

FLAVONOIDS FROM AERIAL PART OF ALGERIAN *AJUGA IVA* (L.) SCHREB.: THE HPLC-UV ANALYSIS AND ANTIOXIDANT CAPACITY

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ABSTRACT. The study deals with the evaluation of the antioxidant capacity of extracts from the aerial part of Algerian *Ajuga iva*. Extraction of flavonoids was carried out by 85% of methanol, then the crude extract was successively separated with ethyl acetate, butanol, and water. The *in vitro* antioxidant activity was assessed by 1,1-diphenyl-2-picrylhydrazyl, reducing power, and thiobarbituric acid reacting substances assays. Extracts are subject to HPLC-UV analysis. The average total phenol contents of extracts vary between 3.87 ± 0.17 and 149.74 ± 3.94 (gallic acid equivalent per gram of dry extract). Furthermore, tested extracts exhibited a broad range of flavonoid contents varying from 1.54 ± 0.09 to 41.18 ± 1.03 (catechin equivalent per gram of dry extract). Butanol and ethyl acetate fractions displayed the highest antioxidant activity. A good correlation between the phenolic and flavonoid contents and the antioxidant activity was observed. Rutin, caffeic acid, quercetin, *p*-coumaric acid, luteolin, and cinnamic acid were present in the extracts. The plant could be a potential source of antioxidant agents.

Keywords: Antioxidant effect, flavonoids, polyphenols, HPLC.

INTRODUCTION

The history of medicinal plants is associated with the evolution of civilizations. In all regions of the world, the history of peoples shows that these plants have always occupied an important place in medicine since antiquity. It was only in the 20th century that scientists became interested in this subject (JANSSEN *et al.*, 1976).

The activities of these curative plants are evaluated by their chemical components. Plants synthesize secondary metabolites, which are molecules that are indirectly essential to the functioning of plants, as opposed to primary metabolites that are vital for vegetal life (FAVIER, 2003). Flavonoids are secondary metabolites and an unusually large group of naturally occurring phenolic compounds ubiquitously distributed in plants. The past two decades has witnessed a renewed interest in the study of flavonoids and of their biological interactions. Flavonoids are known for their pharmacological, biochemical properties, and antioxidant activities (CROZIER *et al.*, 2006).

Several studies have shown that flavonoids have great potential as antioxidants agents. In addition, a lot of research is currently being done on natural antioxidants that act as free radical scavengers. The excessive production of these radicals can be harmful to the body, they damage many cellular components as diverse as proteins, lipids, or DNA, causing oxidative stress (VAN ACKER *et al.*, 1998).

A wide range of analytical techniques is used for the characterization of flavonoids extracted from medicinal plants, including high performance liquid chromatography (HPLC), either single or coupled with mass spectrometry. In recent years, HPLC has been the analytical method that has dominated the determination of flavonoids (ROBBINS, 2003).

Thanks to geographical location Algeria is home to rich and diverse vegetation. The genus *Ajuga* that belongs to the family Lamiaceae comprises at least 301 species (ZAFAR and BADIÀA, 2009). Flavonoids and tannins are found in large quantities in *Ajuga iva*. It also contains anthocyanins, phenolic acids, and other substances, in particular ajugarin (EL HILALY *et al.*, 2004). *Ajuga iva* is an aromatic plant that grows from spring to late summer, in deep soils at 2700 m altitude. It is widespread in the Mediterranean region: southern Europe and northern Africa, especially in Algeria, Morocco, Tunisia, and Egypt (BATANOUNY *et al.*, 1999). This small, bitter-tasting perennial, 5-10 cm long, with creeping, hairy green stems, has oblong to linear, pubescent leaves, 14 to 35 mm long. The flowers are purple, pink, or yellow, 20 mm long (HALIMI, 2004). This plant species has already been the subject of several studies, which show a broad spectrum of biological effects of extracts and essential oils including hypoglycemic (CHABANE *et al.*, 2013), anti-inflammatory (BOUDERBALA *et al.*, 2008), antimicrobial (ZERROUG *et al.*, 2011), and antioxidant (TALEB-SENOUCI *et al.*, 2009).

