Journal of Research in Pharmacy

ResearchArticle

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HPLC-DAD Screening and Antioxidant Activity of Phenolic Compounds of *Salvia balansae* de Noé Leaves Extract

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Received: 01 October 2022 / Revised: 29 January 2023 / Accepted: 29 January 2023

ABSTRACT: *Salvia balansae* de Noé is known to be an important source of a wide variety of non-toxic bioactive compounds with lack of information hinders exploitation in food and pharmaceutical industries as natural antioxidants. In this work, the phenolic compounds from *S. balansae* leaves were extracted using an ultrasound-assisted methodology and were separated and identified by HPLC-DAD. The antioxidant activity was evaluated by ABTS, and DPPH scavenging assays, Iron (Fe^{2+}) Chelating ability and reducing power (FRAP) tests. The total phenolic compounds (TPC) and total flavonoid content (TFC) were found with amounts of 49.63 ± 6.31 mg GAE/g DW and 8.14 ± 0.39 mg QE/g DW respectively. The ABTS, DPPH assays and metal chelating ability test demonstrated a higher antioxidant properties with IC_{50} values of 328.95 ± 5.29 µg/mL, 545.03 ± 3.27 µg/mL and 689.40 ± 0.86 µg/mL respectively. The reducing power at 700 nm increased considerably with concentration. In addition, the HPLC-DAD analysis showed that there were twelve detectable phenolic compounds and the benzoic acid, quercetin, myrecetin, hydroxyflavon, and ascorbic acid have been exclusively identified for the first time in *S. balansae* leaves extract and are rarely determined in other *Salvia* L. species. Therefore, these results provided new data about the main phenolic compounds present in *S. balansae* leaves extract with a strong antioxidant potential, which will be an alternative source for synthetic compounds.

KEYWORDS: Salvia balansae de Noé; phenolic compounds; antioxidant capacity; HPLC-DAD profile.

1. INTRODUCTION

A rising number of chronic diseases over the years has led to the development of food-derived bioactive peptides that can help improve these medical conditions. Some of the biological effects produced by these peptides are antioxidant, anti-inflammatory, anti-thrombotic, anti-hypertensive, and anti-diabetic [1].

The *Lamiaceae* family is widely distributed over almost the entire landmass, with a denser presence in the Mediterranean and subtropical regions [2, 3]. This wide distribution allows these species to occupy different natural ecosystems. These plants are characterized by a penetrating odour, which allows them to be classified as aromatic plants. Historically, the *Lamiaceae* family is known for its traditional values considering their preventive and curative properties. Species belonging to the genus *Lavandula*, *Mentha*, *Stachys*, *Thymus*, *Rosmarinus,* and *Salvia* are used in several fields, such as traditional medicine and gastronomy [2, 4].

Most species in this family contain essential oils. These oils are located mainly in the leaves, but may also be present in other parts of the plant, such as roots, rhizomes, stems, and flowers [5].

Sage appears to be one of the oldest plants used throughout time. The name of the genus *Salvia* is derived from the Latin 'Salveo' (= to save) because of the many healing properties of the plant [6]. The genus

How to cite this article: Mahdjoub MM, Benzitoune N, Maiz Y, Aouadi NEH, Bouhenna MM, Kadri N. HPLC-DAD Screening and Antioxidant Activity of Phenolic Compounds of *Salvia balansae* de Noé Leaves Extract. J ResPharm. 2023; 27(3): 1076-1085.

ISSN: 2630-6344

http://dx.doi.org/10.29228/jrp.400

Salvia L. is one of the aromatic plants rich in essential oils, which are generally located in the glandular hairs found in all aerial parts of the plant [7].

Salvia species such as *S. officinalis* and its constituents were high in anti-oxidant compounds, primarily phenolic compounds like carnosic, caffeic, rosmarinic, and salvianolic acids, as well as other phenolic structure-based compounds [8-10].

A review of the literature showed that the principal components of *Salvia* species are flavonoids and terpenoids. The Aerial- parts of *Salvia* species- generally contain flavonoids and triterpenoids as well as volatile compounds like monoterpenoids, while in the roots the main compounds are diterpenoids [11].

