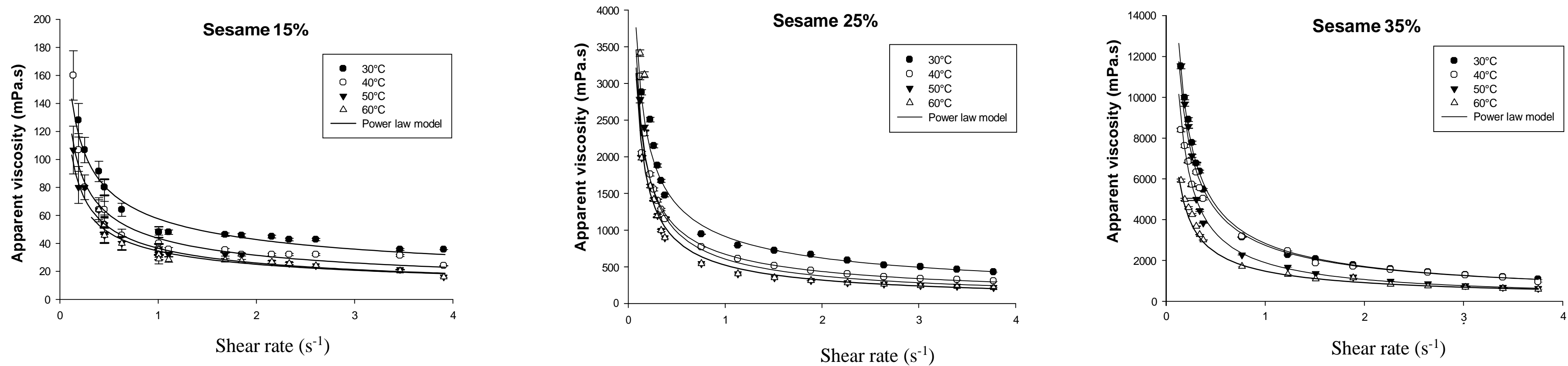

Figure 2. Effects of the rotation speed on the viscosity of sesame gruels at different concentrations and temperatures.

forces (Arslan et al., 2005). Similar to the case of sorghum, this behaviour was observed for the four tested temperatures. Similar behaviours have been observed by other authors on sesame pastes, mixed or not mixed with other products (Arslan et al., 2005; Razavi et al., 2007; Çifçi et al., 2008; Akbulut et al., 2012) and on oil/water emulsion of protein isolates and sesame oil (Lokumcu Altay and Ak, 2005).

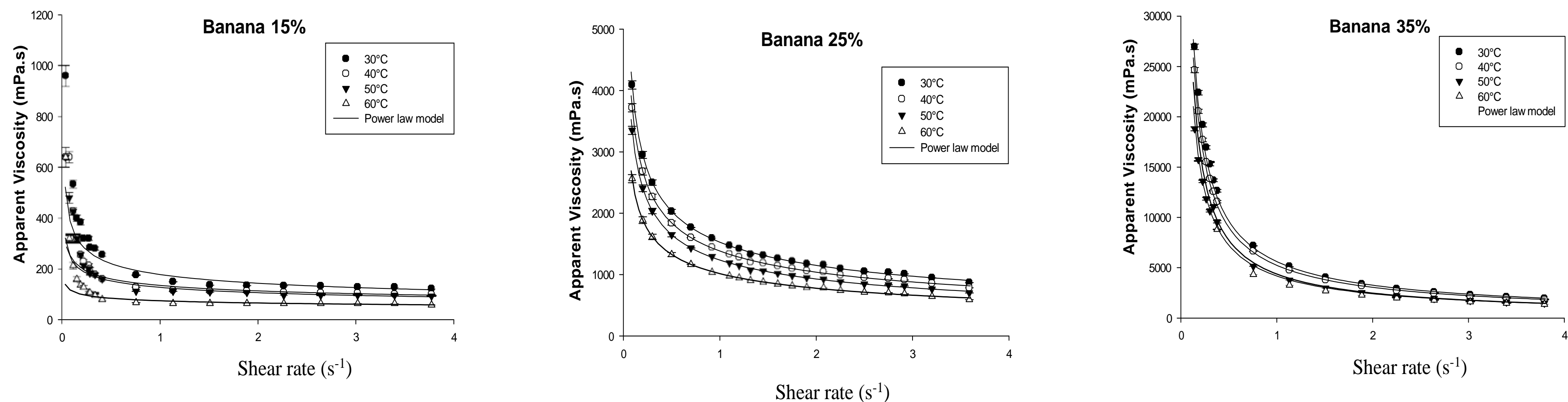

Figure 3. Effects of the rotation speed on the viscosity of banana gruels at different concentrations and temperatures.

Banana gruels showed a similar behaviour to that of sesame gruels (Figure 3). Similar behaviour of banana purée was also observed by Guerrero and Alzamora (1997). The viscosity values for the banana gruels were relatively high, compared to those of sesame and sorghum for each given temperature and concentration. This could be attributed to the differences in composition between the products (Table 2). For all the tested concentrations of the banana gruel, the viscosity as well as the consistency index globally decreased with increase in temperature, which can be attributed to the rupture of intermolecular bonds by thermal energy, leading to a decrease in the torque at a given speed of rotation. Ahmed and Ramaswamy (2007) also observed a decrease of the consistency index with temperature. Concerning the effects of concentration, the viscosity of the banana gruels increased with concentration while the flow behaviour index decreased, explained by the increase in the solid matter with concentration. Similar observations were obtained by Mohamed et al. (2010). Sorghum and sesame gruels presented similar evolution of viscosity, consistency and flow behaviour indices with temperature and concentration. However, the pseudo-stationary viscosity of banana gruels increased by a factor relatively high compared to those of sorghum and sesame gruels, as the concentration increased from 15 to 25 to 35%. Moreover, the flow index values obtained with sorghum gruels at 35% are due to the fact that this concentration (unlike the sorghum gruels at 15 and 25%) didn't present a two-phase behaviour.

Mathematical models for predicting the rheological parameters

The flow curves of all the three gruels showed a good fit with the Ostwald de Waele model (p-values less than 0.01), while the Herschel-Bulkley model was less suitable as it produced negative shear-stress thresholds. The corresponding values of the flow and consistency indices as well as the model statistical parameters are presented in Table 3. However, in the case of banana purée, the values of the flow index ($0.18 < n < 0.82$) were relatively higher than those presented in the literature (Guerrero and Alzamora, 1997) and this could be due to differences in raw material composition and treatment procedures. For each substrate concentration, the values of the consistency index for banana gruels were relatively high compared to those of sorghum and sesame gruels. The effects of concentration on the consistency index were conveniently described by the exponential model (Equation 7), with R^2 values ranging from 0.991 to 0.999 (Table 4) and Figure 4 presents the model fit with respect to experimental data. The values of the model parameter k_0, were comprised between

Table 3. Parameters of the power law model ($\tau = k \cdot \dot{\gamma}^n$) at different temperatures and substrate concentrations.

Substrate	T (°C)	Substrate concentrations (% w/w)								
		15			25			35		
		k (mPa.s^n)	n (-)	R^2_{adj}	k (mPa.s^n)	n (-)	R^2_{adj}	k (mPa.s^n)	n (-)	R^2_{adj}
Sorghum (first phase)	30	24.49 ± 1.92[a]	0.50 ± 0.09[a]	0.735	58.41 ± 6.64[a]	0.34 ± 0.08[ab]	0.650	345.96 ± 7.93[a]	0.70 ± 0.02[a]	0.993
	40	20.34 ± 1.66[b]	0.34 ± 0.07[b]	0.653	48.83 ± 4.61[b]	0.36 ± 0.07[a]	0.753	327.47 ± 11.58[b]	0.72 ± 0.03[a]	0.984
	50	19.26 ± 0.95[b]	0.30 ± 0.05[b]	0.788	41.89 ± 4.07[bc]	0.30 ± 0.07[c]	0.678	229.30 ± 7.29[d]	0.72 ± 0.03[a]	0.988
	60	15.90 ± 1.04[c]	0.17 ± 0.05[c]	0.555	34.83 ± 1.90[c]	0.31 ± 0.04[c]	0.883	272.39 ± 9.66[c]	0.64 ± 0.03[b]	0.976
Sorghum (second phase)	30	20.95 ± 2.07[a]	1.26 ± 0.08[b]	0.982	75.92 ± 5.91[a]	1.00 ± 0.07[b]	0.967	345.96 ± 7.93[a]	0.70 ± 0.02[a]	0.993
	40	21.05 ± 2.53[a]	1.21 ± 0.10[b]	0.970	63.84 ± 5.14[b]	1.10 ± 0.07[ab]	0.973	327.47 ± 11.58[b]	0.72 ± 0.03[a]	0.984
	50	12.33 ± 1.25[b]	1.63 ± 0.08[a]	0.991	54.69 ± 4.91[bc]	1.17 ± 0.08[a]	0.974	229.30 ± 7.29[d]	0.72 ± 0.03[a]	0.988
	60	14.15 ± 2.15[b]	1.31 ± 0.13[b]	0.963	48.63 ± 4.24[c]	1.15 ± 0.07[a]	0.977	272.39 ± 9.66[c]	0.64 ± 0.03[b]	0.976
Sesame	30	55.77 ± 1.55[a]	0.66 ± 0.03[ab]	0.979	914.11 ± 13.27[a]	0.44 ± 0.01[a]	0.989	2836.07 ± 29.23[a]	0.27 ± 0.01[c]	0.984
	40	39.87 ± 1.54[b]	0.72 ± 0.04[a]	0.983	671.09 ± 6.05[b]	0.38 ± 0.01[ab]	0.994	2744.45 ± 36.33[b]	0.29 ± 0.01[b]	0.977
	50	33.77 ± 1.14[c]	0.70 ± 0.03[ab]	0.983	605.00 ± 18.40[c]	0.31 ± 0.03[c]	0.916	1892.62 ± 13.77[c]	0.18 ± 0.01[d]	0.982
	60	31.38 ± 1.71[c]	0.65 ± 0.05[b]	0.959	522.10 ± 13.01[d]	0.29 ± 0.02[bc]	0.915	1477.42 ± 11.35[d]	0.31 ± 0.01[a]	0.993
Banana	30	177.96 ± 5.60[a]	0.69 ± 0.03[b]	0.985	1 628.46 ± 26.25[a]	0.52 ± 0.01[b]	0.998	5603.62 ± 83.07[a]	0.22 ± 0.01[a]	0.971
	40	134.89 ± 3.81[b]	0.75 ± 0.03[b]	0.991	1 458.51 ± 19.57[b]	0.36 ± 0.01[c]	0.992	5234.80 ± 74.68[b]	0.22 ± 0.01[a]	0.973
	50	127.30 ± 4.18[c]	0.74 ± 0.03[b]	0.987	801.50 ± 31.9[c]	0.19 ± 0.01[d]	0.978	4170.64 ± 33.10[d]	0.21 ± 0.01[a]	0.990
	60	74.47 ± 3.28[d]	0.82 ± 0.04[a]	0.983	453.37 ± 6.31[d]	0.75 ± 0.01[a]	0.997	4369.43 ± 100.50[c]	0.18 ± 0.01[b]	0.900

For each substrate and on the same column, data with the same superscript letter are not significantly different according to the Duncan test (P<0.05).