The aim of this work is to study the phenolic content of aqueous methanolic extracts and their solvent fractions of *A. iva* collected from Algeria. As well as the antioxidant potential was evaluated by employing DPPH radical-scavenging, reducing power, and lipid peroxidation assays to establish a possible correlation between polyphenol and flavonoid contents and antioxidant activity.

MATERIAL AND METHODS

Plant material, extraction, and separation

The plant was collected from Mascara, Algeria and was botanically identified by Dr. Righi Kada in the agricultural science department at the University of Mascara Mustapha Stambouli. The plant material was dried at room temperature and ground into powder using a shredder. The plant powder (100 g) was extracted by the classical method of maceration at room temperature with 85% of methanol according to the protocol described by MERGHEM *et al.* (1995) to obtain the crude extract. The hydromethanolic extract was evaporated and then dissolved in distilled water. The solution was successively partitioned with ethyl acetate, butanol, and water.

Total phenolic content

The total phenolic content was determined following the protocol applied by MILIAUSKAS *et al.* (2004). In a test tube, 1 mL of known concentration extract was mixed with 5 mL of Folin-ciocalteu reagent (2M) diluted 10 times and 4 mL of a 75 g/L sodium carbonate solution (Na₂CO₃). The absorbance was measured at 765 nm after incubation for 1 hour at room temperature. The calibration curve is made by gallic acid. The amount of phenolic content was calculated as gallic acid equivalent per gram of dry extract (mg GAE/g DE).

Total flavonoid content

The total flavonoid content was determined according to the method adopted by ARDESTANI and YAZDANPARAST (2007). Each extract solution (500 μL) was mixed with 2 mL of distilled water and 150 μL of NaNO_2 solution (15 %). After 6 min, 150 μL of AlCl_3 solution (10%) was added, the mixture is left for 6 min. After that, 2 mL of NaOH solution (4%) was added. Immediately, the mixture was completed with distilled water until the volume reached 5 mL. After 15 min of incubation, the absorbance was measured at 510 nm against a blank containing all the solutions except extract. The calibration curve is made by catechin. Results were expressed as catechin equivalent per gram of dry extract (mgCE/g DE).

Antioxidant activity

The antioxidant activity of the samples was investigated using three methods: DPPH radical assay, reducing power assay, and thiobarbituric acid reacting substances assay.

DPPH radical assay

A volume of 50 μL of various concentrations of the extracts in methanol was added to 1950 μL of a methanol solution DPPH (6.10^{-5} M), this mixture is strongly stirred for 30 seconds. Then, incubated for 30 min at room temperature in the dark. Absorbance was measured at 517 nm using methanol as blank (SHIMADA *et al.*, 1992). Ascorbic acid and catechin were used as a positive control. The percentage inhibition (*PI*) of free radical formation was calculated according to the following formula:

$$PI = (1 - A_1/A_0) \times 100 \quad (1)$$

where A_1 is the absorbance of the extract and A_0 is the absorbance of the control. The results were estimated in terms of IC_{50} (the concentration of extract which inhibits 50 % of free radicals present in the reaction medium). And it was calculated from the graph plotted of linear regression.

Ferric reducing antioxidant power assay

The reducing power of samples was determined as per the reported method of OYAZU (1996). A quantity of 1 mL of each extract was mixed with 2.5 mL of sodium phosphate buffer (0.2 M, pH 6.6) and 2.5 mL of potassium ferricyanide (1%). After incubation for 20 min at 50°C , 2.5 mL of trichloroacetic (10%) was added. After that, the mixture was centrifuged at 3000 rpm for 10 mn. In a test tube, 2.5 mL of the upper layer fraction was mixed with 2.5 mL of distilled water and 0.5 mL of ferric chloride (0.1%). The absorbance was measured at 700 nm and compared with standards. Increased absorbance of the reaction mixture indicated stronger reducing power. EC_{50} ($\mu\text{g}/\text{mL}$) value is the effective concentration giving an absorbance of 0,5 for reducing power and it was obtained from linear regression analysis.