It has several interesting activities, such as antibacterial (*S. pomifera*), antioxidant (*S. fruticosa*), antidiabetic, and antitumor proprieties [11]. Some species can combine a multitude of biological activities, such as the leaves of *S. officinalis,* which are antibacterial, antifungal, and antiviral [12].

In Algeria, there are 19 species of *Salvia*[13], of which the vast majority of species are centralised in the northern part of the country. Several published works have dealt with phytochemical analyses of sage species in Algeria, like extraction of essential oils, phenolic compounds, antioxidant activity, and antimicrobial activity [14-17]. Polyphenols are a heterogeneous group naturally synthesized by the secondary metabolism of plants in which they are widely distributed. Phenolic compounds, on account to their diverse chemical structures, from simple to complex, are considered as more efficient antioxidant. Their ability to undergo series of reduction and oxidation due to the localized resonance effects on the phenyl rings, allows them to act as reducing agents, hydrogen donators, singlet oxygen quenchers and potential metal chelators. In addition to their antioxidant activity, they are preventive agents for chronic diseases, such as diabetes, obesity, cardiovascular diseases, neurological diseases, cancer, among others. In addition, this group of compounds can be used in other applications, such as food preservation, bioactive packaging, cosmetics, natural dyes, as well as the elaboration of hydrogels and nanocomplexes [18-22].

To the best of our knowledge, no study has gained insight into secondary metabolites composition of *Salvia balansae* and their biological effects. Therefore, the objective of the present study was to investigate bioactive compounds of this rare and endemic species in terms of total phenolic compounds, HPLC-DAD screening and antioxidant activities of this specie's leaves in the order to explore its potential applications in the food and pharmaceutical industries.

2. RESULTS AND DISCUSSION

2.1. Total phenolic and flavonoid contents of *S. balansae* leaves extract

In recent years, polyphenols have gained a lot of importance because of their potential use as prophylactic and therapeutic agents in many diseases. Tremendous works have been presented by the scientific community which focus on their antioxidant effects [23].

The results of TPC and TFC of *S. balansae* leaves extract were shown in Table 1. According to the results, The TPC of *S. balansae* leaves extract is on average of 49.63 ± 6.31 mg GAE/g DW extract. These levels are relatively low compared to those found by Farhat, Landoulsi, Chaouch-Hamada, Sotomayor and Jordán [24] concerning *S. officinalis* (161.37 mg GAE/g DW) and *S. verbenaca* (67.67, 72.02 mg GAE/g DW). On the other hand, it was noted that the obtained TPC of *S. balansae* leaves extract is in range of several *Salvia* species, namely Turkish *S. fruticosa* Miller extracts (41.58–44.60 mg GAE/g DW). They were gathered by maceration at 40°C using aqueous methanol (80%) as a solvent [25] and *S. gilliesi* extract (33.2 mg GAE/g DW) obtained by a simple aqueous infusion [26].

The TFC is influenced by several parameters, such as the extraction conditions [27], the standards used, the agronomic and geographical conditions [28], as well as the varietal character and the degree of maturity [29]. The results of TFC of *S. balansae* leaves extract were presented in Table 1.

Table 1: Total phenolic (TPC) and flavonoid (TFC) contents of *S. balansae* leaves extract.

	TPC (mg GAE/g DW)	**TFC (mg QE/g DW)**
***S balansae* leaves extract**	49.63 ± 6.31	8.14 ± 0.39

All the values in the table are mean ± SD of three independent determinations.

According to the obtained results, the TFC of *S. balansae* leaves extract averaged between 8.14 ± 0.39 mg QE/g DW. These results are higher than those reported for the TFC of *S. officinalis* L. extract, which was 0.44 QE/g DW, and was obtained by maceration at room temperature using aqueous methanol (80%) as a solvent [30].

http://dx.doi.org/10.29228/jrp.400
JResPharm 2023; 27(3): 1076-1085

2.2. Identification of phenolic compounds

Phenolic compounds constitute one of the main classes of secondary metabolites. They display a large range of structures and are responsible for the major organoleptic characteristics of plant-derived foods and beverages, particularly color and taste properties. They also contribute to the nutritional qualities of fruits and vegetables [31]. These compounds are associated with many physiological processes involved in the ability of plant species to resist to attacks by insects and micro-organisms [32].

