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Table of Contents: Volume 11 Number 7 17 February, 2017

ARTICLES

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Full Length Research Paper

Anthelmintic activity of *Artemisia annua* in sheep-model

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Artemisia annua* L. is a well-known source of artemisinin, an antimalarial drug. This compound has been used in traditional medicine to treat malaria for many years. The anthelmintic property of *A. annua* has also been attributed to artemisinin, hence the belief that artemisinin could be useful as an alternative anthelmintic to control *Haemonchus contortus* in livestock. The present study focused on evaluating the anthelmintic activity of *A. annua* against *H. contortus* by oral administration on infected sheep in a controlled clinical trial. The trial was conducted using 24 male sheep of the Santa Inês breed, with average weight of 20 kg, artificially infected with *H. contortus* (Embrapa 2010 isolate). The animals were kept in metabolic cages and randomly divided into four homogeneous groups with six animals each, being: (T1) negative control (untreated), (T2) positive control: 10 mg/kg BW of levamisole phosphate, (T3) fed 10% *A. annua* (0.2% BW), (T4) fed 20% *A. annua* (0.4% BW). EPG counts were conducted on days -3, -2, -1, 0 (treatment day), 3, 7, 10, 14, 17, 21, 24 and 28 post-treatment. After extraction of the artemisinin in plant material, it was quantified in triplicate and analyzed by high-performance liquid chromatography with infrared detection. The average efficacy in the positive control treated with levamisole was 89%. Moderate anthelmintic efficacy against *H. contortus* was observed in the groups fed 0.2 and 0.4% BW *A. annua* for 30 days (32.9 and 14.8%, respectively), mainly from day 21 post treatment (47.1 and 25.2%, respectively). At the highest dose, the animals avoided eating due to the compound's bitter taste. It is concluded that *A. annua* presents moderate anthelmintic activity in sheep in both doses. The lowest dose was accepted by animals and seems to have practical use. Oral administration was safe and after further *in vivo* trials could be introduced in organic farms in tropical countries.

Key words: Artemisinin, sesquiterpenoids, gastrointestinal nematodes, control, veterinary ethnopharmacology.

INTRODUCTION

Parasitism by gastrointestinal nematodes is one of the major problems of small ruminants, being responsible for high mortality. Among all nematode species, *Haemonchus contortus* is the main culprit in tropical countries. In Brazil, this blood-sucking abomasal parasite is responsible for large losses to sheep breeders. Small ruminant infections are controlled by using the commercially available chemicals. However, there are various reports of multidrug-resistant in small ruminant nematodes throughout the world (Veríssimo et al., 2012; Chagas et al., 2013; Gárcia et al., 2016; Keegan et al., 2016). Unfortunately, the strong selection pressure that has been imposed associated with incorrect frequency and doses are responsible for the anthelmintic inefficacy of the available chemical groups (Soares et al., 2009).

Therefore, other approaches to control gastrointestinal nematodes in small ruminants have been studied. These include *refugia* and targeted selective treatment (Van Wyk and Reynecke, 2011), as well as the evaluation of plants' biological effects for use in phytotherapy (Chagas, 2015). *Artemisia annua* L., Asteraceae family, has been studied because it is a well-known source of the antimalarial drug artemisinin, which has been used in folk medicine for the treatment of malaria for many years (Weathers et al., 2014; Ruan et al., 2016). Studies have reported different biological effects of artemisinin as well as for its derivatives dihydroartemisinin (DHA), artesunate (ART) and artemether (ARTE). These substances have presented anti-inflammatory (Salminen et al., 2008; Stebbings et al., 2016), antioxidant (Gupta et al., 2016), antifungal (Santomauro et al., 2016), antibacterial (Tajehmiri et al., 2014) and anti-HIV (Lubbe et al., 2012) activities. The potential anticancer action has been investigated as well (Tajehmiri et al., 2014; Weathers et al., 2014; Humphreys et al., 2016; Ko et al., 2016).

The anthelmintic property of *A. annua* has been attributed to artemisinin and it is believed that this compound can be useful as an alternative to control *H. contortus* in livestock (Ferreira et al., 2011; Sprenger et al., 2015). Studies of the *Artemisia* genus have shown negative and positive results. Different species have been evaluated against gastrointestinal nematodes *in vivo*: crude ethanol extracts of *A. annua*, *Artemisia absinthium* and their essential oils in gerbils artificially infected with *Haemonchus contortus* (Squires et al., 2011); *Artemisia brevifolia* aqueous extract in sheep naturally infected with mixed gastrointestinal nematodes (Iqbal et al., 2004); *A. absinthium* leaves in alfalfa pellet diet against *H. contortus* in lambs (Valderrábano et al., 2010); and ethanol extracts of *A. absinthium* in gastrointestinal nematodes of sheep (Tariq et al., 2009).

Interestingly, neither *A. annua* extracts nor artemisinin have been tested in sheep infected with gastrointestinal nematodes. Therefore, in a previous trial by our research group, naturally infected sheep treated with *A. annua* sodium bicarbonate extract (aqueous 0.1% sodium bicarbonate) in a single dose of 2 g/kg BW in 20 mL of soybean oil had non-significant EPG reduction (19%). However, the extract tested *in vivo* contained no artemisinin, but had high antioxidant capacity (Cala et al., 2014). Lack of artemisinin was expected due to the affinity of this substance to ethanolic solvents instead of aqueous. Authors indicated that the anthelmintic activity of the extract, previously detected *in vitro*, was not due to the presence or lack or artemisinin but, may be due to the high content of antioxidant compounds (e.g., flavonoids) measured by the oxygen radical absorbance capacity (ORAC). So, the objective of this study was to establish a controlled clinical trial to investigate the anthelmintic activity of *A. annua*, rich in artemisinin this time, by oral administration in sheep artificially infected with *H. contortus as* shown in graphical abstract.

MATERIALS AND METHODS

Plant material collection

A. annua variety CPQBA-UNICAMP was cultivated in Paulinia, São Paulo state, Brazil, Lat. 22°48'02.38"S, Long. 47°06'43.10"W, altitude 612 m. The plants were spray irrigated and the weeds were controlled manually until the disappearance of the lines between the plants due to their development. The soil in the experimental area was classified as typical clayey eutroferric red latosol. The seedlings were formed in sleeves under screening with 50% interception and cultivated in the field for approximately 4 months from October, with spacing of 0.6 x1.0 m. Harvesting was done in the state preceding flowering and only the top third of the plant was harvested, resulting in artemisinin content between 1.0 and 1.1%. The leaves were sun-dried with periodic turning, followed by manual separation of the stalks and thick twigs. Thus, the final raw material consisted only of leaves, which were ground into powder.