Thiobarbituric acid reacting substances assay (TBAR)

The anti-lipid peroxidation ability was assessed following the method described by TRIPATHI and SINGH (2001). In a test tube, 0.5 mL of rat liver homogenate (KCl 0.15 M) at a rate of 10%, 1 mL of potassium chloride (0.15 M), and 0.5 mL of extract solution were mixed. Then, 100 μL of ferric chloride (1 mM) was added to initiate the lipid peroxidation. After incubation for 30 min at 37°C , the reaction was stopped by adding 2 mL of ice-cold hydrogen chloride (0.25 N) containing trichloroacetic acid (15%) and thiobarbituric acid

(0.38%), and 0.2 mL of butylated hydroxyl toluene (0.05%). The reaction mixtures were heated at 80°C for 60 min, cooled, and centrifuged at 6900 rpm for 15 min. The absorbance of the upper layer fraction was measured at 532 nm against a blank (contained all reagents without liver homogenate and drug). Synthetic antioxidants (ascorbic acid and catechin) were used as a positive control. The percentage of anti-lipid peroxidation (% ALP) was determined by following formula:

$$\%ALP = (A_{FeCl_3} - A_{extract}) \times 100 / A_{FeCl_3} - A_{normal} \quad (2)$$

A_{FeCl_3} : absorbance that indicates the level of lipid peroxidation (the reaction medium contained all the reagents without FeCl₃ and without extract).

$A_{extract}$: absorbance that indicates the level of anti-lipid peroxidation (the reaction medium contained all the reagents).

A_{normal} : absorbance of the reaction medium which contained all reagents except extract and FeCl₃.

IC₅₀ is the value that corresponds to 50% of anti-lipid peroxidation, and it was obtained from linear regression analysis.

HPLC analysis

HPLC analysis of standards and extracts was performed using a Shimadzu Prominence-I HPLC apparatus equipped with LC-2030 pump and a photodiode array detector (PDA: LC-2030/2040). Flavonoids were separated on a C-18 reverse phase HPLC column (Supelco, 150 mm x 4.6 mm, particle size 5 µm) at 25°C. Eluent A was the acetic acid aqueous solution (1%) and eluent B was a methanol acetic acid solution. Separation was performed in an isocratic step at 2% of B for 2 min followed by a linear gradient from 2% to 20% of B in 10 min, then to 100% of B in 65 min and 100% of B in 68 min with a flow rate at 0.8 mL/min. Flavonoids were identified by comparing their retention times and corresponding UV-Vis absorption spectra with standards.

Correlation analysis

Results were expressed as Mean±standard deviation (SD) of five separate experiments using Excel. Correlation coefficients between the total phenolic and flavonoid contents were determined from linear regression analysis using Excel.

RESULTS AND DISCUSSION

Total phenol and flavonoid content

The results of the levels of phenolic and flavonoid contents, as presented in Table 1, showed that the highest values of polyphenol were recorded in the ethyl acetate (149.74 ± 3.94 mg GAE/g DE) and butanolic fractions (50.09 ± 1.27 mg GAE/g DE), while the aqueous fraction showed the lowest values (3.87 ± 0.17 mg GAE/g DE). This order of fractions was confirmed by the results of ADJADJ (2009) who found that the ethyl acetate fraction is the richest in polyphenols and the aqueous fraction is the poorest. The contents obtained in our study are not in agreement with previous reports that proved that methanol and water are the most effective solvents for the extraction of polyphenols (MAKNI *et al.*, 2013).

Flavonoids content of the investigated extracts varied from 1.54 ± 0.09 to 41.18 ± 1.03 mg CE/g DE (Table 1). The highest total flavonoid contents were found in the butanolic extract followed by the ethyl acetate fraction. Moreover, the lowest flavonoid contents were

registered in the aqueous extract. These results indicate that flavonoids were better soluble in butanol and ethyl acetate. Our results are consistent with those conducted by ANOKWURU *et al.* (2011) where ethyl acetate was found to be the best solvent for the extraction of flavonoids. The variation of the phenolic and flavonoid contents might be due to the variation in procedure extraction, geographical conditions (EBRAHIMI *et al.*, 2008).

Table 1. The total phenolic and flavonoid contents of extracts.

