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Original article

Chemical composition and antioxidant activity of the ethanol extract and purified fractions of cadillo (*Pavonia sepioides*)

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ABSTRACT

Background: Bioactive plant compounds can contribute to prevent diseases related to oxidative stress. Some of these compounds are water soluble and fat soluble vitamins, carotenoids and a huge variety of phenolic compounds associated with the antioxidant activity (AOA).
Objective: The aim of this study was to study the antioxidant activity of *Pavonia sepioides*.
Methods: The AOA was evaluated by spectrophotometric methods using the free radical DPPH* and the ABTS*+ radical cation assays. Total phenolic content (TPC) was determined by Folin–Ciocalteu method and total flavonoid content (TFC) was determined by direct reaction with aluminum trichloride. The phenolic content was evaluated by GC/MS technique.
Results: The greatest antioxidant activity was found in ethyl acetate fraction (Fea), The total ethanol extract (TE) showed good AOA in comparison with hexane fraction (Fhx), dichloromethane fraction (Fdc) and aqueous fraction (Faq). The TPC and TFC analyses provided results consistent with the AOA with highest values for Fea and ET respectively. Different phenolic compounds of low molecular weight were identified by GC/MS (Salicylic acid, cinnamic acid, p-hydroxybenzoic acid, p-hydroxyphenylacetic acid, p-hydrocinnamic acid, vanillic acid, gentisic acid, p-cinnamic acid, o-Cinnamic acid, protocatechuic acid, syringic acid, m-cinnamic acid, ferulic acid, and caffeic acid). These compounds could be associated with the named activity. The Pearson and Spearman association tests indicated a positive linear correlation between the AOA and TPC and TFC results, showing correlation coefficients greater than 0.810 in the Pearson test and greater than 0.700 in the Spearman test ($p < 0.05$).
Conclusion: The AAO of *P. sepioides* (ET and purified fractions) was confirmed. The study provides evidence that *P. sepioides* is potential source of natural antioxidants showing positive correlations between phenolic contents and the AAO.

1. Introduction

Oxygen is an essential molecule for living organisms; however, reactive oxygen species (ROS) and free radicals are inevitably generated during aerobic type metabolic processes.[1–4] Each of these generated species has oxidizing characteristics that produce damage in molecules such as proteins, carbohydrates, lipids, and nucleic acids, among others.[1,2,5–7] Despite the fact that living beings possess natural antioxidant defenses, including different types of enzyme such as peroxidase, catalase and glutathione, the protective effect that these offer may be limited by a high production of ROS, generating cellular oxidative stress as a result of the imbalance between antioxidant and oxidant species of living organisms.[1,7–9]

To contribute to the prevention of diseases related to oxidative stress, some studies have shown that diet plays an important role, due to the presence of bioactive plant compounds that maintain the balance in biological systems. Some of these compounds are water soluble and fat soluble vitamins, carotenoids and a huge variety of phenolic compounds whose antioxidant activity and potential beneficial effects have continued to be widely studied over recent years.[10–19] The consumption of vegetable products has been linked with a lower incidence of such diseases as atherosclerosis, atheromatous disease, arterial hypertension, acute myocardial infarction, carcinogenic and neurological diseases, since these products possess anti-inflammatory, anti-allergic, anti-thrombotic, anti-microbial, and anti-neoplastic activity.[3,4,6,16,17,20–28]

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The Malvaceae family, distributed in temperate and warm regions in both hemispheres, consists of 250 genera and 4200 species. Of the 250 known species, 224 are found in the Americas, distributed throughout the temperate and tropical regions. The plants of this family have been associated with anxiogenic and central nervous system depressant activity, activity against *Plasmodium falciparum*, hypothermic and antioxidant activity, prevent and control malaria and regulate both intestinal transit and the menstrual cycle in women.[6,29–34]

Based on the above, it is very important to seek and identify compounds with antioxidant properties in new natural sources, given their wide applicability. This study seeks to determine the antioxidant activity of the ethanol extract of *Pavonia sepioides*, a plant that, on review of current scientific reports, has yet to feature in phytochemical, biochemical, biological or pharmacological studies, thereby acting as the starting point for the scientific analysis of this traditional medicinal plant.