Quantification of artemisinin by HPLC-RI

Chromatographic analysis was done by high-performance liquid chromatography with a refractive index detector (HPLC/RI) according to a previously described protocol (Celeghini et al., 2009). It was performed with a modular Waters system comprised of a Waters 515 pump, a column oven, a Waters 2414 refraction index detector and an using a LC-CN column (4.6 × 250 mm, 5 µm particle size, Luna Phenomenex, Macclesfield,UK). Separations were made in the isocratic mode, using methanol: water (60:40v/v)

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Table 1. Quantification of artemisinin (ART) from *A. annua* per 100 g of dry weight analyzed by HPLC-RI.

Sample	Weight (mg)	Volume (mL)	Concentration (µg/mL)	(%) w/w	Mean	Standard deviation	CV (%)
Sample-A	250.0	5.0	487,841	0.980			
Sample-B	251.0	5.0	481,932	0.960	0.96	0.010	1.03
Sample-C	251.6	5.0	481,649	0.960			

mL/min at a flow rate of 1 mL/min with 20 µL injection volume. The detector and column temperature was 35°C. *A. annua* (250 mg) was extracted using Ultra Turrax mixture for 2 min, 6000 rpm at room temperature (25°C) with three portions of 5 mL methanol, following clean-up procedures (Celeghini et al., 2006)

Artemisinin was quantified by analytical curve. The artemisinin analytical standard (Sigma-Aldrich, Sant Louis - USA) stock solutions (2491 µg/mL) were prepared in methanol and successively diluted in the range of 50 to 1250 µg/mL, three replicates each in methanol, retention time 7.0 min. All samples were analyzed by HPLC as described above. A graphic correlating area under the curve (AUC) with the respective concentration was plotted and analyzed by linear regression using Empower software (Waters).

Infection of animals

Before the experiment, 24 males of the Santa Inês breed, approximately four months old and with mean live body weight (BW) of 20 kg, were kept indoors in collective stalls, where they were fed with corn silage, concentrated feed, mineral salt and water *ad libitum*. To remove the natural infection by nematodes, the animals received levamisole phosphate (Ripercol® 150F, Fort Dodge, Brazil) at a dose of 10 mg/kg BW (body weight) each 24 h for three consecutive days and albendazole (Valbazen®, Pfizer, Brazil) in a single dose of 10 mg/kg BW on the last day.

After treatment, fecal samples were collected directly from the rectum of each animal to confirm their worm-free status. Then, the sheep were artificially infected with approximately 4,000 larvae of the *H. contortus* isolate Embrapa2010, characterized as resistant to benzimidazole, macrocyclic lactones and imidazothiazoles (Chagas et al., 2013).

The experimental protocols were approved by the Embrapa Pecuária Sudeste (CPPSE - Southeast Livestock Unit of the Brazilian Agricultural Research Corporation) Animal Care and Use Committee. Animals were under the care of a veterinary assistant during the entire experiment.

A. annua evaluation *in vivo*

This *in vivo* study was performed at CPPSE, which is located in the city of São Carlos, São Paulo state. Twenty-eight days after artificial infection, the sheep were weighed and eggs per gram of feces (EPG) were recorded for each animal on days -3, -2 and -1 (Ueno and Gonçalves, 1998). Lambs were allocated into homogeneous experimental groups based on their weight and mean EPG (of the three days). All the rams were placed in metabolic cages to receive their respective treatments (n=6): T1: negative control (untreated), T2: positive control (10 mg/kg BW levamisole phosphate), T3: fed 10% *A. annua* (0.2% BW), T4: fed 20% *A. annua* (0.4% BW). The T3 and T4 animals were given five days to adjust to the diet (1% day 1, 5% day 2, 5% day 3, 10% day 4, 10% day 5) and then received *A. annua* for 30 consecutive days. Samples of faces were collected individually directly from the rectum to count EPG on days 0, 3, 7, 10, 14, 17, 21, 24 and 28 post-treatment.

Data analysis

The results of EPG were natural log-transformed ($\ln^{-1}$) and analyzed by a split-plot design, where α_i, β_j and $(\alpha\beta)_{ij}$ represent the whole plot and ω_k, and δ_{jk} represent the split-plot. The sums of squares for the factors were computed for three-way analysis of variance without replication employing the mixed models method by the *T*-test at 95% confidence, using the R program (R Core Team, 2016). The mean EPG values obtained were used to calculate the efficacy against *H. contortus* as follows: % efficacy = mean control – mean treated/mean control x 100.

RESULTS

Phytochemical analysis

In the quantification of artemisinin, the equation curve was Y = 1.17e+003 X + 4.49e+003, the correlation coefficient $R2$ = 0.9998 ± 0.0005, the detection limit (LOD) = 2.2 µg/mL and, the quantification limit (LOQ) =7.5 µg/mL). The phytochemical analysis showed the presence of artemisinin in a concentration of 0.96% ± 0.010 or 483.87 µg/mL (Table 1). Each 100 g of dried plant material contained 0.96 g of artemisinin. As each sheep received approximately 40 (0.2% BW) and 80 g (0.4% BW) of *A. annua* per day, it can be estimated that they ingested approximately 384 and 768 mg of artemisinin/day, respectively. The quantification showed high concentration of artemisinin (960 mg/100 g).

In vivo trial

The infections were monitored through the experiment and compared with the positive and negative control as extremes. On day zero, the average EPG counts (non-transformed data) for all treatments were as follows: positive control (T1 = 1625), negative control (T2 = 1625), *A. annua* 0.2% BW (T3 = 1908) and *A. annua* 0.4% BW (T4 = 2083) (Figure 1).