Table 4. Effects of substrate concentration on the consistency index of the gruels at different temperatures: Model parameters for the equation: $k = k_0 * \exp(a * C)$.

T (°C)	Substrates											
	Sorghum (first phase)			Sorghum (second phase)			Sesame			Banana		
	k_0	a	R^2_{adj}	k_0	a	R^2_{adj}	k_0	a	R^2_{adj}	k_0	a	R^2_{adj}
30	0.90	0.17	0.994	1.85	0.15	1.000	35.39	0.13	0.967	56.13	0.13	0.992
40	0.55	0.18	0.995	1.26	0.16	0.998	15.37	0.15	0.994	45.23	0.14	0.992
50	0.83	0.16	0.991	1.49	0.14	1.000	22.89	0.13	0.980	12.47	0.17	0.999
60	0.27	0.20	0.994	0.74	0.17	0.999	23.68	0.12	0.970	1.64	0.23	0.999

0.27 and 1.85, 15.37 and 35.39 and 1.64 and 56.13 mPa.s^{-n} respectively for sorghum, sesame and banana gruels. In the case of banana gruels, the values of k_0 decreased with temperature as shown on Table 4. For all the studied gruels the parameter, a was comprised between 0.13 and

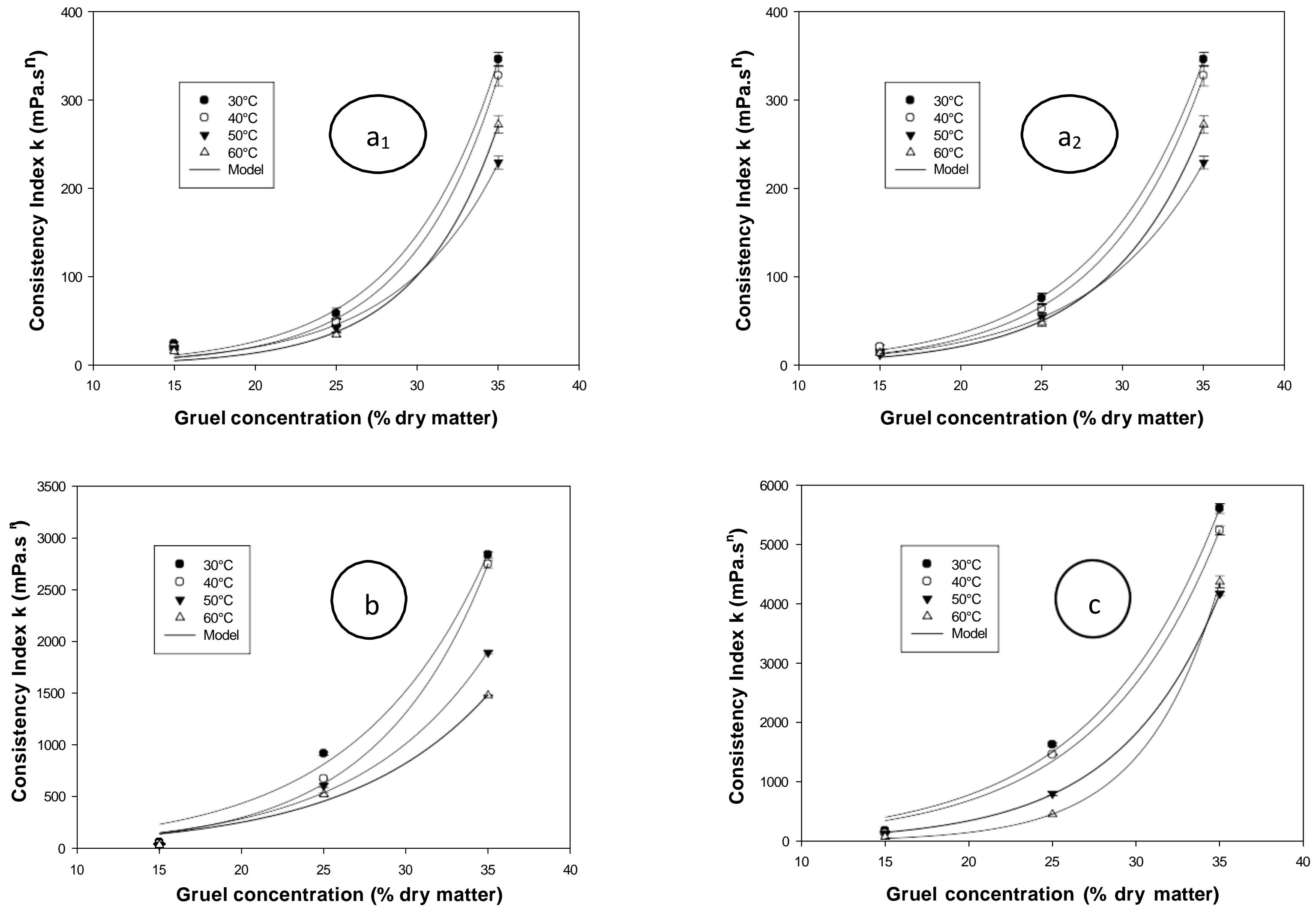

Figure 4. Experimental and model (Equation 7) curves for the gruel consistency index in function of the concentration and temperature: (a1) sorghum-first phase, (a2) sorghum-second phase, (b) sesame and (c) banana.

0.23. Concerning the flow behaviour index, no descriptive trend was observed regarding its variation with temperature and substrate concentration. Similar results were reported by Arslan et al. (2005).

The effect of temperature on the gruels' viscosity

Table 5. Arrhenius-type model parameters for the different gruels.

Substrate	Concentration (% w/w)	A (Pa.s)	E_a ($J.mol^{-1}$)	R² adjusted
	15	1 406 ± 13^a	964 ± 6^c	0.983
Sorghum	25	514 ± 24^c	9 992 ± 17^b	0.825
	35	1 020 ± 39^b	14 144 ± 37^a	0.968
	15	232 ± 2^b	13 225 ± 58^c	0.917
Sesame	25	1 373 ± 7^a	18 552 ± 26^b	0.964
	35	158 ± 8^c	21 070 ± 38^a	0.858
	15	10 043 ± 14^b	10 233 ± 54^c	0.990
Banana	25	34 484 ± 12^a	12 054 ± 12^b	0.946
	35	81 ± 2^c	18 825 ± 17^a	0.821

For each substrate and on the same column, data with the same superscript letter are not significantly different according to the Duncan test (P<0.05).

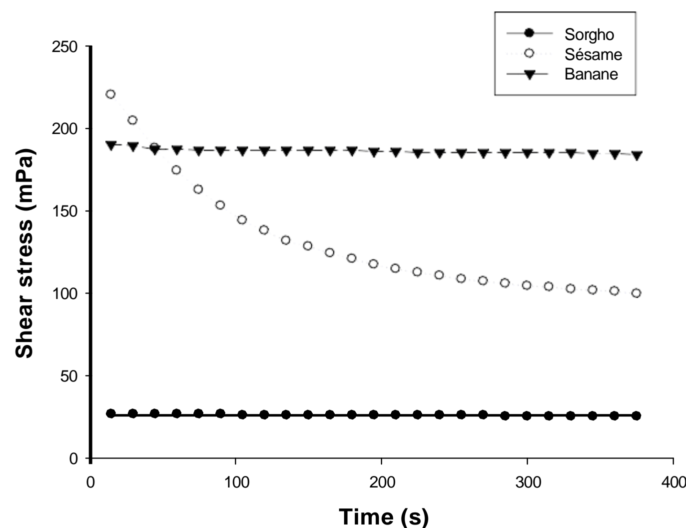

Figure 5. Test of the time sensitivity of sorghum, sesame and banana gruels (at 30% w/w each) at 100 rpm (3.8 s^{-1}) and 30°C.

followed the Arrhenius model (Table 5) with adjusted R² values ranging from 0.820 to 0.990. The value of the constant (A) varied with substrate, ranging from 514 to 1,406 Pa.s for sorghum purée, 158 to 1373 Pa.s for sesame purée, and 81 to 34,484 Pa.s for banana purée. This constant showed no trend regarding its variation with substrate concentration in the three different gruels. These differences could be attributed to the physico-chemical nature of the gruel, mainly their lipid and protein contents for sesame, and carbohydrate content for sorghum and banana; as well as their ease of forming hydrogen bonds. E_a, which measures the sensitivity of the purée viscosities to temperature, was highest for sesame purée (13,225 – 21,070 $J.mol^{-1}$), followed by banana purée (10,233 - 18,825 $J.mol^{-1}$) and then by sorghum purée (964 - 14,144 $J.mol^{-1}$). The value of Ea was observed to increase with flour concentration for all the purées. These results show that the energy required for the fluid to flow increases with concentration (Table 5) and this could be explained by the fact that the number of inter-molecular bonds involved in maintaining the structure of a substrate in a milieu increases as the concentration of the milieu increases. This trend was equally observed by Arslan et al. (2005) on sesame paste.

Effect of time on the rheological behaviour of the gruels

At a constant share rate of 3.8 s^{-1}, the shear stress decreased progressively with time but showed no significant drop for sorghum and banana gruels (Figure 5). To confirm the time dependency of the gruels, a "loop

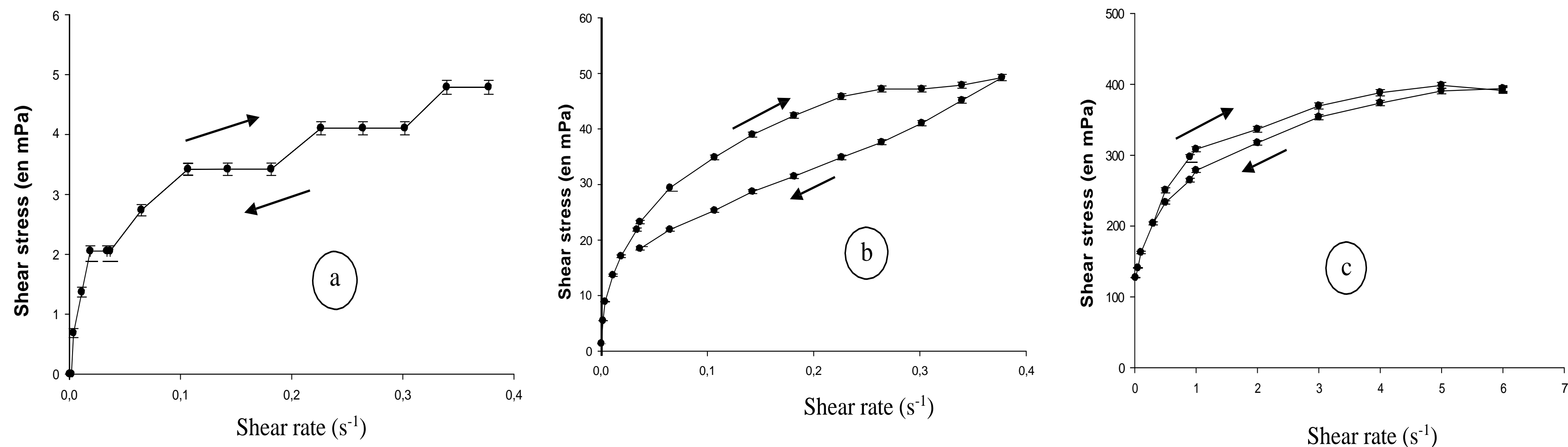

Figure 6. Forward and backward curves of the gruels (30% w/w) at 30°C: (a) Sorghum, (b) sesame and (c) banana.

test" was conducted. The forward-backward curves of the gruels (Figure 6) indicate the absence of a hysteresis loop for sorghum (Figure 6a), confirming thereby the time-independency of sorghum gruel. A hysteresis loop existed for sesame and banana gruels, with ahigher amplitude for the former than for the latter (Figures 6b and c). Lokumcu Altay and Ak (2005) also observed a hysteresis loop on tahin and attributed this behaviour to the thixotropic nature of the substrate. Abu-Jdayil (2003) and Habibi-Najafi and Alaei (2006) also noticed a thixotropic character on sesame-based products.Gruels prepared from infant flours are rich in starchy and protein foods and have a viscosity that increases very rapidly as a function of their dry matter concentration. This makes the gruels difficult to swallow, digest and absorb by children due to reduced activity and capacity of their organs. The rheological study of each of the constituents could make it possible to orient the formulated mixture of these foods, and also to envisage fluidification treatments for the manufacture of infant flour. The gruels derived from these flours should have rheological properties which facilitate the ingestion by the children, while taking into account the nutritional aspects.