To the best of our knowledge, the qualitative-quantitative analysis of phenolic compounds of *S. balansae* leaves extract have not yet been published. The chromatographic identification of phenolic compounds of the mentioned species leads to the assessment of twelve different compounds and are presented in Table 2.

Table 2: Phenolic compounds contents of *S. balansae* leaves extract.

Compound notations	Retention times (min)	Name of phenolic compounds	Quantity (mg/g DW)
1	2.46	Ascorbic acid	19.99 ± 0.63
2	3.80	Gallic acid	21.72 ± 0.54
3	4.61	Tannic acid	71.11 ± 1.03
4	13.09	Catechin hydrate	0.26 ± 0.02
5	20.86	Caffein	14.48 ± 0.36
6	21.94	Vanillin	16.74 ± 0.27
7	24.24	Coumaric acid	0.09 ± 0.01
8	29.08	*p*-Coumaric acid	0.12 ± 0.07
9	31.12	Benzoic acid	70.77 ± 0.98
10	39.69	Myrecetin	04.03 ± 0.08
11	41.61	Quercetin	09.34 ±0.12
12	46.45	Hydroxyflavon	03.42 ±0.1

All the values in the table are mean ± SD of three independent determinations.

The total identified compounds amounts are ranging from 0.26 mg/g DW to 71.11 mg/g DWrepresenting two major classes namely, phenolic acids and flavonoids. TA (71.11 mg/g DW) and BA (70.77 mg/g DW) were recorded in higher amounts followed by GA (21.72 mg/g DW), AA (19.99 mg/g DW), C (14.48 mg/g DW), VL (16.74 mg/g DW) and QU (09.34 mg/g DW). Nevertheless, five other compounds are identified as shown in Table 2 (Catechin hydrate, CMA, *p*-CMA, HF and MC). Other research has reported the identification of these compounds in several Salvia specieswith very lower amounts compared with those mentioned in our study**[25, 33-36]**. It should be noticed that amounts of BA, QU, MC HF and AA have been exclusively identified for the first time in the *S. balansae* leaves extract, and are rarely identified in other *Salvia* species.

2.3. Antioxidant activity of *S. balansae* leaves polyphenolic extract

Various methods have been proposed to study the antioxidant activity of extracts, as a single method cannot fully assess the antioxidant potential of natural extracts due to the complexity of the oxidation process and the chemical diversity of antioxidants, with lipophilic and hydrophilic compounds [21, 37, 38]. In the present study, different *in vitro* assays were used in order to determine the antioxidant activity of *Salvia balansae* leaves polyphenolic extract, namely DPPH and 2,2-azinobis (3-ethylbenzothiazoline6-sulfonic acid (ABTS) radicals scavenging assays, ferric reducing antioxidant power (FRAP) and iron chelation activity**.**

Figure 1 presents the ability of *S. balansae* leaves polyphenolic extract to scavenge DPPH radical, tested at different concentrations. The percentage of inhibition of DPPH was dose-dependent; it increased with the increase of the concentration of the polyphenolic extract reaching a percentage of inhibition of 74.70 ± 1.37 % at a concentration of 1.2 mg/mL.

The ABTS radical scavenging assay of *S. balansae* leaves polyphenolic extract is presented in Figure 1, As with the DPPH assay, the percentage of inhibition of ABTS was dose-dependent; however, the extract showcased a better capacity to scavenge the ABTS radical than the DPPH radical, with the ABTS radical being completely scavenged at a concentration of 1.2 mg/mL.