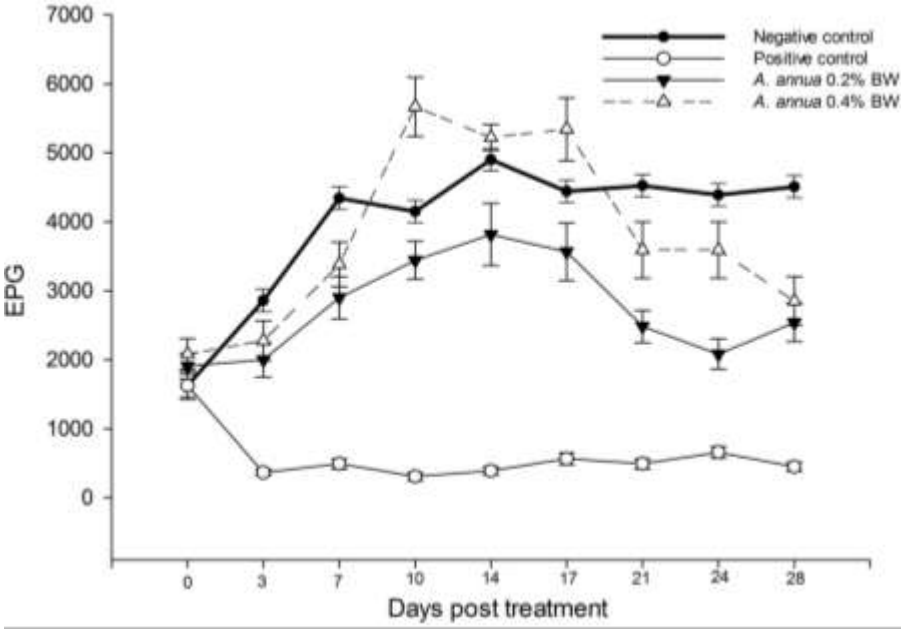


Figure 1. Non-transformed mean EPG in Santa Inês sheep artificially infected with *H. contortus*, in four groups: negative control, positive control, fed with 0.2 and 0.4% BW of *A. annua*.

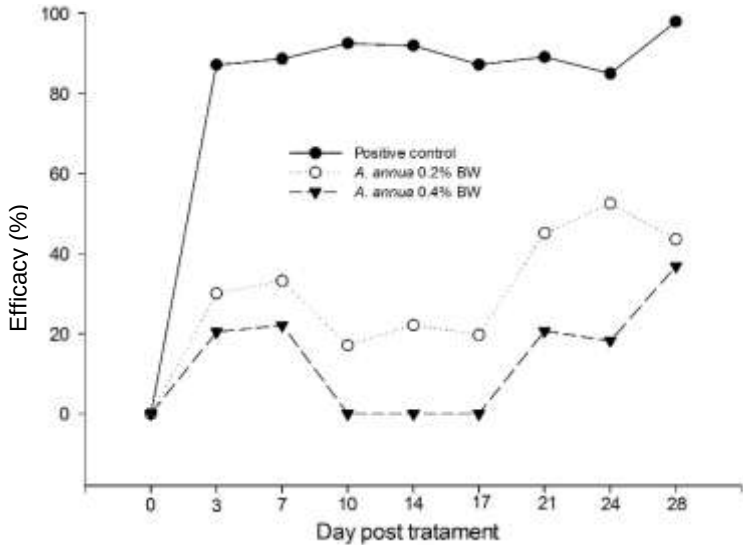
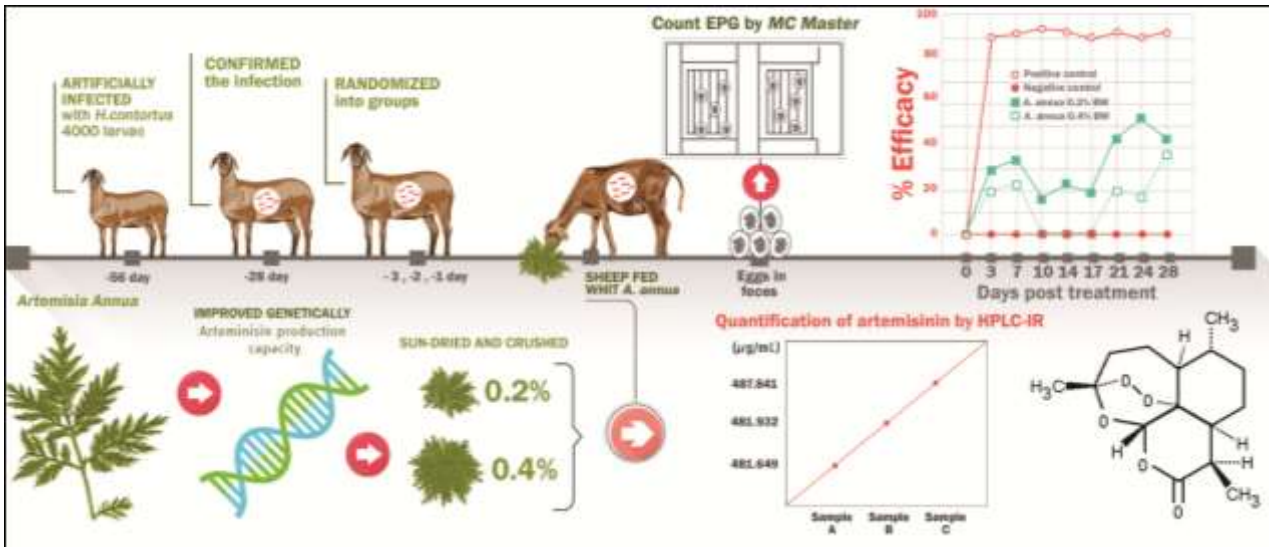


Figure 2. Mean efficacy (%) of the reduction of EPG in Santa Inês sheep artificially infected with *H. contortus*, for all treatments: positive control, fed with 0.2% and 0.4% BW of *A. annua.*

In the negative control, constant EPG from day 7 to 28 post treatment was observed, whereas in the positive control group, levamisole phosphate presented high efficacy (89.0% average) from day 3 (Figure 2). Moderate anthelmintic efficacy against *H. contortus* was observed in the groups fed 0.2 and 0.4% BW of *A. annua* for 30 days (32.9 and 14.8%, respectively), mainly from day 21 after treatment (47.1 and 25.2%, respectively) (Figure 2). Regarding data analysis of the log-transformed EPG values, no significant EPG reduction for both oral doses



Graphical Abstract

of *A. annua* was observed. However, treatment with levamisole statistically differed in all days (from days 3 to 28) of the negative control and treatments. Toxic reactions were not observed in any animal that ingested *A. annua* daily in both concentrations, although at the highest dose animals did not eat all the plant material because of the bitter taste.

DISCUSSION

Knowledge on physicochemical characteristics of plants has contributed to advances in biotech research, leading to the breeding of commercially important plants. In this study, the oral administration of *A. annua* in infected sheep was evaluated. The quantification of artemisinin was performed by HPLC/IR and artemisinin was detected in a mean concentration of 0.96% or 483.87 µg/mL. This denoted that 100 g of dried plant material had 960 mg of artemisinin. Lapkin et al. (2014) investigated the variability of metabolic profiles of *A. annua* grown in different geographical regions and observed differences. According to Charles et al. (1990), there is variation from 0.003 to 0.39% in artemisinin content in leaves of *A. annua* worldwide. The findings in the present study can be explained by *A. annua* genotype used (CPQBA/UNICAMP), produced by genetic breeding and adapted to Brazilian climatic conditions. This content is not normally found.

