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Full Length Research Paper

Phytoconstituents, antimicrobial and antioxidant properties of the leaves of *Persea americana* Mill cultivated in Ghana

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The leaves of *Persea americana* are widely used for ethno-medicinal purposes worldwide. This study assessed the antimicrobial and antioxidant activities of the methanol, ethyl acetate, chloroform and petroleum ether leaves extracts of *P. americana*. Extracts displayed variable antimicrobial activities that were microorganism-specific. The methanolic extract displayed the most potent antimicrobial activities with the largest zones of inhibition (0-1.8 mm) in the agar diffusion assay and with the lowest minimum inhibitory concentration (MIC) in the broth dilution assay against a panel of microorganisms that included *Bacillus subtilis*, *Escherichia coli*, *Salmonella typhi*, *Streptococcus aureus*, *Pseudomonas aeruginosa* and *Candida albicans*. The ethyl acetate extract exhibited the most potent antioxidant potential with the lowest EC₅₀ of 4.15x10⁻³⁰ g/ml for the peroxide radical scavenging activities. The data supports the ethnomedicinal use of the leaves of *P. americana* for the management of infections and for other symptoms whose etiology may be linked to oxidative stress.

Key words: *Persea americana*, antioxidant, antimicrobial, phytochemical screening, minimum inhibitory concentration.

INTRODUCTION

There is emerging general and scientific interest these days for discovering phytochemicals as distinct options for the synthetic substances that are normally used in food, pharmaceutical and cosmetic industries. This interest for natural products as alternatives to synthetic products has been used by medical doctors, scientific researchers, as well as by general public (Borchers et al., 2000; Truiti et al., 2003). This thought is bolstered by the buyer's worry about the safety of items containing

synthetic chemicals in light of the fact that such molecules are suspected to bring about or advance negative health effects (Rodríguez-Carpena et al., 2011).

Therapeutic plants have kept on drawing in consideration worldwide in the quest for powerful antimicrobial drugs that can battle resistant pathogens that have rendered numerous conventional medications out of date in the treatment of diseases (Cox, 1990). Numerous medications utilized in medicine are acquired

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from plants (Idris et al., 2009). The most active of these bioactive constituents of plants are alkaloids, tannins, steroids, terpenoids and phenolics.

The important function of antioxidants is suppressing the oxidation of different molecules by restraining the initiation or propagation of oxidizing chain reactions by free radicals and, accordingly, diminishing oxidative harm (Frankel and Meyer, 2000). Antioxidants act in different ways, which incorporate complexation of redox-catalytic metal particles, scavenging of free radicals, and decomposition of peroxides. Using different methods for the investigation of antioxidant action of food related extracts permit a complete screening of their plausible antioxidant activities (Frankel and Meyer, 2000). Unrefined extracts of herbs, natural products, flavors, and other plant materials rich in phenolics are of expanding enthusiasm for the food industry on the grounds that they hinder the oxidative degradation of lipids, and thus enhance the quality and nutritional estimation of foods (Bastida et al., 2009; Ganhão et al., 2010).

Phenolic compounds in plants function as antioxidants due to their redox properties. They are therefore useful as reducing agents, hydrogen donors, free radical quenchers, and metal chelators. A lot of antioxidants have already been extracted and isolated from different parts of plants and plant materials, such as oil seeds, cereal crops, vegetables, fruits, leaves, roots, spices and herbs (Alothman et al., 2009; Badu et al., 2012; Garcia-Alonso, 2004; Kähkönen et al., 1999; Velioglu et al., 1998).

Persea americana originated from southern Mexico, however, they are now grown worldwide. Countries where *P. Americana* is cultivated include Australia, South Africa and Spain (Rodríguez-Carpena et al., 2011). Because of the high demand for *P. Americana* fruit worldwide owing to its high nutritional value and reported health benefits, including anticancer activity, the food industry has grown tremendous interest in processing this crop and enhancing its value (Lu et al., 2005). *P. americana* leaves' extracts have been used as analgesic, anti-inflammatory, hypoglycaemic, anticonvulsant, antidiabetic and vasorelaxant among other therapeutic uses (Adeyemi et al., 2002; Antia et al., 2005; Gondwe et al., 2007; Ojewole and Amabeoku, 2006; Owolabi et al., 2005).

A few studies have focused on the phytochemical composition of *P. Americana* (Torres et al., 1987). *P. americana* leaves have been reported to possess anti-inflammatory (Adeyemi et al., 2002), antifungal (Prusky et al., 1991) and antibacterial activities (Gomez-Flores, 2008). There is scarce information available in the literature about the total phenolic content and antioxidant capacity of the leaves (Owolabi et al., 2010; Yasir et al., 2010), pulp (Alothman et al., 2009) and residues from *P. Americana* fruit (Wang et al., 2010). Exploiting the phytochemical content of *P. Americana* waste materials such as leaves, peel and seed may lead to new food

products of enhanced quality, and that would have a significant impact on both the *P. americana* and the processed-food industries. Although, the antimicrobial and antioxidant properties of *P. Americana* have been widely reported, there has been no report on the species grown in Ghana.

The aim of the present study was to determine the phytoconstituents of the leaves, the antimicrobial and antioxidant potential of different leaves' extracts (ethyl acetate, chloroform, petroleum ether and methanol) of *P. americana* cultivated in Ghana.

MATERIALS AND METHODS

Chemicals

All chemicals and reagents used for the present work were purchased from Sigma Chemicals (Huge Ltd, Accra, Ghana). All the solvents used for extraction were suitable for industrial food use and were used as received without any further purification or treatment.

Sample collection and preparation

Fresh leaves of *P. americana* Mill were collected from the Kumasi metropolis in The Ashanti region of Ghana. The leaves were plucked from different *P. Americana* trees which weighed a total of 1.5 kg. The leaves were washed with distilled water to remove dirt settled on it and air-dried for seven days. The dried leaves were pulverized into powder using a mill. The powder was stored in a transparent air-tight container, labeled with a permanent marker and stored at room temperature.

Phytochemical Screening

Phytochemical screening was carried out on the powdered sample to determine the presence of eleven pharmacologically active phytochemicals in the leaves using standard methods described by Trease and Evans (1989). The phytochemicals determined were tannins, saponins, general glycosides, terpenoids and steroids, carotenoids, coumarins, alkaloids, anthraquinones, cyanogenic glycosides, flavonoids and anthraquinone glycosides.

Extraction of phytochemicals from *Persea americana* leaves

A soxhlet extraction method was used for the extraction of phytochemicals from the *P. Americana* leaves. 400 ml each of methanol, chloroform, ethyl acetate and petroleum ether was used to extract from 50 g each of the powdered plant sample for 10 h. The solvent was then removed with a rotary evaporator after which the extracts were dried by slow evaporation at room temperature.

Antimicrobial analysis

The methanol, chloroform, ethyl acetate and petroleum ether leaves extracts were tested against *Escherichia coli*, *Salmonella typhi*, *Streptococcus aureus*, *Candida albicans*, *Pseudomonas aeruginosa* and *Bacillus Subtilis* to ascertain their zone of inhibition and minimum inhibitory concentrations. These organisms were chosen because they are the common gram positive, gram negative

Table 1. Phytoconstituents of *P. americana* leaves.

Test	Result
Glycosides	Present
Alkaloids	Present
Tannins	Present
Saponins	Present
Flavonoids	Present
Terpenoids and steroids	Present
Cyanogenic glycosides	Absent
Coumarins	Absent
Anthraquinones	Absent
Anthraquinone glycosides	Absent
Carotenoids	Absent

and fungus.

Zone of inhibition

The zone of inhibition was determined using the nutrient agar method. Twenty-four (24) petri-dishes with each petri-dish corresponding to one test organism for each extract and labeled in all the four different concentrations of the plant extracts were used. A 20 ml of nutrient agar in the petri-dish labeled for the organism. The nutrient agar was allowed to solidify and wells created in them using the cork borer (6 mm). Each well was filled with its respective concentration of the plant extract and left for about one hour for complete diffusion of the extract within the nutrient agar. The petri-dishes containing the nutrient agar were then incubated between 37°C and 42°C for a period of 18 h after which the zone of inhibition was determined.

Antimicrobial activity index

Antimicrobial index (AI) for methanol, ethyl acetate, chloroform, and petroleum ether extracts of *P. Americana* leaves were calculated as the mean value of the antimicrobial activity obtained against the sum of all individual microorganisms. Weight age was assigned to activity of extracts against each microbe. For zone of inhibition up to 10 mm, a weightage of 1 was given and that ranging from 11 to 20 mm, weightage of 2 was assigned. For zone of inhibition greater than 20 mm, weightage of 3 was assigned and for no antimicrobial activity, weightage of zero was assigned. The sum total of weightages obtained by each extract divided by the total number of pathogens tested gave the AI of the extract.

