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ANTIOXIDANT CAPACITY AND PROTECTIVE ROLE OF METHANOLIC EXTRACTS OF *Jatropha platyphylla* AGAINST OXIDATIVE STRESS ON MACROPHAGE CELLS

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ABSTRACT

Jatropha platyphylla is an endemic non-toxic Mexican specie from the Pacific coast, with high nutritional value and bioactive potential. The phenolics, tannins and antioxidant capacity of *J. platyphylla* methanolic extracts and their effect on oxidative stress in macrophages were determined. The antioxidant potential of total phenolic extracts (TPE) and free tannin extracts (FTE) were evaluated on macrophage cells. The total phenolic content ranged from 487.82-1753.89 mg GAE 100 g^{-1}, with the highest proportion of hydrolysable tannins (452.73-904.01 mg GAE 100 g^{-1}). *In vitro* chemical methods showed values of 29-87% inhibition of radical DPPH and 68-844 μmolTEg^{-1} for ORAC, and both methods revealed the influence of tannins content on antioxidant capacity. Besides, the TPE of bound phenolics from kernel and free phenolic from pulp showed 46 and 42% ROS inhibition, respectively. *J. platyphylla* tannins showed a high antioxidant capacity, while TPE from kernel and phenolics from pulp showed a protective role of oxidative stress on macrophages cells.

KEYWORDS: ***bound phenolics, hydrolysable tannins, macrophages, oxidative stress, tannins.***

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INTRODUCTION

Jatropha platyphylla is a Mexican non-toxic *Jatropha* wild species, exclusive of the Pacific coast of Mexico, growing from the state of Sinaloa to Michoacán. *J. platyphylla* is a tree 2-5 m tall, drought resistant, with peltate leaves 15-35 cm, large petioles, round seeds of 15 mm in diameter, weighing about 1.75 g, white flowers.[1] These inflorescences are very different from those of other species of *Jatropha*. Residents of the communities of Sinaloa have consumed *J. platyphylla* as roasted seeds or as preserve made of unripe fruits, due to its high nutritional value, since it contains about 23% protein.[2, 3] This species has been barely studied, to date, there is no report on its nutraceutical potential, in contrast to others species such as *J. unicostata* and *J. curcas*, of which there is scientific evidence of its antioxidant and anti-inflammatory potential.[4-7] *Jatropha* antioxidant capacity was highly associated with the content of phenolic compounds,[5, 8, 9] mainly phenolic acids and flavonoids, although also has been reported the presence of tannins.[10] Tannins are traditionally considered food anti-nutrients for its mineral (iron and zinc) chelating ability, in addition to protein precipitation avoiding its proper absorption. However, recently many studies have found that precisely these characteristics and their structural similarity to the polyphenolic compounds, give antioxidant properties to tannins.[11]The aim of this study was to determine total phenolics and tannins content on *J. platyphylla* kernel, pulp and leaf methanolic extracts; also the antioxidant capacity and the tannin influence on it, and the extracts effect on oxidative stress on macrophages were determined.

MATERIAL AND METHODS

Plant material

For the study pulp, kernel and leaves from wild *Jatropha platyphylla* trees were used, and fruits were collected at ripening mature green stage. *Jatropha platyphylla* was confirmed and authenticated by the botanical researcher J. Manuel Aguilar-Patiño from the Agricultural School at the Sinaloa State University. Samples were collected during August and September 2012, in Mazatlán, Mexico (23,5333°N; 106,4667°W, 60 masl). Samples were washed in chlorinated water solution (150 ppm), lyophilized and ground to be exposed to the extraction process. The kernel flour was defatted before analysis by solvent extraction**.**

Free phenolic extract preparation

A 2.5 g flour sample was blended with 15 mL of chilled methanol/water (80:20, v/v), and agitated for 24 h. After centrifugation at 10,000 rpm, at 4 °C, for 15 min (Beckman™ J2-M1, JA-17 123 rotor), the supernatant was evaporated to 2 mL at 45 °C. The resulting extracts were frozen at -80 °C and stored until use.[12]