http://dx.doi.org/10.29228/jrp.400
JResPharm 2023; 27(3): 1076-1085

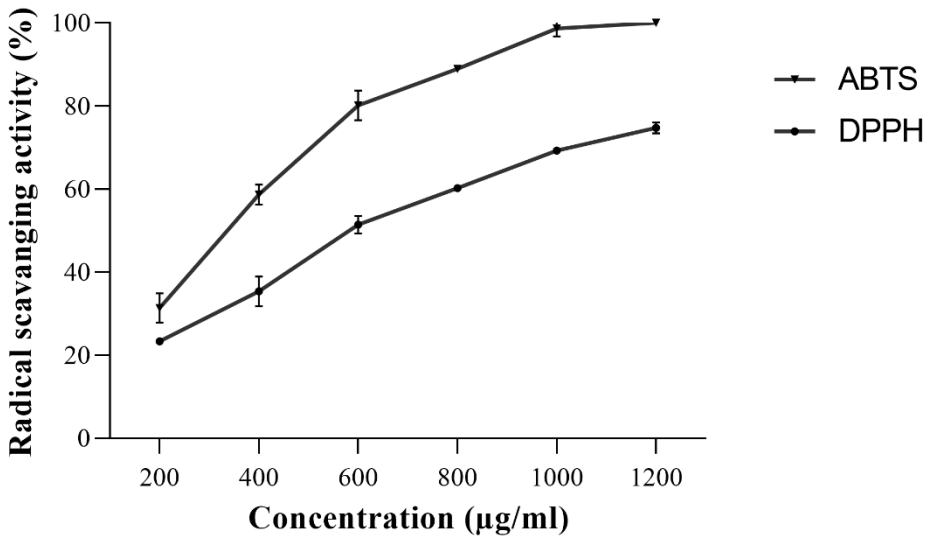

Figure 1: DPPH and ABTS radicals scavenging activity of *S. balansae* leaves polyphenolic extract.

The ability of *Salvia balansae* leaves polyphenolic extract to scavenge both radicals could be attributed to its ability to donate hydrogen. Polyphenols have been found to be strong antioxidants that can neutralize free radicals by donating an electron or hydrogen atom. They suppress the generation of free radicals and therefore reduce the rate of oxidation by inhibiting the formation of or deactivating the active species and precursors of free radicals. More commonly, they act as direct radical scavengers of the lipid peroxidation chain reactions (chain breakers). Chain-breakers donate an electron to the free radical, neutralizing the radicals and becoming stable (less reactive) radicals themselves, thus stopping the chain reactions [39].

Half-maximal inhibitory concentration (IC_{50}), the concentration that reduces 50 % of radicals, was estimated for ABTS and DPPH. The IC_{50} values of the polyphenolic extract were found to be 328.95 ± 5.29 µg/mL and 545.03 ± 3.267 µg/mL for ABTS and DPPH, respectively. Different IC_{50} values were reported: *Salvia albimaculata* (227.4 µg/mL and 50.2 µg/mL for DPPH and ABTS, respectively) and *Salvia nydeggeri* (248.4 µg/mL and 52.3 µg/mL for DPPH and ABTS, respectively) polyphenolic extracts[40].

Along with radical scavenging, polyphenols are also known to be metal chelators. The metal chelating ability is important because it can reduce the concentration of the catalyzing transition metal in lipid peroxidation. In addition, chelation of transition metals such as Fe^{2+} can directly reduce the rate of Fenton reaction and thus prevent oxidation caused by highly reactive hydroxyl radicals [39, 41]. The metal chelating activity of *S. balansae* leaves polyphenolic extract is shown in Figure 2.