Antioxidant assay by hydrogen peroxide decomposition

The antioxidant activity of *P. Americana* leaves was determined using the hydrogen peroxide decomposition method by iodometric titration. A 2 ml, 4 ml and 8 ml aliquots of the methanol extract was prepared by dissolving 1 g of extract in 100 ml distilled water, added to 8 ml of 17 mM H₂O₂ solution in three different conical flasks. The mixture in each conical flask was mixed by gentle swirling motion. A 25 ml of distilled water was added to 1 ml of the mixture from each conical flask to slow the consumption of H₂O₂ by the extract at 60 s interval for a period of 4 min. A 2.2 g of KI and 10 ml of 2M H₂SO₄ were added to each of the mixtures in the conical

flasks and the liberated iodine gas (I₂) titrated against 0.0519M standard sodium thiosulphate solution using starch as indicator. A blank was also titrated against standard sodium thiosulphate and the titre subtracted from that of the extract before its concentration was determined. The decomposition of H₂O₂ for three different concentrations of the extract was plotted against time of decomposition to ascertain the antioxidant activities of the extracts.

Statistical analysis

A one way analysis of variance (ANOVA) was done using Microsoft excel software to establish the presence or absence of variability between the antimicrobial activities of various extracts and same extracts at different concentrations. Student's t-test analysis was carried out for analyzing the results. P values at <0.05 were considered for describing the significant levels.

RESULTS AND DISCUSSION

Phytoconstituents of *P. americana* leaves

Phytochemical screening of the leaves of *P. Americana* showed the presence of tannins, saponins, terpenoids and steroids, alkaloids, flavonoids and glycosides as presented in Table 1. The presence of these phytochemicals has pharmacological and medicinal importance to humans (Yasir et al., 2010). For example, alkaloids can act as antimalarial, anticancer, antiasthma and antibacterial pharmacological constituents in humans. Tannins on the other hand have been used to combat diarrhea (Idris et al., 2009). The presence of tannins also enhances the antioxidant properties of the *P. americana* (Alothman et al., 2009). Saponins have gained grounds as a dietary supplement and nutraceutical (Akinpelu et al., 2014). Saponins have also been used to lower blood cholesterol level and also as an anticancer agent. Furthermore, its amphipathic properties promote the penetration of proteins through the cell membranes. Glycosides are known for their antibiotic properties. The therapeutic properties of *P. Americana* based on the phytoconstituents cannot be overlooked (Cox, 1990). The phytoconstituents obtained in this study can be compares well with other literature reports (Owolabi et al., 2010; Yasir et al., 2010).

Antimicrobial activity of the leaves' extract of *Perseaamericana*

The antimicrobial activity of the leaves extract of *P. americana* was tested against five bacterial strains and one fungal strain. The microorganisms were *B. Subtilis*, *C. albicans*, *P. aeruginosa*, *S. aureus*, *S. typhi* and *E. coli*. Antimicrobial activities were assessed by determining the zone of inhibition (measured in mm) of the various extracts against the test microbes (Figure 1). The size of this zone depends on the effectiveness of the extract against the growth of microbes. Usually, at higher

Table 2. Antimicrobial activity index of extracts of *Persea americana* leaves at different concentrations.

Concentration (%)	Methanol extract	ethyl acetate extract	Chloroform extract	Petroleum ether extract
1.25	0.50	0.17	0.33	0.00
2.5	0.83	0.67	0.50	0.00
5	1.00	1.00	0.67	0.50
10	1.00	1.00	0.67	0.83

Table 3. Minimum inhibitory concentration.

Microbe	Minimum inhibitory concentration (%)			
	Methanol extract	Ethyl acetate extract	Chloroform extract	Petroleum ether extract
<i>B. Subtilis</i>	1.56 x 10 ⁻⁶	0.93	0.62	1.98
<i>C. albicans</i>	0.036	1.48	1.05	6.29
<i>P. aeruginosa</i>	0.942	0.95	No inhibition	0.196
<i>S. aureus</i>	15.96	0.92	No inhibition	1.34
<i>S. typhi</i>	3.16x10 ⁻⁵	0.093	0.077	1.34
<i>E. coli</i>	1.702	1.48	1.67	No inhibition

concentrations of the antibiotics, larger zones are created. The methanol extract was active against *S. typhi*, *C. albicans*, and *B. Subtilis* at low concentrations (1.25%). The extract showed no antimicrobial activity against *E. coli* at concentration $\leq 2.5\%$.

The methanol extract was very potent against *B. Subtilis* whereas very low potency was recorded for *E. coli*. Although, these two microbes are rod-like, the outer membrane of the *E. coli* which is a gram negative bacterium is able to inhibit the antimicrobial activity of the extract. The ethyl acetate extract was only potent against *S. typhi* at higher concentrations. Increasing the concentrations from 1 to 10 % increased the antimicrobial activity against all the microbes. The petroleum ether and chloroform extracts showed no antibacterial activity against *E. coli*. Generally, the polarity index of the solvent had a great impact on the antibacterial activity of the leaves extracts as it was observed that all the extracts showed decreasing antimicrobial activity in order of decreasing polarity index of the extracting solvent.

The methanol, ethyl acetate, chloroform and petroleum ether extracts of the leaves exhibited antimicrobial activity on all the test organisms producing zone of inhibition ranging from 0 to 1.8, 0 to 0.9, 0 to 0.7 and 0 to 0.4 mm respectively. These results are in good agreement with the antimicrobial activity of seed extracts of *P. Americana* on similar test organisms (Idris et al., 2009). The methanol extract inhibited the growth of *C. albicans* at all concentrations whereas for ethyl acetate, chloroform and petroleum ether extracts, the extent of inhibition was concentration dependent. That is, at lower concentrations no inhibitions were observed.

The antimicrobial activity index of extracts of *P. Americana* leaves at different concentrations was also

investigated and is detailed in Table 2. Methanol extract of the leaves recorded the highest antimicrobial activity and achieved the highest activity index among all the extracts. The difference in the activity indices may be due to different phytoconstituents present in the individual extracts. This is because different solvents have different degrees of solubility for different phytoconstituents (Gopalakrishnan et al., 2012). With the exception of the petroleum ether extract, increasing concentration of the extract from 5 to 10% had no effect on the activity index.

The minimum inhibitory concentration (MIC) of the methanol, ethyl acetate, chloroform and petroleum ether extract of the leaves of *P. americana* was tested against all six organisms. The solvents were chosen based on their polarity. Methanol has a polarity index of 5.1, ethyl acetate, 4.4; chloroform, 4.1 and petroleum ether, polarity index of 0.1. From Table 3, it was observed that the extracting solvent had a significant impact on the MIC of the microbes.

The low MIC for the extracts demonstrated the therapeutic potential of the phytoconstituents (Idris et al., 2009). The petroleum ether extract showed a higher MIC against *B. subtilis*, *C. albicans* and *S. typhi* than the other solvent used. It however, showed no inhibition against *E. coli*. This is in agreement with the zone of inhibition for the petroleum ether extract at various concentrations as high MIC indicates low antimicrobial activity.

Antioxidant properties of *P. americana* leaves

In humans, oxidative stress results from a decrease in antioxidant potential or an increase in the production of oxygen radical. These are capable of altering the structure