Conclusion

This study evaluated the rheological properties of infant gruels produced from sorghum, sesame or banana as base constituents. The sorghum gruels showed dilatant properties at high shear rates and pseudoplastic properties at low shear rates, while the sesame and banana gruels were pseudoplastic fluids throughout the range of shear rates. A good correlation was obtained between the consistency coefficient and concentration for each temperature. For all the gruels, the viscosity reduced with temperature and increased with concentration. The effect of temperature on the gruels' viscosity followed the Arrhenius law. The activation energies were highest for sesame gruels, followed by banana gruels and then sorghum gruels. The later presented a time-independent behaviour, whereas banana and sesame gruels had a time-dependent behaviour. The results of this study could be exploited for the formulation and improvement of derived products, as well as for the dimensioning of equipment and for the conception of production units.

CONFLICT OF INTERESTS

The authors have not declared any conflict of

interests.

Abbreviation

M, Mass of sample (g); ***M_g,*** Mass of good grains in the sample (g); ***Wc,*** *W*aste content (%); ***Gr,*** Germination rate (%); ***N_g,*** Number of germinated grains at the end of germination (-); ***N_0,*** Number of good grains (-); ***Mm,*** Mass of 1000 grains (g), ***MS,*** Mass of dry matter in 100 grams of good grains (g), **N,** Rotation speed (rpm); τ**,** Shear stress (Pa), ***R,*** Universal gas constant ($Jmol^{-1}K^{-1}$); $\dot{\gamma}$**,** Shear rate (s^{-1}); T_w**,** Torsion torque (%); μ**,** Apparent viscosity (Pa.s); τ_c**,** Threshold shear stress (Pa); n**,** Flow index (-); **k,** Consistency index ($mPa.s^n$); k_0**,** Consistency index model parameter ($mPa.s^n$); a**,** Consistency index model parameter (100g/g); A**,** Arrhenius constant (Pa.s); E_a**,** Activation energy ($J.mol^{-1}$); ***T,*** Absolute temperature (K).

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Molecular characterization and insecticidal activities of Malian native crystalliferous *Bacillus thuringiensis*

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The objective of this work was to select the most active *Bacillus thuringiensis* (Bt) isolated from agricultural soils of Mali through the molecular characterization and the determination of insecticidal activities of the protein crystals, produced by these native isolates. Crystal proteins were extracted from *B. thuringiensis* culture, and characterized using the SDS-PAGE techniques. Their insecticidal activities were tested using third-instar larvae of *Helicoverpa armigera* in bioassay tests. The results showed that, of 62 *B. thuringiensis* treated, 52 isolates showed fragments varying between 10 and 140 kDa on 12% polyacrylamide gel. Cry1 and Cry2 protein crystals were recognized to be effective against Lepidoptera's larvae, which were found in 21% of the tested isolates. In addition to these two expected crystal protein weights, other molecular weights were observed at different proportions, suggesting the presence of other cry genes in the local *B. thuringiensis* isolates. Four native *B. thuringiensis* isolates were able to kill 95 to 100% of *H. armigera* 3rd-instar larvae. Only one native of *B. thuringiensis* isolate was able to kill 100% of the *H. armigera* larvae. This is the first study for molecular characterization of Malian native *B. thuringiensis* isolates, showing the efficacy of the native *B. thuringiensis* against an important agricultural insect pest.

Key words: Protein crystals, *Bacillus thuringiensis*, cry genes, insecticidal activity, molecular characterization, *Helicoverpa armigera*, Mali.

INTRODUCTION

The introduction and subsequent proliferation of synthetic insecticides has played a key role in increasing agricultural productivity, protecting forest crops, and controlling insect vectors of human diseases (Joung and Côté, 2000). In Mali, the damage caused by insect pests can be as high as 60% crop loss (Hamadoun, 1996). The use of synthetic insecticides has led to the emergence of resistant biotypes that are no longer controlled by major groups of

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chemical insecticides (Pooja et al., 2013).

A promising alternative to synthetic insecticides that has attracted attention is the development of protein toxins, produced by *Bacillus thuringiensis* (Bt) as insecticides (Pooja et al., 2013). *B. thuringiensis* is a sporoform ubiquitous gram-positive bacterium that forms protein crystals (protoxins) during the stationary phase of its growth cycle (Schnepf et al., 1998). These protein exhibit toxious activities against insect larvae (Nazarian et al., 2009; Pardo-Lopéz et al., 2013). *B. thuringiensis* toxins are specific to a limited number of insect species without any toxicity to humans or other organisms (Bravo et al., 2011). Some of them are toxic to a large number of insect species in this order; Lepidoptera, Diptera and Coleoptera in addition to some Homoptera (MacIntosh et al., 1990; Bravo et al., 2007; Porcar et al., 2009; Palma et al., 2014), and Nematodes (Wei et al., 2003).

PCR is a rapid tool for preliminary characterization of *B. thuringiensis*. The reliability of predicting insecticidal activity on the basis of PCR results is dependent on the expression of cry genes. The cry genes that lack promoters or functional genes, which are weakly expressed, will produce an erroneous prediction. So, for a better characterization of Bt strains, essential complements to the identification of cry genes, should be made through the determination of parasporal crystals composition, by polyacrylamide gel electrophoresis using sodium dodecyl sulfate (SDS-PAGE) and biological activity assays (Porcar and Juarez-Perez, 2003). In Mali, over the last three years, there has been an intense interest in the collection and analysis of local Bt strains from various environmental samples in Mali (Kassogué et al., 2015). The aim of this work was to characterize and describe the toxicity of several *B. thuringiensis* toxins (Cry1B, Cry1C, Cry1F and Cry2) for *Helicoverpa armigera* and *Orselia oryzivora*.

MATERIALS AND METHOD

B. thuringiensis strains

Fifty-two (52) *B. thuringiensis* strains isolated from soil and plants of Mali, harboring cry1 and/or cry2 genes (Fané et al., 2015) were analyzed by SDS-PAGE for cry crystal proteins production and distribution.

Production, extraction and separation of crystalline proteins

To produce crystal proteins, *B. thuringiensis* strains were cultured on Luria Bertani (LB) solid medium enriched with salts according to the method described by Valicente et al. (2010), for 24 h at 30°C. From the 24 h culture, the strains were transferred in 50 ml nutrient broth and incubated at 30°C for 72 h with continuous stirring at 200 rpm (Ammouneh et al., 2011).

Proteins were extracted using the modified protocol of Ammouneh et al. (2011). To do this, the suspension obtained was incubated in ice for 20 min and then centrifuged at 2000 *g* for 5 min using a centrifuge (Sigma 2-6 E). 2 ml of the pellet were taken and incubated in an ice for 15 min and then centrifuged with a centrifuge (Eppendorf 5424) at 14500 rpm for 10 min. The pellet obtained was resuspended in 1 ml of 0.5 M iced NaCl and then centrifuged at 14,500 rpm for 5 min. These pellet was resuspended in 1 ml of solution (1% SDS and 0.01% beta-mercaptoethanol) and the mixture was boiled at 95°C for 10 min, and centrifuged at 14500 rpm for 10 min. The supernatant was harvested and analyzed using 12% SDS-PAGE as described by Laemmli (1970). Electrophoresis was performed in a system (Omni PAGE 'WAVE' Electrophoresis Systems) at 90 V and 45 mA for 16 h. The gel was stained with 0.1% coomassie blue R250. Protein masses were determined by comparison with a broad range protein molecular weight marker.

Insect and bioassay

Several larvae of various instars of about 100 *H. armigera* were collected from maize and tomato fields in Mali, July, 2016 and were used for mass rearing under controlled conditions on an artificial diet (Abbasi et al., 2007), in a growth chamber at 25°C by using a relative humidity of 70% in a photoperiod consisting of 16 h of light and 8 h of darkness.

The population was reared for three generations in the laboratory before insects, which were used for bioassays (Fiuza et al., 1996).To determine the insecticidal activity of the spore-crystal mixtures, different *B. thuringiensis* isolates were screened on third-instar larvae of *H. armigera* as described by Aboussaid et al. (2009). In the first screening, the bioassay was performed with non-concentrated spore-crystal suspensions. For each isolate, 30 larvae were used. Mortality was observed after 3 days.

Bioassay was replicated with the isolates showing mortality rate, superior to 30%. For the chosen strains, the spore-crystal suspensions were centrifuged for 15 min at 15 000 *g* and 4°C and the spores and crystals were collected in the pellets. The pellets obtained were washed three times with phosphate buffered saline (PBS) containing 0.005% Triton X-100 in order to promote the bursting of the bacterial cells. The pellets were lyophilized and used to determine their insecticidal activity (Weathersbee et al., 2006). Three dilutions were used for each isolate and 30 third-instar larvae were used for each dilution. Mortality was observed after 3 days as described by Hassani and Gaouar (2008).

RESULTS AND DISCUSSION

Profile and frequency of Cry proteins synthetized by the native *B. thuringiensis* isolates

To determine the ability of the Malian *B. thuringiensis* (Bt) isolates to synthetize Cry proteins (crystal proteins), the 62 isolates were characterized by SDS-PAGE. The results obtained indicate that, some isolates were able to synthesize Cry proteins with molecular weights of about 130 and 70 kDa (Figure 1).

The protein profiles of cry1 B, cry1C, cry1F and cry2 genes observed on the 12% polyacrylamide gel (SDS-PAGE) showed bands of molecular weight between 10 and 140 kDa (Table 1). These results are in agreement with those of Gao et al. (2008) who observed parasporal crystals in 342 *B. thuringiensis* isolates, composed of more than one protein with molecular weights ranging between 28 to 150 kDa which is usually 65 to 140 kDa, with seven isolates producing a single protein Crystal of 50 kDa.

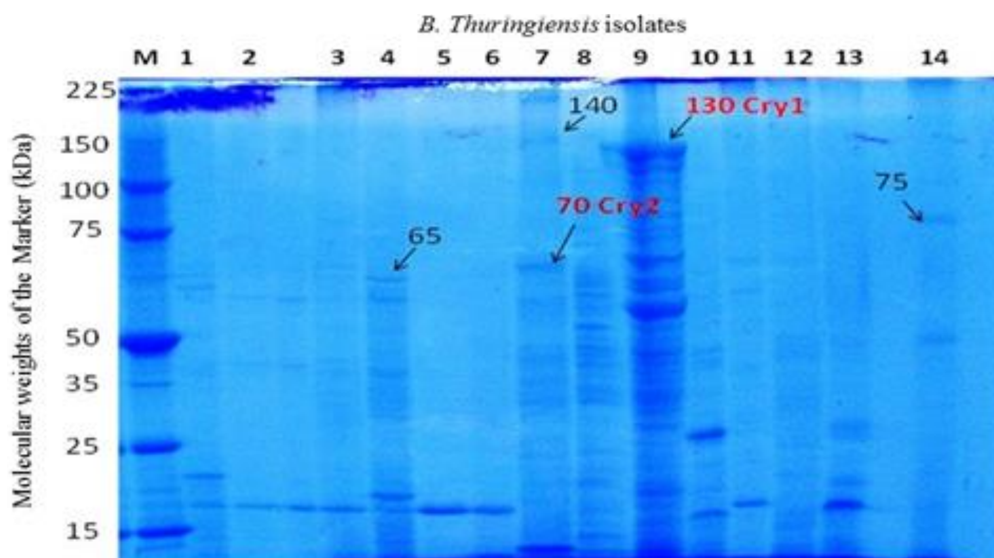

Figure 1. SDS-PAGE of the mixed spore-crystals of the *Bacillus thuringiensis* isolates. M, marker of molecular weight in KDa (broad range protein molecular weight marker); column 1 to 14 strains of Bt (D5, D8, D1G, B1G, B5, C4, I4 '', I4'N, I7, Dr2, DL1, DL2, DL4 and DL5).

Table 1. Molecular weight, number and frequency of protein bands.

Molecular weight (kDa)	Bands	
	Number	Frequency of bands (%)
140	3	6
125	5	10
130-70	11	21
100-110	5	10
80-90	4	8
66-68	5	10
60-65	13	25
50-55	14	27
40	6	10
20-25	5	10
10-17	4	8

B. thuringiensis showed bands with approximately 130 and 70 kDa which were expected in protein sizes as expected during the present study (Table 2). These results can be compared with those of Ammouneh et al. (2011) who obtained protein sizes of about 130 and 65 kDa in the local strains of *B. thuringiensis* of Syria. Similarly, dos Santos et al. (2009) found the presence of two main polypeptides of approximately 130 and 65 kDa of the proteins obtained from the spore-crystal mixtures by SDS-PAGE. Apart from these two sizes, 130 and 70 kDa and other protein molecular masses were observed in several Malian local strains which would indicate the presence of other Cry genes. The results obtained by Fiuza et al. (2012) are different from those obtained by the present investigation because they showed that Cry9 proteins have about 130 kDa.

In addition to these two expected crystal protein weights, other molecular weights were observed at different proportions (Table 1), suggesting the presence of other cry genes in the local Bt isolates analyzed. This result is in agreement with those obtained by Pinheiro (2013), who showed that most of the *B. thuringiensis* analyzed are capable of producing more than one type of crystal. Gonzalez and Carlton (1984) and Lereclus et al. (1989) showed that, crystals can be formed by different Cry and / or Cyt proteins as occurs for example in *B. thuringiensis* sub sp. *Israel* which has 5 genes encoding Cry proteins of cytolysin located on the same plasmid of 72MDa.

Among the *B. thuringiensis* isolates analyzed, 21% possessed the desired band sizes showing the expression of cry1 and cry2 genes which were harbored by these bacteria (Table 1 and 2). In 50% and more of the isolates analyzed, cry1 and cry2 genes were not expressed as shown by the absence of Cry endotoxins (Table 3). In the isolates harboring, only a cry gene and the cry2 gene expresses more than the cry1 (50% for cry2 and 38.46% for cry1), the same phenomenon is observed in the isolates, harboring at the same time cry1 and cry2 genes (27.58% for cry2 and 3.44% for cry1) (Table 3). Contrary to our results showing more than 50% of non-expressed cry genes, Ammouneh et al. (2011) supported the idea of expression of the cry1 and cry2 genes in all their *B. thuringiensis* isolated from Syria.

Table 2. Isolates of *Bacillus thuringiensis*, *cry* gene(s) harbored by each isolate, expected and non-expected Delta-endotoxins sizes (kDa) obtained.

B. thuringiensis isolates	*Cry* genes	Size of Delta-endotoxins (KDa)	
		Expected	Not expected
DL1	*cry1* B, *cry1* C, *cry2*	70*	-
Dr2	*cry1* F, *cry1*C	130**, 70	65, 60, 50
Ch2	*cry1* B, *cry1* C, c*ry2*	130, 70	-
S1	*cry1* B, c*ry*1F, *cry* 2	130,75*	60, 25 10
Dr3'	*cry1* F	130	50, 40, 25
D3P	*cry1* F, *cry2*	-	40
D5	*cry1* F, *cry2*	70	20, 17
DL2	*cry1* B, *cry1*C, *cry2*	140**, 130, 70	-
Ch1	*cry1F, cry2*	130, 70	-
C5	*cry1* F	130	-
I7'	*cry1* F	-	100, 50
D2P	*cry1* F, *cry2*	-	65, 60
C4	*cry1* F, *cry1* B, *cry2*	-	125, 10
B4	*cry2*	-	60
I2	*cry1* C, *cry2*	70,75	125, 90

*CryII delta-endotoxin; **CryI delta-endotoxin.

Table 3. Percentage of Cry Delta-endotoxins (crystal proteins) produced by *Bacillus thuringiensis* harboring different cry genes.

Bt with cry genes	Cry delta-endotoxins (%)				
	Total	Cry1	Cry2	Cry1+cry2	No Cry endotoxin
cry1+cry2	29	3.44	27.58	17.24	51.72
cry1	13	38.46	-	-	61.54
cry2	2	-	50	-	50

Insecticidal activity of the *B. thuringiensis* isolates on *H. armigera* larvae

Morphology and distribution of the B. thuringiensis crystal (Cry) proteins

Microscopic observation of bacterial smears colored with coomassie blue, at 1000 magnification using oil immersion, made it easy to distinguish the spores from the crystals which will appear in blue. Thirty-five (35) of the *B. thuringiensis* isolates studied, produced crystals (Delta (δ)-endotoxins). Three types of crystals (Bipyramidal, spherical and cubic) were found alone or in combination (Table 4). The types of crystals, the number and identity of the strains producing these crystals as well as the numbers and percentages of these strains are presented in Table 4.

Analysis of the data in this table shows that, only DL4 and D8 isolates produce bipyramidal crystals alone. *B. thuringiensis* isolates producing spherical crystals alone or combined with bipyramidal crystals represent respectively, 22.85 and 25.71% of the crystal-producing *B. thuringiensis* isolates. The isolates producing Cry1 and Cry2 proteins as mixture of spherical-cubic crystals represent 20% of the total crystal-producing bacteria. *B. thuringiensis* isolates producing cubic crystals alone (14.28%) and others producing a mixture of bipyramidal-cubic crystals (11.42%) were also identified.

Mortality rate of H. armigera larvae caused by the B. thuringiensis crystal proteins

The bioassay tests were carried out on a total of 30 larvae per isolate. Data of insecticidal activity of the tested *B. thuringiensis* isolates on *H. armigera* larvae are presented in Table 5. Of the isolates, producing Cry1 and/or Cry2 proteins tested 47% had a mortality rate greater than 80% while 35.30% had a mortality rate between 80 and 75%, and 17.64% of the isolates killed

Table 4. Morphology of crystals, identity and percentages of the *B. thuringiensis* isolates producing these crystals.

Crystals	Morphology of the Delta-endotoxin crystals					
	Spherical	**Bipyramidal**	**Cubic**	**Spherical-Bipyramidal**	**Spherical- cubic**	**Bipyramidal-Cubic**
***B. thuringiensis* isolates**	DL5	DL4	C3'	Dr2	D1P	I2
	I7	D8	S1	DL1	DL5'	C5'
	CH3	-	I4″	DL2	B1G	D1G
	B1P	-	CH1	AM2'	C5	D11
	Dr3	-	C3″	I7'	Dr5	-
	D3P	-	-	D5	D1P	-
	AM1	-	-	B4	B5	-
	DL3	-	-	D2P	-	-
	-	-	-	D3G	-	-
Total	**22.85**	**5.71**	**14.29**	**25.71**	**20**	**11.43**

Table 5. Efficacy of *B. thuringiensis* isolates as expressed by the mortality rate of *Helicoverpa armigera* larvae (%).

	Mortality rate of *Helicoverpa armigera* larvae (%)									
	0	100	95	90	85	80	75	70	60	55
Isolates	Control	DL_1	Dr_2 L_2 CH_2	S_1	$Dr_{3'}$ D_3P D_5	DL_2 CH_1 D_1P	C_5 I_7' D_2P	C_4	B_4	I_2

Figure 2. Insecticidal activity of *B. thuringiensis* isolate Dily1 against *H. armigera* larvae.

between 70 and 55% of *H. armigera* larvae (Table 5). As observed in this work, Bravo et al. (1998) and Crickmore et al. (1998) indicate that, the most known proteins on Lepidoptera are encoded by the Cry1, Cry2, and Cry9 genes.

B. thuringiensis producing spherical (D3P), or spherical combined to other Cry endotoxins morphology (DL1, D5…) were identified as more actives. The results showed an efficacy ranging from 55 to 100%, obtained with the isolate Dily1 (Figure 2). These results are clearly superior to those of Lalitha et al. (2012) who obtained a Bt mortality rate on *H. armigera* in stage 2, ranging from

5.56 to 94.4%.

Conclusion

Fifty-two (52) isolates showed fragments varying between 10 and 140 kDa on 12% polyacrylamide gel. Cry1 and Cry2 protein crystals recognized to be effective against Lepidoptera when found in 21% of the isolates.

Out of the 52 Bt isolates tested, 4 were able to kill 95 to 100% of *H. armigera* third-instar larvae. Only one native Bt isolate was able to kill all the *H. armigera* larvae. This study constitutes to our knowledge, the first molecular characterization of Malian native of *B. thuringiensis* isolates, showing the efficacy of the native Bt isolates against the important agricultural insect larvae.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interest.

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Full Length Research Paper

Computational antioxidant capacity simulation assay of *Garcinia kola* (Heckel) seed extracts

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Polypharmacological activities of the biflavonoid fraction of *Garcinia kola* seed justify its development as a nutraceutical, however, quality assurance of active nutraceutical ingredient (ANI) requires conformance to appropriate standards of composition and quality. It was hypothesized that variation in extraction protocols, as previously reported for the biflavonoid fraction, would lead to variation in extract composition and potency. Computational antioxidant capacity simulation (CAOCS) assay of *G. kola* extracts obtained by different extraction protocols tested the hypothesis, through incremental addition (250 and 100 µL) of standard antioxidant (AOX) extract solutions in a photometric titration. Preferred model fitting was then statistically selected between mono- and bi-exponential decay. Best - fit reaction constant (k_{ptt}) was integrated into a metric for ranking antioxidant capacity (AOC) of the extracts. The AOC metric is a molecular descriptor for kinetics of phenolic bond cleavage. Three AOX extracts, namely, ethyl acetate seed extract, kolaviron and acetone seed extract were found to vary in composition, and produced optimal AOC values of 1500/g, 1150/g, and 1050/g respectively. Our findings demonstrated that the composition and potency of biflavonoid fraction of *G. kola* seed are critically dependent on solvent extraction protocol, and hence, consistent with the hypothesis. CAOCS assay is a suitable analytical tool for ensuring batch–to–batch sameness of ANI prepared from *G. kola* seed.

Key words: *Garcinia kola* seed extracts, biflavonoids, active nutraceutical ingredient, antioxidant capacity, quality assurance.

INTRODUCTION

The seed of *Garcinia kola* (Heckel, Fam: Guttiferae/ Clausiaceae) is a very popular adaptogen in West African countries, and Nigeria in particular. There are many folkloric usages, which include treatment of bronchitis, laryngitis, oral infections, colic, dysentery, bacterial and viral infections (Irvine, 1961; Farombi, 2003). The seed has been clinically evaluated as treatment for knee osteoarthritis (Adegbehingbe et al., 2008), while its potential as an aphrodisiac was also demonstrated in male Wistar rats (Sewani-Rusike et al., 2016; Farombi et al., 2013b).

The biflavonoid fraction, originally described as

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"kolaviron" (KV) by Iwu reportedly contain a mixture of structurally related biflavonoids (Iwu, 1985). KV was shown, mechanistically, to have significant hepatoprotective (Farombi, 2000; Farombi et al., 2009), antiinflammatory (Farombi et al., 2013a; Abarikwu, 2014; Onasanwo et al., 2016), antioxidant (Farombi et al., 2002; Farombi et al., 2004a, b) and immunomodulatory activities (Nworu et al., 2008). The bioactivity profile of KV therefore justifies its usage as dietary supplement for chemoprevention of diseases and health promotion.

Despite the well documented usefulness of *G. kola* seeds, its use as dietary supplement is still only prevalent among older adults (over age 50 years) in Nigeria, because the bitter taste precludes its adoption by a younger generation. This usage pattern is consistent with the pattern of supplement usage in general in the United States as revealed by National surveys conducted over three decades (Bailey et al., 2011; Block et al., 1988; Radimer et al., 2004; Koplan et al., 1986). It is therefore desirable to promote its usage among younger adults (under age 50 years) and wider adoption among older adults.

The argument for increased usage of proven supplements across adult age groups is supported by an emerging paradigm in healthcare: “integrative healthcare”, which aims to prevent disease in the first place, and emphasize attacking underlying causes of disease rather than symptoms, in treatment modalities (Mister, 2012).

A wider use of *G. kola* - based supplement is achievable through careful preparation of active nutraceutical ingredient (ANI) comprised of the biflavonoids, for use in appropriate and more palatable formulations. We observed that variations of solvent - solvent extraction protocol were reported for extraction of the biflavonoids described as KV, by different investigators. Iwu initially used chloroform fraction of defatted methanol extract of powdered *G. kola* seeds (Iwu, 1985). In a later report by Iwu et al. (1987), the combined chloroform and *n*-butanol fraction of defatted acetone extract, was described as KV. Olaleye et al. (2000) reported KV as the ethyl acetate fraction of defatted acetone extract of *G. kola*. Nwaneri-Chidozie et al. (2014) reported extraction with methanol and subsequent partitioning with ethyl acetate, while Farombi et al. (2005) reported chloroform fraction of defatted methanol extract. It is obvious from literature that the mixture of biflavonoids were assumed to be extracted by these varied protocols, albeit, without any validation of the assumption. Therefore, we hypothesize that the relative amounts of the individual polyphenols (Figure 1) and overall composition would be somewhat different in the various extracts obtained, due to variation in solubilization capacity of organic solvents for a given class of compounds. Furthermore, purity of the biflavonoid extracts would be dependent on relative amounts of other non-biflavonoid secondary metabolites extracted along with the polyphenols.

Quality assurance considerations for manufactured dietary supplements require that they conform to appropriate standards of purity, quality and composition (TABD - DSEG, 2002). It is therefore the aim of this study to quantitatively evaluate the quality of biflavonoid extracts of *G. kola* seed in addition to qualitative evaluation of purity and composition. This will serve as first step towards standardizing the extraction protocol, to ensure consistent purity and optimal potency. Quality - assured ANI is critical to successful commercial manufacturing of dietary supplements. Computational antioxidant capacity simulation (CAOCS) assay is a bespoke assay developed for antioxidant capacity profiling of polyphenols and phenol-like compounds (Idowu et al., 2009; Idowu, 2014). The systematic workflow and associated informatics required to implement the two photometric assays that constitute CAOCS assay is displayed in Figure 2.

In this paper, we report a quantitative study, using CAOCS assay, of the antioxidant capacity (AOC) of KV and two other *G. kola* extracts obtained by variants of the extraction protocol. A primary molecular descriptor for kinetics of phenolic bond cleavage was integrated into a metric for ranking antioxidant capacity (AOC), as a measure of extract potency and quality.

MATERIALS AND METHODS

Chemicals and reagents

Sodium hydroxide pellets (LobaChemie, India, 98%), Phenolphthalein (Wardle Chemicals, UK, 98%), Methanol, Ethyl acetate, *n*-Hexane, Chloroform, and Acetone; all were from Sigma, USA Borax (LabTech, India, 98%).

Equipment

Analytical balance (Kern, Germany), Digital colorimeter (Jenway, Model 6051, U.K.), Magnetic stirrer (Gallenkamp, U.K.), Laboratory oven (Astell Hearson, U.K.), Rotary evaporator (Heidolph, Germany), pH meter (PHS-3C, China).

Plant material

The seeds of *G. kola* were purchased in a local market at Ibadan, Oyo State, Nigeria. The specimen was authenticated at the Forestry Research Institute of Nigeria, (FRIN), Ibadan, where a voucher specimen was deposited with the voucher specimen number; FHI 110593.

Preparation of reagent stock solutions

i) Preparation of 0.10% w/v Phenolphthalein solution: Phenolphthalein (0.10 g) was weighed and dissolved with little quantity of methanol in a beaker and upon complete dissolution, transferred into a 100 mL volumetric flask and made up to volume with methanol.

ii) Preparation of 0.025 M sodium hydroxide solution: Sodium

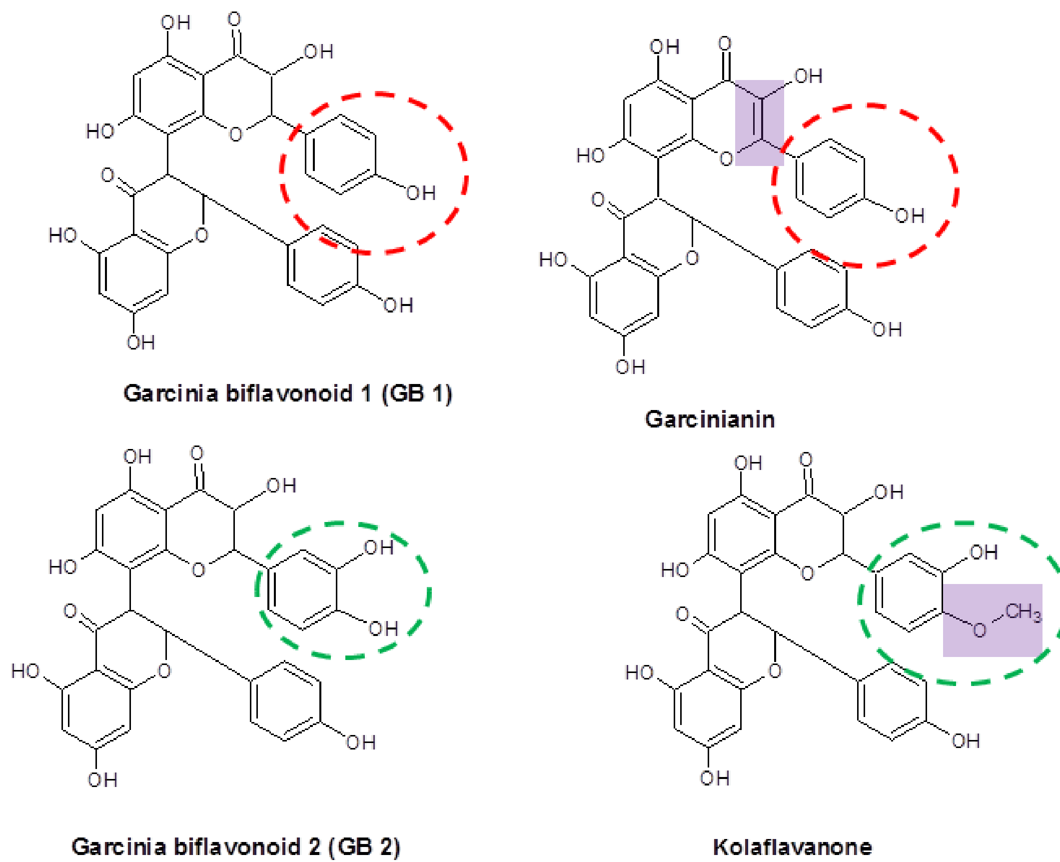

Figure 1. Chemical structures of biflavonoids isolated from *Garcinia kola* seed show structural similarity. Garcinianin is shown to be a derivative of GB 1, while kolaflavanone is a derivative of GB 2. The difference in the structures is highlighted in purple.

hydroxide pellets (0.10 g) was weighed and dissolved with little quantity of distilled water in a beaker, and upon complete dissolution, allowed to cool. The solution was then transferred to a 100 mL volumetric flask, and made up to volume with distilled water.

Preparation of *G. kola* extracts

Seeds of *G. kola* were peeled, cut into thin slices and dried in the oven at 50°C for 24 h. The dried seed were afterwards ground into powder with a milling machine. Various extracts were prepared from the powdered seed by following various extraction protocols.

i) Kolaviron (KV): KV was prepared by adapting the method of Iwu (1985) and Olaleye et al. (2000). Powdered seed (1 kg) was defatted exhaustively by cold maceration in *n*-hexane. This was followed by exhaustive extraction by cold maceration in methanol (2 L per cycle). The methanol extract was concentrated (300 mL) and diluted with twice its volume of water. The alcoholic-aqueous mixture was then partitioned into ethyl acetate (6 x 250 mL). The combined ethyl acetate fraction was concentrated by rotary evaporation and dried *in vacuo* at 40°C. The dried extract was stored in a clean beaker.

ii) Acetone seed extract (ASE): The crude extract from which kolaviron was prepared by Olaleye et al. (2000) is the acetone seed extract. Powdered seed (1 kg) was defatted exhaustively by cold maceration in *n*-hexane. The dried defatted marc was exhaustively extracted by cold maceration in acetone (2 L per cycle). The combined acetone extraction was concentrated to dryness by using a rotary evaporator and dried *in vacuo* at 40°C. The dried extract was stored in a clean beaker (ASE).

iii) Ethyl acetate seed extract (EASE): An alternative to solvent-solvent extraction procedure was devised to prevent loss of the biflavonoids to the aqueous phase. Powdered seed (1 kg) was defatted exhaustively by cold maceration in *n*-hexane. This was followed by exhaustive extraction of dried marc by cold maceration in Chloroform (2 L per cycle), which was followed by exhaustive extraction of dried marc by cold maceration in ethyl acetate (2 L per cycle). The combined ethyl acetate extract was concentrated to dryness by using a rotary evaporator and dried *in vacuo* at 40°C. The dried extract was stored in a clean beaker.

CAOCS assay - Photometric Phenolphthalein Assay (PPA)

250 µL increment

Phenolphthalein solution in methanol (0.50 mL, 0.10% w/v) was pipetted into a volumetric flask (5.0 mL). To this was added sodium hydroxide solution in water (1.0 mL, 0.025 M) in order to generate

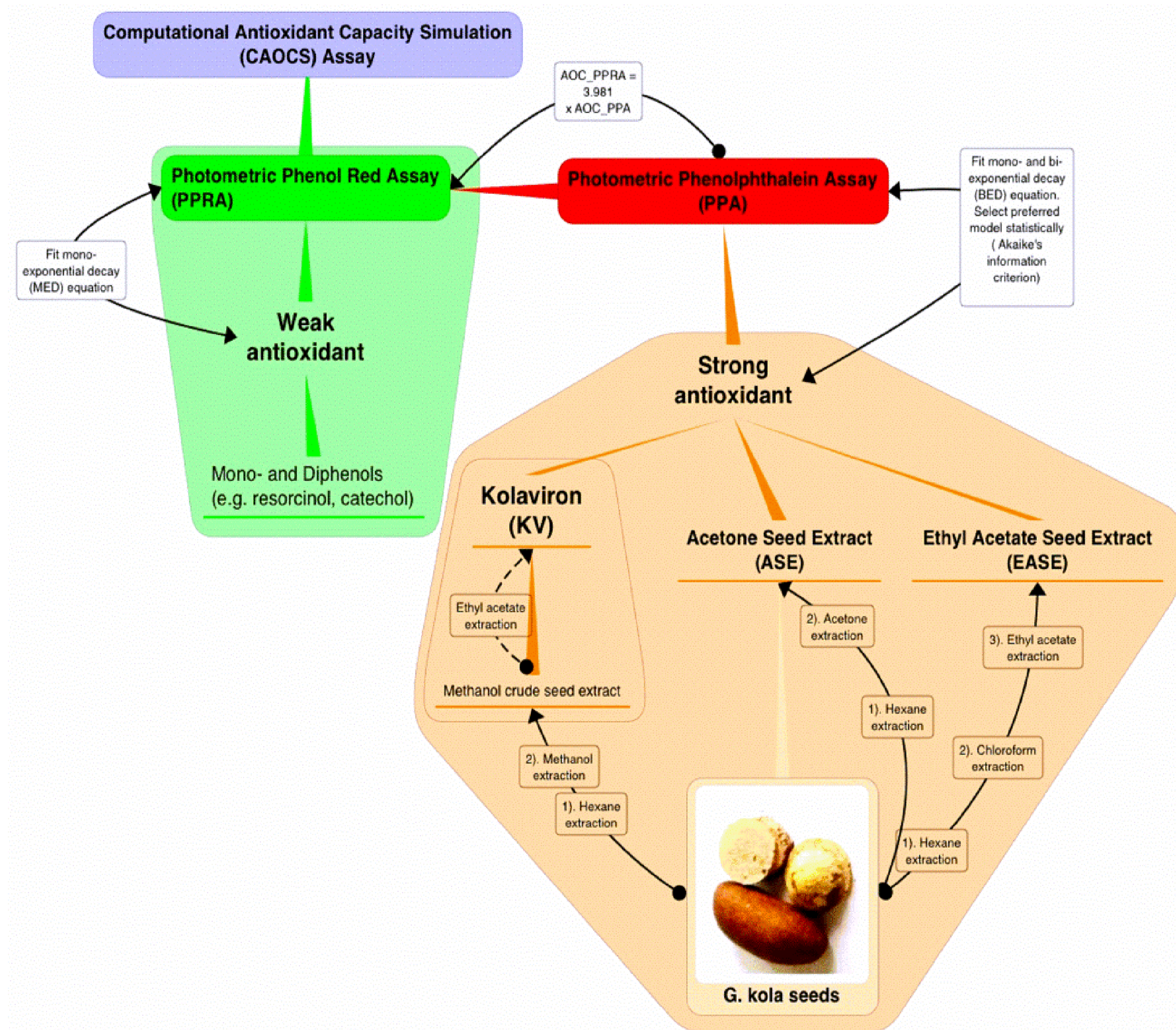

Figure 2. The systematic workflow of two photometric assays (Photometric Phenol Red Assay (PPRA); Photometric Phenolphthalein Assay , PPA) and associated informatics that constitute Computational Antioxidant Capacity Simulation (CAOCS) assay, a bespoke assay for antioxidant capacity profiling of polyphenols and phenol - like compounds. The application of PPA to the assay of *Garcinia kola* seed extracts is depicted in a tree diagram.

the oxidized specie of the probe molecule. This solution was titrated by incremental addition of the test antioxidant (AOX) solution (250 µL increment), up to a maximum of 1.75 mL), and the volume was made to mark with methanol. The absorbance of the solution was measured at 540 nm on a digital colorimeter after each increment. All measurements were performed in duplicate. A plot of absorbance against volume of antioxidant was made and both bi-exponential decay (BED) and mono-exponential decay (MED) model were fitted to the data. The preferred model was statistically selected after fit-comparison, by using Akaike's Information Criterion. This assay is based on proton transfer kinetics modeling (PTKM) (Idowu, 2014).

100 µL increment

Phenophthalein solution in methanol (0.20 mL, 0.10% w/v) was pipetted into a volumetric flask (2.0 mL). To this was added sodium hydroxide solution in water (0.40 mL, 0.025 M) in order to generate the oxidized specie of the probe molecule. The solution was then titrated by incremental addition of the test antioxidant (AOX) solution (100 µL increment) up to a maximum of 0.70 mL (50 µL increment was adopted in some cases for 0.80 and 1.00% w/v standard solutions, when plateau was reached after addition of 0.40 mL, with 100 µL increment), and the volume was made to mark with methanol. The absorbance of the solution was measured at 540 nm

on a digital colorimeter after each increment, using a micro-cuvette. All measurements were performed in duplicate. A plot of absorbance against volume of antioxidant was made as a form of PTKM. Preferred mathematical model was statistically selected between BED and MED, after fit comparison, using AICc.

CAOCS assay of *G. kola* extracts as AOX

i) KV: The assay was performed as described above, by using test solutions of KV in methanol (0.20, 0.30, 0.40, 0.60, 0.80 and 1.00% w/v).

ii) ASE: The assay was performed as described above, by using test solutions of ASE in methanol (0.20, 0.30, 0.40, 0.60, 0.80 and 1.00% w/v).

iii) EASE: The assay was performed as described above, by using test solutions of EASE in methanol (0.20, 0.30, 0.40, 0.60, 0.80 and 1.00% w/v).

Mathematical modeling and statistical analysis

Mono-exponential decay (MED) model

$$Absorbance = Ae^{-kV} + C \quad (1)$$

(A = Span, k = reaction constant, V = volume of antioxidant, C = plateau).

Bi-exponential decay (BED) model

$$Absorbance = A_1 e^{-k_1 V} + A_2 e^{-k_2 V} + C \quad (2)$$

(A_1 = Span 1, A_2 = Span 2, k_1, k_2 = reaction constants, V = volume of antioxidant, C = plateau)

Digital signal processing (DSP)

Digital signal processing of data was performed by using a complex multiplier, K_{cf}, which is different for each data set, to filter out random error:

$$K_{cf} = \frac{\left[\sum_{i=1}^{n} A_i\right] / n}{A_i} \quad (3)$$

A_i = mean of initial absorbance values for each test solution
n = number of test solutions that makes up a family of data sets).

Calculation of AOC (/g)

The AOC metric was computed from the following relationship (Idowu, 2014):

$$Slope = \frac{K_{ptt}\left(mL^{-1}\right)}{Concentration\left(\%, {}^{w}/_{v}\right)} = \frac{mL^{-1}}{g/100mL} = \frac{100}{g} \quad (4)$$

$$AOC = \frac{\left(Slope \times 100\right)}{g}$$

$$AOC_{PPRA} = 3.981 \times AOC_{PPA} \quad (5)$$

Akaike's information criterion

Where there are several competing models, the Akaike's information criterion (AICc) is defined by the model and the maximum likelihood estimates of the parameters, which give the minimum of AICc defined by:

AIC = (-2) Log_{10} (maximum likelihood) + 2 (number of independently adjusted parameters)

The model with the lowest AIC value is the one that fits the data with minimum loss of information, and hence with the highest "probability it is correct", which is thus selected as the preferred model (Akaike, 1974).

All mathematical and statistical analyses were performed by GraphPad Prism version 4.03 for Windows (GraphPad Software, San Diego, CA, www.graphpad.com, 2005).

Preferred model selection by fit comparison

The fit comparison was implemented for the assay carried out with 250 µL increment by using global curve - fitting method. For the BED model, plateau was shared and must be > 0, k_1 and k_2 were constrained and must be > 0. For the MED model, plateau was shared and must be > 0, and *k* was constrained and must be > 0. For the assay carried out with 100 µL increment, global curve-fitting was not adopted, and the difference was that plateau was not shared, though constrained as > 0, while all other constraints were maintained as described for the 250 µL increment assay.

The curve -fitting exercise started with absorbance signal of the first increment, excluding the initial absorbance signal. The model with the highest "probability it is correct" was selected as the preferred model. Regression coefficient, R^2 was computed for each standard solution and a global shared R^2 was computed (where relevant) to reveal goodness -of- fit. In addition, best - fit reaction constant was computed for each standard solution. A linear regression of the reaction constant (k_{ptt}) with concentration of standard solutions produced a slope which was used for computation of the antioxidant capacity (AOC) for the extracts. AOC on PPA platform was converted to the AOC on Photometric Phenol Red Assay (PPRA) by a specific conversion factor.

Thin layer chromatographic analysis of *G. kola* extracts

The composition of the three extracts was examined by performing thin layer chromatographic analysis on the extracts. Methanolic solution (2 µL, 0.2% w/v) of each sample was spotted on thin layer chromatographic plates, stationary phase is silica gel, (10 × 5 cm, 0.2 mm, GF_{254}), and mobile phase is ethyl acetate: *n*-hexane (65:35). The plates were spotted 1 cm apart at the origin, which is 1 cm from the base and allowed to run a 7 cm path. The developed plates were air dried, and visualized under UV lamp short wavelength, 254 nm.

RESULTS AND DISCUSSION

PTKM of data obtained from 250 µL increments, and computation of AOC for KV is as displayed in Figure 3A, and the corresponding model parameters are shown in Table 1a. MED was shown to be the preferred model,

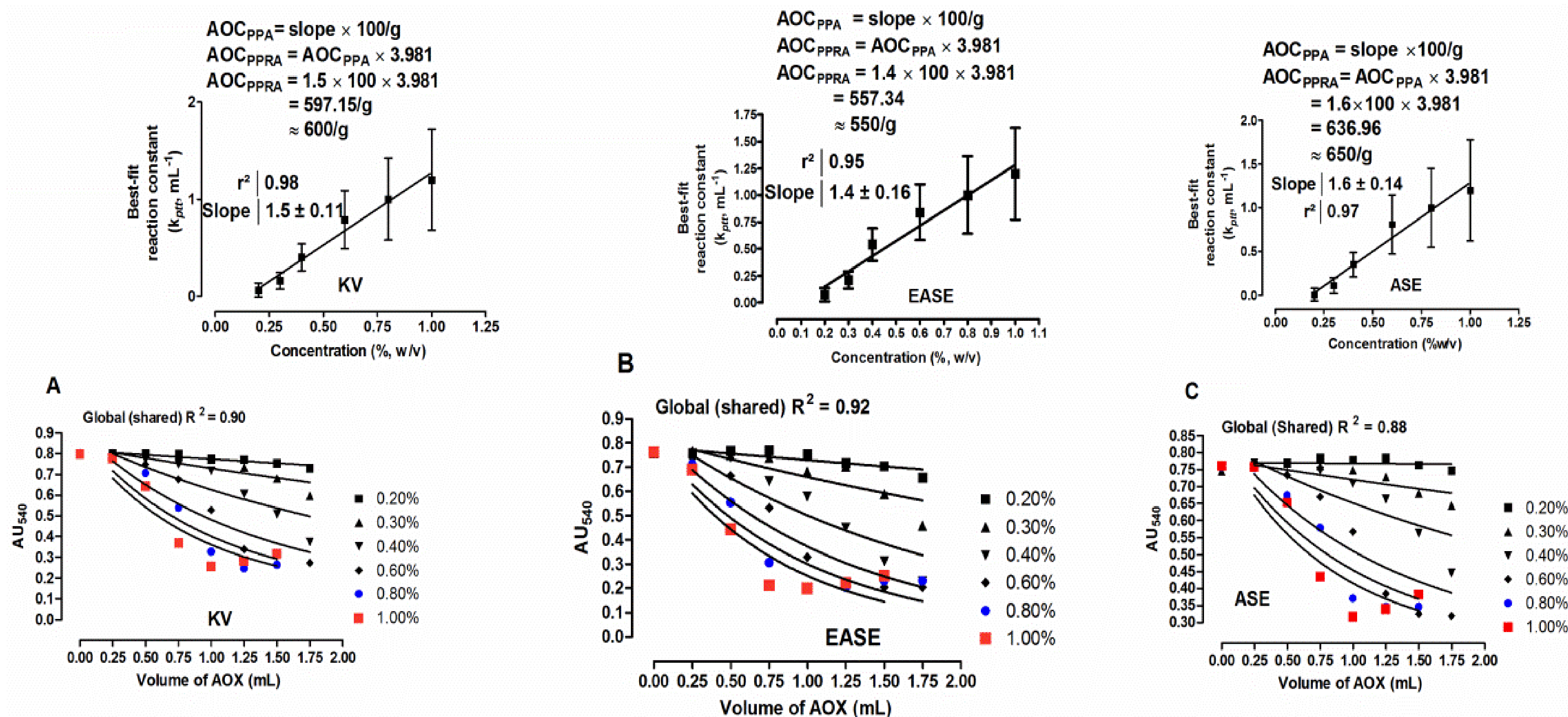

Figure 3. Graph of kinetic data from standard solutions of A) KV, B) EASE, and C) ASE on PPA showing concentration-dependent response obtained from 250 µL incremental addition of antioxidant solution preferably fitted by mono-exponential decay (MED) and the associated computation of antioxidant capacity. Assay sensitivity is diminished by similarity of the best-fit reaction constant for 0.8 and 1.0% AOX solutions across the three seed extracts. Potency ranking follows the sequence: ASE > KV > EASE.

with a global R^2 value of 0.90, computed AOC value of 597.15/g, reported to the nearest fifty as 600/g. PTKM and computation of AOC for EASE are displayed in Figure 3B, and the model parameters are shown in Table 3a. MED was shown to be the preferred model, with a global R^2 value of 0.92, computed AOC value of 557.34/g, reported to the nearest fifty as 550/g. Also, the PTKM and computation of AOC for ASE are displayed in Figure 3C, and the model parameters are shown in Table 2a. MED was found to be the preferred model, with a global R^2 of 0.88, computed AOC value of 636.96/g, reported to the nearest fifty as 650/g.

The data set obtained by 100 µL increments afforded a more sensitive assay. PTKM with MED fit, and AOC computation for KV is displayed in Figure 4A, and the model parameters are shown in Table 1b. Computed AOC value is 1154.49/g, reported to the nearest fifty as 1150/g.

PTKM, with preferred MED fit and AOC computation for EASE is displayed in Figure 4B, the model parameters are shown in Table 3b, computed AOC value is 1512.78 /g reported to the nearest fifty as 1500 /g. Similarly, PTKM, with preferred MED fit and AOC computation for ASE is displayed in Figure 4C, the model parameters

Table 1. Model parameters for CAOCS assay (PPA) of KV from a fit of classic mono-exponential decay (MED) using a) 250 µL and b) 100 µL increments.

Concentration (%, w/v)	**Best-fit values**	
(a)*250 µL increment**	**Proton-transfer reaction constant (k_{ptt}, mL^{-1}± S.E)**	**Regression Coefficient (R^2)**
0.2	0.062 ± 0.074	0.86
0.3	0.16 ± 0.086	0.72
0.4	0.40 ± 0.140	0.78
0.6	0.79 ± 0.30	0.85
0.8	1.00 ± 0.42	0.88
1.0	1.20 ± 0.52	0.82
(b)*100 µL increment***		
0.2	0.22 ± 0.62	0.98
0.3	0.75 ±1.80	0.86
0.4	1.20 ± 2.20	0.83
0.6	1.80 ± 1.50	0.93
0.8	2.00 ± 3.40	0.87
1.0	2.70 ± 3.10	0.88

*Global shared parameters:
R^2 = 0.90, Plateau = 0.13 ± 0.15; Absolute sum of squares = 0.19, $S_{y \times x}$ = 0.076, Constraints: k > 0; plateau > 0 and shared.

**Constraints: k >0; plateau >0; preferred model = MED, >99.99% probability it is correct.

Table 2. Model parameters for CAOCS assay (PPA) of ASE from a fit of classic mono-exponential decay (MED) using a) 250 µL and b) 100 µL increments.

Concentration (%, w/v)	**Best-fit values***	
(a)*250 µL increment**	**Proton-transfer reaction constant (k_{ptt}, mL^{-1}± S.E)**	**Regression Coefficient (R^2)**
0.2	0.0054 ± 0.077	0.019
0.3	0.11 ± 0.089	0.63
0.4	0.35 ± 0.14	0.72
0.6	0.81 ± 0.34	0.82
0.8	1.00 ± 0.45	0.86
1.0	1.20 ± 0.58	0.85
(b)*100 µL increment***		
0.2	0.081± 5.10	0.44
0.3	0.29 ± 2.80	0.73
0.4	0.72 ± 2.50	0.78
0.6	1.30 ± 2.20	0.86
0.8	1.80 ± 2.40	0.87
1.0	2.10 ± 3.20	0.87

*Global shared parameters:
R^2 = 0.88; Plateau = 0.24 ± 0.13; Absolute sum of squares = 0.15; $S_{y \times x}$ = 0.066

**Constraints: k >0; plateau >0; preferred model = MED, > 99.99% probability it is correct.

are shown in Table 2b, computed AOC value is 1035.06 /g, reported to the nearest fifty as 1050 /g.

Thin layer chromatogram for the three *G. kola* extracts is as displayed in Figure 5. Spotting equal volume of identical solution concentration for each extract allowed a rough estimate of the relative composition of the extracts

Table 3. Model parameters for CAOCS assay (PPA) of EASE from a fit of classic mono-exponential decay (MED) using a) 250 µL and b) 100 µL increments.

Concentration (%, w/v)	Best-fit values*	
(a)*250 µL increment**	**Proton-transfer reaction constant (k_{ptt}, mL^{-1}± S.E)**	**Regression Coefficient (R^2)**
0.2	0.073 ± 0.064	0.68
0.3	0.21 ± 0.08	0.72
0.4	0.54 ± 0.15	0.85
0.6	0.84 ± 0.26	0.91
0.8	1.00 ± 0.36	0.90
1.0	1.20 ± 0.43	0.88

**Global shared parameters:*
R^2 = 0.92; Plateau = 0.011± 0.13; Absolute sum of squares = 0.19, $S_{y\times x}$ = 0.074, Constraints: k > 0, plateau > 0 and shared.

(b)*100 µL increment***		
0.2	0.31 ± 1.40	0.91
0.3	0.88 ± 1.90	0.86
0.4	1.50 ± 2.00	0.86
0.6	2.20 ± 1.80	0.90
0.8	2.60 ± 1.70	0.90
1.0	3.50 ± 3.30	0.87

**Constraints: k >0; plateau >0; preferred model = MED, 99.99% probability it is correct.

with respect to individual compounds that constitute the biflavonoid fraction. EASE was shown to contain trace amounts of the front - running compound, which is present in significant amounts in the other two extracts. It is apparent that this compound, being the most lipophilic, has been largely removed by chloroform which was used to extract the defatted powdered seed sample and discarded, before obtaining the ethyl acetate fraction that was labelled EASE (Figure 2).

Polypharmacological effect profile of *G. kola* biflavonoid extracts

Polypharmacology is a concept that reflects the high complexity in the mechanisms of action of drugs. It underscores the multitarget nature of interactions that describes the effect profile of many drugs. Correlation between interaction profiles of drugs with target proteins and clinical effect profile of drugs was shown to validate this concept (Simon et al., 2012). Furthermore, it is an emerging paradigm in drug discovery that suggests that more effective drugs can be developed by specific modulation of multiple targets (Anighoro et al., 2014). The various bioactivity profile exhibited by *G. kola* extract, and the various underlying mechanisms corroborates multitarget interactions. In addition, the extracts consist of at least 4 structurally similar polyphenol compounds (Figures 1 and 5). Good TLC resolution of the constituents, however, underscores the differences that yet exist in their overall physicochemical properties. This would imply variations in their binding affinities with target proteins and contribute to synergism in the effect profile. This broad effect profile justifies the development of the extract as a nutraceutical, which is intended for disease prevention and health promotion. It is thus well documented that the overall effect profile of *Garcinia* biflavonoids (KV and its variants) comprises both antioxidant and non - antioxidant mechanisms.

Quality assurance requirements for nutraceuticals

Manufacturing of ANI requires application of quality assurance principles as enunciated by Good Manufacturing Practice:

> "To assure batch uniformity and integrity of dietary products, written procedures shall be established and followed that describe the in-process controls, and tests, or examinations to be conducted on appropriate samples of in-process materials of each batch" (TABD-DSEG, 2002).

Several techniques have been applied to the analysis of nutraceuticals including, chromatographic, spectroscopic and hyphenated techniques (Bernal et al., 2011). Optimization of pressurized liquid extraction has been reported for the extraction of zeaxanthin from *Chlorella ellipsoidea,* (Koo et al., 2012), and the extraction of

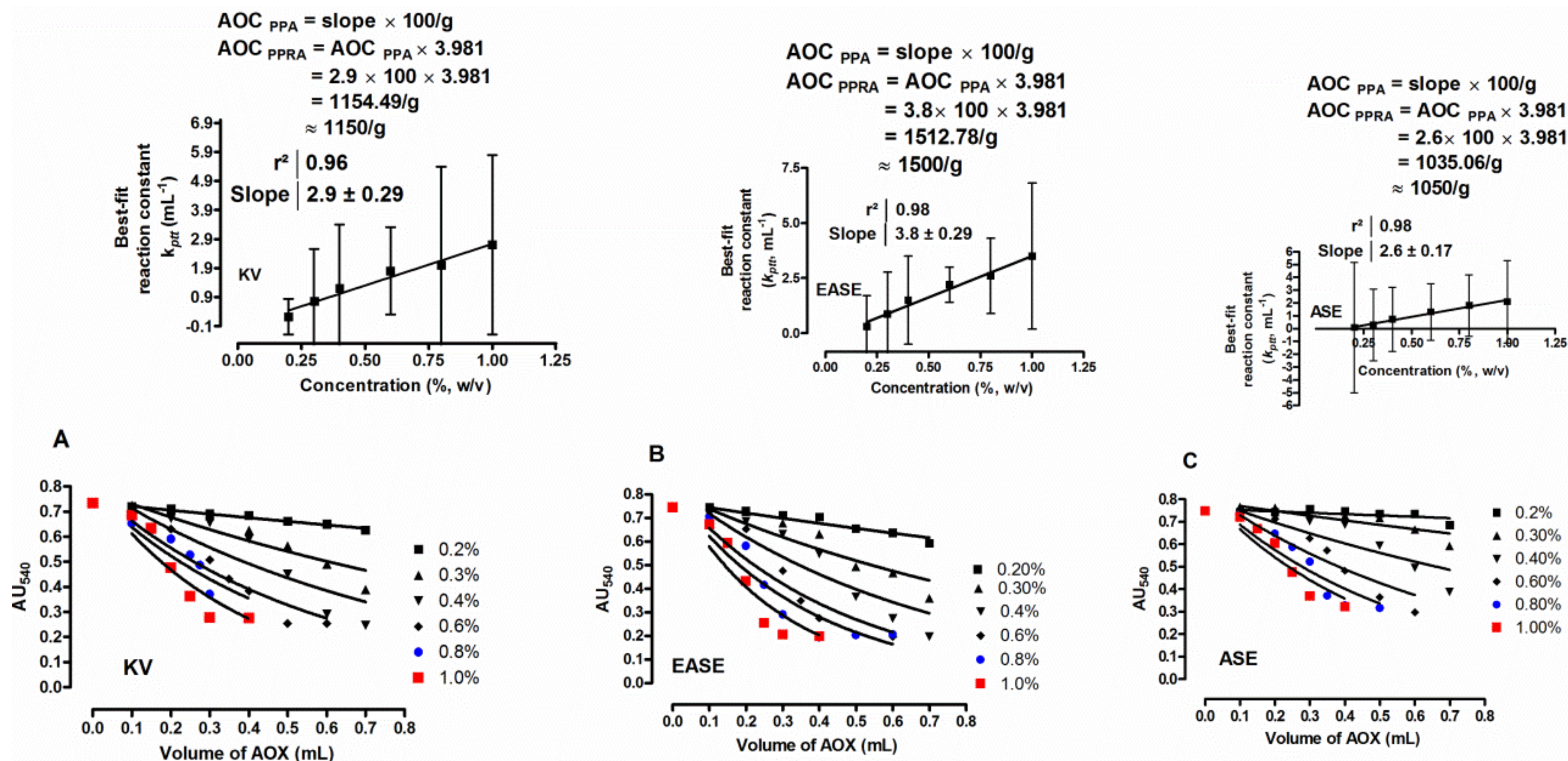

Figure 4. Graph of kinetic data from standard solutions of A) KV, B) EASE, and C) ASE on PPA, showing concentration-dependent response obtained from 100 µL incremental addition of antioxidant, preferably fitted by MED and the associated computation of antioxidant capacity. Assay sensitivity is enhanced by significant difference between the best-fit reaction constant for 0.8 and 1.0% AOX solutions across the three seed extracts. Potency ranking follows the sequence: EASE > KV > ASE.

anthocyanins and biflavonoids from *Schinus terebinthifolius* Raddi (Feuereisen et al., 2017). The optimized variables that influence the yield are; extraction time, extraction temperature, and solvent.

The individual *Garcinia* biflavonoids have been isolated by counter-current chromatography (Okunji et al., 2007) for investigation as tyrosinase inhibitor, but there was no quantitative estimation of the extract as a whole. The individual biflavonoids were also isolated in order to investigate their relative antimalarial potency against strains of *Plasmodium berghei* infected mice and *Plasmodium falciparum*, *in vitro* (Konziase, 2015), but there was no quantitation of the whole extract in this study, either. Derbre et al. (2014) reported a total polyphenol assay of an ethanol extract of *Garcinia* biflavonoids by high performance liquid chromatography (HPLC) method with UV detection. The extract reportedly contains 48% biflavonoids (reported as equivalent

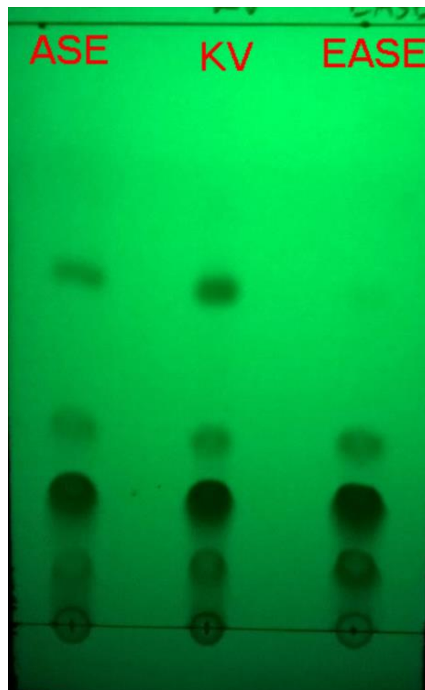

Figure 5. Thin layer chromatogram of ASE, KV and EASE where 2 µL of 0.2% w/v methanolic solution of each sample was spotted. Stationary phase is silica gel, 10 × 5 cm, 0.2 mm, GF_{254}; mobile phase is ethyl acetate: hexane (65:35) and visualized under UV lamp short wavelength (254 nm). EASE is shown to contain only traces of the front running compound (most non-polar component), which is present in significant amount in the other two samples. Various solvent extraction protocols obviously result in some variation in composition and relative amounts of constituents.

of the pure GB 2, used as an external standard). It was shown that the biflavonoids are the predominant components of the ethanol extract. Other compounds present in trace amounts relative to the biflavonoids were detected by HPLC – MS. These include; alpha acids, humulinone, garcinoic acid and polar compounds identified as polyols. The biflavonoid extract was used to formulate cosmetic cream demonstrated to have anti-glycation effect. To the best of our knowledge, there is no previous report addressing the quantitative evaluation of the biflavonoid extract, using the "total chemistry" of the whole extract, for the purpose of standardizing the extraction protocol, towards preparation of a quality-assured nutraceutical.

In addition to thin layer chromatography fingerprinting which reveals the composition of the biflavonoid constituents, quantitative assay is valuable to confirm batch-to-batch sameness. CAOCS assay, which is based on the total (phenolic bond cleavage) chemistry of the biflavaonoid extracts, is a suitable quality assurance procedure. The relative potency of the three extracts was evaluated to guide extraction protocol optimization, and development of standard operating procedure (SOP) for manufacturing ANI. MED was found as the preferred model for fitting the absorbance decay data for the three extracts (Tables 1 to 3). The potency of *G. kola* seed extracts determined by CAOCS assay and AOC reported to the nearest fifty was shown to be inversely related to the size of the photometric titration increment.

Assay sensitivity and result accuracy

When 250 µL increment was used for the assay, the ranking obtained was ASE > KV > EASE, with AOC of 650, 600, and 550/g respectively. The values are generally below 1000/g, although the 650/g obtained for ASE is a significant increase from 300/g obtained for the same extract, when 1000 µL increment was used for the assay in a previous study (Idowu et al., 2009). The sensitivity of the assay was diminished by the fact that the best-fit reaction constant (k_{ptt}) of 0.80 and 1.00% standard solutions was similar for the three extracts (Tables 1 to 3a).

On the other hand, when a smaller increment (100 µL) was used for the assay, the ranking obtained was EASE > KV > ASE with AOC of 1500, 1150, and 1050/g respectively. The assay sensitivity was enhanced by a significant difference between the k_{ptt} of 0.80 and 1.00% standard solutions for the three extracts (Tables 1 to 3b) and a higher slope of the linear regression of k_{ptt} and concentration of standard solutions, across the board (Figure 4A-C).

In summary, the use of 100 µL increments gave the optimal, more sensitive and more accurate results. The ranking obtained; EASE > KV > ASE is corroborated by domain knowledge about the preparation of the extracts. ASE is a crude acetone extract of the whole seed hence it has the least biflavonoid concentration. KV is ethyl acetate fraction of a crude extract like the ASE and is therefore more concentrated in biflavonoids, especially because of the medium polarity of ethyl acetate that favours solubility of the polyphenols. In contrast to these two extracts, the preparation of EASE avoided solvent - solvent extraction protocol involving an aqueous phase altogether. The highest yield of biflavonoids in this extract is partly due to the fact that loss of polyphenols through their partial solubility in water was completely avoided, and the relative amount of lipophilic non-biflavonoid constituents are much lower, having been removed by chloroform. Our findings are thus shown to be consistent with our hypothesis.

Conclusion

Qualitative evaluation of extract purity and composition

suggested that some variations exist in the relative amounts of polyphenols in the biflavonoid fraction of *G. kola* seed obtained by different solvent extraction protocols. The difference in extraction efficiency and composition of extracts was corroborated by quantitative estimate of potency, which evaluated the “total chemistry” of biflavonoids, with respect to kinetics of phenolic bond cleavage. The use of a smaller increment (100 µL rather than 250 µL), unambiguously, led to enhanced assay sensitivity and accuracy of results. A multivariate optimization studies guided by CAOCS assay, to maximize biflavonoid extraction yield is ongoing in our laboratory.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interest.

ABBREVIATIONS

AICc, Akaike's information criterion; **ANI,** active nutraceutical ingredient; **AOC,** antioxidant capacity; **AOX,** antioxidant; **ASE,** acetone seed extract; **BED,** bi-exponential decay; **CAOCS,** computational antioxidant capacity simulation; **DSP,** Digital signal processing; **EASE,** ethyl acetate seed extract; **KV,** kolaviron; **MED,** mono-exponential decay; **PPA,** photometric phenolphthalein assay; **PPRA,** photometric phenol red assay; **PTKM,** proton transfer kinetics modeling; **TLC,** thin layer chromatography.

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