2. Material and methods

2.1. Chemicals

All chemical used were of analytical grade. 2,2-Diphenyl-1-picrylhydrazyl (DPPH), 3,5-Di-tert-4-butylhydroxytoluene (BHT) and potassium persulfate were from Sigma–Aldrich (St. Louis, USA). DL-α-Tocopherol, 2,2′-Azino-bis(3-ethylbenzthiazoline-6-sulfonic Acid) (ABTS), 6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), hesperidin, ascorbic acid, sodium carbonate, sodium phosphate dibasic, potassium phosphate monobasic, potassium chloride and sodium chloride were from Merck (Darmstadt, Germany). Gallic acid and Folin–Ciocalteu's phenol reagent were from Panreac (Barcelona, Spain). Methanol and Ethanol were from Supelco (Bellefonte, PA, USA).

2.2. Plant material

The leaves were collected in Popayan (Cauca, Colombia), between 02°26'260'' north latitude and 076°36'01 0.7'' west longitude, W: 16°. The plant was identified by Ms.C. Bernardo Ramirez (Departamento de Biologia, FACNED, Universidad del Cauca), A voucher specimen (CAUP-01) was deposited at the Herbarium CAUP of the University. The leaves were dried at 40 °C and stored in a tightly closed container for further use.

2.3. Preparation of extracts

The process was carried out based on the methodology described in literature,[35] with some modifications. Cadillo leaves were cut into pieces and dried at 40 °C. The sample was subjected to Soxhlet extraction for 24 h with 96% ethanol. The extract was dried and a 1 g aliquot of the total ethanol extract (TE) was dissolved in 30 mL of distilled water obtaining the crude extract (CE_1), stirred on a Vortex machine and placed in a separatory funnel to undergo liquid–liquid extraction (LLE). 10 mL of hexane was added to the separatory funnel. The mixture was gently shaken for 1.5 min and allowed to stand for 2 min, permitting the separation of the two phases.

The procedure was performed twice more with 10 mL of hexane (successive extraction method). The hexane fractions (F_{hx}) were mixed, and the crude extract (CE_2) was then partitioned successively with dichloromethane and ethyl acetate, obtaining the fraction in dichloromethane (F_{dc}), ethyl acetate fraction (F_{ea}) and the aqueous fraction (F_{aq}) respectively. Each of the fractions was dried and stored at 4 °C.

2.4. Evaluation of antioxidant activity

The evaluation of antioxidant activity was performed by the DPPH[35] and ABTS[36] methods, with some modifications.

2.4.1. DPPH radical scavenging activity

The methanolic solution of the DPPH• radical was prepared dissolving 2.9 mg of DPPH in 100 mL of methanol; the absorbance value was adjusted to 0.800 ± 0.03 ($\lambda = 515$ nm). A calibration curve was prepared with a DPPH• radical solution, in a concentrations range from 2×10^{-5} M to 8×10^{-5} M.

2.4 mL of the radical solution was mixed in with 1.2 mL of the test sample (10–1000 μg/mL). These were stirred and kept at room temperature and in darkness until measurement of absorbance was carried out in a spectrophotometer at 515 nm. Measurements were taken after 15 min of reaction. The same procedure was followed for the analysis of the antioxidant standards: BHT, ascorbic acid, and DL-α-tocopherol. The results were expressed as TEAC (trolox equivalent antioxidant capacity) values.