Bound phenolic extract preparation

After extraction of free phenolic compounds, the residue was digested with 10 mL of 2 M sodium hydroxide in a water bath at 95 °C for 30 min with the previous removal of oxygen using nitrogen gas. Finally, the sample was agitated for 1 additional hour at room temperature. The mixture was acidified (pH<2.0) with 2 mL of hydrochloric acid and extracted with hexane to remove lipids. The final solution was extracted five times with 10 mL of ethyl acetate for each extraction. The ethyl acetate fraction was evaporated to dryness under vacuum at 35 °C. Bound phenolic compounds were reconstituted in 2 mL of methanol/water (80:20, v/v), were frozen at -80 °C and stored until use.[13, 14]

Determination of total phenolic compounds

Phenolic content was determined by Folin-Ciocalteu assay,[15] 15 µL of extracts plus 240 µL distilled water were oxidized with 15 µL of Folin-Ciocalteu reagent 2N in 96 well plate (Costar™, Waltham, MA USA); after 3 min, 30 µL Na_2CO_3 4N were added and incubated for 2h. Finally, absorbance (Abs) was measured at 725 nm using a microplate reader (Synergy HT, BioTek™, Inc, Winooski, VT, USA), using methanol 80% as blank. A calibration curve was prepared using gallic acid as standard and total phenolics were expressed as milligrams of gallic acid equivalents (mg GAE) per 100 g of dry weight (DW) of sample.

Determination of tannins

Tannins were precipitated by 100 mg polyvinyl pirrolidone (PVPP) adding to 1 mL distilled water and 1 mL of extract.[16] The mixture was agitated and kept at 4 °C for 15 min, after centrifugation at 10,000 rpm, 4 °C, 15 min. The supernatant was collected, and the phenolic content was determined as above. Total tannins were determined by subtracting from total phenolics. For condensed tannins,[17] 20 □L of extract were placed in a 96-well plate, 200 □L dimetilamin cinamaldehyde (DMCA) 0.1 % were added and after 5 min absorbance was measured at 640 nm using a microplate reader. A calibration curve was prepared using catechin as standard. Finally, hydrolysable tannins were determined subtracting condensed to total tannins.

Antioxidant capacity

Antioxidant capacity was determined using the oxygen radical absorbance capacity (ORAC) and scavenging of 1,1-diphenyl-2-picryl-hydrazyl (DPPH) radical assays. For the ORAC assay,[18] aliquots of 25 µL of free and bound phenolic extracts were diluted in 75 mM phosphate buffer and transferred in a 96-well round opaque bottom microplate. A microplate reader was programmed to dispense 200 µL 0.96 µM fluorescein and 75 µL 95.8 µM AAPH (2,2'-azobis[2-amidinopropane]dihydrochloride) free radical. Reaction fluorescence was µlmeasured using a wavelength of 485 nm (excitation) and 580 nm (emission) during 70 min at intervals of 70 s. Protective effects of samples and control were calculated subtracting the net integrated area under the curve of the control from the area under the curve of the sample [AUC_{sample}-$AUC_{control}$]. Results were expressed as mg of Trolox equivalents per kg of dry weight (mg TEk g^{-1}). For DPPH assay,[19] aliquots of 20 µL of free and bound phenolic extracts and 280 µL DPPH (200 µM) were added in a 96-well plate, after 30 min (25°C) absorbance was measured at 540 nm using a microplate reader, methanol 80% was used as a blank. Scavenging of DPPH radical was calculated using the next equation:

$$\text{Scavenging of DPPH (\%)} = \frac{Abs_{blank} - Abs_{sample}}{Abs_{blank}} \times 100 \quad (1)$$