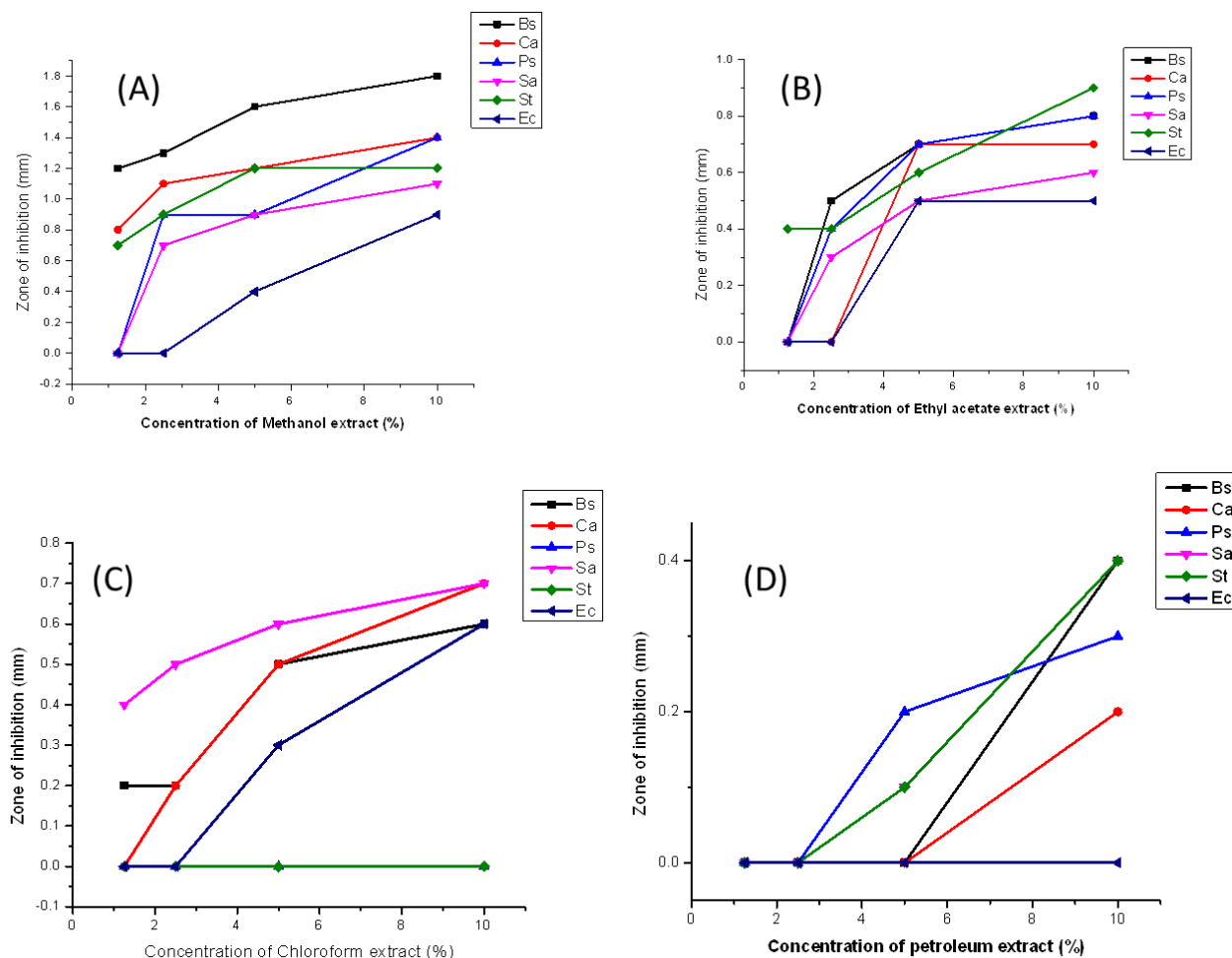


Figure 1. Zone of inhibition of micro-organisms against (A) methanol, (B) ethyl acetate, (C) chloroform and (D) petroleum ether extracts of *P. Americana* leaves. Bs, Ca, Ps, Sa, St and Ec represent *B. subtilis*, *C. albicans*, *P. aeruginosa*, *S. typhi*, *S. aureus* and *E. coli* respectively.

structure and functions of many biomolecules. *P. Americana* leaves' extracts have shown strong antioxidant activities (Figure 2). Generally, for all the extracts, increasing concentration corresponded to an increase in their antioxidant properties. There were however, no significant differences between % H_2O_2 decomposition at different concentrations of a particular extract $P > 0.05$ and different extracts of similar concentration. The petroleum ether extract of the leaves showed no % H_2O_2 decomposition at all the concentrations used. This indicates no antioxidant activity of the extract.

The methanol extract showed an increase in the % H_2O_2 decomposition with respect to increasing concentration of the extract at constant time. However, at lower concentrations of the extract (0.02 g/ml), increasing time from 60 to 120 seconds resulted in a constant antioxidant activity. Increasing concentration of the extract from 0.02 to 0.08 increased the % H_2O_2

decomposition at constant time. Also, for all the three different concentrations used, increasing time corresponded to an increase in the % H_2O_2 decomposition. At low concentration of the extract (0.02 g/ml), a constant % H_2O_2 decomposition was recorded when the time was increased from 60 to 120 s.

At any particular concentration of the ethyl acetate extract, increasing time resulted in an increase in the % H_2O_2 decomposition. At a concentration of 0.08 g/ml, increasing time from 60 to 240 s resulted in a proportional increase in the % H_2O_2 decomposition. Comparing 0.02 and 0.04 g/ml concentrations of the ethyl acetate extract, the 0.04 g/ml concentration showed increase in % H_2O_2 decomposition when the time was increased from 60 to 120 s.

However, increasing the time further from 180 to 240 s resulted in a decrease in the % H_2O_2 decomposition as compared to the 0.02 extract concentration. Of all the extracts, ethyl acetate extracts showed the highest

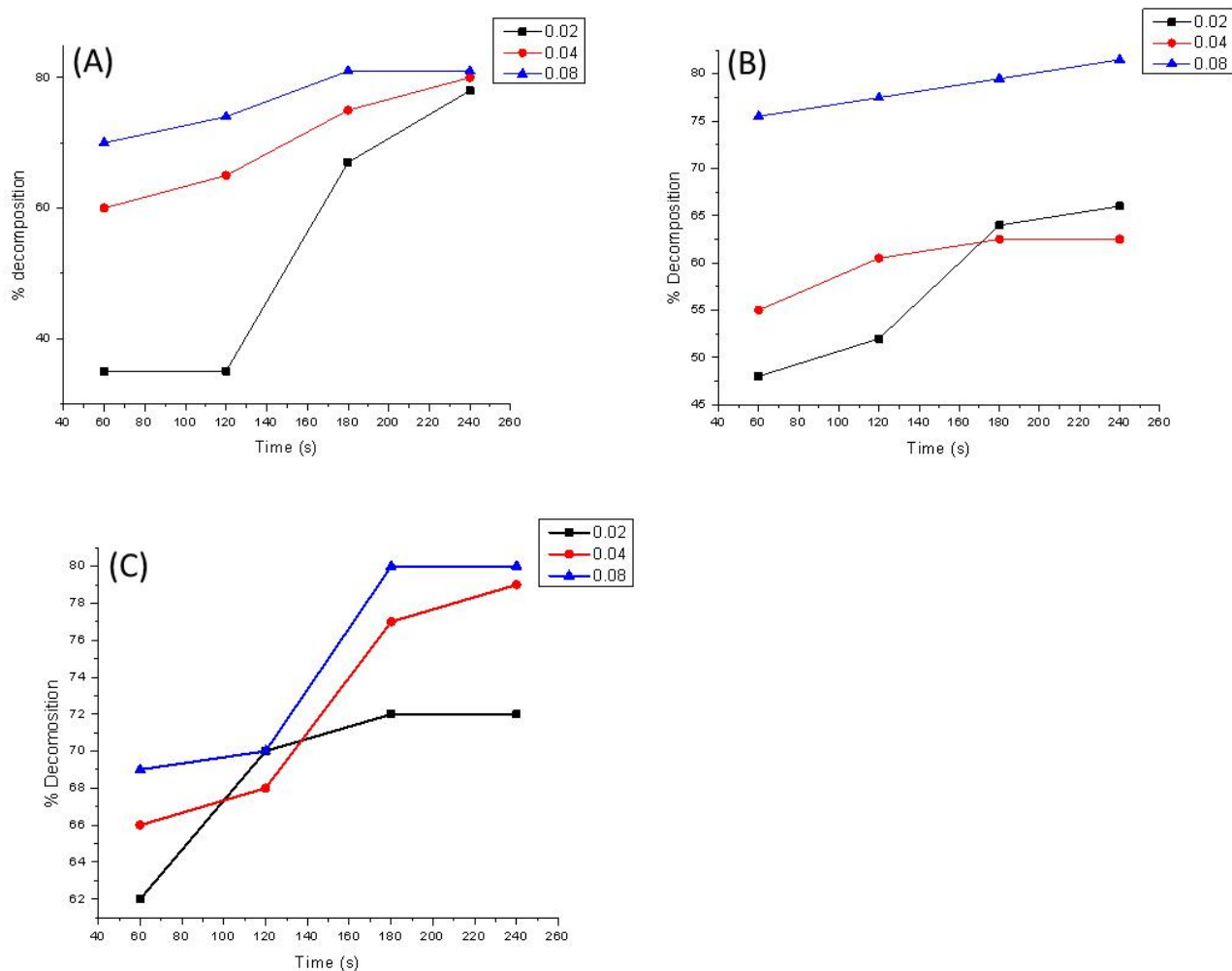


Figure 2. % H₂O₂ decomposition for (A) methanol, (B) ethyl acetate and (C) chloroform extracts.

antioxidant activity with EC₅₀ of 4.15×10^{-30} g/ml. Methanol extract followed with EC₅₀ of 5.52×10^{-10} g/ml and chloroform extract with EC₅₀ of 6.57×10^{-5} g/ml. The antioxidant property of the leaves extract is in good agreement with earlier report by Owolabi et al. (2010).

This study used the % H₂O₂ decomposition method to determine the antioxidant of the leaves of *P. americana* and concluded that the leaves of *P. americana* contain antioxidant which can help prevent stress related diseases. Also, Ikpeme et al. (2014) evaluated the antioxidant efficacy of fresh and dried fruits of *P. Americana* and reported that the fresh fruit is more efficient than the dry fruit.

Conclusion

Phytochemical screening of the leaves of *P. americana* showed the presence of tannins, flavonoids, saponins, terpenoids, steroids, alkaloids and glycosides.

The antimicrobial activities of methanol, ethyl acetate, chloroform and petroleum ether extracts of *P. Americana* leaves have been investigated. The extracts were tested against *B. subtilis*, *E. coli*, *S. typhi*, *S. aureus*, *P. aeruginosa* and *C. albicans*. All the extracts were potent against the test organisms with the methanol extract exhibiting the highest zone of inhibition.

Also, the antioxidant properties of the extracts have been investigated. All the extracts except petroleum ether showed antioxidant activities. Of all the extracts, methanol extracts showed the highest antioxidant activity. This research has confirmed the antimicrobial and antioxidant properties of the leaves' extracts of *P. americana*.