http://dx.doi.org/10.29228/jrp.400
JResPharm 2023; 27(3): 1076-1085

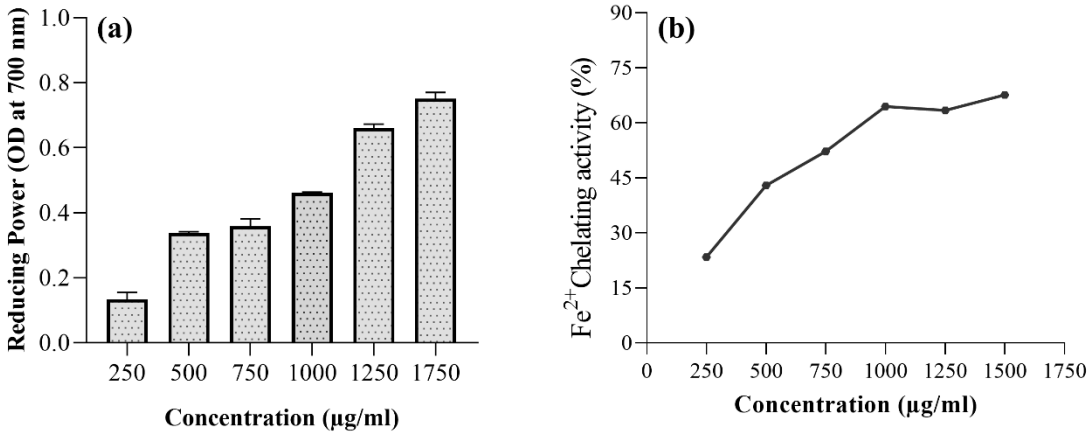

Figure 2: (a) Reducing power, (b) Ferrous iron chelating activity of *S. balansae* leaves polyphenolic extract.

The increase in the metal chelating ability of *S. balansae* leaves polyphenolic extract with increasing the concentration indicates its ability to chelate iron and capture iron before Ferrozine. The latter forms a complex with Fe^{2+} and produces a red chromophore with absorbance measured at 562 nm, the presence of metal chelators disrupts the ferrozine/Fe^{2+} complex and the red color fades [41]. Moreover, *S. balansae* leaves polyphenolic extract presented an IC_{50} value of 689.40 ± 0.86 µg/mL, which was promising compared to those reported for *Salvia officinalis* ethanolic extract (1185.54 µg/mL) and *S. fruticosa* ethanolic extract (1582.53 µg/L) [42].

The reducing power of a compound can serve as an indicator of its potential antioxidant capacity. The reducing power of *S. balansae* leaves polyphenolic extract was estimated at different concentrations and the results are presented in Figure 2. The absorbance at 700 nm increased considerably with the increase of the concentration, indicating that the extract possesses a reducing power ability. Generally, the reducing capacity of a compound depends on the presence of reductones, which, by donating a hydrogen atom, break the free radical chain. The presence of antioxidant reductants in the polyphenolic extract causes the reduction of Fe^{3+}/ferricyanide complex to the ferrous form (Fe^{2+}), resulting in the formation of Perl's Prussion blue, measured at 700 nm [41, 43].

2.4. Correlation between antioxidant activity and total polyphenols, and total flavonoid contents

Correlation analysis was used to explore the relationships between total phenolic, flavonoid content and the different antioxidant variables measured for *Salvia balansae* polyphenolic extract (Table 3).

Table 3: Linear correlation between antioxidant capacities and phenolic and flavonoid contents of *S. balansae* leaves extract.

	Correlation coefficients			
	DPPH	**ABTS**	**FRAP**	**Fe $^{2+}$ Chelation**
TPC	y = 0.7638x + 11.091 R^2 = 0.9151	y = 0.9623x + 24.261 R^2 = 0.8062	y = 0.0074x – 0.0402 R^2 = 0.8586	y = 0.5812x + 13.53 R^2 = 0.909
TFC	y = 2.7222x + 24.744 R^2 = 0.9318	y = 3.4294x + 41.463 R^2 = 0.8209	y = 0.0265x + 0,106 R^2 = 0.8823	y = 2.0103x + 26.219 R^2 = 0.9079

http://dx.doi.org/10.29228/jrp.400
JResPharm 2023; 27(3): 1076-1085

There was a significant linear correlation between the Antioxidant activity determined by using the DPPH, ABTS, FRAP and Fe^{2+} chelation assays, and total polyphenolic compounds (Phenolic and flavonoids). Phenolic compounds are considered to be the most important antioxidants found in plant materials as they have an ideal structural characteristic for free radical scavenging properties [51]. Various reports have been published in the literature, and some authors suggest a correlation of all these values, whereas others have not found such a relationship [44].

3. CONCLUSION

Salvia balansae de Noé is a rare endemic medicinal plant species, limited to northwest Algeria. Based on HPLC-DAD, several phenolic compounds were identified for the first time in *S. balansae* leaves. DPPH, ABTS, Fe^{2+} chelating activity and FRAP assays showed the high antioxidant activity of the phenolic extract of leaves. These results may play a major key role in preventing oxidative stress and several health issues related to it. Twelve phenolic compounds were determined in *S. balansae* leaves, which are probably related to the environmental factors that characterize the area of distribution of this endemic species. Nevertheless, these results are promising and can be applied to understand the environmental adaptation of the species to its region, and on the other hand to make it a vital source for therapeutic and physio-pharmacolagical applications. In the meantime, further studies on the different parts of the plant such as flowers, stems and roots are highly recommended for better application in the pharmaceutical and food industry.