Cell culture

Macrophages were grown in the DMEM-low glucose (pH 7.2 – 7.4) including 4 g L^{-1} glucose, 3.7 g L^{-1} sodium bicarbonate, 10% fetal bovine serum (FBS) and antibiotics (100 units mL^{-1} penicillin and 100 µg mL^{-1} streptomycin) in a humidified atmosphere with 5% CO_2 at 37 °C. *Tests for cell proliferation and lps-induced lactate dehydrogenase (ldh) activity.* Macrophages (10^5 per well) were plated in a 96-well clear bottom plates (Costar) and cultured overnight. The cells were stimulated by LPS (1µg mL^{-1}) for 19 h either with or without the 5 h pre-treatment of *J. platyphylla* extracts and were subjected to the tests for cell proliferation and LPS-induced mitochondrial dehydrogenase activity, which were evaluated in macrophages using the MTT assay kit (Promega, Madison, WI), according to the manufacturer's instructions. The quantity of formazan product was measured at 490 nm and is directly proportional to the mitochondrial dehydrogenase activity.

Cellular oxidative stress on macrophages

Macrophages Raw 264.7 were split and counted by trypan blue exclusion. The cells were harvested the day before the experiment, seeded in a dark 96-well microplate with 25,000 cells per well and allowed to attach overnight. The following day, the cells were washed with 100µL per well of PBS once. After 100µL per well of DCFDA were added and incubated in 5% CO_2/95% air at 37°C for 45 min in the dark. The cells were washed with 100 µL per well of PBS again. The cells were incubated for 45 min with the pulp, leaf and kernel extracts (80 µg mL^{-1}), after they were incubated with AAPH 500 µM for 20 min. The fluorescence of the cells from each well was measured using a microplate reader (Synergy HT, BioTek™, Inc, USA) with temperature maintained at 37°C. The excitation filter was set at 485 ± 10 nm and the emission filter was set at 528 ± 10 nm. Results are expressed as percent of relative production of ROS with respect to the cells treated with AAPH only.[20]

Statistical analysis

Multifactorial ANOVA procedure was used for the analysis of the experimental results. Results are expressed as the mean of triplicate ± standard error. Differences among treatments were determined using Tukey's test ($p<0.05$).

RESULTS

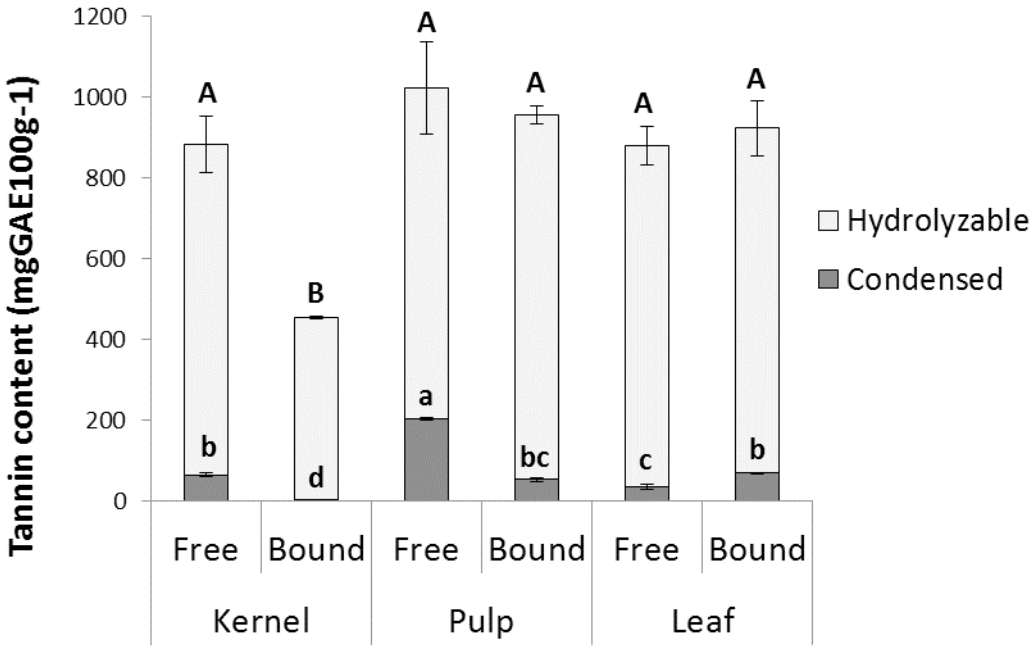

Figure 1

Hydrolysable and condensed tannin content from free and bound phenolic extracts of kernel, pulp and leaf of Jatropha platyphylla. Letters within columns provide statistical significance. Lower cases (a, b, c) for condensed tannin content. Capital letters (A, B, C) for hydrolysable tannin content. Columns not sharing a letter are significantly different.

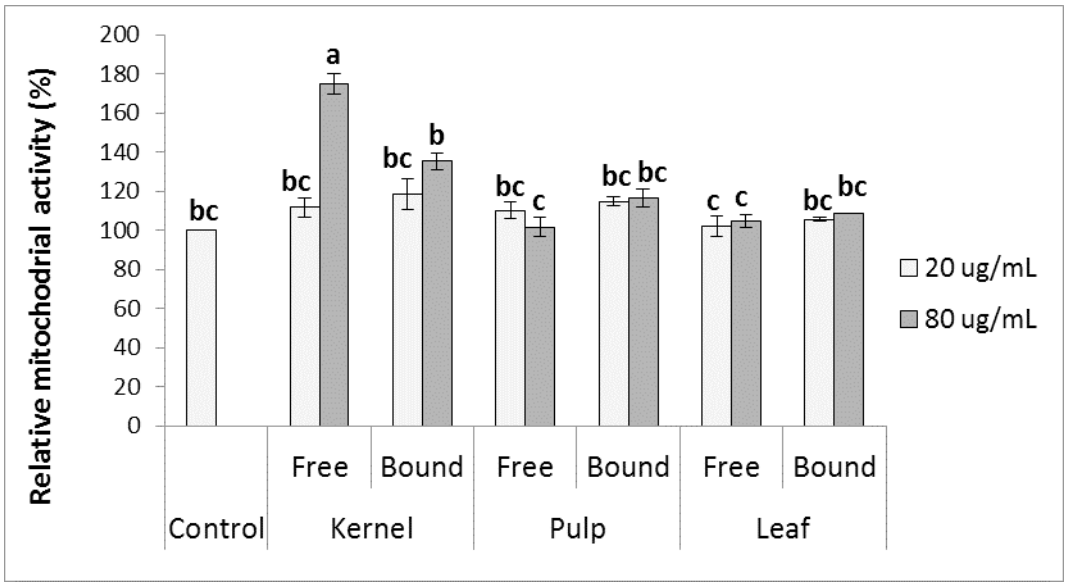

Figure 2

Relative mitochondrial activity in macrophages-LPS stimulated treated with free and bound phenolic extracts of kernel, pulp and leaf of Jatropha platyphylla. C: control. Letters (a, b, c) within columns provide statistical significance. Columns not sharing a letter are significantly different.

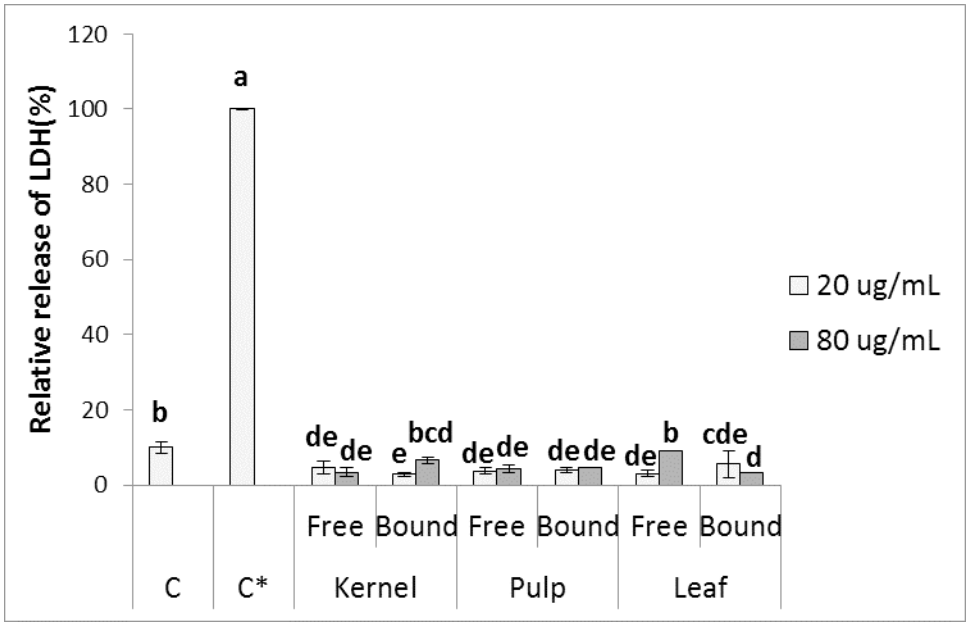

Figure 3

Relative release of LDH in macrophages-LPS stimulated, treated with free and bound phenolic extracts of kernel, pulp and leaf of Jatropha platyphylla. C: spontaneous cellular death; C*: maximum death. Letters (a, b, c, d, e) within columns provide statistical significance. Columns not sharing a letter are significantly different

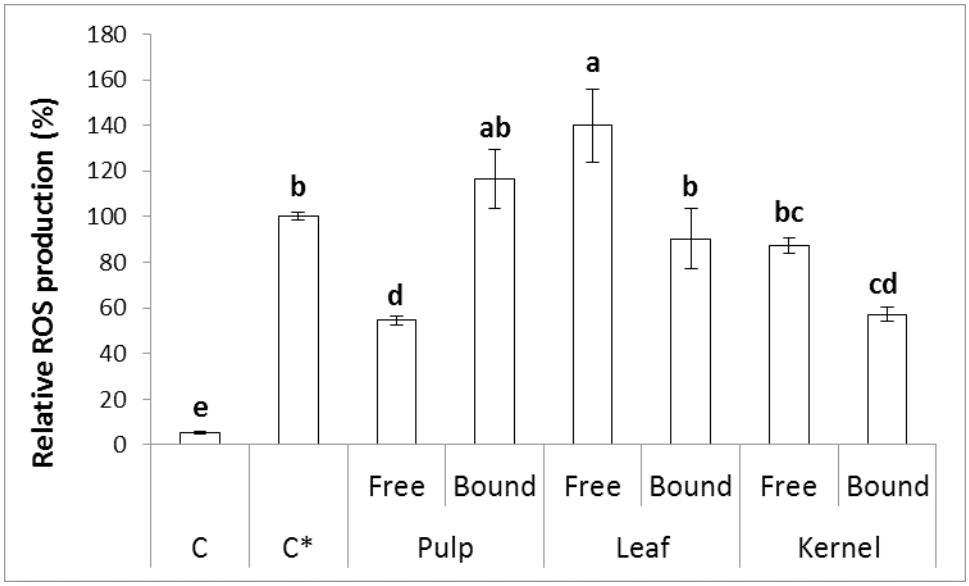

Figure 4

Relative reactive oxygen species production in macrophages-AAPH stimulated, treated with free and bound phenolic extracts of kernel, pulp and leaf of Jatropha platyphylla. C: Non-treated cells; C*: cells AAPH-stimulated. Letters (a, b, c, d, e) within columns provide statistical significance. Columns not sharing a letter are significantly different.

Table 1

Phenolic content of extracts of Jatropha platyphylla plant materials.

Material	Phenolic type	Total phenolic content (mgGAE100g^{-1}DW)	
		TPE	FTE
Kernel	Free	1175.56 ± 33.62 b	385.96 ± 13.50 a
	Bound	450.80 ± 35.32 c	154.62 ± 4.85 c
Pulp	Free	487.82 ± 23.09 c	371.80 ± 18.84 a
	Bound	1753.89 ± 86.53 a	203.03 ± 5.08 bc
Leaf	Free	1329.97 ± 38.79 b	449.14 ± 15.39 a
	Bound	1131.09 ± 55.81 b	278.10 ± 36.74 b

TPE: Total phenolic extract; FTE: Free tannin extract; GAE: Gallic acid equivalent. DW: dry weight. Mean of triplicate ± standard error. Tukey test p<0.05. Letters (a, b, c) within columns provide statistical significance. The means not sharing a letter in the same column are significantly different.

Table 2

In vitro antioxidant capacity of Jatropha platyphylla extracts.

Material	Phenolic type	Inhibition of DPPH radical (%)*		ORAC (μmolTEg^{-1}DW)	
		TPE	FTE	TPE	FTE
Kernel	Free	78.92 ± 1.51 b	5.7 ± 0.19 d	388.41 ± 4.25 d	38.24 ± 4.93 d
	Bound	76.64 ± 1.56 b	12.4 ± 1.73 b	67.96 ± 6.69 f	39.80 ± 1.12 d
Pulp	Free	73.07 ± 0.20 b	7.5 ± 0.26 cd	309.98 ± 5.97 e	4.80 ± 0.20 e
	Bound	87.70 ± 0.94 a	30.5 ± 0.28 a	443.13 ± 13.62 c	72.24 ± 1.78 c
Leaf	Free	85.15 ± 0.58 a	6.64 ± 0.28d	795.89 ± 6.82 b	496.82 ± 5.38 a
	Bound	29.48 ± 2.07 c	11.33 ± 1.23 bc	844.42 ± 13.41 a	114.97 ± 1.28 b

TPE: Total phenolic extract; FTE: Free tannin extract; DW: Dry weight of sample. *Inhibition of DPPH radical at 400 mgGAE/100g DW. Mean of triplicate ± standard error. Tukey test p<0.05. Letters (a, b, c, d, e, f) within columns provide statistical significance. The means not sharing a letter in the same column are significantly different.

Total phenolic content

The phenolic content, which is expressed as mg GAE100g^{-1} for dry weight (Table 1), was determined for two types of extracts: total phenolic extract (TPE) and free tannin extract (FTE), the tannins were removed from TFE. The removal of tannins allows indirectly determining the amount of these, observing that most of phenolic compounds present in the *J. platyphylla* extracts correspond to tannins. It is assumed that the phenolic compounds present in TFE correspond to

flavonoids and phenolic acids. The phenolic content in TPE ranged from 487.82 to 1753.89 mgGAE100g^{-1}, showing similarities with the 1080 mgGAE100g^{-1} reported for *J. curcas*.[5] The experimental design consisted of two factors: material (kernel, pulp and seed levels) and type of phenolic (free and bound levels). Resulting from that design, the two factors were significant ($p<0.00$). The bigger values were for pulp and leaf levels in the material factor, while bound level was bigger for the type of phenolic factor. This implies that for the purpose of phenolic compounds extraction are preferable extracts of bound compounds from pulp and leaf.

Tannin content

The total tannin content ranged from 455.03 to 1024.30 mgGAE100g^{-1}. According to Georgiev et al.[21] the safe amount of tannins in a food product is about 3.84%, because of this, all the materials tested could be considered as edible, due to the tannin levels were below 2%. About hydrolysable and condensed tannin content (Figure 1), it was observed higher content of hydrolysable (452.73-904.01 mgGAE100g^{-1}) than condensed tannins (2.30- 69.17 mgGAE100g^{-1}) in all the materials tested. The *J. platyphylla* hydrolysable tannin content (0.45-0.9%) is similar to *J. curcas* bark (0.5%), however, condensed tannin content in *J. curcas* is bigger (0.2%).[22]

Antioxidant capacity

The antioxidant capacity was determined in TPE and FTE, in order to attribute some antioxidant properties to the presence of tannins, either by direct inhibition of radical or a synergistic effect with other phenolic compounds such as flavonoids and phenolic acids. Phenolic compounds can exert antioxidant capacity by different mechanisms. To asses two of them, we used two methods of evaluation: DPPH radical inhibition and ORAC (Table 2). The DPPH radical inhibition assay measures the ability of the phenolic compounds to inhibit DPPH free radicals by transferring one hydrogen atom and the sequential proton loss electron transfer donating electrons, this method has been often adopted to inspect the radical-scavenging properties of plants and medicinal herbs extracts.[23,24] While ORAC assay provides a direct measure of the hydrophilic and lipophilic chain-breaking antioxidant capacity versus peroxyl radicals, by donating protons and thereby to stabilize free radicals and reactive species.[19] The results obtained with the DPPH method for TPE were in the range 29-87% DPPH radical inhibition, finding higher values than those reported for *J. unicostata* (43.8%), but lower than Vitamin C (96%), which is known as a powerful antioxidant.[4] Statistical analysis indicated that there is significant difference between free and bound levels, having bigger values for free level. The TPE antioxidant capacity by ORAC method was in the range 68-844 µmolTEg^{-1}, these values were higher than those reported for *J. curcas* seed shell (8.84 µmolTEg^{-1})[25] and some fruits rich in phenolics as strawberry (35.41 µmolTEg^{-1}) and blackberry (47.5 µmolTEg^{-1}).[26] Similarly, TPE showed bigger antioxidant capacity than reported for grains such as white corn (193.12 µmolTEg^{-1}).[14] Nevertheless, it is important to mention that most of the studies reported, evaluated the antioxidant capacity of extracts of free phenolic compounds without regard to bound phenolics. In this study, it was observed statistically significant differences between free and bound ($p<0.00$), having larger values for free level. It is important to remark that bound phenolics extracts have great antioxidant capacity, these results mention above indicate that others studies underestimate the antioxidant potential of materials.

Cell proliferation and LPS-induced lactate dehydrogenase (LDH) activity

Prior to evaluating the effect of *J. platyphylla* extracts on ROS production, we examined its effect on cell proliferation in RAW 264.7 macrophages. We found that TPE of kernel, pulp and leaf affect cell proliferation at concentrations 100-400 µgmL^{-1}, so we proved 20-80 µgmL^{-1} an evidence that TPE did not have a negative effect on proliferation at lower concentration; even the free phenolics extract of kernel raise the mitochondrial activity (Figure 2). Some phenolic compounds have shown to protect against mitochondria injury and greatly increase the activity of succinate dehydrogenase.[27] The high LPS-induced LDH activity implies cell damage. The TPE of kernel, pulp and leaf did not show cell damage at concentrations 20-80 µgmL^{-1} (Figure 3). In this sense, it has been well-known that phenolic compounds play a protective role for altered mitochondrial function and free radical-mediated tissue damage because their free radical scavenging and antioxidant potential.[28] The results mentioned above indicate that the TPE can be used in concentrations up to 80 µgmL^{-1} without affecting cell proliferation or causing cell damage, and they are suitable for to evaluate their effect over oxidative stress.

Cellular oxidative stress on macrophages

Oxidative stress was evaluated by relative ROS production, having as a reference a control consisting of macrophages AAPH-stimulated. The TPE exhibit a decrease from 9 to 45% on ROS production, presenting the best values bound phenolics from kernel and free phenolic from pulp (Figure 4). These results are similar to those reported for caffeic, syringic, and protocatechuic acids which presented ≈20 and 15% on ROS inhibition in HEL and HUVEC cells, respectively.[29]

DISCUSSIONS

It is difficult to compare the phenolic content of TPE with other *Jatropha* species or other plant materials, as most of the found studies are focused only on the analysis of free phenolics. Among them, Wong-Paz et al.[30], reported 234 mgGAE100g^{-1} in *J. dioica,* while Igbinosa et al.[31] presented 2887 mgTAE100g^{-1} in *J. curcas* bark, and Oskoueian et al.[32] showed 390 mgTAE100g^{-1} in *J. curcas* kernel. All extracts tested had a content of free phenolic compounds positioned within the range of fruits which are considered with high phenolic content as strawberry (363.70 mgGAE100g^{-1}) and blackberry (1515.90 mgGAE100g^{-1}).[33] About tannin content, Haslam[34] mentioned that considerable amounts of tannins could be found in virtually all-structural parts of the plant. However, the increased production of tannins was highly associated to insect attack. This study was performed on wild *J. platyphylla* fruits, so naturally have