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Conflicts of interest

The authors have none to declare.

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Full Length Research Paper

Antioxidant and toxicological studies of ethanolic root extract of *Byrsocarpus coccineus*

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***Byrsocarpus coccineus* (syn. *Rourea coccinea*) Schum. and Thonn. (Connaraceae) is used in traditional medicine to treat several ailments in which reactive oxygen species are involved. This study aims to investigate the *in vivo* antioxidative properties and moreover the toxicological potential of ethanolic root extract of *B. coccineus* (EEBc). Antioxidant activity was measured using ferric reducing antioxidant power (FRAP) and nitric oxide (NO) assays, respectively on serum and on bronchoalveolar lavage fluid and moreover by quantifying malondialdehyde (MDA) in rat model ovalbumin-induced airway inflammatory. Toxicological screening was performed using single oral administration at 5000 mg/kg and sub-chronic (4 weeks) administration at 400 and 800 mg/kg to rats. Results indicated that EEBc increases antioxidant potential in the blood. EEBc significantly reduced the NO level ($P < 0.05$) and MDA concentration ($P < 0.01$). The extract at a single dose did not produce the signs of toxicity or mortality during 14 days. The sub-chronic tests showed no alterations in animals. The results did not show any biochemical and hematological abnormalities. This study shows that EEBc may be used as natural antioxidant and may help to prevent pathological conditions related to oxidative stress.**

Key words: Antioxidant, malondialdehyde, toxicity, *Byrsocarpus coccineus*.

INTRODUCTION

Plants have been the source of natural products used, since earliest times, in non-conventional medicine known as traditional medicine through communities worldwide. Today, medicinal plants have continued to play an important role in the primary health care for more than 80% of people living in poor communities in the developing countries (Bennett and Brown, 2000; Nath et

al., 2011). During the last decades, the use of medicinal plants in therapeutics has increased substantially (Castro et al., 2009; Lee et al., 2012) due to the increasing interests for natural substances. Among medicinal plants used in traditional medicine, some have antioxidant property and are used rightly or wrongly to prevent premature aging.

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Antioxidants have been reported to prevent oxidative damage caused by reactive oxygen species (ROS) which readily attack and induce damage to various biologic compounds, including proteins, lipids, sugars and DNA (Farber, 1994; Büyükkuroğlu et al., 2001; Kaur et al., 2006). These oxidative damages are considered as crucial etiological factor implicated in the initial phase of several chronic diseases, such as diabetes mellitus, pulmonary diseases, cancer, neurodegenerative diseases and also in the ageing process (Pong, 2003; Roussel, 2009). There is a growing interest of antioxidant considered as alternative opportunity to prevent chronic diseases. Plants rich in phenolic compounds like flavonoids have been demonstrated to have anti-inflammatory, anti-allergic, anti-viral, anti-aging, and anti-carcinogenic activities which can be attributed to their antioxidant properties (Agil et al., 2006).

Throughout popular knowledge, *Byrsocarpus coccineus* (syn. *Rourea coccinea*) Schum. and Thonn. (Connaraceae) is used in the traditional medicine in Togo (West Africa) to alleviate various diseases including dysmenorrhea, swellings, muscular and rheumatic pains, sore, wounds, hemorrhage, hypertension, primary and secondary sterility, abscess and anemia. Previous studies reported that the leave of *B. coccineus* have various pharmacological activities such as analgesic activities (Akindele and Adeyemi, 2006a), antidiarrhea activities (Akindele and Adeyemi, 2006b), antipyretic activities (Akindele and Adeyemi, 2007a), anti-inflammatory activities (Akindele and Adeyemi, 2007b), anxiolytic/sedative activities (Akindele and Adeyemi, 2010) and antidiabetic activity (Dada et al., 2013). Recently, we have shown that ethanolic root extract of *B. coccineus* (EEBc) has anti-inflammatory and *in vitro* antioxidant activities (Dosseh et al., 2014).

Although, the use of herbal medicine may be considered to be safe, some natural products are known to be toxic at high doses and others may have potential adverse effects after prolonged use. Many data concerning the safety of herbal medicine has been reported and frequently these reports are related to hepatotoxicity (Saad et al., 2006; Park et al., 2010) and nephrotoxicity (Cheng et al., 2006; Debelle et al., 2008). Hence, toxicological assessment of medicinal plants is required even if these plants are used many centuries ago. Despite intensive use in traditional medicine, the fruits of *B. coccineus* are known to be popularly highly toxic. Based on this consideration, toxicological studies had been undertaken on the leaves of *B. coccineus* (Akindele and Adeyemi, 2006b; Adeyemi et al., 2010; Akpan et al., 2012), however and according to our knowledge, any similar investigation had not been conducted on the root of this plant. Thus, this present study is aimed at investigating the antioxidant activity and toxicological screening of EEBc in rats. In addition, the content of total

flavonoids was measured, in the extract in order to correlate them with antioxidant activity.

MATERIALS AND METHODS

Plant

Matured roots of *B. coccineus* were collected around the campus of University of Lomé in February 2013. The plant was authenticated by Dr. Kokou Kouami of the Botany Department (University of Lomé) and a voucher specimen has been deposited in the herbarium of the department under reference Number Togo 15075.

Ethanolic extract

The root bark was dried under air-conditioning and reduced into powder. The powder (100 g) was extracted with continuous agitation in ethanol 95° (1000 ml) at room temperature for 72 h based on traditional practice in which alcohol is used frequently. The filtrate was concentrated to dryness under vacuum in a rotary evaporator at 40°C and yielded a residue of 13.84% (w/w). The extract was stored at -4°C.

Experimental animals

Male and female Wistar rats weighing 130 to 160g were produced by the Department of Physiology/Pharmacology of University of Lomé. Animals were kept under ambient temperature, with a 12 h light and dark cycle and had free access to food and water. Before each experiment, the animals were fasted overnight with free access to water. All animal procedures were performed after approval from the Ethics Committee of the University of Lomé (Togo) and in accordance with the recommendations of the proper care and use of laboratory animals (No. SBM/UL/2015/SN 0005).

In vivo antioxidant activity

Serum ferric reducing antioxidant power (FRAP)

Animal were randomly divided into three groups of 5 animals (3 male and 2 female) each. The first group (control) received distilled water (10 ml/kg) and the second and third groups received, respectively EEBc at 400 and 800 mg/kg. The animals were daily treated orally (p.o.) for 21 days. At the end of EEBc administration, blood was collected from overnight fasted rats under anaesthesia by retro-orbital bleeding into tubes without ethylenediaminetetraacetic acid (EDTA). Blood samples were then immediately centrifuged at 3000 rpm for 10 min and the serum was extracted and then stored at -20°C for further use.

The antioxidant power of serum was determined by measuring its ability to reduce Fe^{3+} into Fe^{2+} by FRAP assay (Nair et al., 2007). Briefly, 300 μl of a daily working reagent (prepared by mixing 25 mL of acetate buffer at 300 mM; 2.5 ml of Fe^{3+} -triipyridyl-s-triazine (Fe^{3+} -TPTZ) at 10 mM in 40 mM of HCl and 2.5 ml of $\text{FeCl}_6\text{H}_2\text{O}$ at 20 mM was mixed with 10 μl of serum sample and 30 μl of distilled water. The change in absorbance at 593 nm was measured against blank after 10 min of incubation. Aqueous solutions of $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ was used for calibration and antioxidant power was expressed as μM ($y = 0.000539x + 0.01227$; $r^2 = 0.999$).

Measurement of nitric oxide (NO) and malondialdehyde (MDA)

The assays for NO and MDA content were performed in bronchoalveolar lavage (BAL) fluid and lung tissue using ovalbumin (OVA)-induced airway inflammatory model in rats as described by Morris et al. (1989).

Sensitization, challenge and experimental protocol

Wistar rats (145 to 160 g) were divided into four groups (n = 5): non-sensitized or normal control group (NS), sensitized non-treated or OVA-control group (SNT), sensitized treated groups with EEBc at 400 mg/kg (ST400) and 800 mg/kg (ST800). The animals, except those of NS group were actively sensitized by an intraperitoneal injection of 10 mg/kg OVA (grade V; Sigma, St. Louis, MO, USA) mixed with 40 mg/kg aluminum hydroxide as adjuvant in normal saline (0.9%). Non-sensitized animals were injected with aluminum hydroxide (40 mg/kg) only. Sensitizations were performed 4 times at days 0, 3, 7 and 21. On days 24 to 27, SNT group and sensitized treated with EEBc groups under light ether anesthesia were challenged with intranasal instillations of 50 μ l of 20% OVA in normal saline, while normal saline was administered to the NS group in a similar manner.

Rats in the EEBc-treated groups were administered orally with 10 ml/kg of 400 and 800 mg/kg 30 min prior OVA challenge, while the others were administered with normal saline. The rats were sacrificed on day 28. BAL was performed by cannulating the trachea with polyethylene catheter PE-240 (ID: 1.67 mm, OD: 2.41 mm) and infusing the lung with 5 ml of sterile 0.9% saline. BAL fluid was obtained by two aspirations via tracheal cannulation and recovery rate of BAL fluid was approximately 78 to 84% (Agbonon et al., 2005).

Measurement of NO production

The pulmonary production of NO was spectrophotometrically determined by assaying BAL fluid for nitrite using the Griess reagent (1% sulfanilic acid, 0.1% N-1 naphthylethylenediamine dihydrochloride, 2.5% phosphoric acid). Absorbance was measured at 570 nm and nitrite concentration was determined using sodium nitrite as a standard (Fermor et al., 2001).

Determination of lipid peroxidation

MDA concentration was determined as an indicator of lipid peroxidation. Whole lung samples were dissected out 24 h after the last OVA challenge and washed immediately with ice cold saline to remove as much blood as possible. They were weighed and 2 g of tissue were homogenized in 5 ml of a cold KCl solution (1.5%). The homogenate was centrifuged at 3000 rpm for 10 min. Subsequently, MDA content in the supernatants was measured according to the method described by Agbonon and Gbeassor (2009). Briefly, 200 μ l of supernatant was exposed to 0.6 ml of phosphoric acid (1%) and 1 ml of thiobarbituric acids (1%) and the mixture was heated to 100°C for 50 min. At the end of the incubation period, the mixture was cooled in ice for 10 min and 2 ml of 1-butanol was added and the mixture was centrifuged as indicated earlier. After centrifuging, the supernatant was removed and the absorbance was read at 535 nm using an UV-visible recording spectrophotometer (Genesys 10S UV-Vis Spectrophotometer, France). 1,1,3,3-tetramethoxypropane (MDA)

was used as a standard to obtain the standard curve (0-60 nM; $y = 0.03860x + 0.04205$; $r^2 = 0.998$).

Total flavonoids content of the extract

Total flavonoids content in the extract was determined according to colorimetric method using Aluminum Chloride ($AlCl_3$) (Kim et al., 2003). The extract or standard (100 μ l at 1 mg/ml) in ethanol was mixed with 0.4 ml of distilled water and 0.03 ml of 5% $NaNO_2$ solution. After 5 min, 0.02 ml of a 10% $AlCl_3$ solution was added. To the mixture was added 0.02 ml of 1 M Na_2CO_3 and 5 min later, 0.25 ml of distilled water was added. The solution was well stirred and the absorbance was read at 510 nm using UV-visible spectrophotometer (Genesys 10S UV-Vis Spectrophotometer, France). Quercetin was used as a standard to obtain the standard curve (0 to 500 μ g/ml; $y = 255.9x + 0.01850$; $r^2 = 0.998$). Result was expressed as μ g quercetin equivalent per mg (μ g QE/mg) of the extract.

Acute and subchronic toxicity

Acute toxicity test

Acute oral toxicity assay was performed using the limit test dose (Lorke, 1983; Bakoma et al., 2013). Five female Wistar rats were individually administered a single oral dose of 5000 mg/kg of extract (one after the other at a grace observation period of 24 h). The control group received water vehicle (10 ml/kg). Animals were observed individually for a period of 4 h for immediate signs of toxicity and mortality and at least once daily for 14 days for delayed mortality and toxic symptoms, such as changes in skin and fur, eyes, mucous membranes, convulsion, salivation, diarrhea, lethargy, sleep and coma. On the 15th day after administration, the survivor animals were weighed and sacrificed and then the vital organs including heart, lungs, livers, kidneys, spleen, and sex organs were grossly examined.

Sub-chronic toxicity test

Experimental design

Repeat-dose oral toxicity study was carried out according to Bakoma et al. (2013) and Diallo et al. (2010). Rats were randomly divided into three groups of 10 animals (5 males and 5 females) each. The first group (control) received distilled water (10 ml/kg); the second and third groups received, respectively EEBc at 400 and 800 mg/kg representing the pharmacological active doses in rats from our previous study (Dosseh et al., 2014). The animals were daily treated orally (p.o.) at the same time for 28 days. They were observed at least twice daily for morbidity and mortality. Body weights of the animals were evaluated weekly. On the 29th day, blood samples were collected from overnight fasted rats under anesthesia by retro-orbital bleeding into tubes with and without EDTA for hematological and biochemical analyses and the rats were sacrificed. The blood tubes without EDTA were centrifuged at 3000 rpm for 10 min and serum was separated and stored at -20°C for biochemical analyses. On the sacrificed rats, internal organs including liver, kidneys, spleen, heart, lung, ovaries and testicles were carefully collected and observed for macroscopic lesions or signs of apparent toxicity. Then, these organs were weighed to determine their relative weights [(organ weight/total body weight) \times

Table 1. Effect of ethanolic root extract of *B. coccineus* on the FRAP values in the blood of rats administered during 21 days.

Traitements groups	FRAP (μM)	
	Before treatment	After treatment
Control	447.98 \pm 17.157	468.64 \pm 15.04
400 mg/kg	444.52 \pm 31.01	752.25 \pm 83.47**
800 mg/kg	450.46 \pm 21.83	769.44 \pm 115.75

**Values are expressed as mean \pm standard error of mean (SEM, n = 5); Rats were administered orally during 21 days by EEBC. ** $P < 0.01$ when compared 400 mg/kg and 800 mg/kg treated groups to control after treatment (one way analysis of variance (ANOVA) followed by Tukey's multiple comparisons test). No statistical difference between the control and treated groups before treatment ($P > 0.05$).

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Blood analysis

White blood cell count (WBC), red blood cell count (RBC), hemoglobin (HGB), hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular haemoglobin concentration (MCHC), mean corpuscular hemoglobin (MCH) and platelet count (PLT) were determined as hematological parameters using an automatic hematological analyzer (BC-2800, Mindray- China). The following marker enzymes were measured in the serum as biochemical indicators for liver injury/dysfunction: alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), total bilirubin (TB), total protein (TP), cholesterol and triglycerides. Kidney dysfunction was indicated by creatinine and blood urea levels. Glucose was assessed to evaluate pancreatic function. Standardized diagnostic kits purchased from Human GmbH, D-65205, Wiesbaden, Germany were used for spectrophotometric determination of the biochemical parameters.

Statistical analysis

The results are expressed as mean \pm standard error of mean (SEM). Data were analysed by one-way analysis of variance followed by Tukey post-hoc test. Results were considered to be significant at $P < 0.05$. All statistical analyses were carried out using GraphPad Prism 5.00 (GraphPad Software Inc., CA, USA).

RESULTS

Antioxidant activity of ethanolic root extract of *B. coccineus*

FRAP analysis

The FRAP assay in blood sera of rats treated with EEBC (400 and 800 mg/kg) showed increased levels of Fe^{2+} as compared to the control group. The initial FRAP values varied considerably after 21 daily gavage ($P < 0.01$) (Table 1).

Effect of ethanolic root extract of *B. coccineus* on NO and MDA level

The NO level in the BAL fluid was significantly increased ($P < 0.05$) in the SNT group ($0.071 \pm 0.015 \mu\text{M}$) as compared to the NS group ($0.020 \pm 0.011 \mu\text{M}$). EEBC at the dose of 800 mg/kg significantly restored the level of NO ($0.021 \pm 0.009 \mu\text{M}$; $P < 0.50$) (Figure 1). The MDA level in the lung tissue was significantly increased ($P < 0.001$) in the SNT group as compared to SN group ($36.30 \pm 4.02 \text{ nM/ml}$ vs. $15.60 \pm 2.31 \text{ nM/ml}$). The MDA level was significantly decreased ($P < 0.01$) by EEBC at 400 ($17.0 \pm 2.52 \text{ nM/ml}$) and 800 mg/kg ($16.7 \pm 2.45 \text{ nM/ml}$) when compared with the SNT (Figure 2).

Total flavonoids content

The total flavonoids contents of EEBC were $414.16 \pm 1.15 \mu\text{g QE/mg}$ of extract.

Acute toxicity study

After the rats were orally given a single dose of EEBC at 5000 mg/kg, no death of rats was recorded for a period of 4 h. Also any behavioral changes and lethargy was not observed in treated groups for 14 days post-treatment. Neither body weight nor internal organ weight in treated rats was significantly changed relative to that of the control group. No abnormality was found in organs at necropsy (data not shown).

Subchronic toxicity

Effect of extract on physical parameters

Daily oral administration of EEBC for 28 consecutive days did not induce any obvious symptom of toxicity in rat. No

Table 2. Mean relative organ weights of control and daily treated rats with ethanolic root extract of *B. coccineus* in sub-chronic oral toxicity test (g \pm SEM per 100 g body weight).