In the present study, the animals that ate *A. annua* at 0.2 and 0.4% BW received daily 384 and 768 mg of artemisinin, respectively. However, it is noticed that at the highest dose, the plant material was not totally ingested due to the bitter taste, explaining the worse response than at the lower dose. Adverse reactions were not observed in any animal that ate *A. annua* daily at both concentrations. Keiser et al. (2008) evaluated semi-synthetic artemisinin at dosages of 40 and 80 mg/kg orally and at 40, 80 and 160 mg/kg intramuscularly in sheep. They reported that even the highest dose of 160 mg/kg was well tolerated. In the present study, at least at the lower dose, which was totally ingested by the animals, this plant species and artemisinin content (384 mg) were safe. This is the first report of daily administration of *A. annua* against *Haemonchus* infection.

A. annua produces a wealth of monoterpenes and sesquiterpenes, including the well-known sesquiterpene lactone artemisinin (Ruan et al., 2016). Artemisinin is pointed out as the main compound responsible for the anthelmintic activity of *A. annua*. The mechanisms of action attributed to this metabolite include interference with parasite transport proteins, disruption of parasite mitochondrial function, modulation of host immune function and inhibition of angiogenesis (Golenser et al., 2006). In the present study, at the half dose, the average efficacy levels were 32.9% and 47.1% for 21 days after treatment. Different studies have already proved the anthelmintic effect in sheep with plants of the genus *Artemisia* (family Asteraceae). The crude ethanol extract of *A. absinthium* demonstrated EPG reductions of 90.46% in sheep at 2.0 g/kg BW and 82.85% at 1.0 g/kg BW, both on day 15 post treatment (Tariq et al., 2009). Maximum reduction (67.2%) in EPG was recorded on day 14 post treatment in sheep with mixed gastrointestinal nematode infection treated with *A. brevifolia* crude aqueous extract at 3 g/kg BW (Iqbal et al., 2004). Cala et al. (2014) evaluated *A. annua* sodium bicarbonate extract

(2 g/kg BW) and artemisinin (100 mg/kg BW) in naturally infected sheep. Artemisinin-treated and extract-treated sheep had non-significant EPG reductions of 28 and 19%, respectively, but this extract had no artemisinin. Irum et al. (2015) evaluated *Artemisia vestita* and *Artemisia maritima* methanol extracts on *H. contortus* in artificially infected sheep. The EPG reductions were 87.2 and 84.5%, respectively, at 50 mg/kg, 4 weeks after treatment.

The differences found among those studies are related to plant species, artemisinin content and administration form (extract, plant material) and the relationship with bioavailability and absorption. Studies have shown that artemisinin has poor bioavailability in small ruminants. Ferreira and Gonzalez (2008) studied the stability in bovine rumen fluid and kinetics in goats. Artemisinin recovery rates from rumen culture ranged from 67 to 92% at pH 6.8 and were 95% at pH 3.0. The kinetics data showed that artemisinin was metabolized to dihydroartemisinin by goats, while unabsorbed artemisinin was eliminated in feces. Dihydroartemisinin peaked in the blood (0.7 µg/mL) at 12 h, and decreased to 0.18 µg/mL at 24 h. After 24 h, artemisinin concentration in feces was 2.4 µg/g, indicating artemisinin's poor bioavailability in goats when provided orally and as capsules. Posner et al. (2004) observed high time-dependent first-pass metabolism in the gut and liver, this drug are conjugates such as glucuronides and can be eliminated through phase-II metabolism. Therefore, artemisinin has low water solubility, resulting in poor and erratic absorption upon oral administration, so the artemisinin conjugation reactions and hydrolysis are the principal reasons these derivatives have a short half-life. The authors decided to evaluate the dry plant mixed in the food because this seems to be the most practical way for future use of this technology by organic farmers, since the oral administration of artemisinin did not present good results in other studies.

There is a need to develop new approaches to find potential anthelmintic plants that can be used to formulate commercial products. Natural compounds can also provide more structural diversity than synthetic anthelmintic drugs, to reduce the occurrence of resistance (Irum et al., 2015). In light of these aspects, the results reported here allow us to infer that *A. annua*, administered orally presented moderate anthelmintic activity in sheep at both doses. The lowest dose was accepted by animals and seems to have practical use. The oral administration was safe and therefore the use in organic farms in tropical countries would be a good option after been more accurate *in vivo* trials.

Conflict of Interests

The authors have not declared any conflict of interests.

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Mangiferin content, carotenoids, tannins and oxygen radical absorbance capacity (ORAC) values of six mango (*Mangifera indica*) cultivars from the Colombian Caribbean

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Mango is one of the tropical fruits of greater production and consumption in the world, and a rich source of bioactive compounds, with various functional properties such as antioxidant activity. In Colombia, mango's market is very broad and diverse. However, there are very few studies that determined the content of bioactive secondary metabolites. The objective of this study was to evaluated the content of different metabolites like Mangiferin, carotenoids, tannins, and the antioxidant capacity by oxygen radical absorbance capacity (ORAC) methodology of six cultivars from the Colombian Caribbean region, with total carotenoid values ranging from 24.67 to 196.15 mg of β-carotene/100 g dry pulp; 84.30 to 161.49 mg Catequine eq./100 g dry pulp for the content of condensed tannins, and 91.80 to 259.23 mg/100 g dry pulp for mangiferin content. The ORAC methodology showed important antioxidant activity results, such as the Chancleta variety with the highest value (2163.78 µmol Trolox/100 g dry pulp). In conclusion, the evaluated mango varieties had promising results as functional food of high nutraceutical value, being Chancleta, Criollo and Jobo varieties, the fruits with highest content in bioactive compounds that expressed the best antioxidant activity.

Key words: Mangiferin, antioxidant activity, mango, nutraceutical.

INTRODUCTION

Mango (*Mangifera indica* L.) from *Anacardiaceae* Family, is a tree 15 to 30 m high, with quick growth in shallow and well drained soils, and pH oscillating from 5.5 to 7.5 (Shah et al., 2010). In tropical zones of Colombia, it grows up to 1200 m high, with a high fruit production within 3 months of the year in low rain seasons (Bally, 2006). In Colombia, mango has more than 200 ecotypes or genetically differentiated cultivars. However, there is

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no much information on its properties as functional food.