4.MATERIALS AND METHODS

4.1. Plant material

The fresh plant samples were collected in February 2022 in Mostaganem, Algeria. *Salvia balansae* de Noé was identified according to [Quezel and Santa [45], 46, 47], and the voucher specimens kept at the Ecological and Environmental Laboratory at the University of Bejaia, Algeria. The plant samples consisting of *Salvia balansae* leaves were separated from stems and washed with distilled water. The clean leaves were then dried in a vacuum oven at 40 °C until a constant weight was attained. The dried samples were ground to a fine powder using an electric grinder and sieved through a 200 µm.

4.2. Ultrasound-assisted extraction of *S. balansae* leaves polyphenols

The polyphenolic extract of *Salvia balansae* leaves was prepared as described by Khan, Abert-Vian, Fabiano-Tixier, Dangles and Chemat [48]. The leaf powder was solubilized in aqueous ethanol (70% v/v) at a solvent-to-sample ratio of 1:10 (w/v). The mixture was placed in an ultrasonic bath and sonicated for 30 min at 40 °C, followed by filtration through Whatman filter paper. The resulting filtrate was concentrated using a rotary evaporator as well as lyophilized, and then stored at -4 °C until analysis.

4.3. Determination of polyphenol and flavonoid contents of the extract

4.3.1. Total phenolic content (TPC)

TPC was determined using Folin–Ciocalteu reagent according to the method described by Singleton and Rossi [49]. A volume of 150 µL of sample (1mg/ml) were mixed with 750 µL of Folin–Ciocalteu reagent (10N) and 600 µL of 7.5% (w/v) sodium carbonate. After incubation at room temperature for 30 min, the absorbance of the reaction mixture was measured at 750 nm, TPC was expressed as mg Gallic Acid Equivalents per gram of dried weight sample (mg GAE/g DW).

4.3.2. Total flavonoid content (TFC)

TFC was determined spectrophotometrically according to Quettier-Deleu, Gressier, Vasseur, Dine, Brunet, Luyckx, Cazin, Cazin, Bailleul and Trotin [50], using a method based on the formation of a flavonoid-aluminum complex, having an absorbance maximum at 430 nm. A volume of 1 mL of diluted sample (1mg/mL) was mixed separately with 1 mL of 2% (w/v) methanolic aluminum chloride solution. After incubation at room temperature for 15 min, the absorbance of the reaction mixture was measured at 430 nm with a UV-Vis spectrophotometer and the TFC was expressed as mg quercetin equivalent per gram of dried weight sample (mg QE/g DW).

http://dx.doi.org/10.29228/jrp.400
JResPharm 2023; 27(3): 1076-1085

4.4. HPLC analysis of total phenolic compounds

The phenolic compounds identification was carried out using HPLC experiment (Agilent 1260 series HPLC system equipped with a DAD Agilent Technologies, Santa Clara, CA, USA), and the operating conditions were as follows. Chromatographic column: Agilent KNAUER -C18 column (4.6 mm × 250 mm, 5.0 µm, 100 A°), column temperature: 30 °C, injection volume: 20 µL, wavelength: 254 nm. Mobile phase condition was obtained using a mixture of two solvents: mobile phase (A) was acetic acid in ultrapure water (1%) and mobile phase (B) was HPLC grade methanol. The elution was performed by a linear gradient of 5 to 95% (v/v) of solvent (B) during 58.0 min elution program with a flow rate of 1.00 mL/min. The standard curve, was prepared at 100 µg/mL in methanol using a standard solution containing gallic acid (GA), ascorbic acid (AA), tannic acid (TC), catechin acid (CA), caffeine (C), vanilline (VL), coumaric acid (CMA), *p*coumaric acid (pCMA), benzoic acid (BA), myricetin (MC), quercetin (QU), hydroxyflavone (HF). The concentration of extract in methanol solution was 1 mg/mL. Peak integration, data acquisition, and calibrations were carried out by CHEMSTATION software.