2.4.2. $ABTS^{\bullet+}$ radical cation scavenging activity

A solution of $ABTS^{\bullet+}$ was prepared in phosphate buffer (PBS – pH 7.4) by reaction of 50 mL of ABTS (2 mM) in PBS with 200 μL of $K_2S_2O_8$ (70 mM) in deionized water. The mixture was kept in

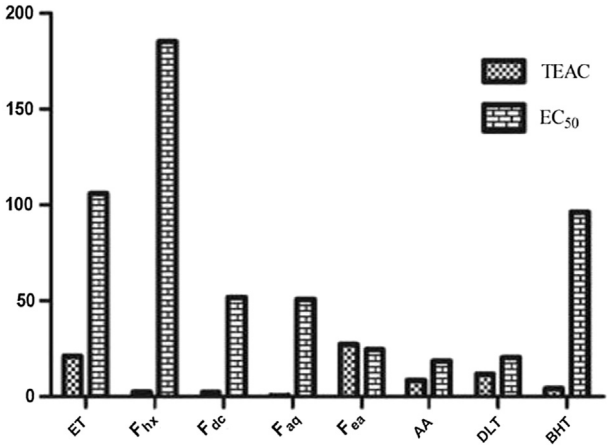

Fig. 1. TEAC and EC_{50} values found by DPPH method.

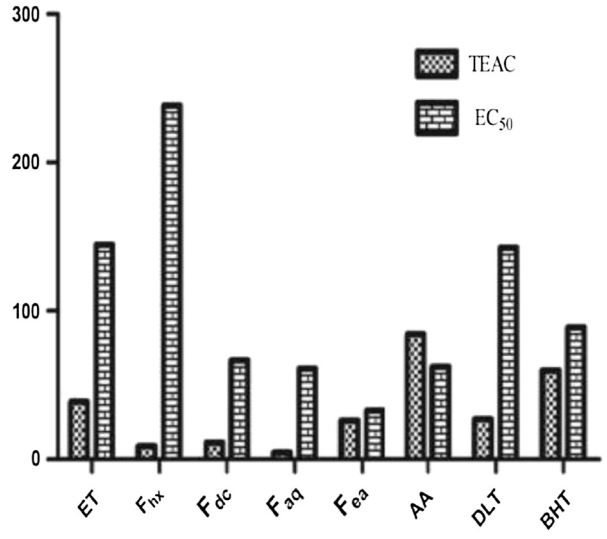

Fig. 2. TEAC and EC_{50} values found by ABTS method.

Table 1
Results of the evaluation of the AOA by the methods of DPPH and ABTS, TPC and TFC analysis.[e]

Method	DPPH		ABTS		TPC	TFC
Sample	TEAC[a]	EC_{50}[b]	TEAC[a]	EC_{50}[b]	GAE[c]	EHSP[d]
TE	21.2 ± 0.8	106 ± 1	38.9 ± 0.4	144.7 ± 0.4	38.7 ± 0.2	8.9 ± 0.1
F_{hx}	2.4 ± 0.3	185.3 ± 0.3	8.8 ± 0.3	238.4 ± 0.3	5.2 ± 0.1	0.6 ± 0.1
F_{dc}	2.2 ± 0.3	51.8 ± 0.9	11.3 ± 0.6	66.6 ± 0.6	21.4 ± 0.1	1.8 ± 0.1
F_{ea}	27.2 ± 0.4	24.6 ± 0.4	26.11 ± 0.3	32.8 ± 0.3	49.1 ± 0.1	13.8 ± 0.1
F_{aq}	0.37 ± 0.02	51 ± 1	4.6 ± 0.5	61.1 ± 0.5	8.4 ± 0.2	0.33 ± 0.02
Ascorbic acid[f]	8.5 ± 0.1	18.6 ± 0.1	84.3 ± 0.1	62.4 ± 0.1	–	–
DL-α-Tocopherol[f]	11.7 ± 0.1	20.4 ± 0.1	27.1 ± 0.1	142.7 ± 0.1	–	–
BHT[f]	4.2 ± 0.1	96.2 ± 0.1	59.8 ± 0.1	88.8 ± 0.1	–	–