In other hand, mango in currently known as one of the most important tropical fruit. It has been cultivated since prehistoric times and its tree has been object of high veneration in India. Different countries cultivate this fruit, among them are, Indonesia, Florida, Hawaii, Mexico, South Africa, Egypt, Israel, Brazil, Cuba and Philippines. Probably, India has more commercial plantations than the rest of the world. However, mango's economic importance is due to the great local consumption in Caribbean countries, especially in Colombia (Michel et al., 2000).

The consumption of fruits rich in biologically active compounds brings important benefits for human health; mango is considered a fruit with nutraceutical properties due to presence of carotenoids, ascorbic acid, fiber, polyphenols, among others; which are associated to antioxidant and anti-inflammatory properties, important in treatments of metabolic disorders such as obesity and diabetes (Ibarra-Garza et al., 2015; Septembre-Malaterre et al., 2016). Also, knowledge of concentration of bioactive structures in fruits, allows obtaining information to implement extraction and drying techniques to give it greater added value as a functional food and to compete in the international market with high quality products.

One of the bioactive compounds of greatest interest in the species *M. indica* is Mangiferin, a polyphenol type glycosyl xanthone that presents pharmacological actions with antioxidant, anti-inflammatory, and neuroprotective properties (Takeda et al., 2007). Studies have suggested that Mangiferin also has an *in vitro* anticancer effect in cellular lines of acute myeloid leukemia (AML) (Shoji et al., 2011). Matkowski et al. (2013) reported Mangiferin as a promising natural product with benefits as analgesic, antidiabetic, anti-sclerotic, antimicrobial, antiviral, cardio and hepatoprotective, anti-allergic, monoamine oxygenase (MAO) inhibitor, and protector against UV radiation.

Carotenoids are pigments found in fruits and vegetables such as mango. Chemically, carotenoids are classified as tetraterpenes, of which more than 600 natural structures of different size, shape and polarity have been identified. Carotenoids are associated with various biological processes like antioxidant defense, photosynthesis and, some of them, as precursors of vitamin A. Also, they determine the orange and yellow colors of mango fruits. Many studies reported that an adequate intake of carotenoids is related to the decrease of various types of cancer (Melendez-Martínez et al., 2007).

In other hand, condensed tannins are polymers formed by flavan-3-ol units. Also, they are one of the principal antioxidant metabolites in products like tee and cocoa which content can be up to 35% of total polyphenols. Tannins are associated to the astringent flavor of fruits and enzymatic browning reactions in mango bark (Pierson et al., 2014).

In Colombia, mango is consumed mostly fresh and it is used in pulps, nectar, juices, jellies and jams elaboration (Mora et al., 2002). Due to the low agro-industrial development, sale in fresh is privileged. However, post-harvest losses reach approximately 40% of annual production (Sumaya-Martinez et al., 2012).

Mango is one of the most consumed tropical fruit in the world, especially for its flavor, texture and versatility (Oliveira et al., 2016). However, the studies that have characterized its nutraceutical power in Caribbean Colombian zone cultivars are few. That is why the present work wants to know the metabolites content, specifically Mangiferin, carotenoids and total polyphenols, associated with antioxidant activity in six mango cultivars from Córdoba, Colombia.

MATERIALS AND METHODS

Reagents and equipment

Formic acid (p.a.), iron trichloride, sodium carbonate and Follin-Ciocalteu reagent were obtained from Merck (Germany). Methanol and other solvents were acquired from Fisher Scientific Co. (Fair Lawn, NJ, USA); 2,2'-Azinobis(2-aminopropane) hydrochloride (AAPH), fluorescein, Trolox®, 2,4,6-tri-(2-pyridyl)triazine (TPTZ) were bought to Sigma-Aldrich Chem.Co (Millwakee, WI). Ultraviolet-visible measurements were done in a spectrophotometer Multiskan Spectrum (Thermo Scientific). The decrease in the intensity of fluorescein measured in ORAC assay was done in a spectrofluorimeter Perkin-Elmer LS-55, (U.K.). Chromatographic assays were done in a liquid chromatographer Shimadzu®, Prominence® UFLC series.

Vegetal material

Vegetal material was collected in the Department of Córdoba, Colombia, especially in Monteria's local market (18 M. ASL and a mean temperature of 28°C in June 2016). Specimens in optimum conditions of each variety were collected randomly in mature state. After being stored in perforated polypropylene bags, they were taken to the Laboratory Food Science, Universidad Nacional de Colombia, Medellin, for analysis.

Sample preparation

3.0000 g of pulp were homogenized with 20 ml of deionized water in Ultra-Turrax (IKA-WERK©) homogenizer. The extract obtained from that was centrifuged at 5000 rpm for 10 min at room temperature. The supernatant recovered was stored at 4°C until the analysis.

Color determination

The color was measured with a colorimeter Minolta (Minolta Co. Ltd., Osaka, Japan) on the basis of the color system CIELAB (L*, a*, b*). In this system L*, a* and b* describe a tridimensional space, where L* is the vertical axis and its value ranges from 100 for perfect white to zero for black. Values a* and b* specify the axis green-red and yellow-blue, respectively. That values range from -60 to +60 or from -a (green) to +a (red) and from -b (blue) to +b (yellow). To determinate the color, a portion of one side of each fruit

was obtained and measured longitudinally in three equidistant points (Garza et al., 1996).

Assessment of antioxidant capacity

Assessment of reducing power by FRAP method

This method assesses the reducing power of a sample according to its capacity to reduce ferric iron (Fe^{+3}) with TPTZ to its ferrous form (Fe^{+2}), which has its maximum absorbance at 593 nm, according to the design done by Benzie and Strain (1996). 50 µL of sample were added to 900 µL of a FRAP solution, acetate buffer pH 3.4, TPTZ and $FeCl_3$ in relation 10:1:1. The results were expressed as ascorbic acid equivalent antioxidant capacity (AEAC: mg of ascorbic acid/100 g dry weight).

Oxygen radical absorbance capacity (ORAC) assay

The method described by Prior et al. (2005) and Romero et al. (2010) was used. 30 µL of sample were added to 21 µL of fluorescein $1x10^{-2}$ M in PBS (75 mM), 2.899 µL of PBS (75 mM), and 50 µL of AAPH 0.6 M in PBS (75 mM), temperature was controlled to 37°C and pH was kept at 7.4. The readings were done at an excitation ʎ 493 nm and excitation slit 10 nm. It was compared to the primary pattern Trolox® curve. The results were expressed as TEAC, µmol trolox equivalent /100 g of dry weight according to Equation 1.

$$ORAC = \frac{AUC - AUC^{\circ}}{AUC_{Trolox} - AUC^{\circ}} f[Trolox] \quad (1)$$

Where AUC is the area under the curve of the sample, AUC° is the area under the curve for control, AUC_{Trolox} is the area under the curve for Trolox, and f is the dilution factor for the extracts.