4.5. Determination of antioxidant activities

4.5.1. DPPH free radical scavenging activity

The DPPH (1,1-diphenyl-2-picrylhydrazyl) radical scavenging activity of *S. balansae* leaf extract was measured according to the method described by Alara, Abdurahman, Ukaegbu and Azhari [26]. A volume of 0.2 mL of extract prepared at different concentrations was added to 1 mL of 0.1 mM freshly prepared DPPH solution. The decrease in absorbance was determined at 517 nm, after incubation for 30 min. the percentage inhibition was determined using the following equation:

$$\%\ DPPH\ inhibition = \frac{A\ control - A\ sample}{A\ control} \times 100$$

Where A control is absorbance of the mixture of methanol and DPPH solution; and A sample is absorbance of the mixture of sample extract and DPPH solution.

The IC_{50} value was calculated from the linear regression between the percentage of inhibition and concentrations and expressed in µg/mL.

4.5.2. ABTS free radical scavenging activity

The ABTS radical scavenging activity of the plant extract was determined according to the method described by Ilaiyaraja, Likhith, Babu and Khanum [27]. The radical cation ABTS was prepared by mixing 7 mM ABTS and 2.45 mM potassium persulfate and incubating in the dark at room temperature for 14 h. A fresh working solution of ABTS was prepared by diluting 1 mL of stock solution in 60 mL of ethanol to give an absorbance of 0.71 ± 0.001 at 734 nm. A volume of 0.1 mL of extract was allowed to react with 1 mL of ABTS solution for 10 min, and the absorbance was measured at 734 nm. Data were expressed as percentage of scavenging activity and calculated as for the DPPH assay. The IC50 value was calculated from the linear regression between percent inhibition and concentrations and expressed in µg/mL**.**

4.5.3. Iron (Fe^{2+}) chelating ability

The ability of the extract to chelate iron (II) was determined by the method described by Pavithra and Vadivukkarasi [51]with a slight modifications. In brief, a volume of 50 µL of 2 mM $FeCl_3$ was added to 1 mL of extract prepared at different concentrations. After 5 min, the reaction was initiated by the addition of 200 µL of 5 mM ferrozine. The reaction mixture was shaken vigorously and left standing at room temperature for 10 min and the absorbance of the reaction mixture was measured at 562 nm against a reagent blank. The chelating activity was calculated using the following equation:

$$\%\ Iron\ chelating\ activity = \frac{A\ control - A\ sample}{A\ control} \times 100$$

Where A control is absorbance of the mixture of ethanol, 2 mM $FeCl_3$ and 5 mM of Ferrozine; and A sample is the absorbance of the mixture of extract, 2 mM $FeCl_3$ and 5 mM of Ferrozine. The IC50 value was calculated from the linear regression between the percentage of inhibition and concentrations and expressed in µg/mL.

http://dx.doi.org/10.29228/jrp.400
JResPharm 2023; 27(3): 1076-1085

4.5.4. Reducing power ability

The reducing power of the extract was evaluated according to the method described by Le, Chiu and Ng [52]. The reaction mixture contained 160 µL of extract prepared at different concentrations, 500 µL of 1% (w/v) potassium ferricyanide (K_3Fe^{3+} $(CN)_6$) and 500 µL of 0.2 M phosphate buffer, pH 6.6. The mixture was incubated at 50°C for 20 minutes and the reaction was stopped by the addition of 500 µL of 10% (w/v) trichloroacetic acid, followed by centrifugation at 3000 rpm for 10 minutes. A volume of 500 µL of supernatant was mixed with 500 µL of distilled water and 100 µL of 0.1% (w/v) ferric chloride ($FeCl_3$), and the absorbance was measured at 700 nm against a blank containing distilled water and phosphate buffer. The increase in absorbance indicates an increase in the reducing power of the sample.

4.5. Statistical analysis

Data were analyzed using Graph Pad Prism 8.0.2 software. All analyses were performed in triplicate and experimental data were expressed as means ± standard deviation. The correlation between TPC/TFC values and antioxidant activities was determined based on Pearson's correlation coefficient.

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