Medicinal plant		Total phenolics	Flavonoids
<i>Ajuga iva</i>	Extract/Fractions	(mg GAE/g DE)	(mg CE/g DE)
	Crude	10.75 ± 0.13	5.43 ± 0.45
	Ethyl acetate	149.74 ± 3.94	34.61 ± 3.01
	Butanolic	50.09 ± 1.27	41.18 ± 1.03
	Aqueous	3.87 ± 0.17	1.54 ± 0.09

Each value represents the mean ± SD (n=5)

Antioxidant activity

DPPH radical scavenging

In this study, the DPPH method was chosen to evaluate the antioxidant activity of the extracts, because it is one of the fastest, simplest, and most effective methods due to the great stability of the DPPH radical (BOZIN *et al.*, 2008).

As demonstrated in Table 2, all the extracts proved to have high antioxidant activities (between 39.53 µg/mL and 512.25 µg/mL). The ranking of IC₅₀ values is: ethyl acetate extract > butanolic extract > crude extract > aqueous extract. Our results confirmed previous studies showing an important antioxidant activity of *Ajuga iva* extracts in other regions of its area (BAGHIANI *et al.*, 2011). The essential oils extracted from Tunisian *A. iva* exhibited a potent DPPH radical scavenging effect (AYARI *et al.*, 2013).

The mechanism of the reaction between antioxidant and DPPH radical depends on the structural conformation of the antioxidant (KOURI *et al.*, 2007). According to the literature, polyphenols and flavonoids are very effective as agents reducing oxidative stress (Molyneux, 2004). From our results, it is clear that all the extracts can donate an electron to DPPH radical, so the DPPH radical scavenging effect could depend on the number of hydroxyl substituents (CAO *et al.*, 1997).

Ferric reducing antioxidant power assay

In this assay, the presence of reducing agents causes the reduction of the Fe⁺³ ferrocyanide complex to the Fe⁺² form. Indeed, the formation of Fe⁺² can be monitored spectrophotometrically by measuring the absorbance of the blue color of the ferrous complex at 700 nm (KARAZOGLER *et al.*, 2008). An increase in absorbance at this wavelength means stronger reducing power of the extracts (OZTURK *et al.*, 2007).

Table 2 shows the reductive capability of extracts compared to ascorbic acid and catechin as standards. The ethyl acetate extract showed stronger reducing power (EC₅₀ = 780.16 ± 1.56 µg/mL) than other extracts. However, the reduction power of ascorbic acid (EC₅₀ = 49.09 ± 0.23 µg/mL) and catechin (EC₅₀ = 46.82 ± 1.01 µg/mL) was more pronounced than that of different extracts of *Ajuga iva*. This activity confirms the results of the DPPH radical-scavenging activity. BAGHIANI *et al.* (2011) found that extracts of *Ajuga iva* give a reduction power better than standards.

A study conducted on the ability of iron reduction by phenols indicated that the catechol nucleus is the only structure that is positively associated with reducing power. According to this study, this structure increases the reducing power of a compound to 36% compared to another which does not contain one. They suggested that this activity was due to the participation of the groups –OH linked to the catechol nucleus (DEGRAFT-JOHNSON *et al.*, 2007).

Table 2. Antioxidant activities for extracts and standards

Medicinal plant	Extract / Fractions	IC ₅₀ /DPPH (µg/mL)	EC ₅₀ /Reducing power (µg/mL)	IC ₅₀ /ALP (µg/mL)
<i>Ajuga iva</i>	Crude	55.04 ± 2.23	2352 ± 1.43	1569.85 ± 2.38
	Ethyl acetate	39.53 ± 1.39	780.16 ± 1.56	419.63 ± 1.71
	Butanolic	44.17 ± 0.43	1173.52 ± 2.89	507.88 ± 0.47
	Aqueous	512.25 ± 1.56	4531.83 ± 1.09	2231.83 ± 2.53
Standards	Ascorbic acid	17.21 ± 0.67	49.09 ± 0.23	208.85 ± 1.55
	Catechin	14.26 ± 0.49	46.82 ± 1.01	49.25 ± 1.02

Thiobarbituric acid reacting substances assay

In this assay, malondialdehyde (MDA) is one of the final products of fatty acid peroxidation. MDA was measured spectrophotometrically at 532 nm by a thiobarbituric acid reactive substance (TBARS) method (FARAHMAND *et al.*, 2013).