TE, Total extract; F_{hx}, hexane fraction; F_{dc}, dichloromethane fraction; F_{ea}, ethyl acetate fraction; F_{aq}, aqueous fraction; TEAC, total equivalent antioxidant capacity; EC_{50}, effective concentration of 50%; TPC, total phenols content; GAE, gallic acid equivalents; TFC, total flavonoids content; EHSP, equivalents of hesperidin.
[a] mg trolox/g of extract.
[b] μg/mL.
[c] mg gallic acid/g of extract.
[d] mg hesperidin/g of extract.
[e] Values are expressed as mean ± standard deviation of three determinations.
[f] TEAC mg trolox/g of substance.

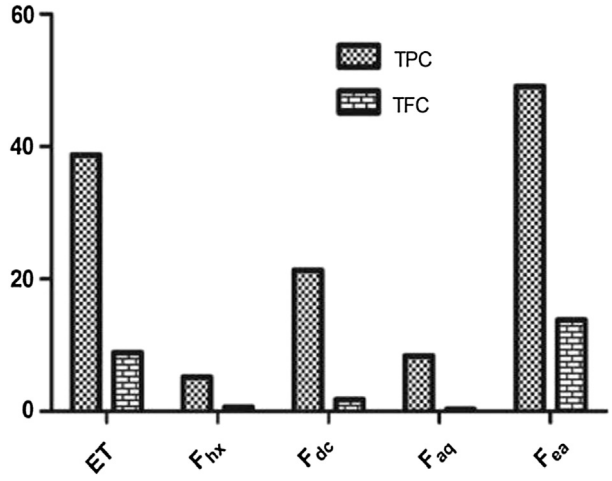

Fig. 3. TPC and TFC values of the samples studied.

darkness at room temperature for 16–17 h before being used. The radical solution was diluted with buffer to adjust the value of the absorbance 0.700 ± 0.03 (λ = 734 nm).

950 μL of a radical cation solution was mixed with 50 μL of a methanolic solution of the test sample (5–250 μg/mL), stirred and incubated at room temperature and in darkness for 5 min. The absorbance value recorded in the spectrophotometer at 734 nm. The blank was prepared from 950 μL of solution in PBS of the $ABTS^{\bullet+}$ and 50 μL of PBS. The same procedure was carried out for the analysis of the standards. The results were expressed as TEAC values.

2.4.3. Determination of total phenols content (TPC)

The TPC value was determined by the colorimetric method of Folin–Ciocalteu,[37] using gallic acid as a phenolic compound standard (15–75 μg/mL). The absorbance value was recorded in a spectrophotometer at 760 nm.

2 mL of an aqueous solution of the Folin–Ciocalteu reagent (0.2 equiv/L) and 0.4 mL of methanolic solution of the extract (100–1000 μg/mL) were mixed and incubated in darkness for 5 min 1.6 mL of sodium carbonate (0.075 μg/mL) was then added, the solution stirred and incubated in the dark for 1 h and the absorbance recorded at 760 nm. The content of phenolic compounds was expressed in mg of gallic acid equivalents (GAE).

2.4.4. Determination of total flavonoids content (TFC)

TFC was determined by direct reaction with aluminum trichloride,[38] with some modifications, using hesperidin as a flavonoid standard (15–75 μg/mL). Thus, 500 μL of sample were reacted with an equal volume of $AlCl_3$ solution. After 1 h incubation at room

Table 2
Compounds identified from *Pavonia sepioides*.