been subjected to various types of stress, such as predators attack. Therefore, a highly tannin content is expected, besides the influence of other determinant factors like environmental conditions,[35] genetic or ripeness of the fruit or plant[36]. According to Bate-Smith and Metcalfe,[37] the hydrolysable tannins are characterized by a restricted taxonomic distribution and are mainly associated with dicotyledonous plants, such as *J. platyphylla*. Moreover, most plants that are capable of synthesizing hydrolysable tannins are unable to synthesize condensed and vice versa,[34] which can explain the low presence of condensed tannins.Hydrolyzable tannins are compounds with a central core of glucose or another polyol esterified with gallic acid (known as gallotannins) either with hexahydroxydiphenic acid or ellagic acid (kwnow as ellagitannins); while condensed tannins consist of polymers formed by the condensation of flavans without any sugar residue, and are known as proanthocyanidins.[38] It was observed in both methods of assessing antioxidant capacity, a significant decrease in antioxidant capacity by subtracting tannins. Tannins possess high antioxidant capacity such in the case of the hydrolysable tannins, which according to Zhang et al.[39], a concentration of 100 □LmL^{-1} is able to neutralize reactive species like superoxide and hydroxyl radicals, and to reduce lipid peroxidation in cell membranes. Condensed tannins have been studied most extensively because they are easier to extract and quantify.[40] These are effective scavengers of free radicals, and inhibit oxidation of tissues even better than vitamin C, vitamin E and □-carotene, as well they are able to inhibit oxidases and prevent oxidation of low-density lipoproteins. Hydrolysable and condensed tannins have a high antioxidant potential due to their high molecular weight and high degree of hydroxylation of aromatic rings[8]. Tannins have shown different mechanisms of antioxidant capacity as free radical scavenging, transition metal chelation, inhibition of pro-oxidative enzymes and lipid peroxidation; among those mechanisms, metal chelation is probably the most significant because the inhibition of Fenton's reaction, besides the stability of metal-tannin complex increase with polymerization degree.[41] We observed that *J. platyphylla* extracts showed decrease on oxidative stress, phenolic compounds may not merely exert their inhibitory effect on ROS production as free radical scavengers, but also by modulating cellular-signal processes, like in NF-□b and Nrf2 pathways;[42] this could explain differences among the evaluation of antioxidant capacity by chemical and cell line *in vitro* methods. Also, we observed an increase in ROS production in macrophages treated with bound phenolics from pulp and free phenolic from leaf. It is well known that phenolic compounds are powerful antioxidants; however, polyphenols may be regarded as xenobiotics by animal cells and are to some extent treated as such. Actually, xenobiotics can enhance the ROS production if they are capable to penetrate into mitochondria and turn on the stimulated production of ROS called "the respiratory burst", as the result of the intentional production of reactive oxygen species in response to external factors.[43] In addition, phenolics are conjugated to various sugar molecules in food matrix, which influence their intestinal absorption, transport, and entry into cells. Some glycosides are totally inactive in tissue cell cultures.[44] Also, because of the chemical characteristics of phenolics they cannot only be considered as antioxidants, since under certain conditions they can also display pro-oxidant activity.[45] Therefore, this could explain, in part, the observed increment in ROS production.

CONCLUSIONS

J. platyphylla methanolic extracts are a good source of phenolic compounds, mostly hydrolysable tannins, besides it is important to remark that *J. platyphylla* materials tested have safe tannins content. Also, it was observed by *in vitro* chemical methods that tannins have a marked influence on the antioxidant capacity, either by direct inhibition of radical or a synergistic effect with other phenolic compounds such as flavonoids and phenolic acids. In addition, we conclude that it has been underestimated the antioxidant potential of bound phenolics, which actually showed a good antioxidant capacity. TPE of bound phenolics from kernel and free phenolic from pulp showed an important decrease on ROS production in macrophages. Finally, we considerate that it is very important to carry out *invivo* experiments, where the activity of tested phenolics reflects the bioavailability, metabolism and cellular activity, which cannot be determined by *in vitro* chemical methods. Also, we recommend to determine the phenolic profile of *J. platyphylla* extracts due to their different performance on antioxidant capacity.

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CONFLICT OF INTEREST

Conflict of interest declared none.

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