The butanolic and ethyl acetate phases exhibit a good lipid anti-peroxidation capacity. The strongest activity was exhibited by the ethyl acetate fraction (IC₅₀ = 419.63 ± 1.71 µg/mL) and the weakest anti-lipid peroxidation activity was exhibited by the crude and aqueous extract (1569.85 ± 2.38 µg/mL, 2231.83 ± 2.53 µg/mL respectively). Values obtained in our study are lower than the data reported by TALEB-SENOUCI *et al.* (2009), when studied the Tunisian *Ajuga iva* aqueous extract.

According to RATTY and DAS (1988), the flavonoid aglycones were more potent in their anti-lipid peroxidation ability than their corresponding glycosides. The structure-activity analysis demonstrated that the flavonoid molecule with polyhydroxylated substitutions on ring A and B, A 2,3-double bond, a free 3-hydroxyl substitution, and a 4-keto moiety, would confer upon the compound potent anti-lipid peroxidation effect.

Correlation analysis

Several researchers have studied the correlation between polyphenol content and antioxidant properties. Some of them found a correlation between the phenolic content and the antioxidant activity, while others did not find a relationship.

There is a good correlation between the total phenol and flavonoid contents and the DPPH assay (R² = 0.68, R² = 0.87, respectively). VELIOGLU *et al.* (1998) had also reported good correlations for fruits and vegetables grain products between the total phenolic amount and the antioxidant capacity. On the other hand, our results are not in agreement with the data reported by WOJDYLO *et al.* (2007).

Regarding ferric reducing antioxidant power assay, there is good correlation between this method and the total phenols and flavonoids contents (R² = 0.80 and R² = 0.85 respectively). This finding agrees with the study of WONG *et al.* (2006), which reported a good relationship between total phenolic content and antioxidant activity when studied the antioxidant activities of aqueous extracts of some medicinal plants using ferric reducing power assay.

Correlation analysis reveals an excellent correlation between the total polyphenols and the anti-lipid peroxidation capacity of extracts with $R^2 = 0.99$ and a moderate correlation between this assay and the levels of flavonoids with $R^2 = 0.53$. The same results were found by VAMANU and NITA (2013) when studying the correlation of inhibition of lipid peroxidation with phenolic and flavonoids contents of extracts from wild edible *Boletus edulis*.

Since the chemical composition of extracts and structures of active compounds identified in extract are important factors governing the efficacy of natural antioxidants, the antioxidant ability of an extract could not be explained based on their phenolic and flavonoid contents (HEINONEN *et al.*, 1998). For instance, it has been demonstrated that phenol compounds with para-dihydroxylation or a hydroxyl and a methoxy group are more effective than simple phenolics (SHAHIDI and WANASUNDARA, 1992).

HPLC analysis

As depicted in Figure 1 and Table 3, the HPLC chromatogram of butanolic extract of *Ajuga iva* showed the presence of caffeic acid, luteolin, and quercetin whereas the aqueous extract contained rutin, *p*-coumaric acid and quercetin. On the other side, ethyl acetate extract revealed the presence of caffeic acid, rutin, *p*-coumaric acid, luteolin, and quercetin, however, gallic acid, cinnamic acid and caffeic acid were found in the hydromethanolic extract.

Many reports have been published on the chemical composition of *Ajuga iva*, mentioning the evidence of numerous bioactive compounds, among them terpenoid, sterols, essential oils, and flavonoids (EL HILALY *et al.*, 2004; ISRAILI and LYOUSSI, 2009). However, there are few references in the literature on the phenolic profile of this species. BOUDJLEL *et al.* (2015) showed that two flavonoids appeared to predominate, apigenin, and naringenin, in the aqueous infusion of *Ajuga iva*. However, KHATTELIA *et al.* (2020) reported the presence of caffeic acid, *p*-coumaric acid, rutin, and luteolin in the extract of the aerial part of *Ajuga iva*. These results are in accordance with our findings.

Table 3. Retention time (Rt), UV-Vis wavelengths of maximum absorption (λ_{max}), and concentration of phenol compounds.