Organs	Control	Extract doses (mg/kg/day b.w.)	
		400	800
Liver	3.49 \pm 0.05	3.61 \pm 0.14	3.47 \pm 0.11
Spleen	0.28 \pm 0.02	0.30 \pm 0.02	0.30 \pm 0.02
Kidneys	0.71 \pm 0.04	0.77 \pm 0.06	0.79 \pm 0.05
Heart	0.42 \pm 0.02	0.38 \pm 0.01	0.37 \pm 0.01
Lung	0.75 \pm 0.02	0.68 \pm 0.01	0.76 \pm 0.02
Ovaries	0.10 \pm 0.00	0.10 \pm 0.01	0.11 \pm 0.01
Testicles	1.50 \pm 0.03	1.38 \pm 0.01	1.38 \pm 0.03

Values are expressed as mean \pm standard error of mean (SEM, n = 5); No statistical difference between the control and treated groups ($P > 0.05$) (one-way ANOVA).

Table 3. Mean hematological value of control and daily treated rats with ethanolic root extract of *B. coccineus* in sub-chronic oral toxicity test.

Parameter	Control	Extract doses (mg/kg/day b.w.)	
		400	800
WBC ($10^9/L$)	9.48 \pm 0.53	10.15 \pm 0.63	9.50 \pm 0.56
RBC ($10^{12}/L$)	6.96 \pm 0.18	7.46 \pm 0.09*	7.59 \pm 0.11**
HGB (g/dl)	13.77 \pm 0.36	14.48 \pm 0.15	14.76 \pm 0.21*
HCT (%)	40.90 \pm 1.19	41.34 \pm 0.84	41.84 \pm 0.41
MCV (fL)	58.08 \pm 1.08	56.40 \pm 1.07	56.16 \pm 1.06
MCH (pg)	19.05 \pm 0.19	19.21 \pm 0.36	18.92 \pm 0.26
MCHC (g/dl)	33.48 \pm 0.21	34.15 \pm 0.18	33.49 \pm 0.79
PLT ($10^3/\mu l$)	524.20 \pm 25.12	535.50 \pm 23.15	564.10 \pm 24.50

Values are expressed as mean \pm standard error of mean (SEM, n = 5); ** $P < 0.01$, * $P < 0.05$ vs. control (one way analysis of variance (ANOVA) followed by Tukey's multiple comparison test).

mortality was recorded during the 28 days. No differences in general behavior were observed between the groups of rats. The body weight in EEBC treated rats was normal in comparison with vehicle treated rats (Figure 3). The means relative organ weights show that there were no significant changes between control and treated groups ($P > 0.05$) (Table 2). At necropsy, no macroscopic change was observed in the internal organs in treated rats.

Effect of extract on hematological parameters

Hematological parameters of rats showed that there were no significant difference between control and treated groups ($P > 0.05$) except the RBC count (400 mg/kg and 800 mg/kg) and HGB (800 mg/kg), which were significantly increased in treated groups (Table 3).

Effect of extract on serum biochemical parameters

Single daily oral administration of EEBC at 400 and 800 mg/kg throughout the treatment period (28 days) did not cause any significant changes ($P > 0.05$) in serum levels of ALT, AST, PAL, creatinine, urea, TP, TB in treated animals compare to the OVA-control group. However, results showed significant decrease in cholesterol and triglycerides levels at the dose of 800 mg/kg ($P < 0.05$) (Table 4).

DISCUSSION

Growing interest for natural products from medicinal plants is evidence nowadays and many of these plants products are used for their antioxidant potential, but this practice may be expose to the toxicological risks. The

Table 4. Mean blood clinical chemistry value of control and daily treated rats with ethanolic root extract of *B. coccineus* in sub-chronic oral toxicity test.

Parameter	Control	Extract doses (mg/kg/day b.w.)	
		400	800
AST (U/L)	123.17 ± 4.73	120.28 ± 5.88	116.92 ± 4.71
ALT (U/L)	86.60 ± 8.53	80.68 ± 6.46	80.50 ± 4.48
ALP (U/L)	160.92 ± 5.69	144.00 ± 12.25	144.85 ± 9.77
Creatinine (mg/L)	7.00 ± 0.21	6.60 ± 0.22	6.50 ± 0.17
Urea (g/L)	1.01 ± 0.01	0.97 ± 0.02	0.96 ± 0.01
Total bilirubin (mg/dl)	1.76 ± 0.15	1.73 ± 0.17	1.65 ± 0.11
Total protein (g/L)	59.52 ± 0.56	59.68 ± 0.31	59.76 ± 0.39
Glucose (g/dL)	0.76 ± 0.05	0.65 ± 0.04	0.64 ± 0.05
Triglycerides (g/L)	0.83 ± 0.02	0.80 ± 0.01	0.76 ± 0.01*
Total cholesterol (g/L)	0.96 ± 0.04	0.90 ± 0.02	0.85 ± 0.01*

Values are expressed as mean ± standard error of mean (SEM, n = 5); *P < 0.05 vs. control (one way analysis of variance (ANOVA) followed by Tukey's multiple comparison test).

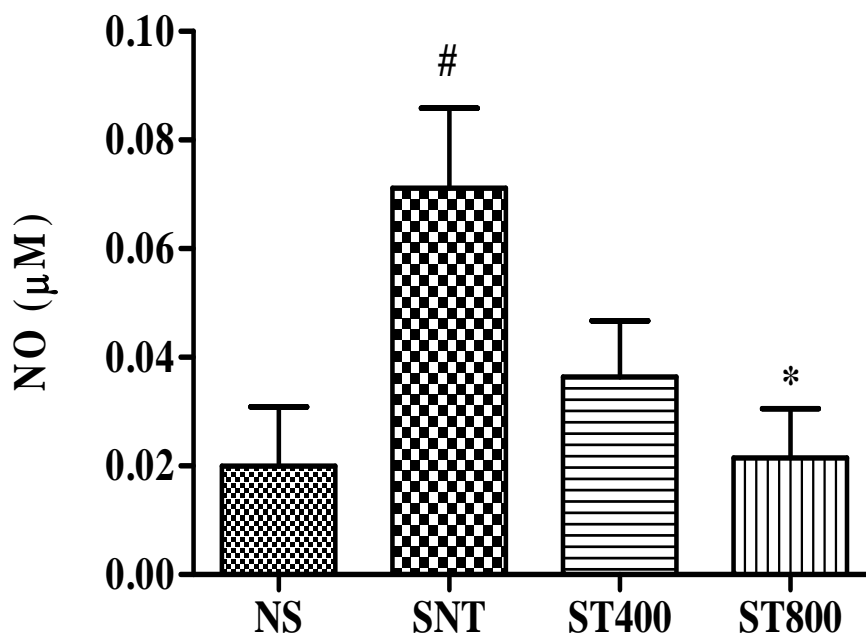


Figure 1. Effect of ethanolic root extract of *B. coccineus* c on NO in BAL. Data are expressed as Mean ± standard error of mean (SEM) n=5; NS = Non-sensitized group, SNT = Sensitized non-treated group with; ST400, ST800: Sensitized treated groups with EEBC at 400, 800 mg/kg, p.o.; #P < 0.05 compared with non-sensitized group; *P < 0.05 compared with sensitized non-treated group (one way analysis of variance (ANOVA) followed by Tukey's multiple comparisons test).

main objective of the present study was to investigate the antioxidant activity and the safety of EEBC.

Oxidative stress results from an imbalance caused by the excessive production of ROS or a reduction in the

antioxidant defenses of the organism (Kaur et al., 2006). Generally, the reducing properties of antioxidant are associated with the presence of compounds which exert their action by breaking the free radical chain by donating

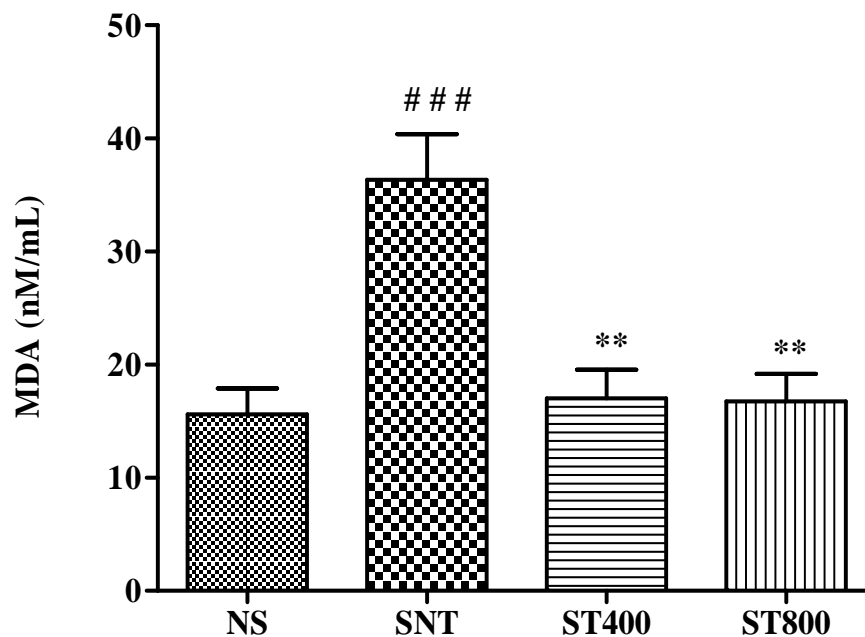


Figure 2. Effect of ethanolic root extract of *B. coccineus* on MDA in lung tissue. Data are expressed as Mean \pm standard error of mean (SEM), n=5; NS = Non-sensitized group, SNT = Sensitized non-treated group with; ST400, ST800: Sensitized treated groups with EEBC at 400, 800 mg/kg, p.o.; ###P < 0.001 compared with non-sensitized group; **P < 0.01 compared with sensitized non-treated group (one way analysis of variance (ANOVA) followed by Tukey's multiple comparisons test).

a hydrogen atom (Duh et al., 1999). Our results suggest the possibility that EEBC is useful for prevention of the phenomenon of the oxidative stress. Increasing the serum antioxidant status has been suggested as a possible method of reducing the risk of many chronic diseases such as asthma.

In this study, OVA was used as an antigen in rats to provoke asthmatic symptoms (Schuster et al., 2000). NO plays a crucial role in the pathogenesis of airway inflammation in allergic asthma (Shin et al., 2012). At physiological concentration, NO functions as vasodilator, neurotransmitter and immune regulator (Valko et al., 2007; Shin et al., 2012). Excess of NO can react with superoxide radicals leading to peroxynitrite generation, a powerful oxidizing agent (Zhu and Li, 2012). In this study, EEBC significantly reduced the production of NO in BAL fluid and thus avoiding the damaging effect of excess NO production. MDA, the decomposition products of lipid peroxidation, reflects the severity of cell attack by free radicals (Feng et al., 2010; Du et al., 2013). MDA content is commonly regarded as a marker of oxidative stress and antioxidant status (Del Rio et al., 2005; Du et al., 2013). Pretreatment with EEBC showed a decrease in

MDA level meaning that EEBC may effectively reduce oxidative burden during the inflammatory response to OVA.

Regarding the role of oxidative stress in the pathogenesis of inflammatory diseases, the *in vivo* antioxidant activities of EEBC in this study seem reasonable. The phenolic compounds included flavonoids present in EEBC may be responsible for *in vivo* antioxidant activities observed in this study. However, the involvement of other secondary metabolites present in the plant cannot be ruled out. These results confirm a previous antioxidant activity of EEBC, using the *in vitro* 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical model (Dosseh et al., 2014).

Many investigations on *B. coccineus* have reported that the plant has numerous pharmacological properties (Akindele and Adeyemi, 2007b; Dada et al., 2013). It is necessary to evaluate the toxicity of this plant to determine its safety for human use. In oral acute toxicity, the LD₅₀ of the extract is above 5000 mg/kg. Thus, referring to the Hodge and Sterner scale (Hodge and Sterner, 1943), the orally administered EEBC could be considered practically non-toxic. This result supports the

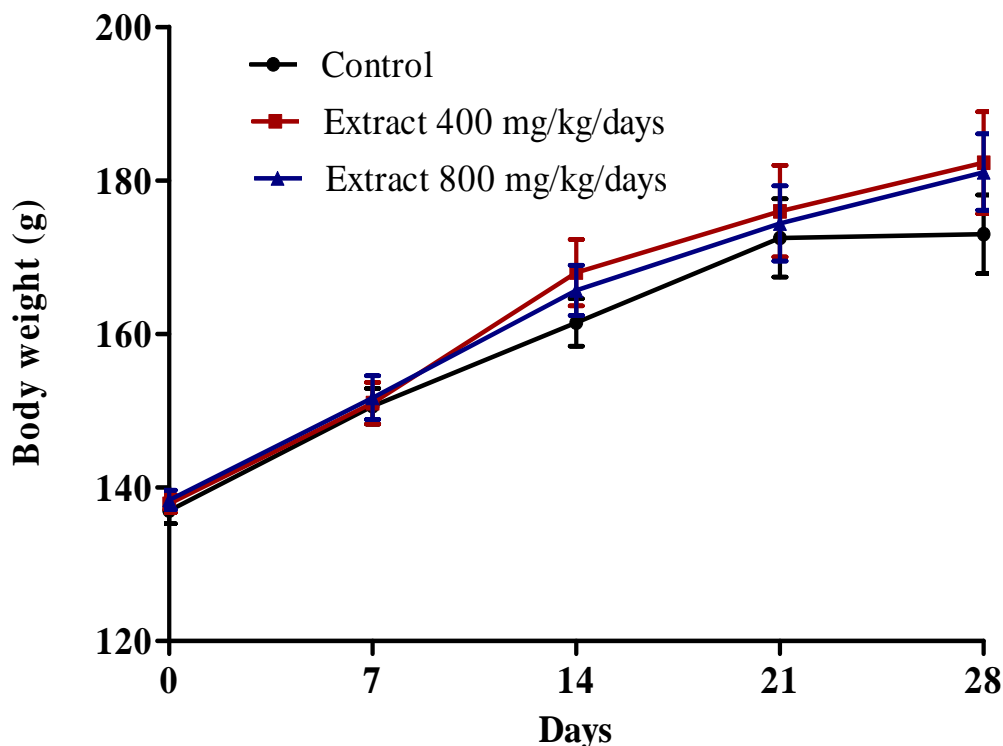


Figure 3. Mean body weight of rats during 28 days treatment with ethanolic root extract of *Byrsocarpus coccineus*. No statistical difference between the control and treated groups ($P > 0.05$).

findings of Akindele and Adeyemi (2006b). Adeyemi et al. (2010) who demonstrated that aqueous leaf extract of *B. coccineus* did not cause oral toxicity, respectively in rats and mice at the dose of 10000 mg/kg. The same trend was observed by Akpan et al. (2012) and Dada et al. (2013) using respectively ethanolic leaf extract of the plant at the dose of 5000 mg/kg in mice and hydro-ethanolic leaf extract at the dose of 10000 mg/kg in rat.

Generally, reductions in body weight gain and internal organs weights are sensitive indices of toxicity after exposure to toxic substances (Teo et al., 2002; Ouedraogo et al., 2013). The results obtained in the sub-chronic toxicity study indicated that EEBc did not affect neither the weight of the whole animal nor the weight of the specific vital organs, as previously described by Adeyemi et al. (2010).

Hematological indices in animals are important to determine the toxicity risk since the changes in the blood system have a higher predictive value for human toxicity (Ouedraogo et al., 2013). The hematological indices obtained in this study suggest that the EEBc is not toxic on hematological parameters. However, the increase in the level of RBC and HGB in treated groups confirms the traditional use of this plant against anemia.

The serum biochemical parameters were studied to evaluate the possible alterations in hepatic, renal and pancreatic functions influenced by EEBc. ALP, AST and ALT are usual markers of liver toxicity (Costa-Silva et al., 2008). Results obtained in this study indicated that EEBc did not induce liver injury.

Serum level of bilirubin was also not altered significantly. Oboh (2005) has reported that increase in bilirubin levels suggests increase in hemolysis intensity. The water solubility of bilirubin allows the bilirubin to be excreted in the bile; the bile is then used to digest food. As the liver becomes irritated, then TB may rise (Muhammad et al., 2011). Results indicated that the extract did not interfere with the metabolism of TB and TP in the liver. The liver is also the site of cholesterol degradation and an increase in cholesterol levels is considered as a sign of hepatic damage (Subhangkar and Rana, 2012). In this study a significant decrease in cholesterol and triglycerides levels was observed. These results may be attributed to the presence of hypolipidemic agents in the extract.

It is well known that almost all drugs, chemicals, xenobiotics are eliminated through renal excretion (Biswas et al., 2010); hence, it was found necessary to

estimate the effects of EEBc on kidney functions. EEBc had no adverse effect on the concentration of creatinine and urea. This is suggestive of no kidney damage specifically by renal filtration mechanism (Crook, 2006).

Conclusion

It can be concluded from the aforementioned results that the ethanol extract from roots bark of *B. coccineus* has a good antioxidant activity and do not produce any toxic signs or evident symptoms in acute and sub-chronic oral toxicity. Hence, *B. coccineus* root may be exploited as a natural antioxidant and health promoting agent for the treatment and prevention of free radicals associated diseases. These results confirm the utilisation of *B. coccineus* in traditional medicine many centuries ago. However, more investigations are needed before its use for clinical purpose.

Abbreviations

Nitric oxide (NO) and Malondialdehyde (MDA)

Conflict of interest

The authors have not declare conflict of interest.