Secondary metabolites content with antioxidant properties

Total phenols

Determination of phenols was done by the colorimetric method *Folin-Ciocalteu* designed by Singleton and Rossi (1965). 50 µL of sample were added to 125 µL of Folin reagent and 400 µL of sodium carbonate 7.1% (w/v), adjusting with distilled water until 1000 µL. The spectrophotometric reading was done at 760 nm and it was compared to the pattern curve using gallic acid as standard. The results were expressed as mg of gallic acid equivalent: GAE/100 g dry weight.

Mangiferin content

Mangiferin content was found by liquid chromatography with diode array detector (brand Shimadzu, Prominence line, Japan). As a mobile phase, a mixture in gradient of 2% acetic acid was solvent A and 0.5% (v/v) acetic acid plus acetonitrile (50:50) was solvent B. The gradient program was: 10-55% B for 50 min, from 55 to 100% B for 10 min and finally from 100 to 10% B for 5 min. Column of C-18 LiChrospher® 100 RP-18 (5 µm) 250*4 mm (Merck, Germany), 25°C of oven temperature, sample injection volume 10 µL, flux 1 ml/min, detection wavelength: 280 nm were used (Schieber et al., 2000). The results were expressed as mg of Mangiferin/100 g dry weight.

Carotenoids determination

It was done by Ultraviolet-Visible (UV-Vis) spectrophotometry. In a test tube, 1.0000 g of sample was added to 5.0 mL of cold acetone and it was left to stand for nearly 15 min in refrigeration (4°C). The mixture was centrifuged at 1370 gravities for 10 min, the supernatant was collected in another test tube. The pellet was re-extracted with 5.0 mL of cold acetone. Both acetone extracts were mixed, and then, were filtered in Whatman paper No. 42 and its absorbance was determined at 449 nm. Carotenoid concentration was obtained by the respective calibration curve, with β-carotene as pattern substance. Data was obtained by the Software SkanIt 2.4.2 RE for Multiskan Spectrum (Biswas et al., 2011). The results were expressed as mg of β-carotene equivalent/100 g dry weight.

Ascorbic acid determination

It was established by HPLC. The aqueous supernatant was filtered (pore size 0.45 µm) and dilutions in super pure water, before injection to chromatographer. A liquid chromatographer Shimadzu® model LC-20AD was used, equipped with an auto-injector SIL-20A/HT, a communication module CBM-20A and a (PDA) SPD-M20A, calibrated to 245 nm. Ascorbic acid quantification was done with a C-8 (5 µm, 250 mm x 4.6 mm) column. Formic acid 0.1% was used as mobile phase, at a flow rate of 0.8 mL min^{-1}, at 35°C in isocratic conditions (Kelebek et al., 2009). The results were expressed as mg of ascorbic acid/100 g dry weight.

Condensed tannin determination

This method is based on reaction of condensed tannins with vanillin under acid conditions, using catechin as standard. A 2 ml aliquot of freshly prepared vanillin solution (1 g/100 mL) in sulfuric acid 70% was added to 500 µL of mango extract. The mixture was incubated at 20°C for 15 min and its absorbance was read at 500 nm. (+) Catechin was used to build the reference curve, and the results were expressed as mg catechin/100 g dry weight (Hagerman and Butler, 1989).

Statistical analysis

All the experiments were performed in triplicate. The regressions were calculated with a 95% significance level ($p<0.05$), using the Statgraphics Plus program version 5.0 (Statistical Graphics Corp., Rockvilee, MD).

RESULTS

Color determination

Color measure is a fundamental parameter in climacteric fruit analysis, given that the changes observed by the naked eye are associated with variations in the fruit's chemical composition. According to Nambi et al. (2015) in the mango's mature state, the coordinates of CIELAB system are characterized by presenting values between 51 to 60, 16 to 29, and 48 to 62 for L*, a* and b* respectively (Table 1).

Mango's pulp composition varies depending on many factors such as variety, location, weather, and maturity stage. However, previous studies have shown that the

Table 1. Color determination system CIE-L* a* b*.

	L	a	b
Criollo	66	16	50
Zapote	56	25	50
Azúcar	76	7	50
Corazón	72	26	46
Jobo	76	19	43
Chancleta	66	16	50

Table 2. Secondary metabolite results in mango samples.

Variety	Total phenols mg gallic acid/100 g dry base	Total carotenoids mg β-carotene/100 g dry base	Ascorbic acid mg ascorbic acid/100 g dry base	Mangiferin mg Mangiferin /100 g dry base	Condensed tannins mg catechin equivalent /100 g dry base
Criollo	196.6±7.8	105.9±9.1	8.7	259.2	144.2±13.1
Zapote	86.3±4.3	39.7±7.5	14.5	187.4	142.2±4.7
Azúcar	90.4±1.9	24.7±6.6	9.7	174.2	122.5±3.0
Corazón	137.5±6.8	25.5±5.5	20.6	246.7	161.5±9.5
Jobo	160.4±0.8	196.2±0.2	24.2	174.0	139.6±8.2
Chancleta	238.1±8.1	60.4±2.8	99.6	91.8	84.3±1.0

stage of maximum expression of bioactive compounds is presented in mature mango. Polyphenols, reducing agents like ascorbic acid, and carotenoids found in fruits and vegetables are the most studied metabolites due to its antioxidant potential, which are associated with health benefits such as protection against cardiovascular diseases and cancer (Masibo and He, 2009).

Total phenols and Mangiferin contents were superior in the Criollo variety. Tannins were higher in Criollo and Corazón varieties. While the ascorbic acid content was expressed better in Chancleta variety (99.6 mg ascorbic acid/100 g dry base). The variety Jobo reported the highest carotenoid content (196.15 mg β-carotene/100 g dry base) (Table 2).

Polyphenolic compounds present in fruits, have antioxidant activity because of their property to interact with different oxygen radical species by a reducing action. The mechanisms to capture free radicals are classified into two types. In both cases, they are blockers of the initial stage in the oxidative process of lipid or protein. The first mechanism is the transferability of a hydrogen atom, called HAT, which is measured by ORAC methodology; and in the second case, it is the transfer of a polyphenol electron, SET mechanism, which is measured by FRAP technique. In Table 3, it is observed that Criollo mango has high values for ORAC and FRAP, in such way that these antioxidant properties by both mechanisms are due to its composition of phenols and condensed tannins fundamentally. Chancleta mango has the highest values for ORAC in accordance with its total phenol content.