Compound	Fea TMS			Faq TMS			Fdc TMS		
	RT[a]	RI[b]	Area[c]	RT[a]	RI[b]	Area[c]	RT[a]	RI[b]	Area[c]
Salicylic acid 2TMS	8614	1596.0	55445785				8.606	1596.0	166824
Cinnamic acid 1TMS							8.922	1622.4	99390
p-Hydroxybenzoic acid 2TMS	9.811	1693.8	119739900						
p-Hydroxyphenylacetic acid 2TMS	9.947	1705.5	40973792						
p-Hydrocinnamic acid 2TMS	11.485	1824.6	28797951						
Vanillic acid 2TMS	11.561	1829.5	94180384				11.551	1829.0	168893
Gentisic acid 3TMS	11.774	1843.6	21728669						
p-Cinnamic acid 2TMS	11.901	1852.2	137560207						
o-Cinnamic acid 2TMS	12.190	1872.2	81078376						
Protocatechuic acid 3TMS	12.394	1885.7	396946590	12.371	1884.4				
Syringic acid 2TMS	13.524	1961.8	68968568						
m-Cinnamic acid 2TMS	14.093	2000.4	264109233						
Unknown				14.118	200.2	253517920			
Ferulic acid 2TMS	16.506	2153.3	116961118						
Caffeic acid 3TMS	17.304	2204.0	501953221	17.253	2200.5	56556964			

[a] RT, Retention time.
[b] RI, Retention index.
[c] Area expressed as arbitrary units.