Rt	Compound	λ_{max} (nm)	Concentration ($\mu\text{g/g}$)			
			Crude	Ethyl acetate	Butanolic	Aqueous
15.30	Gallic acid	272	148 221	nd	nd	nd
25.21	Cinnamic acid	277	389 955	nd	nd	nd
27.22	Caffeic acid	219 239 323	420 098	133 9041	855 52	nd
30.08	Rutin	259 355	nd	126 1426	nd	237 027
39.70	<i>p</i> -coumaric acid	224 309	nd	6.284	180 2917	252 826
41.52	Luteolin	260 348	nd	8,637	224 440 6	nd
49.01	Quercetin	256 372	nd	8.118	154 055	937 28

nd – not detected

Several studies have been demonstrating the role of flavonoids and phenolics as antioxidant agents. SALUCCI *et al.* (2002) reported that gallic acid possesses a very high antioxidant capacity. In this order, YANG *et al.* (2008) reported that rutin exhibited strong DPPH radical scavenging activity and had effective inhibition of lipid peroxidation. On the other hand, luteolin exhibits a potent quenching effect on Fenton-induced 8-OHdG formation (CAI *et al.*, 1997). According to the study of CHANG *et al.* (2017), caffeic acid and *p*-coumaric acid inhibited LDL oxidation and quenched radicals. Quercetin has a great antioxidant activity as shown by ZHANG *et al.* (2011).

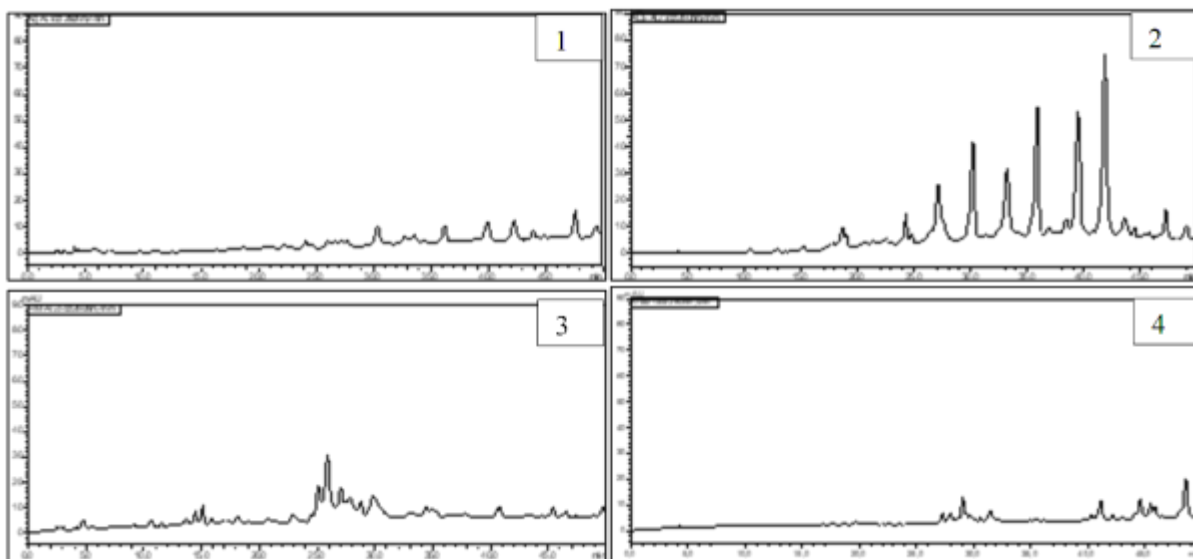


Figure 1. Representative high performance liquid chromatography profile of *Ajuga iva* extracts: Aqueous (1), Ethyl acetate (2), Hydromethanolic (3), Butanolic (4).

CONCLUSIONS

This study confirmed that *Ajuga iva* is a rich source of polyphenols and flavonoids and possesses antioxidant capacity mainly its ethyl acetate and butanol extracts. The antioxidant power of *Ajuga iva* extracts can be associated with their free radical scavenging properties. These data confirm their use in traditional medicine and encourage their application as alternative antioxidants. In addition, there is a need to isolate the compounds responsible for the activity, which may allow the development of a modern drug from this plant.

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