DISCUSSION

Various studies report similar values of color measurement to those found in this research, concluding that the carotenoid content like β-carotene, 9-*cis*-violaxanthin and lutein, is correlated positively ($r^2 > 0.9$) to the measurements of these parameters (Ayour et al., 2016). Thus, the studied mango varieties could have important bioactive pigment content. During maturation, climacteric fruits suffer different physiological changes at physical and biochemical level. Among the phenomena associated with fruit maturation, there is color change from green to yellow, orange, or even red. This change is caused by carotenoids' synthesis and chlorophyll's degradation as the first observable sign of maturation (Merzlyak et al., 1999).

Total phenols

Phenolic compounds constitute a group of secondary metabolites that are considered natural antioxidants with multiple biological benefits for human health. According to the results from Table 3, the varieties with greater polyphenol content are Chancleta, Criollo and Jobo, with 238.14, 196.58 and 160.44 mg of gallic acid/100 g dry matter, respectively. Different authors say that the main

Table 3. Antioxidant activity results in mango samples.

Variety/Technique	FRAP (AEAC*), dry base	ORAC (TEAC**), dry base
Criollo	237.9±20.7	1624.8±119.6
Zapote	81.0±5.9	926.7±72.6
Azúcar	115.5±8.1	848.7±47.1
Corazón	144.5±8.4	865.4± 43.5
Jobo	111.8±8.9	1203.6± 109.3
Chancleta	81.0±5.9	2163.8 ± 157.1

*AEAC: mg ascorbic acid/100g dry sample; **TEAC: µmol Trolox/100g dry sample.

phenols found in mango are chlorogenic, gallic, vanillin and protocatechuic acid, in order of abundance (Wall-Medrano et al., 2014). On the other hand, in the mango's peel and seed, values between 3.8 and 13 GAE/100 g dry matter have been found (Dorta et al., 2012). Chong et al. (2013) reported values between 120.70 and 210.24 mg gallic acid/100 g for mango cultivated in Malaysia. Also, Lobo et al. (2017) showed that there is a great oscillation in total phenol content in the Tommy Atkins mango pulp cultivated in Brazil (46.18 – 116.93 mg gallic acid/100 g). Thanaraj et al. (2009) reported 1055 and 1691 mg gallic acid/100 g dry weight as the interval for five mango varieties cultivated in Sri Lanka. A high variability in total phenol content was reported for mango, due to the different weather and growing conditions of each region. However, the evaluated Colombian pulps presented a significant content of phenolic compound as compared to what was reported by most authors.

Total carotenoids

Carotenoids are the compounds responsible for coloration in most food, some of them are provitamin A, like α and β-carotene, and β-cryptoxanthin. Recent studies have made these pigments' antioxidant properties manifest, as well as their effectiveness in the prevention of certain human diseases, such as atherosclerosis or even cancer (Müller et al., 2011).

Criollo and Jobo varieties presented the higher total carotenoid content with 105.88 and 196.15 mg β-carotene equivalent/100 g dry matter, respectively. Silva et al. (2014), who quantified bioactive compounds in different fruit from Brazil, found 0.954 mg β-carotene equivalent/100 g dry matter in mango; Seok et al. (2010) reported that the carotenoid content in Malaysia's mango pulp was 0.650 equivalent β-carotene/100 g dry matter, which are much lower values than the ones found in this study. It is worth noting that there are 17 important carotenoids found in mango, among them β-cryptoxanthin, zeaxanthin, isomers of luteoxanthin, violaxanthin, neoxanthin and β-carotene, which is the last one, the one with the highest prevalence (Rungpichayapichet et al., 2015). The above, suggests Colombian mango as an important source of these pigments, not only for fresh consumption, but for its use as a colorant additive in food matrices.

Vitamin C

Mango is recognized for its important contribution in vitamins like ascorbic acid, thiamin, riboflavin, and niacin. In this study, the varieties with the most vitamin C content were Chancleta and Jobo. However, the reported content for the six cultivars was lower than the one for other fruits studied in countries like Ecuador, Brazil and Colombia (Contreras-Calderón et al., 2011; Vasco et al., 2008). Seok et al. (2010) found that ascorbic acid content in fresh mango from Malaysia was 136.8 mg/100 g dry matter, a value close to the one found for Chancleta. The variations in vitamin C content are due to the fact that this compound is cataloged as the most unstable nutrient and prone to immediate loss after harvest. Therefore, its content depends on the changes during the post-harvest handling, storage conditions, transformation and elaboration (Spínola et al., 2013).

Condensed tannins

Polyphenols present in fruits and vegetables can include simple structured compounds (phenolic acids), oligomers (flavonoids, xanthones, stilbenes), or polymers (condensed tannins) (Pierson et al., 2014). Condensed tannins are oligomeric and polymeric proanthocyanidins that are widely distributed in the vegetable kingdom. It has been reported that they are used to stop small local bleeding, decrease buccal cavity inflammations, colds, bronchitis, burns, hemorrhoids, etc. (Piovesan et al., 2017; Kasay et al., 2013). Also, the condensed tannins' chemical nature makes them a natural source of organic compounds, with an application potentially wide for medicinal and industrial uses (Aguilar-López et al., 2012). According to the results in Table 2, all mango fruits presented a high condensed tannin content, highlighting

the varieties Corazón and Criollo. Gorinstein et al. (2011) evaluated the bioactive compound content in different exotic fruits, reporting 27.0 mg of catechin equivalent/100 g dry weight as the condensed tannin content in mango. Arogba (2014) reported a content of 135.0 mg of catechin equivalent/100 g dry weight in mango seed. The above shows that the evaluated mango varieties presented an important condensed tannin content, compounds whose antioxidant activity has been widely reported (Dobrecky et al., 2014; Ocampo et al., 2014).

Mangiferin

Mangiferin is the main polyphenol that constitutes mango's leaves, fruit and bark. The literature has reported its wide pharmacological, antidiabetic, antitumor, immunomodulatory and antioxidant activity (López et al., 2015). According to the results presented in Table 2, mangos cultivated in Córdoba present similar values and even higher for Mangiferin content reported in different research. For example, Luo et al. (2012) reported that the Mangiferin content in 11 varieties of mango pulp from China, was between 0.2 and 20 mg/100 g dry matter. Other authors suggested that Mangiferin is mainly found in mango's peel, leaves and bark, and in smaller measure, in pulp, reporting values from 169 mg/100 g dry matter in peel, and 4.2 mg/100 g dry matter in seeds (Rymbai et al., 2015). This shows that the evaluated Colombian mangos have a promising Mangiferin content, highlighting their nutraceutical quality and encouraging industrialization of this fruit in functional products. The high Mangiferin content in the studied varieties can be explained since this xanthone is synthesized by the phenylpropanoid pathway in presence of high solar radiation, which is a representative condition of the Caribbean Colombian region (Ruiz and Romero, 2001).