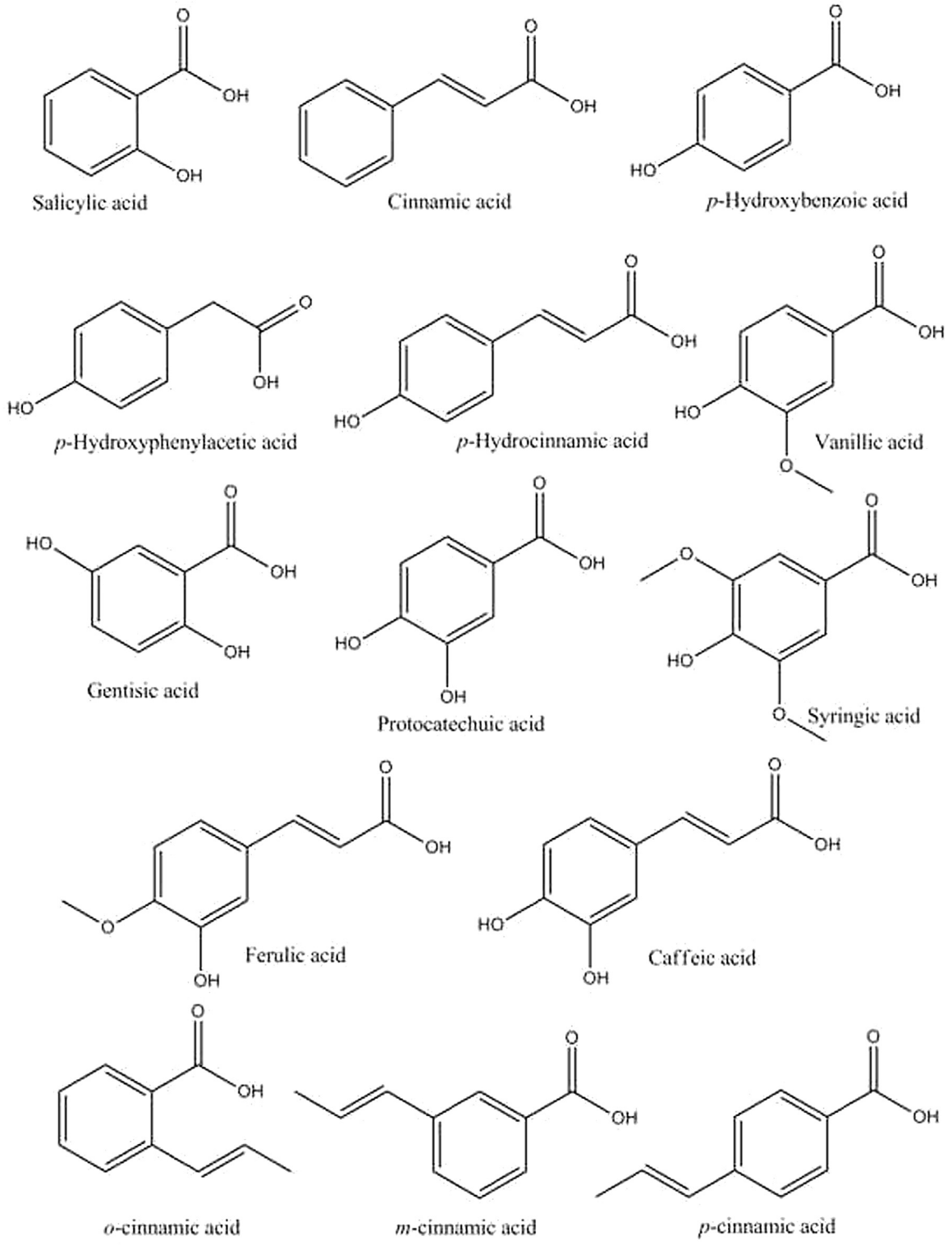

Fig. 4. Phenolic acids composition of *Pavonia sepioides*.

temperature, the absorbance was recorded at 376 nm. TFC content was expressed in mg equivalents of hesperidin (EHSP).

2.4.5. CG/MS analysis

The samples were dissolved in 200 μL of pyridine. 100 μL of the derivatizing reagent BSTFA (N,O-Bis(trimethylsilyl)trifluoroaceta mide) were added and heated at 40 °C for 3 h. The samples were analyzed directly by GC–MS (Hewlett Packard 6890, MSD 5975), the type of ionization being electron impact (EI) at 70 eV. The chromatographic column was an HP-5 (30 m × 0.25 mm, 0.25 μm). The temperature gradient was 100–180 °C at 15 °C/min, 180–300 °C at 5 °C/min and 10 min held at 300 °C. The injector temperature was 250 °C, and the flow of gas (helium) 0.8 mL/min 1 μL was injected in splitless mode. The analysis and deconvolution of

the data was performed using the AMDIS software and a phenolic acids spectra library.

2.5. Statistical analysis

The statistical analysis was performed using the statistical package SPSS (Statistical Package for the Social Sciences) version 15.0.

3. Results and discussion

The methods of evaluation of antioxidant activity using the $ABTS^{\bullet+}$ radical cation and free radical $DPPH^{\bullet}$ reflect the ability of an antioxidant to donate protons in a physiological or in an alcoholic medium.[36,39,40] Both methods are able to differentiate the ability of one or more antioxidants to trap free radicals in a reaction medium.[41] On conducting the AOA analysis using the DPPH and ABTS methods, Figs. 1 and 2 respectively, F_{hx} showed the highest EC_{50} and the lowest value for F_{ea}. F_{dc} and F_{aq} showed intermediate AOA. The antioxidant efficiency of these fractions is determined by the lowest concentration capable of inhibiting 50% of the radical within a given time.[36]

The levels of extractable material from each of the fractions showed variability, so that the yields expressed as percentage by weight relative to the dry plant material were 32.4%, 0.93%, 15.61% and 51.21% for F_{hx}, F_{dc}, F_{ea} and F_{aq} respectively. The total solids content in ET was 9.66%. Table 1 summarizes the AAO, TPC and TFC results.

The TEAC values found by both methods showed a behavior similar to those of an effective concentration, recording highs for Fea and ET. F_{aq} showed the lowest TEAC value comprising a fraction low in antioxidant compounds. The standards compounds showed a good TEAC values. The results are represented in Figs. 1 and 2. The best antioxidant response with DPPH radical was shown by BHT, whose activity may be associated with the phenomenon of hyperconjugation in the phenoxyl radical formed in the proton transfer process, due to the nature of the tert-butyls in the ortho-position on the hydroxyl group, giving the radical a high stability.[42–44]

It can be shown that by both methods (DPPH and ABTS), which rely on electron capture,[8,45–48] the level of antioxidant activity presented by each extract and fraction studied can be corroborated, because both methods obtained the same results in terms of activity level. By comparing the AOA results it is found that to achieve an inhibition of 50% of the DPPH radical a concentration of extract is necessary that is lower than the concentrations needed to inhibit 50% of the radical $ABTS^{\bullet+}$.