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Full Length Research Paper

***In vitro* antibacterial activities of pomegranate extract against standard microorganisms of bovine mastitis**

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Bovine mastitis is characterized by inflammation of the mammary gland, usually due to bacterial infection, compromising quantity and quality of milk production. This study aimed to determine the antibacterial activity, *in vitro*, of the hydroalcoholic extract of the pomegranate peel at 10% against standard strains of bovine mastitis. The colonies were adjusted to the concentration of 10^7 ml⁻¹ using UV-visible spectrophotometry, and the extracts were evaluated in quintuplicate in concentrations of 1000, 500, 250, 75, 50 and 25 µg ml⁻¹. The sensitivity of the strains was determined using the minimum inhibitory concentration and disk diffusion test. Additionally, antioxidant activity and total phenolic content was evaluated. The extract, at concentrations of 500 and 1000 µg ml⁻¹, inhibited *Staphylococcus aureus* (ATCC 25923), *S. Saprophyticus* (ATCC 15305), *S. Epidermidis* (ATCC 12228), *Escherichia coli* (ATCC 11229), *Enterobacter cloacae* (ATCC 23355) and *Bacillus cereus* (ATCC 33018), but it was not effective for *Pseudomonas aeruginosa* (ATCC 27853) and *Salmonella enterica* subspecies, *enterica* serovar Typhi (ATCC 19214). Antioxidant activity was observed from 50 µg ml⁻¹ reaching a plateau at 500 mg ml⁻¹ with 64.90%, and the concentration that causes 50% of the inhibition (IC₅₀) corresponded to 378.80 µg l⁻¹. Perhaps the presence of other substances in the extract may have been responsible for the antioxidant activity detected. That way, the antioxidant and antibacterial activities of EHPG 10% may represent an important therapeutic potential, particularly for animal health in organic and agroecological production systems.

Key words: Agroecology, medicinal plants, organic animal production, *Punica granatum* Linn.

INTRODUCTION

Bovine mastitis is an inflammation frequently caused by bacterial infection which determines important economic impact (Deb et al., 2013). The clinical presentation is mainly caused by *Escherichia coli*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa*, and the

subclinical form is mainly caused by *Staphylococcus aureus*, *Streptococcus agalactiae*, *Streptococcus dysgalactiae* and *Streptococcus uberis*. Treatment usually occur by the use of antimicrobial chemicals, however, during lactation the use of these drugs is rare,

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Table 1. Average values of inhibition halo (mm) of growth of *S. aureus* (ATCC 25923), *S. saprophyticus* (ATCC 15305), *S. epidermidis* (ATCC 12228), *E. coli* (ATCC 11229), *E. cloacae* (ATCC 23355) and *B. cereus* (ATCC 33018), submitted to different hydroalcoholic extract of *P. granatum* Linn at 10%.

Extract ($\mu\text{g ml}^{-1}$)	<i>S. aureus</i> ATCC 25923	<i>S. saprophyticus</i> ATCC 15305	<i>S. epidermidis</i> ATCC 12228	<i>E. cloacae</i> ATCC 23355	<i>E. coli</i> ATCC 11229	<i>B. cereus</i> ATCC 33018
500	10.2	9.6	-	15.8	-	-
1000	16.6	17.6	17.6	20.6	11.8	13.6

due to the low efficiency and the presence of milk residues (Vlieghe et al., 2012). In addition, this kind of drug can determine residue in milk, and for this reason the use of chemical antibiotics are not allowed in organic or biodynamic systems of production. Thus, searches were determined for natural solutions that approach the problem without milk disposal. There was significant increase in production of organic and use of medicinal plants as encouraged by the World Health Organization (WHO) because it is accessible economically.

Punica granatum Linn., known as pomegranate, is cultivated worldwide and could be considered a functional food because it has compounds in different parts that display functional and medicinal effects (Bhandari, 2012; Ismail et al., 2012). Its constituents are alkaloids (peletierin, isopeletierin, metilpeletierin), tannins, phenolic compounds (anthocyanins, quercetin, phenolic acid) and flavonoids (Duman et al., 2009; Moorthy et al., 2013; Silva et al., 2013). Antimicrobial properties have been demonstrated (Moorthy et al., 2013), on *S. aureus* (Silva et al., 2013; Moorthy et al., 2013; Santos et al., 2014; Moreira et al., 2014). Antibacterial properties are particularly important for the treatment of bovine mastitis. So this study aimed to determine the antibacterial activity, *in vitro*, of the hydroalcoholic extract of the pomegranate peel at 10% (HEP10%) against standard strains of bovine mastitis.

MATERIALS AND METHODS

A sample of the plant material was identified in the Forest Institute of Assis/SP- Brazil, and a voucher specimen was deposited in the Herbarium of the Institute under number SPSF 40136. For the preparation of the extract, 30 g of fruit peels dry with kiln forced ventilation air at 40°C was used, 80 ml of distilled water, and 190 ml of absolute ethyl alcohol (99.5%). The extraction occurred by constant mechanical stirring in 24 h, vacuum filtration and adding new hydroalcoholic solution in the same volume. This procedure was repeated for three consecutive times. After that, the extract was concentrated using rotary vaporizador (60°C), frozen and lyophilized. The antimicrobial activity was evaluated by using Kirby-Bauer disk diffusion technique (CLSI, 2011). 1000 $\mu\text{g ml}^{-1}$ of the extract was added in nutrient broth and the following dilutions were held: 500, 250, 100, 75, 50 and 25 $\mu\text{g ml}^{-1}$. The disk diffusion test was performed in five replications by impregnating an aliquot of 40

μl of each concentration of HEP 10%, in filter paper discs (7 mm).

After drying, the discs were fixed in agar plates Mueller - Hinton previously seeded by 10^7 CFU ml^{-1} of *S. aureus* (ATCC 25923), *S. saprophyticus* (ATCC 15305), *S. epidermidis* (ATCC 12228), *E. coli* (ATCC 11229), *P. aeruginosa* (ATCC 27853), *S. enterica* subsp. *enterica* serovar *Typhi* (ATCC 19214), *E. cloacae* (ATCC 23355) e *B. cereus* (ATCC 33018). The incubation was performed in microbiological incubator at 37°C for 24 to 48 h. To determine the Minimum Inhibitory Concentration (MIC), the extract was resuspended in nutrient broth under concentrations of 1000, 500, 250, 100, 75, 50 and 25 $\mu\text{g ml}^{-1}$. The bacterial inoculation (5×10^5 CFU ml^{-1}) proceeded for 20 h at 35°C (CLSI, 2011). The antioxidant activity was evaluated in triplicate, using the same concentrations above. The antioxidant activity was determined according to Blois (1958) and the determination of the sample concentration that causes 50% of the inhibition of the initial concentration of DPPH (IC_{50}) was calculated by linear regression of the points plotted graphically. The mean values obtained by the DPPH test were used to plot the points as Di Mambro and Fonseca (2005).

RESULTS AND DISCUSSION

The disk diffusion test and the MIC test demonstrated that 500 and 1000 $\mu\text{g ml}^{-1}$ of the HEP 10% presented an antimicrobial activity against Gram positive bacteria. Regarding the dosage of 250 $\mu\text{g ml}^{-1}$, only the MIC test was able to demonstrate antimicrobial activity (Table 1, 2). Santos et al. (2014) also examined the inhibitory effects of pomegranate peel extract on *S. aureus* isolates from cases of bovine mastitis, which has also been checked by us in preliminary studies (Moreira et al. 2014). However, for the conditions evaluated by the present study, antimicrobial activity for Gram-negative bacteria was not observed. These bacteria are more resistant to antimicrobials based on natural extracts (Carvalho et al., 2013) because they have phospholipid external layer that is impermeable for solute lipophilic. Additionally, the porins create a barrier against hydrophilic solutes, restricting the penetration of antimicrobial compounds. On the other hand, the Gram-positive bacteria have only peptidoglycan on the cell wall (CLSI, 2003; Rabêlo et al., 2014). Moorthy et al. (2013) reported antibacterial activity of pomegranate extract for Gram positive and negative bacterias, but they used the pericarp for making the extract. In this study, the use of the peel was prioritized as residue in order to promote the

Table 2. Minimum Inhibitory Concentration (MIC) for the growth of *S. aureus* ATCC 25923, *S. saprophyticus* (ATCC 15305), *S. epidermidis* (ATCC 12228) and *B. cereus* (ATCC 33018) and antioxidant activity in different concentrations of hydroalcoholic extract of *P. granatum*.

Extract ($\mu\text{g ml}^{-1}$)	<i>S. aureus</i> ATCC 25923	<i>S. saprophyticus</i> ATCC 15305	<i>S. epidermidis</i> ATCC 12228	<i>B. cereus</i> ATCC 33018	Antioxidant (%)
250		G	G	G	35.59
500				G	64.90
1000					78.35

G: Bacterial growth / I: Growth Inhibition

economic sustainability of the activity.

This extract showed antioxidant activity with values corresponding to 4.62% in the concentration of $50 \mu\text{g ml}^{-1}$, reaching 64.90% in the concentration of $500 \mu\text{g ml}^{-1}$, and the IC_{50} corresponding to $378.80 \mu\text{g ml}^{-1}$ (Table 2). Karaaslan et al. (2014) found phenolic compounds, but they use the fruit to do the extract. Silva et al. (2013) also evaluated the extract confectioned from the pomegranate peel, and just as the present study, they found a high antioxidant activity, but without correlation to phenolic content. The pomegranate has complex composition; perhaps other alkaloids may have been responsible for the antioxidant activity. Noda et al. (2002) and Duman et al. (2009) associated the antioxidant activity with anthocyanins. Moorthy et al. (2013) reported the punicalagin as a major antimicrobial constituents that was found in pomegranate.

CONCLUSION

The inhibition of Gram positive bacteria allows us to conclude that the hydroalcoholic extract of pomegranate showed therapeutic potential for bovine mastitis control.

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Conflicts of interest

Author has none to declare.

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