Antioxidant capacity

FRAP and ORAC-Total

Different methodologies have been proposed to evaluate fruit's antioxidant capacity, of which ferric ion reducing antioxidant power (FRAP) and oxygen radical absorbance capacity (ORAC) methods are widely used. These methods measure different mechanisms of antioxidant activity; while ORAC is related to capacity for neutralizing a free radical using HAT mechanism, FRAP assay measures sample's capacity for reducing the ion Fe^{3+} to Fe^{2+} (SET mechanism) (Rodríguez *et al.*, 2010; Botero et al., 2007). In this research, the variety with the best reducing capacity was Chancleta with 296.06 mg of ascorbic acid/100 g dry sample, followed by Criollo and Azúcar. In general, low values of reducing capacity have been reported for mango (Paz et al., 2015).

FRAP values found in the evaluated mango pulps, match with low quantity of vitamin C analyzed by HPLC. However, ORAC unities from Chancleta, Criollo and Jobo varieties are higher than the ones reported by Singh et al. (2015) in dried Tommy mango chunks (408 to 651 µmol TE/100 g dry sample). In other hand, ORAC values between 0.46 and 1.33 mmol Trolox/100 g dry simple were found in a study that was done on 10 cultivars of Mediterranean diet fruits (Wojdylo et al., 2016). This shows that the analyzed varieties in this research exhibit a considerable antioxidant capacity measured with ORAC methodology. It is worth to mention that this fluorometric technique is the one endorsed by the United States Department of Agriculture (USDA) for measuring antioxidant capacity in food and nutritional supplements (Rojano et al., 2012).

Correlations between polyphenols and antioxidant activity

As shown in Figures 1 and 2, a positive correlation was found between total phenol content and antioxidant capacity evaluated with ORAC and FRAP (r^2 = 0.87 and r^2 = 0.84), which shows that polyphenols are the main contributors to mango's antioxidant activity. Also, the synergism between antioxidants could explain why the fruit's antioxidant capacity is higher than the individual content in each antioxidant, like vitamin C. Similar results have been found in other researches (Thaipong et al., 2006; Silva and Sirasa, 2016).

Conclusion

The present study showed the antioxidant potential of six mango varieties cultivated in the Department of Córdoba, being Chancleta, Criollo and Jobo, the varieties that showed the highest antioxidant activity *in vitro.* As regards mangiferin content, all varieties stood out above fruits from other countries. These results prove that Colombian mangoes have a high nutraceutical potential due to its Mangiferin content and to the antioxidant expression of the other bioactive compounds like carotenoids and tannins. It is necessary to conduct studies *in vivo* to determine bio-accessibility and/or bio-availability of mango's extracts with higher antioxidant potential and Mangiferin as the main bioactive.

CONFLICT OF INTERESTS

The authors declare there is no conflict of interest.

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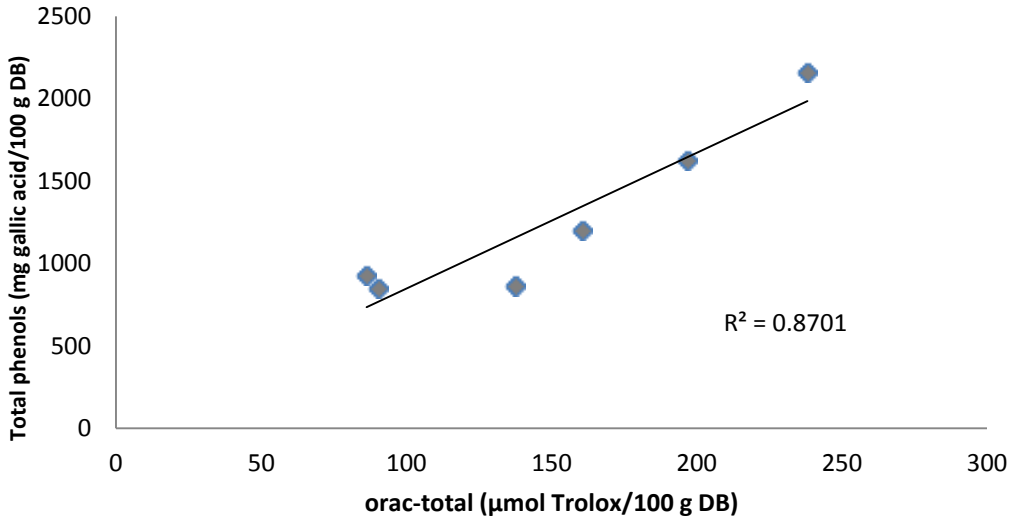

Figure 1. Correlation total phenols vs. ORAC-total.

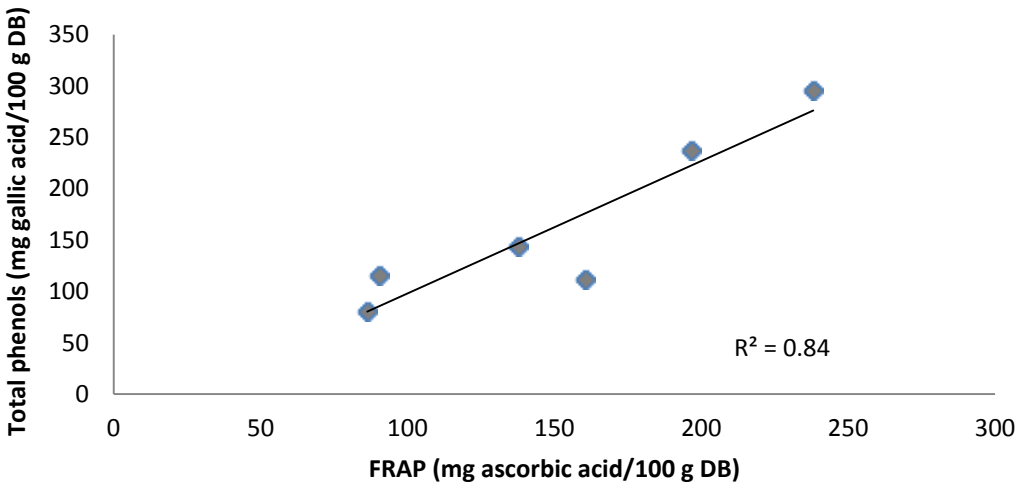

Figure 2. Correlation total phenols vs. FRAP.

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