In the TPC assay a calibration curve prepared with gallic acid showed a correlation coefficient (r) of 0.998, while the calibration curve prepared with hesperidin in TFC assay showed r value of 0.997. TPC and TFC results are represented in Fig. 3. Both values were highest for F_{ea} and ET. The results are consistent with the highest AOA obtained by the DPPH and ABTS methods.

The GC/MS Analysis was made in the purified fractions with the best antioxidant activity. Table 2 shows the compounds identified using GC/MS. In each compound identified, the number of trimethylsilyl (TMS) groups, bonded to the hydroxyl as TMS, was specified.

A total of 13 phenolic acids compounds of low molecular weight were identified, Fig. 4, F_{ea} presented an unknown compound with a retention time of 14.118. The AOA can be associated to the presence of phenolic nature compounds, biologically active components, who are the main agents that can donate hydrogen to free radicals and thus break the chain reaction of biomolecules oxidation at the first initiation step.[23,49,50] This high potential of phenolic compounds to scavenge radicals may be explained by their phenolic hydroxyl groups, in addition to synergistic effects of the compounds present in ET and each fraction.[13,50] In some of them, the AAO has been studied both in models In Vitro or In Vivo, showing good results, as in the cases of the salicylic acid,[51,52] cinnamic acid,[53,54] p-Hydroxybenzoic acid,[55,56] vanillic acid,[57] gentisic acid,[58] protocatechuic acid,[59–61] syringic acid,[49,62] ferulic acid[62–65] and caffeic acid.[17,58,64,66,67]

The statistical analysis using the Pearson and Spearman association tests indicated a positive linear correlation between the DPPH and ABTS assays, enabling evaluation of the AOA, the total phenols content (TPC) and total flavonoids content (TF), in agree with other reports.[3,11,23,68] The analyses were statistically significant ($p < 0.05$), showing correlation coefficients greater than 0.810 in the Pearson test and greater than 0.700 in the Spearman test.

4. Conclusion

The antioxidant activity of the ethanol extract of *P. sepioides* and its purified fractions was confirmed by the methods of DPPH and ABTS. Although the values differ in each technique, the behavior is similar, with an equal activity level being found for each of the samples. The AOA results agree with the TPC and TFC values, showing correlation coefficients greater than 0.810 in the Pearson test and greater than 0.700 in the Spearman test ($p < 0.05$ in both cases). The AOA could be associated to the presence of phenolic nature compounds as phenolic acids identified in purified fractions of the plant by GC/MS.

Conflicts of interest

All authors have none to declare.

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Abbreviations

ROS: Reactive oxygen species
AOA: Antioxidant activity

TPC: Total phenolic content
TFC: Total flavonoid content
TE: Total ethanol extract
F_{ea}: Ethyl acetate fraction
F_{hx}: Hexane fraction
F_{dc}: Dichloromethane fraction
F_{aq}: Aqueous fraction
GC/MS: Gas chromatography-mass spectrometry
LLE: Liquid–liquid extraction
CE: Crude extract
PBS: Phosphate buffer
TEAC: Trolox equivalent antioxidant capacity
GAE: Gallic acid equivalents
EHSP: Equivalents of hesperidin
r: Correlation coefficient
EC50: Effective Concentration
DPPH: 2,2-diphenyl-1-picrylhydrazyl
BHT: 3,5-di-tert-4-butylhydroxytoluene
ABTS: 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid)
Trolox: 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid