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Full Length Research Paper

Physico-chemical and bacteriological characterization of sachet water sold in Yaounde City, Cameroon

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The objective of this study is to take an inventory of all the sachet water brands sold in Yaounde and to assess their sanitary quality. This study was carried out on 100 samples, representing 20 sachet water brands sold in the urban city of Yaounde, Cameroon. Selected physico-chemical and bacteriological analyses of the sachet water were done. The results showed that their pH values and nitrate level were in line with the WHO guideline values. The chloride ion contents were significantly lower than the limit values for drinking water, *Escherichia coli* was absent in all brands and 75% of the samples contained intestinal *enterococci*. Sachet water brands can be grouped into 3 homogeneous classes based on *streptococcal* contamination, chloride and nitrate content, and the level of pH. Packaging material and storage conditions (exposure to sun) can aggravate this contamination. Sachet water can be contaminated and become undrinkable.

Key words: Sachet water quality, potability, Yaounde, Cameroon, physico-chemical parameters, bacteriological analyses.

INTRODUCTION

Water is a source of life due to its indispensable characteristics and the place it occupies in our daily life. Accessibility and availability of fresh clean water is a key to sustainable development and an essential element for health, food production and poverty reduction (Adetunde and Glover 2010). Water is a widely consumed commodity, hence it is necessary to study its potability. Because

of its affordable price (5 and 10 CFAF in Ivory Coast and 50 CFAF in Cameroon), its availability and accessibility, water packaged in sachets is highly consumed. Several brands from drinking water production companies (legal or illegal) have thus emerged in several big cities in Africa. But this abundance is not without problems, particularly in terms of quality of products. Lack of

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hygiene and the questionable quality of these waters mean that the risk of water-borne infections is serious and frequent. The consumption of soiled water most often begins gastroenteritis or even dysentery, typhoid fever and cholera (Mossel et al., 1993; WHO, 1994). According to WHO (1994) and PNUD (1990), 80% of diseases in the world are waterborne, and drinking water is very often responsible for this. In developing countries, more than 25 million people die every year due to consumption of contaminated water (GRET, 1994).

With this situation, some studies have been conducted to assess the health risk, quality and potability of these waters distributed in some African big cities (Akely, 1994; Samake, 2002). In four municipalities of Abidjan, N'diaye (2008) and Aboli et al. (2007) conducted a bacteriological study of drinking water sold in sachets. These studies highlighted the existence of some pathogenic and non-pathogenic germs (*Enterococcus faecalis*, *Escherichia coli*, *Pseudomonas* sp., *Staphylococcus* sp. and *Salmonella enterica* serovar *typhimurium*), indicators of faecal and environmental contamination of sachets water. The analyses also revealed that sachet water offered to consumers is of low quality. In a similar study, Kouadio et al. (1998) studied the potability of drinking sachet water sold around the public primary schools of Abidjan and they observed that all the samples collected had poor microbiological and chemical quality. Rutz (1996) reported that sachet water vending machine may not be free of microorganisms, because a bacterium, *Streptococcus faecalis* was isolated from sachet water producing machines. Obiri-Danso et al. (2003), who examined the microbiological quality of sachet drinking water and bottled water sold in the streets of Kumasi, Ghana, concluded that bottled water sold in the Ghanaian market is of good microbiological quality. Hoteyi et al. (2014) analyzed the risks of consuming sachet water in the city of Porto-Novo in South Benin. This study showed that sachet water produced from borehole and traditional wells contained 95% pathogens while that from tap had 5% pathogens. The borehole and traditional wells tested samples had high contents of nitrates ranging from 75 to 97 mg/l concentrations.

Looking at the recurrence of the diarrheal diseases and serious health risks due to the consumption of sachet water, an inventory of all the water sachet brands sold in the city of Yaounde, Cameroon was taken and their sanitary quality was assessed. More specifically, a survey of water sachet sellers or producers, collection of various sachets water brands sold in the markets of Yaounde, as well as the study of the potability of the collected samples was done.

MATERIALS AND METHODS

Collection of samples

Different sachet water brands available in the main markets of the city of Yaoundé were collected from the vendors. In the main

markets of the 7 subdivisions of Yaoundé (Etoudi, Mokolo, Mvog-Mbi, Mvog-Ada, Essos, Acacia and Nkolbisson), all sachet water brands available were sampled. The analyses covered 100 water samples corresponding to 20 sachet water brands (5 samples per brand). The vendors were also interviewed on their sources of supply and the storage conditions of the sachet water.

Physicochemical analysis

Some physico-chemical parameters of the sachet water were analyzed at the soil Laboratory of the Institute of Agricultural Research for Development (IRAD), which is certified ISO 12025. The pH was determined using the potentiometric method. The quantities of nitrates (NO_3^-) were determined by the automated colorimetric method (ISO 7890-3 December, 1998). Chloride was measured by titration with silver nitrate according to AFNOR NF ISO 9297.

Microbiological analysis

Two types of microbiological analysis were carried out at the 'Centre Pasteur Laboratory' of Yaounde, Cameroon which is also certified ISO 12025. They are:

1. The coliform bacteria analysis: Enumeration of *Escherichia coli* through membrane filtration method according to NF ISO 9308-1 norm.
2. The Intestinal Enterococci analysis: The membrane filtration method according to NF ISO 7899-2 norm was used for the enumeration of the faecal coliforms.

Statistical analysis

The statistical analysis was done using three soft wares: 1. EXCEL 2010: Descriptive statistic; 2. Graphpad Prism 5.0: Variance analysis (ANOVA); and 3. XL STAT 2015 for Hierarchical Ascending Classification (HAC) and Principal Components Analysis (PCA). Descriptive statistics helped to graphically illustrate changes in physico-chemical parameters for the different brands surveyed. Analysis of variance (ANOVA) was used to assess the significance of changes in the physico-chemical parameters for the different brands studied. Factorial analysis of the different sachet water brands and their physico-chemical parameters allowed the grouping of the water brands into homogeneous classes. The groupings, according to the degree of similarity (Pearson correlation coefficient), were performed using a hierarchical clustering (Dendrogram). The representation of sachet water brand and physico-chemical parameters on a multi-factorial design was carried out by a principal component analysis (PCA). PCA was performed according to average values of the physico-chemical parameters measured for each of the brands. The proximity between the different brands was shown.

RESULTS

Inventory of the different brands of sachet water sold in the markets of Yaoundé

In Yaoundé City, a total of 20 brands of sachet water were recorded: Crys Water, Alpha, Omega, Aquarrel, Faro Nostra, Max water, Roi, Kanadienne, Charone, Eau de montagne, Golden, Equina, Bonatura Water, Pomi

Water, Bonheur, Natural, Ilma, Boni Water, Prince and Lerex Water. The large number of sachet water brands shows that markets are areas of great affluence where there are several economic activities and where the need to drink is needed. Sachet water sellers are unaware of the chain of production, their role being limited only to sales. For further work, and for confidentiality reasons, the various water sachet brands were coded.

Physical description of sachet water brands

The physical examination of the sachet water allowed us to notice the absence of some conventional information which must appear on food package, namely the manufacturing date and the serial number which did not appear in any brand of the sachet water. The absence of the serial number on the sachet water made it difficult to respect the FIFO (First In, First Out) principle which is essential in the food industries. Ten brands out of 20 had an expiration date, and only 6 brands out of 20 had an operating license. Some bits of information concerning the volume and physico-chemical parameters were wrong.

The physical characterization of the sachet water, like the absence or presence of an operating license made us to group the water into semi-industrial and artisanal types. Out of the 20 brands collected, 6 were of a semi-industrial operation (SW-18, SW-13, SW-12, SW-7, SW-4 and SW-3), and 14 of an artisanal type (SW-1, SW-2, SW-5, SW-6, SW-8, SW-9, SW-10, SW-11, SW-14, SW-15, SW-16, SW-17, SW-19 and SW-20).

The absence, insufficiency, or the wrong character of the information contained in certain sachet water collected may reflect a fraudulent activity that takes place without any regulatory compliance.

Conservation of sachets water during marketing

In general, the average duration of evacuation of 100 sachets water varied from 1 to 2 days depending on whether it is raining or dry season. According to the actors, types of conservation varied. The itinerant vendors kept them essentially in buckets and coolers (60%) while vendors who had shops kept them in refrigerators (40%).

These pre-sale conservation conditions, which in most cases were unsuitable (exposed in buckets or coolers), were likely to favour certain biological reactions (microbial proliferation and container-contents migration) which may reduce the sanitary quality of sachet water.

Physico-chemical and bacteriological characterization of sachet water

Some physico-chemical parameters of pollution were analysed, namely pH, contents of chlorides and nitrates

ions (Table 1). The pH of the different water sachets varied between 6.5 and 8.5. Their chloride contents were between not detectable and 3.2 mg/l. There was no significant difference between the semi-industrial and artisanal type. Fifty percent of the water brands were not detectable for chloride ions.

The sachet water nitrates levels were between not detectable and 18 mg/l. Seventy percent of the water brands had also zero nitrates content, against 40% of brands which had positive values but remained largely lower than 50 mg/L.

The bacteriological analysis of the sachet water showed high faecal Enterococci contamination levels. In fact, 14 out of 20 brands were contaminated with levels varying between 2 and 56 CFU/100 ml.

The distribution of the sachet water contamination by faecal Enterococci varied according to the type, be it semi-industrial or artisanal (Table 2): Sixty-seven percent contamination was from the semi-industrial type against 71.5% contamination from the artisanal type.

Statistic interpretation of results

Analysis of variance of factors

The analysis of variance of two factors revealed highly significant p values (<0.05), indicating a significant variation in the physico-chemical and bacteriological parameters for the different sachet water brands. Variation in the parameters themselves for the different brands was also observed and 99% chance of the identified sachet water brands was very different.

Classification of different sachet water brands

Hierarchical ascendant classification

The dendrogram in Figure 1 illustrates the hierarchical ascending classification of different sachet water brands according to the evaluated physicochemical and microbiological parameters. The dendrogram was created on the basis of similarities between these quality parameters according to Pearson correlation coefficient.

The discriminant analysis of the dendrogram revealed that the different identified brands can be grouped into 3 homogeneous classes. The first class comprised 9 brands: WS-1, WS-8, WS-3, WS-19, WS-16, WS-6, WS-12, WS-4 and WS-7; the second class included 4 brands: WS-17, WS-2, WS-13 and WS-15 and finally, the third class included 7 brands: WS-11, WS-14, WS-20, WS-5, WS-18, WS-9 and WS-10. These classes comprised brands with close or similar physico-chemical and microbiological parameters.

Principal component analysis

Figure 2 illustrates the principal component analysis of

Table 1. Summary sheet of physicochemical and bacteriological analysis results of sachets water.

S/N	Water brands	Faecal coliforms (<i>E. coli</i>) (VG = 0/100 ml)	Faecal streptococci (Enterococci) (GV = 0/100 ml)	pH (6.5<GV< 8.5)	Nitrate (GV = 50 mg/l)	Chloride (GV = 250 mg/l)
1	SW- 1	nd	02±00	7.5±0.12	nd	2.12±0.04
2	SW-2	nd	02±00	7.5±0.04	18±1.1	nd
3	SW-3	nd	nd	7.7±0.10	nd	0.71±0.22
4	SW-4	nd	02±00	7.5±0.02	nd	0.71±0.01
5	SW-5	nd	52±02	7.24±0.03	nd	3.19±0.05
6	SW- 6	nd	nd	7.47±0.01	nd	Nd
7	SW-7	nd	02±00	7.81±0.05	nd	nd
8	SW-8	nd	01±00	7.43±0.02	nd	2.13±0.02
9	SW-9	nd	24±01	7.29±0.07	nd	nd
10	SW-10	nd	20±02	8.23±0.06	nd	nd
11	SW- 11	nd	11±01	7.87±0.90	nd	nd
12	SW-12	nd	nd	7.62±0.40	nd	nd
13	SW-13	nd	03±00	7.58±0.20	12±0.7	2.13±0.08
14	SW-14	nd	06±01	7.06±0.06	nd	2.29±0.07
15	SW-15	nd	2±00	6.73±0.07	10±	nd
16	SW- 16	nd	nd	7.5±0.04	nd	nd
17	SW-17	nd	nd	7.02±0.05	8±0.9	2.29±0.10
18	SW-18	nd	56±02	8.3±0.06	4±0.6	nd
19	SW-19	nd	nd	7.5±0.19	nd	nd
20	SW-20	nd	17±01	7.58±0.07	9±0.8	2.49±0.09

GV, Guide value; nd, not detectable.

Table 2. Distribution of the contamination of sachets water by faecal Enterococci.

Type of manufacturing	Contamination	Number	Percentage
Semi-industrial (N=06)	Positive	4	67.0
	Negative	2	33.0
Artisanal (N=14)	Positive	10	71.5
	Negative	4	28.5

sachet water brands based on the physicochemical and microbiological parameters measured. It is the representation of the water brands as well as the quality parameters evaluated in a multi-factorial plan. The graph thus revealed that some brands were characterized by a high content of streptococci (WS-11, WS-14, WS-20, WS-5, WS-18, WS-9 and WS-10) and showed that all these brands were those of class 3 in the dendrogram. A second group of brands was characterized by similar contents of chlorides and nitrates (WS-17, WS-2, WS-13 and WS-15); all these brands belong to class 2 in the dendrogram. Finally, one last group of brands was characterized by close levels of pH (WS-1, WS-8, WS-3, WS-19, WS-16, WS-6, WS-12, WS-4 and WS-7). These brands were those of class 1 in the dendrogram.

However, the classification of water according to their quality parameters (physicochemical and bacteriological characteristic) made us to notice that waters of class 3 were the most unfit for consumption.

DISCUSSION

Water is the basic daily beverage of human beings. The human body needs daily and generous amount of water to function properly and avoid dehydration. Unfortunately, this water so important to life is not always safe for consumption. When it is of poor quality, it can affect our health and eventually the human life (Livre bleu de Belgaqua, 1998). The concentration of certain physico-

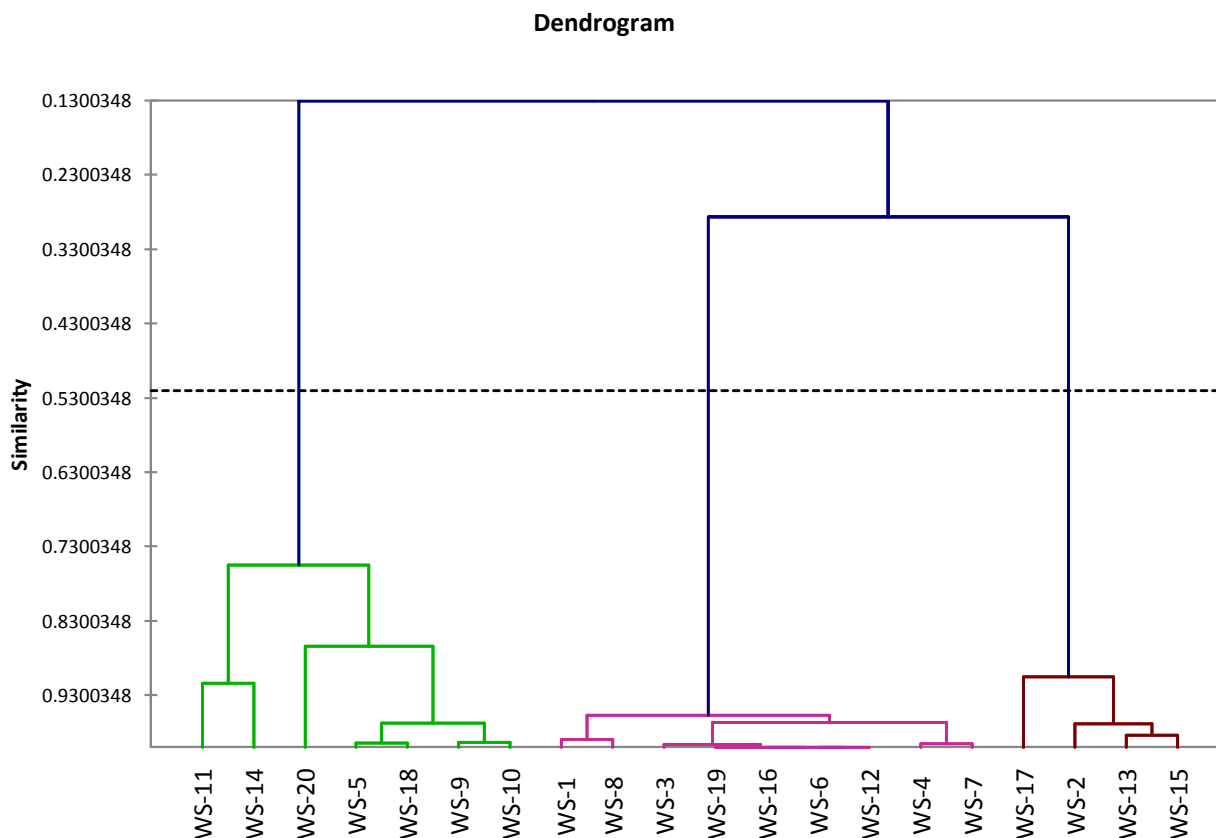


Figure 1. Hierarchical ascending classification (HAC) of water brands based on physicochemical and microbiological parameters measured.

chemical parameters or the presence of certain germs in water can serve as factors of pollution.

pH is one of the most operational water quality parameters, thus it needs to be controlled in all stages of water treatment to ensure water clarification and disinfection. According to WHO (1994), pH values for drinking water should range between 6.5 and 9.5. This range is in conformity with the study results (7.02 - 8.3) as it fell within the WHO drinking water-guideline. In addition, these sachet water brands are unlikely to cause health problems such as acidosis (Asamoah and Amarin, 2011).

Chlorides are important inorganic anions contained in variable concentrations in natural water usually in the form of salts of sodium (NaCl) and potassium (KCl); an excess amount of which, if taken over a period of time, can constitute a health hazard (Oyelude and Ahankorah, 2012). Chlorides are often used as pollution index in surface and ground waters (WHO, 2000) and high concentrations may result in taste problems (APHA, 1998). WHO (1986) recommended 250 mg/l as the maximum chloride ion level allowable in drinking water. This guide value helps to conserve the water aseptic. The chloride content found in the sachet water was lower (between 0 and 3.19 mg/l) than that of the WHO

guideline. This is as a result of the sachet water being exposed to sunlight during sales. Solar radiations promote the conversion of chlorine into inactive chloride ions. Moreover, solar heat, by raising the temperature of the sachets favours microbial growth (Kouadio et al., 1998).

At elevated concentrations, nitrate ion is known to result in cyanosis in infants. Nitrates constitute the final stage of the oxidation of nitrogen and represent the form of nitrogen in the highest degree of oxidation in water. Nitrates can be at the origin of the formation of nitrites and nitrosamines, responsible for two potentially pathological phenomena: The methemoglobinemia and the risk of cancer disease (WHO, 2000). Except for 6 sachet water brands (WS-2, WS-13, WS-15, WS-17, WS-14 and WS-20), there was no nitrates in the other sachet water brands analysed (14 brands). The measured contents remained well below the threshold for drinking water according to WHO standards (50 mg/L). This indicated that the water studied was not subjected to nitrate pollution.

Faecal coliforms, such as *Escherichia coli*, are indicative of faecal contamination in drinking water (Bourgeois et al., 1990; WHO, 2000; N'diaye, 2008). The presence of *E. coli* in drinking water reflects in most cases the

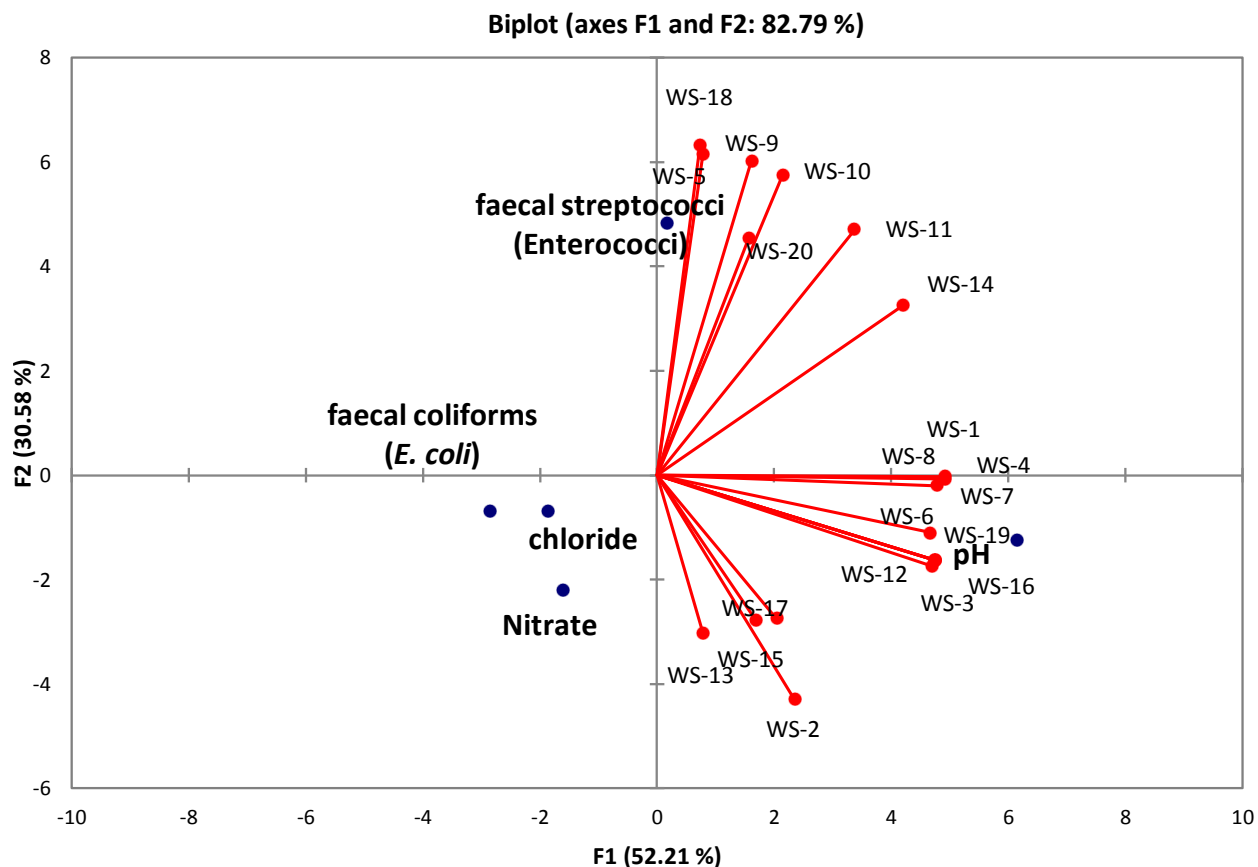


Figure 2. Principal component analysis of water brands according to the physicochemical and bacteriological parameters.

existence of risk of the presence of enteric pathogenic micro-organisms (Quebec, 2004). WHO (2000) Standard recommends 0 CFU/mL of faecal coliforms in drinking water. However, the bacteriological analysis of sachet water showed a strong contamination by the faecal coliforms (70%) and a complete absence of contamination of sachet water by *E. coli*. The presence of these coliforms in the water revealed either an ineffective treatment or a contamination after treatment due to lack of hygienic care. Similar results were obtained by Navou (2000), Diop (1995), and Degbey (2009) when studying the quality of drinkable water in Bobo-Dioulasso City (Burkina Fasso), Khombole City (Senegal), and Godomey City (Benin) respectively. According to Obiri-Danso et al. (2003), the standard of hygiene in the various stages in the production of the factory plastic-bagged sachet water is similar to that of bottled water. It was difficult to independently verify this claim with regard to the factory-filled sachet water brands. But these results did not allow us to thoroughly confirm the complete absence of contamination of sachet water by *E. coli*. According to Vaurette and Le Duc (2014), the lifespan of *E. coli* in natural waters and sediments can vary from few hours to several days, depending on environmental conditions. However, given the fact that the manufacturing

date of this sachet water was not known, it became difficult to estimate the duration of storage.

Depending on the types of process, the industrial-type sachets as well as the artisanal ones were unfit for consumption because of the presence of germs which showed faecal contamination that resulted in poor bacteriological quality.

During sales, sachet water brands are kept in buckets and coolers and exposed to temperature and sunlight. Storage conditions and the environment are also important factors of contamination. Several authors have demonstrated that temperature and x-rays can promote the migration of plastic elements to food, and also promote microbial growth in food (Limm and Hollifield, 1996; Etienne and David, 2002; Tehrany and Desobry, 2004). Residual odours of storage place and the trace element of outer wall of sachet (plastic) can also pass through the container and deteriorate the food, both organoleptically and toxicologically (Cristina, 2011).

Conclusion

This study aimed to take an inventory of all the water sachet brands sold in Yaounde City and to assess their

potability. The results obtained highlighted that contaminations were essentially of faecal origins: 75% of the samples contained intestinal *enterococci*. Majority of them were not fit for human consumption. However, further work is needed to complete the microbial and physico-chemical contaminations. These sachet water brands do not only meet a vital need, but are a source of income for vendors. Their ban without adequate replacement measures is not feasible. To protect consumers' health by reducing health risks, a number of recommendations can be made: The sensitization of consumers about the health risks they incur, the education of sellers and the monitoring of their activity by the municipal hygiene services.

CONFLICT OF INTERESTS

All authors declare that there are no conflicts of interest.

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Full Length Research Paper

Toxicity of powdered and ethanolic extracts of *Uvaria chamae* (Annonaceae) Bark on selected stored product insect pests

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A study was carried out on the insecticidal effects of the powdered stem bark extract of *Uvaria chamae* and its ethanolic extract on three most devastating stored products pests (Coleopterous) in Nigeria, namely: *Callosobruchus maculatus* F. (Bruchidae), *Rhizopertha dominica* F. (Bostrichidae) and *Sitophilus zeamais* Motschulsky (Curculionidae). Graded concentrations of each formulation of the powdered bark and ethanolic extracts were in exposure chambers of each insect in laboratory bioassays under ambient conditions (25±2°C). *S. zeamais*, *R. dominica* and *C. maculatus* were exposed to the following concentrations 0.00, 0.10, 0.20, 0.40, 0.80, and 1.60 mg/L grains in three replicates per treatment and control. The mortality of the insects was used to compute mean lethal concentration (LC₅₀) values by probit analysis. All the concentrations tested showed appreciable toxicity against each test insect species. The computed LC₅₀ values for powder formulation gave significantly (P≤0.05) higher toxicity against *C. maculatus* (1.281 g/kg) than either *S. zeamais* (2.145 g/kg) or *R. dominica* (5.189 g/kg). However, the ethanolic extract was more toxic on *C. maculatus* (0.134 mL/L), *S. zeamais* (0.173 mL/L) or *R. dominica* (0.359 mL/L). It was found that the higher the concentration of the ethanolic extract, the higher the mortality. The result implies that, *U. chamae* powdered and ethanolic stem bark extracts have potentials for use during storage of grains, ensuring food security, profit maximization and availability of seeds for the next planting season without being damaged by these test insect species. The presence of high concentration of steroids and terpenes may be responsible for the observed high insecticidal activity of the test extracts.

Key words: Bioassay, *Callosobruchus maculatus*, storage insect, *Uvaria chamae*.

INTRODUCTION

The storage of crops is a deliberate policy in most countries to guarantee the populace freedom from hunger, malnutrition and deprivation through actions that ensures adequate and consistent food supply at affordable prices. According to Ihimodu (2004), the food

self-sufficiency ratio has fallen from 98% in the early 1960s to less than 54% in 1986. In 1990, 18% of the Nigerian population (14.4 million) was estimated to be critically food insecure and this has increased to 36% (32.7 million) in 1992 and further increased to 40.7% in

1996 (Babatunde and Oyatoye, 2005). Presently, over 40% of Nigeria's estimated population of 133 million people is food insecure (Idachaba, 2004). Food security in sub-Saharan Africa largely depends on improved food productivity through the use of sustainable Good Agricultural Practices (GAPs) and the reduction of postharvest losses caused by pests and diseases (Babatunde and Oyatoye, 2005).

Insects form more than 75% of the population of known animals and constitute the major factor limiting agricultural food production. About 10.84 million metric tons of cereals and almost a million tons of legumes are produced annually and an average of between 1.5 and 2 million tons are lost to heavy insect pest infestations and mould within poor storage system (FAO, 2011). Losses of about six million tons of grains per annum are incurred by insects both in the field and stores translating to five billion naira per annum (Bogunjoko, 1987). Ahmed (2013) reported that post-harvest losses is making Nigerian farmers poorer and Patrick (2013) reported that Nigeria records over 40% post-harvest losses, which has led to an unprecedented hike in food importation in the country. According to FAO (2011), about one third of food for human consumption is lost or wasted globally to about 1.3 billion tons per year. Also, 30 to 40% of the food crops produced worldwide is never consumed as a result of damage, rotting as well as pest and diseases which affect the crops after harvest (Meena et al., 2009).

Nigeria is the largest producer and consumer of cowpea in the world (Lowenberg-Deboer and Ibro, 2008; Pereira et al., 2001) and it was estimated by FAO that 3.3 million tons of cowpea dry grains were produced in 2000 (IITA, 2001), but only a small proportion enters international trade due to losses by insects pests during storage. Similarly, maize is also one of the most important cereal crops grown from the coast to the savannah (IITA, 2009); however, weight losses of 10 to 30% have been recorded in maize stored for 3 to 6 months (Samuel et al., 2011) due to insect pests. Although synthetic insecticides are effective and quick in action, they are not eco-friendly and are mostly toxic if consumed. Safer and more environment-friendly alternative methods of controlling insect pests on stored grains are therefore needed. Some studies have shown that botanicals may serve as such alternatives (Denloye et al., 2007). Botanical insecticides remain important in insect pest management because they are believed to provide the most effective control against insect pests that have become resistant to other insecticides (Weinzierl, 2000). They may provide sustainable, safe, available and cheap alternative to synthetic insecticides in the control of storage insect pests threatening stored food and these have led to the belief that plant-derived

insecticides are safer and more ecofriendly than synthetic products. However, there is little information on the use of *Uvaria chamae* bark as biopesticide. This study was therefore aimed at investigating the insecticidal efficacy of the powdered and ethanolic stem bark extracts of *U. chamae* against *Callosobruchus maculatus* F. (Bruchidae), *Rhizopertha dominica* F. (Bostrichidae) and *Sitophilus zeamais* Motschulsky (Curculionidae).

MATERIALS AND METHODS

Plant

Fresh bark of *U. chamae* was procured from vendors at Oyingbo market in Lagos State and identified at the herbarium of Botany Department, University of Lagos, Nigeria. The test plant bark was dried to constant weight in the oven at 50°C for 8 days, and then pulverized by first pounding in a mortar before using a micro-hammer mill to grind into powder. The powder was passed through a sieve of 0.1 mm mesh size in order to standardize particle size.

Preparation ethanol extracts

Test plant powder (500 g) was wrapped in a clean dry muslin cloth and then placed inside the thimble of a Soxhlet apparatus and 1 L of 80% ethanol then poured into a round bottom flask. The apparatus was heated at 60°C using a heating mantle. The experiment was left to run through several refluxes for 6 h until a colourless liquid was observed in the capillary tube. The resultant filtrate was then concentrated over a water bath at 40°C, kept in specimen bottle and refrigerated until needed for bioassay. For the ethanol extraction, 0.5 and 10 mg/L concentrations of the various test plant materials were used, while the controls were carried out exactly the same way except that the grains were treated with ethanol.

Disinfestation of cowpea and maize seeds

Cowpea seeds (*Vigna unguiculata* [L.] Walp. var. Tvu 3629) and maize grains (*Zea mays* var. TZESR-20) were obtained at the Bariga market, Lagos. They were identified at the International Institute of Tropical Agriculture (IITA), Ibadan. All damaged seeds and debris were sorted out from the grains after which disinfestation was carried out in an oven at 50°C for 6 h to kill all life stages of insects within the grains. The grains were then left for 24 h to stabilize at ambient conditions.

Culture of test insects

C. maculatus was maintained on the disinfested cowpea seeds. 500 g of cowpea seeds in five replicates were weighed into clean 1 L Kilner jars. Fifty 0 to 3 day old unsexed adults were introduced into the jar and covered with muslin cloth held in place by rubber band. *S. zeamais*, *R. dominica* and *C. maculatus* were exposed to the following concentrations 0.00, 0.10, 0.20, 0.40, 0.80, and 1.60 mg/L grains in three replicates per treatment and control. After 5 days, the insects were removed and left undisturbed until insect

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Table 1. Toxicity of *Uvaria chamae* seed powder and Actellic dust against test insects (df=3).

Treatment	Test insects	48 h LD ₅₀ (g/kg)	95% Confidence limits	Regression equation (Y)	Slopes (Standard error)
<i>U. chamae</i> powdered seed	<i>Callosobruchus</i>	1.281	4.424-3.429	-0.823+0.96x	0.955±0.310
	<i>Sitophilus</i>	2.145	0.894-5.826	-0.286+0.865x	0.865±0.307
	<i>Rhizopertha</i>	5.189	3.258-9.880	1.614+0.38x	-1.1541±0.614
Actellic dust	<i>Callosobruchus</i>	0.048	0.010-0.127	1.816+1.380x	1.380±0.330
	<i>Sitophilus</i>	0.142	0.001-0.388	0.935+0.105x	1.105±0.390
	<i>Rhizopertha</i>	0.201	0.016-0.401	-1.204+0.727x	1.727±0.532

emergence was observed. At each peak of emergence, the insects were sieved out and new cultures were set up to ensure regular supply of adult insects of known age for experimentation. Similarly, *S. zeamais* and *R. dominica* were separated and maintained on disinfested maize grains. Fifty unsexed 7 to 14 day old adults of *S. zeamais* were introduced into 500 g of the disinfested maize grains in 1 L Kilner jars in five replicates in the laboratory. All adult insects were left for 7 days to allow for oviposition, after which they were removed. They were then left undisturbed until adults emerged. At each peak of emergence, the adults were removed and used to set up new cultures. Series of fresh cultures were made from these ones to ensure regular supply of adult insects of known age for use in subsequent experiments. Similar method was used for preparation of *R. dominica* cultures.

Laboratory bioassays

Powdered and ethanolic extracts of the bark of *U. chamae* were respectively screened against each experimental insect species following the method of Denloye et al. (2007). For these series of experiments, the prepared plant powder and extracts were respectively tested against each test insect species in elaborate bioassays to measure acute toxicity levels dependent on 48 h LC₅₀ values. For the test plant powder, 20 unsexed 2 to 3 day old adult insects were exposed per replicate of each treatment and control. The insects were treated to admixture of plant powder and cowpea (for *C. maculatus*) or maize (for *S. zeamais* or *R. dominica*) at concentration of 0.125 to 8.00 g/kg grain. For the ethanolic extract, the grains were dipped in extracts of 0.10 to 1.60 g/L concentrations. Dipping was carried out by completely immersing seeds in each extract concentration for 30 s. All dipped grains were allowed to drain on filter paper for 5 min before transferring them into bioassay containers. Several sets of 40 cowpea seeds were at these concentrations with untreated seeds used as control. All experiments and control were set up at the same time. Each treatment and control was replicated four times. In all treatments and control, mortality of each of the exposed test insect species was assessed every 24 h for 2 days.

Quantitative determination of chemical constituents of test plant materials

This was carried out on the plant extracts obtained following standard procedures of the Association of Official Agricultural Chemists (AOAC, 2000) for alkaloids, flavonoids, tannins, cardiac glycosides, cyanogenic glycosides, anthraquinone, glycosides, saponins, anthocyanides, anthocyanin pigments, reducing sugar compounds and phlobatannins.

Gas chromatography-mass spectrometry (GC-MS)

The test ethanolic extract of *U. chamae* (5 g) was soaked in 50 mL hexane for 2 days, and extracted again with 20 mL hexane three times concurrently. The extract was then concentrated in water bath. GC-MS analysis was performed using an Agilent 5975C gas chromatograph apparatus equipped with an Agilent mass spectrometric detector, a direct capillary interface and fused silica capillary column HP-5MS (30 m × 0.32 mm, film thickness 0.25 µm). Helium was used as carrier gas at approximately 1.0 ml/min, pulsed split less mode. The solvent delay was 4 min and the injection size was 1.0 µL. This pushed the samples injected into the columns, being the stationary phase which has been conditioned to 320°C for 2 h for separation. The column was pumped down to allow for stability. The analyses were carried out in duplicate for each sample batch. The individual peaks were identified by comparison of their retention indices to those of available authentic samples using the Wiley and pesticides mass spectral database library.

Data analysis

The number of dead test insects at all treatments in acute toxicity experiments were corrected using Abbott's (Abbott, 1925) formula. Probit analyses were then carried out following Finney (1971) protocol and median lethal concentrations (LC₅₀) values were obtained based on a computer programme. Values with overlapping confidence limits were not significantly different.

RESULTS

Toxicity of *U. chamae* powder stem to the test insects relative to Actellic dust

The test plant powder and actellic dust, respectively were toxic to all test insect species, although actellic demonstrated a much higher insecticidal effect against each of the test insects than *U. chamae* powder (Table 1). The LC₅₀ values computed for *C. maculatus* (1.28 g/kg), *S. zeamais* (2.15 g/kg) and *R. dominica* (5.19 g/kg) in Table 1 indicate that the test plant powder was more potent against *C. maculatus* than any of the other insects. There was however no significant difference in the toxicity of the powder to the test insects due to overlap in the 95% confidence limits. The toxicity factor of actellic dust when compared with the effects of *U. chamae* on the

Table 2. Toxicity of *Uvaria chamae* ethanolic extract against test insects (df=3).

Test insects	48 h LC ₅₀ (mg/L)	95% Confidence limits	Regression equations	Slopes (± Standard error)
<i>Callosobruchus</i>	0.134	0.025-0.245	Y= 0.939+1.076x	1.076±0.334
<i>Sitophilus</i>	0.173	0.049-0.301	Y= 0.840+1.103x	1.103±0.328
<i>Rhizopertha</i>	0.359	0.079-0.626	Y=0.617+1.387x	1.387±0.370

Table 3. Percentage mortality of *S. zeamais* adults treated with test plant ethanolic extracts.

Organism	Concentration	%Mortality
<i>S. zeamais</i>	0.1	22.0 (4.74%) ^a
	0.2	28.5 (4.89%) ^a
	0.4	41 (6.44%) ^a
	0.8	56.5 (7.55%) ^{ab}
	1.6	82.0 (9.08%) ^c
<i>R. dominica</i>	0.1	18.5 (4.56%) ^b
	0.2	26.5 (5.19%) ^b
	0.4	40 (6.36%) ^b
	0.8	51.5 (7.21%) ^b
	1.6	76.0 (8.75%) ^c
<i>C. maculatus</i>	0.1	18.5 (4.36%) ^a
	0.2	30 (5.5%) ^a
	0.4	50 (7.11%) ^a
	0.8	61.0 (7.84%) ^b
	1.6	88 (9.41%) ^c

Each datum is a mean of three replicates. Values in parenthesis are square roots ($\sqrt{x + 0.5}$) transformed. Mean values bearing the same letters are not significantly different by LSD at P = 0.05.

test insects showed that it was 15.1, 25.8 and 26.7 times more toxic to *S. zeamais*, *R. dominica* and *C. maculatus*, respectively.

Toxicity of *U. chamae* ethanolic extract to the test insects

The test plant extract was more toxic to *C. maculatus* than either *S. zeamais* or *R. dominica* (Table 2). The LC₅₀ values computed for *C. maculatus* (0.134 mg/L), *S. zeamais* (0.173 mg/L) and *R. dominica* (0.359 mg/L g/kg) in Table 1 indicate that the test plant powder was more potent against *C. maculatus* than any of the other insects. There was however no significant difference in the toxicity of the extract to the test insects due to overlap in the 95% confidence limits. The computed LC₅₀ values shows that the *U. chamae* ethanolic extract was significantly more toxic to *C. maculatus* (0.134 mg/L) than *R. dominica* (0.359 mg/L) with confidence limits not

overlapping (Table 2).

However, the result for the rate of mortality induced by various ethanolic concentrations of *U. chamae* to the organisms is shown in Table 3. The result indicated high percentage mortality among insects treated with higher concentrations of the ethanolic extracts of *U. chamae*. The effect increases with increase in concentration.

Chemical constituents in *U. chamae* ethanolic extract

The qualitative analysis indicated that *U. chamae* contained alkaloids, catechol tannins, condensing tannins, cardiac glycosides, flavonoids, reducing sugar, saponins, anthraquinone, phlobatannins, hexose's'-sugar, Keto-sugar Pento-sugar and monosaccharide (Table 3). The major constituents identified by Gas Chromatography/Mass Spectrometer analyses in *U. chamae* were benzene derivatives (52.9%), followed by aliphatic compounds dominated by higher acids, alcohols

Table 4. Quantitative determination of constituents present in *U. chamae* extracts.

Analysis	Constituents
Alkaloid analysis	
Dragendoff's reagent	+
Meyer's reagent	+
Test for Tannins	
Ferric chloride test	+
Ferric bromine water	+
Cardiac-Glycoside analysis	
Legal test.	+
Kedde test	-
Steroid test	
Lieberman's test	+
Salkowski test	+
Flavonoid analysis	
Ferric chloride test	+
Lead acetate test	+
Saponins	
Benedict's test	+
B. Frothing test	+
Anthraquinones analysis Bortrager's test	
Phlobatannin's test	
Reducing sugar analysis	
Barfoed test	-
Resorcing test	+

and hydrocarbons (38.6% fatty acids), terpenoids with total percentage peak area of 7.39% and quinolines 1.09% (Table 4).

DISCUSSION

The biological activities of the dusts and ethanol extracts of the test plants were different in their efficacies, with the ethanolic extracts being the most effective followed by the dust formulation. This corroborates the reports of Obi and Onuoha (2000) and Ogueke et al. (2006) who independently reported that ethanol is the best solvent for the extraction of most plant active ingredients. The differences in toxicity between the ethanolic extract and dusts formulation agree with the findings of Benner (1993) who observed that the active materials in plant extracts are more concentrated than in the plant dusts,

hence the reason for their higher potency. Lale and Mustapha (1999) also reported that extracts of neem were significantly more potent in reducing oviposition and adult emergence of *C. maculatus* than the powdered form.

The effect of dust on *C. maculatus* might be due to their action as physical barriers that deter free movement or access of ovipositing adults to suitable sites on the seeds or clog the insect spiracles and trachea causing suffocation or may be that the cuticle of the insect might have suffered abrasion which could result in dehydration therefore causing stress and death which has a direct relationship with particle size of dusts as stressed by Sousa et al. (2005). This could probably be the case with *C. maculatus* with a thinner and less sclerotized cuticle than *S. zeamais* and *R. dominica*, making it more susceptible to the dust formulation than the other test insects.

However, mortality in *S. zeamais* and *R. dominica* might be due to feeding on treated grains as suggested by Wolfson et al. (1991) although this does not apply to *C. maculatus* adults since they do not feed except there is proof that the developing larvae fed on the treated plant materials. This could have only been the case if the extract penetrated into the seed, since the larvae feeds within the seeds. The toxicity of *U. chamae* against the adult test insects agrees with the work of Lajide et al. (1998) who reported that pulverized seeds of *Uvaria afzelli* (although a different species from the one used in this study) were found to be highly toxic to maize weevil when used as surface treatment of maize grains subsequently infested with the weevils.

The presence of saponins, terpenes and cardiac glycosides in *U. chamae* is similar to the findings of Oluremi et al. (2010), Okon et al. (2013), Kone et al. (2013) and Osoagwu and Ihenwosu (2014) as they all advocated that toxicity to various effects of terpenes in plants to insects as stressed by Adebowale and Adedire (2006). The presence of high proportion of steroids and terpene alcohols in *U. chamae* is probably responsible for its insecticidal activity.

Conclusions

The insecticidal activity of *U. chamae* extracts was found to be effective against *S. zeamais*, *R. dominica* and *C. maculatus*. It was found that both the ethanolic extracts and powdered extracts can be used to control these devastating pests. However, for easy usage, the use of powdered extract is recommended as it can easily be prepared and highly effective ecofriendly insecticide to the synthetic insecticides.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interest.

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Full Length Research Paper

Chemical composition of *Citrus limon* (Eureka variety) essential oil and evaluation of its antioxidant and antibacterial activities

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The study evaluates the chemical composition, antioxidant and antimicrobial effects properties of essential oil of *Citrus limon* (Eureka) extracted by hydrodistillation. The composition of this oil was analysed by GC/MS for 30 constituents, which accounted for 97.8% of total oil. The main components were limonene (61.3%) followed by β -pinene (9.7%), α -citral (4.2%) and α -terpinene (3.8%). Antioxidant activity of the *C. limon* essential oil was evaluated by using DPPH radical scavenging and β -carotene-linoleic acid bleaching. In both tests, the oil showed antioxidant property close to that of positive control (α -tocopherol). The essential oil was tested against nine bacteria (two Gram+: *Bacillus cereus*, *Staphylococcus aureus* ATCC 29213 and seven Gram-: *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Salmonella enterica*, *Klebsiella pneumoniae*, *Enterobacter aerogenes*, *Serratia marescens*, *Proteus mirabilis*) by using disc diffusion and microdilution methods. *C. limon* essential oil showed antimicrobial effect against all microorganisms tested. Maximum activity (MIC = 180 $\mu\text{g}\cdot\text{ml}^{-1}$) was observed against *Staphylococcus aureus*.

Key words: Chemical composition, diffusion technique, microdilution technique, hydrodistillation, percentage of inhibition.

INTRODUCTION

Citrus is produced in abundance in several areas worldwide (Thomas and Spreen, 2010). The amount of residue obtained from *Citrus* fruits account for 50% of the original amount of the whole fruits (Chon and Chon, 1997). Essential oils have diverse and high biological

applications. For instance, they are used in the medical field like biocidal activities (bactericidal, virucidal and fungicidal) and medicinal properties (Mayaud et al., 2008). The use of essential oils as food preservatives has been described (Burt, 2004; Tiwari et al., 2009). Because

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of their complex chemical composition which composed of more than 100 different terpenic compounds, essential oils have a broad biological and antimicrobial activity spectrum (antibacterial, antifungal, antimoulds, antiviral, pest control, insect repellents).

Recently, the essential oils and various extracts of plants have been of great interest as they have been important sources of natural products. In order to prolong the storage stability of foods in industrial processing, synthetic antioxidants and antibacterials are used. Otherwise, side effects of some synthetic conservatives used in food processing have been documented (Ames, 1983; Baardseth, 1989). For this reason, governmental authorities and consumers are concerned about the safety of the food and potential effects of synthetic additives on human health (Reische et al., 1998).

Despite their wide uses and fragrances, essential oil constitutes an effective alternate to synthetic compounds produced by chemical industry without having any side effects (Faixova and Faix, 2008). The objective in this present study is to evaluate the antioxidant and antibacterial properties of essential oil of *Citrus limon* (Eureka variety) extracted by hydrodistillation.

MATERIALS AND METHODS

Essential oil

Process of extraction

The essential oil of *C. limon* (Eureka) peel is extracted by steam distillation for 2 h and 30 min using a Clevenger-type apparatus. The supernatant was separated by decantation after addition of 50 % NaCl. The essential oil was collected, dried over anhydrous sodium sulfate, and stored in glass vials covered with aluminum foil at 4°C until used.

Gas chromatography-mass spectrometry

The essential oils were analyzed by gas chromatography coupled to mass spectrometry (GC/MS) (using a DB-5 fused-silica-capillary column polar (30 m × 0.25 mm, 0.25 µm film thickness). The oven temperature program was 60°C and held for 8 min, then increased from 45 to 250°C at a rate of 2°C/min which was held at 250°C for 20 min. Helium gas was used as the carrier gas at a constant flow rate of 3 ml/min. Injector and MS transfer line temperatures were set at 250°C and 280°C, respectively. The temperature of electronic impact at 70 eV source was 230°C. Samples (0.2 µl) were injected at 250°C at a split ratio 50:1. Identification of the components was made by determination of their retention indices (KI) relative to homologous series of n-alkanes (C₈-C₂₈) (Fluka, Buchs SG, Switzerland) by matching recorded mass spectra with those stored in spectrometer database (NIST MS Library v. 2.0) and bibliography (Adams, 2001). Component relative percentages were calculated based on GC peak areas.

Evaluation of the antioxidant and antiradical activities

Scavenger effect on DPPH

The ability of the essential oil to donate hydrogen atom or electron

and scavenge 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical was determined by slightly modified method of Brand (Brand-Williams et al., 1995). The concentrations of the tested essential oil ranged from 2 to 0.05 mg/ml. A portion of sample solution (200 µl) was mixed with 3 ml of 5.25 × 10⁻⁵ mol/l DPPH in absolute ethanol. The decreasing absorbance of the tested mixtures was monitored every 1 min for 30 min at 515 nm using ultraviolet and visible (UV-Vis) spectrometer.

Absolute ethanol was used to zero the spectrophotometer. The DPPH solution was used as a blank sample and α-tocopherol was used as a positive probe. The radical scavenging activity of the tested essential oil, expressed as percentage inhibition of DPPH, was calculated according to the following formula:

$$IC (\%) = [(A_0 - A_t)/A_0] \times 100$$

Where, A_t is the absorbance value of the tested sample and A₀ is the absorbance value of the blank sample, in a particular time (t). The percentage of inhibition was plotted after 30 min against concentration, and the equation for the line was used to obtain the IC₅₀ value.

B-carotene bleaching assay

Antioxidant capacity is determined by measuring the inhibition of the volatile organic compounds and the conjugated diene hydroperoxides arising from linoleic acid oxidation (Dapkevicius et al., 1998). A mixture of 2 mg β-carotene and 25 µl linoleic acid was prepared in 10 ml of chloroform and 200 mg Tween 40. The chloroform evaporated completely at 40°C under vacuum. 50 ml of oxygenated distilled water was subsequently added to the residue and mixed gently to form a yellowish emulsion. The essential oil and α-tocopherol (positive control) were individually dissolved in methanol (2 mg/ml), 350 µl volumes of each were added to 5 ml of the above emulsion in test tubes and mixed thoroughly. The test tubes were incubated in a water bath at 50°C for 2 h together with a negative control (blank) which contained the same volume of methanol instead of the essential oil. The absorbance values were measured at 470 nm on an ultraviolet and visible (UV-Vis) spectrometer. The antioxidant activities (inhibitions percentage, I%) of the samples were calculated using the following equation :

$$A_{\beta\text{-carotene after 2h assay}} / A_{\text{initial } \beta\text{-carotene}} \times 100$$

Where, A_{β-carotene after 2 h assay} is the absorbance value of β-carotene after 2 h assay remaining in the sample and A_{initial β-carotene} is the absorbance value of β-carotene at the beginning of the experiments.

Antimicrobial activity

Bacterial strains

The essential oil was tested against nine strains of food borne pathogenic bacteria: two Gram+: *Bacillus cereus*, *Staphylococcus aureus* ATCC 29213 and seven Gram-: *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Salmonella enterica*, *Klebsiella pneumoniae*, *Enterobacter aerogenes*, *Serratia marcescens* and *Proteus mirabilis*. Bacterial strains were cultured overnight at 37°C on Mueller Hinton broth and adjusted to a final density of 10⁶ CFU/ml, used as an inoculum.

Diffusion assay

In vitro, antibacterial activity of the essential oil was evaluated against the nine bacterial strains by the disk diffusion method (Rota

et al., 2004). The test was performed in sterile Petri dishes containing solid and sterile Mueller-Hinton agar medium. The essential oil (5 μ l) absorbed on sterile paper discs (Whatman disc of 6 mm diameter), were placed on the surface of the media previously inoculated with 100 μ l of microbial suspension (10^6 CFU/ml), then the Petri dishes were incubated at 37°C for 24 h after staying at 4°C for 2 h. The inhibition zone diameters around each of the disks (diameter of inhibition zone including the disc diameter) were measured in millimeters.

Determination of minimal inhibitory concentration (MIC)

The minimal inhibition concentration (MIC) values were studied for the bacterial strains which were sensitive to essential oil in disc diffusion assay. Minimal inhibition concentration (MIC) values were determined by broth micro dilution method (Carson and Riley, 1995). The test was performed in Mueller Hinton broth (MHB) supplemented with Tween 80 (concentration of 0.5% (v/v) and 1 ml of different concentrations of essential oil (1000 to 10 μ g/ml with a range of 10 μ g/ml) diluted in DMSO).

Bacterial strains were cultured overnight at 37°C in Mueller Hinton Agar (MHA). Test strains were suspended in MHB to give a final density of 10^5 CFU/ml. The mixture (various dilutions of the essential oil + MHB + Tween 80) was placed in Petri dishes, after solidification bacterial strains are inoculated (1 μ l containing 10^5 CFU/ml) and the negative control was set up with it.

The MIC is defined as the lowest concentration of the essential oil at which the microorganism tested, does not demonstrate visible growth in the broth after 24 h of incubation at 37°C (Bassole et al., 2003). The MBC is the lowest concentration of essential oil inhibiting any growth visible to the naked eye after 5 days of incubation at 37°C (Mayachiew and Devahastin, 2008). The tests were performed in duplicate and were repeated twice.

Statistical analysis

The obtained yield, antioxidant and antibacterial results were stated in mean \pm standard deviation. Significant differences means were determined by Student t-test. Probability values lower than 5% were regarded as significant.

RESULTS AND DISCUSSION

Chemical composition of the essential oil

The oil extracted (2.04 ± 0.34 %) is yellowish in color and has an aromatic odor characteristic of lemon. The yield cited by Himed and Barkat (2014) of the Eureka variety, extracted by cold pressure is 1.02 ± 0.04 %. The two yields represent a significant difference; this means that the method of extraction affects the yield.

Regarding the chemical composition of the essential oil tested, this was shown to be a complex mixture of many components. Table 1 show the identified 30 compounds which accounted for 97.8% of the total oil. The essential oil was dominated by limonene (61.3%) followed by β -pinene (9.7%), α -citral (4.2%) and α -terpinene (3.8%). Limonene concentration is an essential oil which may vary between 32 and 98%, and that depend on: 32 to 45% in bergamot, 45 to 76% in lemon and 68 to 98% in sweet orange (Moufida and Marzouk, 2003).

Antioxidant property

As shown in the Table 2, the free radical scavenging activity measured by DPPH assay of the essential oil tested is inferior to α -tocopherol with IC_{50} (1.01 ± 0.420 and 0.78 ± 0.130 mg/ml respectively). Oxidation of linoleic acid, in β -carotene/linoleic acid system, is effectively inhibited by the essential oil of *C. limon* with a value close to α -tocopherol (72.2 ± 0.014 % and 72.8 ± 0.079 % respectively). No significant differences were reported, it means that *C. limon* essential oil has significant antioxidant property close to that of α -tocopherol.

Wei and Shibamoto (2007) showed the presence of a significant antioxidant potential of essential oils rich in hydrocarbon monoterpenes (limonene and β -pinene). Ruberto and Baratta (2000) reported that γ -terpinene could also be taken into account for the antioxidative activity observed, which is for 3.8% oil studied. This activity can also be attributed to the presence of oxygenated sesquiterpenes (Cherrat et al., 2014). Aoyama et al. (1988) reported that terpenes showed a synergistic effect in antioxidant with other antioxidants.

Therefore, by inference the higher antioxidant activity in the essential oils might be due to their higher contents of terpenes (with basic structure of isoprene) (Mau et al., 2003). The aldehyde monoterpenes (citral) and hydrocarbon sesquiterpenes (trans-caryophyllene) were responsible for DPPH neutralization (Mimica-Dukic et al., 2004).

Antimicrobial property

The disc diameters of zone of inhibition (DIs), minimum inhibitory concentrations (MICs) and minimum bactericidal concentrations (MBCs) of *C. limon* essential oil for the bacteria tested are shown in Table 3. The correlation between the two different screening methods was examined, and generally have larger zones of inhibition correlated with lower MIC and MBC values.

The essential oil of *C. limon* showed inhibition zones against all microorganisms tested. This was confirmed by both MICs and MBCs data, where the essential oil exhibited significant antibacterial activity against the microorganisms tested, particularly against gram-positive bacteria (*S. aureus* and *B. cereus*) which have the lowest MIC (240 and 300 μ g.ml⁻¹ respectively). As cited by Burt (2004) and Hussain et al. (2010), the test Gram-positive bacteria were found to be more susceptible to antimicrobial agents than Gram-negative bacteria. The weaker antimicrobial activity against Gram-negative compared to Gram-positive bacteria is ascribed to the structure of their cellular walls mainly with regard to the presence of lipoproteins and lipopolysaccharides in Gram-negative bacteria that form a barrier to hydrophobic compounds (Inouye et al., 2001). It is well known that the composition, structure, as well as functional groups of

Table 1. Chemical composition of *Citrus limon* (Eureka variety) essential oil obtained by hydrodistillation.

N°	RT min	Compound	Area percentage (%)	I
1	10.243	α -Thujene	0.24	931.196
2	10.645	α - Pinene	1.47	938.523
3	13.452	β -Pinene	9.65	981.960
4	14.420	β -Myrcene	1.35	994.856
5	17.666	Limonene	61.26	1044.186
6	18.542	β -Ocimene	0.13	1056.486
7	19.292	γ -Terpinene	3.84	1066.498
8	21.232	α -Terpinolene	0.19	1090.692
9	22.621	Linalool	0.39	1108.870
10	24.073	Trans -Limonene oxide	0.15	1131.072
11	24.555	Cis-Citronellol	0.61	1138.016
12	24.926	Trans-Citronellol	0.78	1143.268
13	25.263	Camphor	0.26	1147.971
14	25.434	Citronellal	0.25	1150.334
15	28.039	α -Terpineol	0.37	1184.485
16	29.260	Linalyl propionate	1.07	1199.414
17	31.355	Trans-carveol	0.20	1231.182
18	31.940	Cis-Carveol	0.20	1240.145
19	32.541	Cis-Citral	2.39	1248.850
20	33.700	Geranial	0.17	1265.193
21	34.732	α -Citral	4.22	1279.279
22	40.394	Piperitenone oxide	1.11	1363.013
23	40.721	Neryl acetate	1.40	1368.244
24	42.023	β -Elemene	2.22	1386.968
25	44.021	Trans-caryophyllen	0.11	1417.241
26	45.063	Trans- α -bergamotene	0.85	1435.896
27	46.571	Germacrene	0.22	1460.544
28	49.705	γ -Cadinene	1.21	1501
29	50.906	Myristicine	0.81	1531.537
30	54.111	Caryophyllene oxide	0.71	1584.571

Table 2. Effects of *Citrus limon* essential oil and positive control (α -tocopherol) on DPPH and β -carotene/linoleic acid systems.

Parameters	Essential oil	α -tocopherol
DPPH assay (IC ₅₀ , mg/ml)	1.01 \pm 0.420	0.78 \pm 0.130
β -carotene – linoleic acid assay (I %)	72.81 \pm 0.014	73.18 \pm 0.079

essential oil play an important role in determining their antimicrobial activity. It has been demonstrated that, essential oils exercise their antimicrobial activity by causing structural and functional damages to the microbial cell membrane (Goni et al., 2009).

Limonene was present at a very high concentration in the *Citrus* essential oil. According to Espina et al. (2011), the greater antimicrobial activity of essential oil might not be attributed to limonene, but should be related to the

presence of other essential oil constituents; unlike Ruiz and Flotats (2014) who reported that, the documented antimicrobial effect of *Citrus* essential oil can be attributed to the essential oil or limonene as well, as its main component.

The strong antimicrobial activity of the essential oil against the susceptible microorganisms can be attributed to the presence of high concentration of monoterpenes (Reza et al., 2014), where 78.1% are monoterpenes in

Table 3. Antibacterial property of *Citrus limon* (Eureka) essential oil.

Bacteria tested	DI (mm)	MIC ($\mu\text{g.ml}^{-1}$)	MBC ($\mu\text{g.ml}^{-1}$)	MIC/MBC
<i>Bacillus cereus</i>	14.5 \pm 0.710	210	260	1.23
<i>Staphylococcus aureus</i> ATCC 29213	16 \pm 0.130	180	180	1
<i>Escherichia coli</i> ATCC 25922	9.1 \pm 0.217	760	/	/
<i>Pseudomonas aeruginosa</i> ATCC 27853	8.9 \pm 0.801	790	/	/
<i>Salmonella enterica</i>	12.7 \pm 0.112	410	500	1.21
<i>Klebsiella pneumoniae</i>	10.1 \pm 0.621	620	620	1
<i>Enterobacter aerogenes</i>	10.7 \pm 0.171	580	700	1.20
<i>Serratia marcescens</i>	11.2 \pm 0.431	500	640	1.28
<i>Proteus mirabilis</i>	9.8 \pm 0.278	700	/	/

DI (mm), Diameter of inhibition zones of essential oil including diameter of disc 6 mm; MIC ($\mu\text{g.ml}^{-1}$), minimum inhibitory concentration; MBC ($\mu\text{g.ml}^{-1}$), minimum bactericidal concentration.

the essential oil of *C. limon* tested. Moreover, oxygenated monoterpenes might be involved in higher antimicrobial activity of studied essential oil. Some authors (Carson and Riley, 1995; Burt, 2004) have demonstrated that oxygenated monoterpenes had an important antimicrobial activity. Nevertheless, the antimicrobial activity of essential oil might also be due to the synergistic interaction of other constituents present in smaller amounts.

Conclusion

In conclusion, essential oil of *C. limon* (variety Eureka) is a rich source of antioxidant which can be used as powerful herbal antioxidant and the antibacterial property can be considered as an additional health promoting factor. Antioxidant and antibacterial properties are directly related to its chemical composition which is rich in monoterpenes.

Finally, this essential oil could play a beneficial role as a natural preservative ingredient in food and pharmaceutical industries.

Conflict of Interests

The authors have not declared any conflict of interests.

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Full Length Research Paper

Thermal, physical and chemical analysis of *Eisenia andrei* flour for supplementary diet

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The search for sources of animal feed has been well-regarded for farmers. In spite of this, the objective of this study was to show the thermal, physical and chemical properties of earthworms flour from *Eisenia andrei* for supplementary food for animals. The species was studied by simultaneous thermogravimetric analysis, differential scanning calorimetry, combustion calorimetry, moisture, ash, fat, protein, carbohydrate and fibre analysis. The samples were dried, ground, screened and stored in suitable vials. After the analysis of physical and chemical characterization, it was possible to verify that *E. andrei* flour presents higher calorific power and levels of protein, fiber, carbohydrate, essential metals and low fat. It can be a promising product as an ideal alternative protein source for better growth performance of animals.

Key words: Earthworm, vermicomposting, physical and chemical analysis.

INTRODUCTION

Vermicomposting is a biotechnological process that uses organic wastes as a compost rich in macronutrients, through the combined actions of microorganisms and earthworms (Edwards, 1972; Gomez et al., 2011). Earthworms are usually used for vermicomposting because they possess higher composting activity in different wastes, such as municipal solid waste (Soobhany et al., 2015), sewage sludge and cow manure

(Lazcano, 2008; Xing et al., 2012), pig waste (Wong and Griffiths, 1991) and vegetables (Huang et al., 2013; Hussain, 2015). Their growth is fast and the biomass can be used in the animal feed industry (Dynes, 2003; Sogbesan and Madu, 2008; Sogbesan and Ugwumba, 2008; Sinha et al., 2010; Tiroesele and Moreki, 2012). Sogbesan and Madu (2008), evaluated earthworm meal as protein feedstuff in diets for fishes and the results

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showed the highest mean weight and relative growth rate were recorded in fish fed 25% earthworm inclusion diet.

The use of earthworm flour was related by Isea et al. (2008), for fish food. In comparison of wheat bran and soy cake, the earthworm flour showed higher digestible protein (Isea et al., 2008). In spite of the search for sources of animal feed, earthworm flour has been well-appreciated by the farmers. But there are fewer researches about the physical, chemical and thermal analysis using earthworms flour from the *Eisenia andrei* species and its importance from the point of view of the quality of the bioproduct regarding to dietary aspects (bioenergetics, protein digestibility, weight gain) (Raemy, 2003; Tamilmani and Pandey, 2015). Therefore, the aim of this study was to evaluate the properties of flour of *E. andrei* by applying simultaneous thermogravimetric analysis (TG/DTG), differential scanning calorimetry (DSC), combustion calorimetry and conventional physical and chemical analysis to contribute to the quality of this bioproduct for animal's diet.

MATERIALS AND METHODS

Earthworms

The *E. andrei* earthworms were cultivated in commercial plastic boxes (0.20x0.35x0.5 m). Half of the box volume was filled with 50% soil (physical and chemical characteristics are shown in the Supplementary data), 20% fruit peels, 10% dry leaves, and 20% cow manure. The experiment was carried out in the Tecnovates Laboratories at the Centro Universitário UNIVATES. The process took 60 days to be performed, in closed room and was conducted under controlled temperature (25°C) and humidity (40%).

Earthworm flour production

Worms (1 kg) were immersed in water for 24 h and then washed in ultrapure water and stored. The biomass was put in a forced air circulation heater for 24 h at 40°C so it could dry faster. The dry samples were ground and sieved with 250 mesh and then stored for further analysis.

Earthworms flour physical and chemical characterization

Composition analysis

For the composition analysis of the earthworms flour, its moisture, ash, protein and lipid contents were determined according to the Association of Official Analytical Chemists (AOAC) protocols (AOAC, 2010). To measure moisture, the sample was put on a direct drying in an oven at 105°C for 24 h. For ash analysis, incineration was done in an oven at 550°C for 4 h. To measure lipids, the technique used was based on solvent extraction with ethyl ether followed by removal by evaporation or distillation of the solvent employed; and for protein, it was determined by classical Kjeldahl method.

Metals analysis

The dried samples were weighed and then a 100 mg aliquot was transferred to an acid-rinsed digestion vessel and 5 mL HNO₃ was

added. Digestion of the samples was carried out using a commercial microwave oven, Multiwave Pro, Anton Paar. The heating program was performed in three steps (temperature/C, ramp/min, hold/min): 1 (100, 5, 1); 2 (120, 4, 5); and 3 (150, 4, 10). There is a fourth step for cooling down the system through forced ventilation for a period of 20 min. After the digestion, samples and blank solutions were transferred to plastic flasks and made up to 10 ml with deionised water. The digestion procedure was done in triplicate for each sample. The conditions utilized were based on Naozuka et al. (2011).

Determination of Ca, Na, K, Mg, Cr, Cu, Al, Cd, Pb, Ba, Ni, Co and Mn was conducted using an inductively coupled plasma optical emission spectrometer (Optima 8000, Perkin Elmer) equipped with a Scott-type double pass spray chamber, a cross-flow nebulizer, a torch and a quartz injector. The operational parameters were in accordance with the literature (Muller et al., 2016).

The standard of multi-element elements from Merck (Darmstadt, Germany) and Perkin Elmer (Shelton, USA) were treated in the same way as the samples and were used for equipment calibration step.

Thermal analysis

For combustion, calorimetry analysis, an oxygen bomb calorimeter (C2000 Basic, IKA Works GmbH and Co., Staufen, Germany) was used for the gross energy determination of the earthworm flour. About 0.5 mg of the sample was put in the pressurized vessel (2 atm O₂) and the procedure was performed in accordance with the fabricant.

TG/DTG was carried out using 3.0 mg dried samples in a thermogravimetric analyser (Model 4000, Perkin Elmer) from 25 to 800°C at a rate of 10°C/min. Nitrogen was used as the purge gas at a flow rate of 50 mL/min (Nuthong et al., 2009).

Differential scanning calorimetry (DSC)

Thermal transition measurements of earthworm flour were determined using a Modulated Differential Scanning Calorimeter (model DSC 4000, Perkin Elmer) equipped with a thermal analysis data station and data recording software. The sample was adapted according to the procedure of Wang et al. (2014), with a slight modification. Briefly, about 2 mg of samples were weighed. The sample was heated from 25 to 445°C at a heating rate of 10°C/min with nitrogen flow of 50 mL/min.

RESULTS AND DISCUSSION

Protein, moisture, ash, fat, carbohydrate and fibre are important tools to food quality. Protein is important because it improves the animals growing. The amino acid composition of the flour had a variation in the contents of total essential and non-essential amino acids. Moisture is important because of the knowledge of the sorption properties of foods which is of great importance in food dehydration, especially in the quantitative approach to the prediction of the shelf life of dried foods. In the ashes, there was a mineral composition such as macro- and micro-elements essential and non-essential for life. Fat and carbohydrate are essential to provide energy and have additional functions in animals system and fibers contribute to the better functioning of the intestinal system (Brewer et al., 1985; Filho et al., 1999; Maihara et

Table 1. Physico-chemical characters of flours (%) derived from different animals.

Parameter	<i>Eisenia andrei</i> flour	Fishbone Tilapia flour ^a	Poultry by-products meal ^b
Moisture	14	14	*
Ash	5	18	*
Fat	9	25	*
Protein	55	40	43
Carbohydrates	15	*	*
Fibre	2	*	1,7

*No information. ^aPetenuci et al. (2010). ^bBoscolo et al. (2005).

Table 2. Results of metals composition of different flours.

Metal (mg/g)	<i>Eisenia andrei</i> flour	White rice ^c	Fish flour ^d
Ca	357±6	162 ± 0.65	50
Na	564±7	-	5.43
K	667±9	-	0.025
Mg	166±4	-	-
Cr	0.012±0.001	-	510
Cu	0.166±0.034	2.31±0.07	-
Al	31±2	-	-
Fe	26±5	<0.1	0.6
Cd	0.304±0.006	-	-
Pb	0.116±0.095	-	-
Ba	1.045±0.030	-	-
Ni	0.081±0.010	-	-
Co	0.474±0.022	-	0.000011
Mn	18±1	8.3±0.07	-

^cLin et al. (2016). ^dFilho et al. (1999).

al., 2006; Sogbesan and Madu, 2008; AOAC, 2010). In this sense, it is important for the analysis of these parameters. The physico-chemical characters of earthworm's flour are shown in Table 1.

Results of moisture (%) of *E. andrei* are in accordance with the observations of Petenuci et al. (2010) for fishbone Tilapia flour. *E. andrei* flour has lower fat in comparison with fishbone Tilapia flour (Petenuci et al., 2010). Fibre was higher than poultry by-product (Boscolo et al., 2005). There was a minimal value of ash but proteins were higher in the *E. andrei* flour, so it can be used as supplementary food rich in protein.

Metals concentration

Minerals are known to be very important for the product quality. Table 2 shows the metals composition of the earthworm flour and other products. The major concentrations of minerals in the *E. andrei* flour are higher in comparison with white rice (Lin et al., 2016) and fish flour (Filho et al., 1999). Calcium content was double

as compared to the white rice. Potassium was 600 times and Iron was 30 times more concentrated when compared with the fish flour. All the elements analyzed are very important for animal nutrition, since metals are involved in a great number of metabolic pathways. It is important to highlight that the mineral composition from *E. andrei* being presented in this table is more complete. This demonstrates the importance of this product in the support of animal nutrition (Brewer et al., 1985; Mageed and Oehme, 1990; Allen, 1994; Aggett et al., 2015).

Thermal analysis

The results show that the caloric value of *E. andrei* flour sample was 4850 kcal/kg. The digestible energy values of the product were compared with those found for Nile tilapia, soybean meal and meat and bone meal by Pezzato (1995), where the results were compared for soybean oil by Sintayehu et al. (1996), for poultry by-products and corn by Degani et al. (1997), and silkworm meal by Pezzato (1999); the results were around 3000

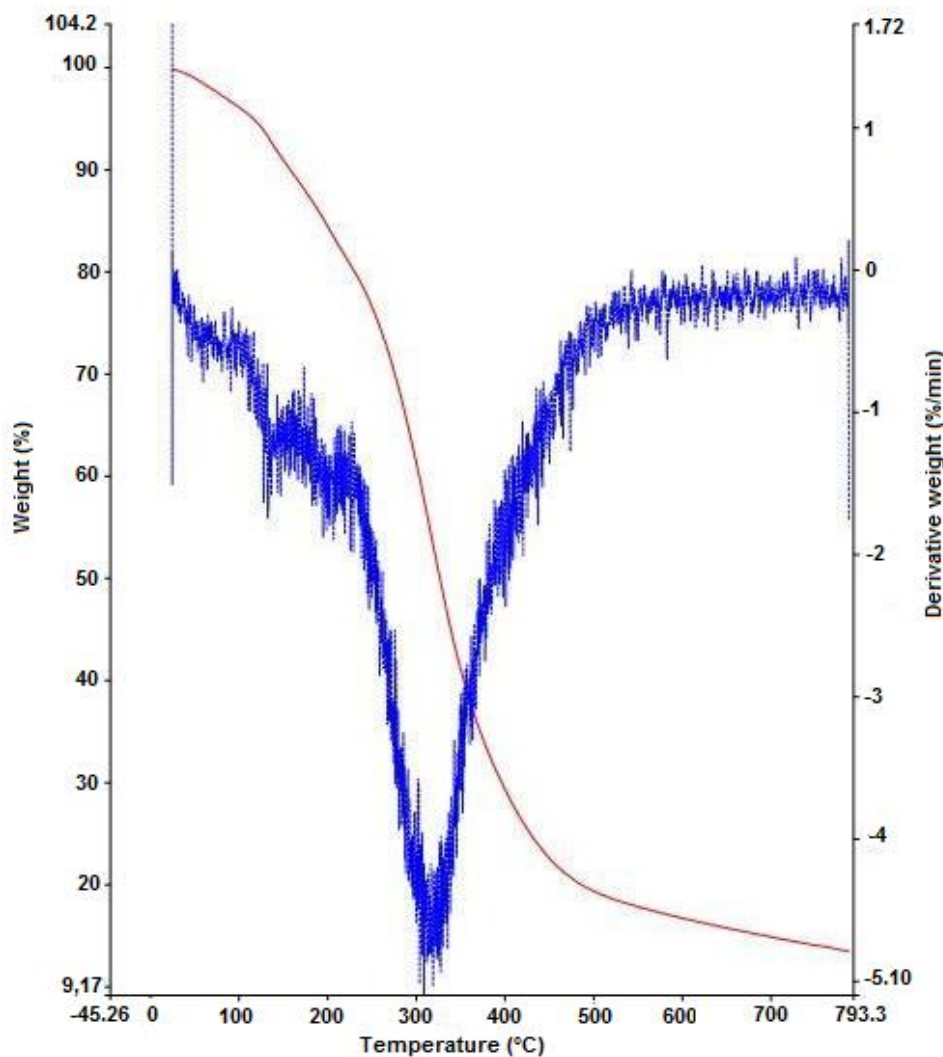


Figure 1. TG/DTG analysis from *Eisenia andrei* flour.

kcal/kg (Boscolo et al., 2005). Therefore, *E. andrei* flour presents higher calorific value when compared with the other types of animal feed cited. And it can improve weight and relative growth rate for the consumers.

TG/DTG analysis

The profiles of TG/DTG curves (Figure 1) were different from those observed by Tomassetti et al. (1987) and Leivas et al. (2013). TG curves show mass loss in one step. Dehydration of organic matter started at about 80°C. A loss of 20% of total original mass was observed. The curve also revealed that the earthworm flour contains 10% residue content until 300°C. The results are in accordance with those of Mothés et al. (2005), who used corn meal flour with loss of 14% of original mass. The profile of DSC analysis curves of earthworm flour are

as shown in Figure 2. It is possible to verify the denaturation of the protein, which is an endothermic event, at 60°C.

In accordance with the results, earthworm flour can be consumed with security until the temperatures cited earlier.

Conclusion

Thermal, physical and chemical analysis was used as tool to analyze the quality of the *E. andrei* flour. Through these, it was possible to conclude that the flour of earthworm presents high protein content, calorific power, fiber, phosphate and essential metals. This product can be utilized to feed animals because of its promising characteristics for agriculture use. Further tests may be conducted, such as shelf live.

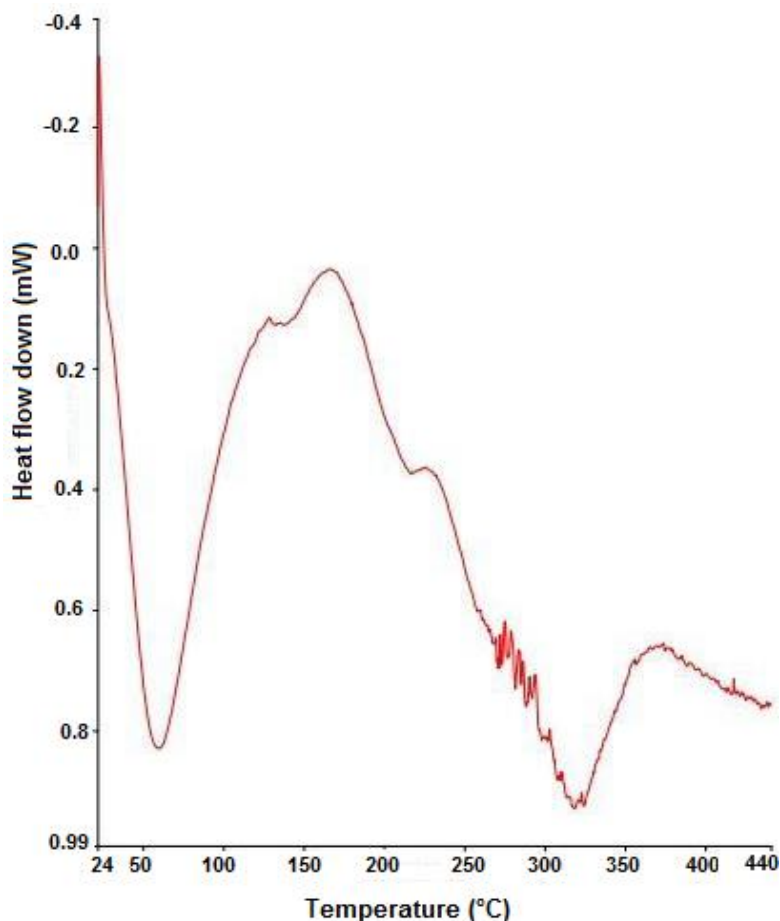


Figure 2. DSC analysis from *Eisenia andrei* flour.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Supplementary data. Soil and vermicompost nutrient analysis.

Parameter	Clay	OM	pH	P	K	Al	Ca	Cd	Cr	Pb
	(%)		(H ₂ O)	(g/kg)		(cmol/dm ³)			(mg/kg)	
Soil	18	1.5	5.8	2.1	18.3	0.0	11.5	<0.2	25.0	14.0

OM, Organic matter; P, phosphorous; K, potassium; Al, aluminium; Ca, calcium; Cd, cadmium; Cr, chromium; Pb, lead.

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