

**PHYTOCHEMICAL STUDIES AND ANTIOXIDANT ACTIVITIES OF
ADIANTUM CAPILUS-VENERIS, *LAVANDULA STOECHAS* AND
*AJUGA IVA***

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ABSTRACT

The present study is to investigate the phytochemical and antioxidant activity of the main constituents of *Adiantum capilus-veneris*, *Ajuga iva* and *Lavandula stoechas*. Preliminary phytochemical screening revealed that alkaloid bases and salts, polyterpenes, flavones aglycones, volatile oils, fatty acids, catechin tannins, cardiac glycosides, flavonoids, reducing compounds, sterol glycosides and terpenes, amino acids and saponins were found to be present in all species studied. The polyuronides are present only in *Lavandula stoechas*, while coumarins, anthocyanins and gallic tannins are characterized only in *Ajuga iva*. Sterols are present in *Lavandula stoechas*, *Ajuga iva* and absent in the *Adiantum capilus-veneris*. Quantitative phytochemical study shows that our plants have good potential nutrient. *Lavandula stoechas* has the highest content of polyphenols and flavonoids followed by *Adiantum capilus veneris* and *Ajuga iva*. The present study demonstrated that the flavonoids extract of all plants possesses a strong antioxidant capacity through different

mechanisms, which could be derived from compounds like phenolic acids and flavonoids. These results provide evident indicators that the free radical scavenging and strong reducing power of this extract may contribute to the prevention of Fe²⁺-induced lipid peroxidation. Essential oils of all plants have not presented an antioxidant activity.

KEYWORDS: Medicinal plants, Phytochemicals, Antioxidant, essential oils, Flavonoids.

INTRODUCTION

Medicinal plants are successful natural source of materials for the treatment of various infectious diseases of human^[1] and therefore scientists are vigorously focusing their attention to discover natural compounds from medicinal plants with the aim of introducing new drugs which would be more effective than those available in the market.^[2] The knowledge of the chemical constituents of plants would further be valuable in discovering the actual value of folk remedies. In recent year, essential oil and herbal extracts have attracted a great scientific interest due to their potential use as a source of natural antioxidants and biologically active compounds.^[3] There is a growing tendency all over the world, to shift from synthetic to natural based products including medicinal and aromatic plants.

Adiantum capillus-veneris belonging to the *Adiantaceae* family is one of the most common and widely distributed species^[4], which consists of 150 to 200 species worldwide distributed in North America, United States, South Dakota, British Columbia, Canada and India.^[5] Ethnomedicinally, the genus has been used as tonic and diuretic; in treatment of cold, fever, cough and bronchial disorders, as stimulant, emollient, purgative, demulcent, general tonic and hair tonic, in addition to skin diseases, tumors of spleen, liver and other viscera^[4], in treatment of jaundice and hepatitis^[6] and many other uses.^[7,8]

The *Lavandula* genus consists of about 20 species of small evergreen shrubs having aromatic foliage and flowers. Lavenders (*Lavandula* spp.) belong to the family *Labiatae* (*Lamiaceae*) and have been used either dried or as an essential oil for centuries for a variety of therapeutic and cosmetic purposes. The plants are useful as decorative hedges in the garden, while the dried flowers are used in potpourris, as cooking herbs^[9] and as insect repellents.^[10] Lavender essential oil is produced, usually by steam distillation, from both the flower heads and foliage, but the chemical composition differs greatly, with the sweeter and most aromatic oil being derived from the flowers.^[11] The oil is traditionally believed to be antibacterial, antifungal, carminative (smooth muscle relaxing), sedative, anti depressive and effective for burns and insect bites.

Ajuga species are used in folk medicine of different parts of the world for the treatment of rheumatism, gout, asthma, diabetes, malaria, ulcers and diarrhea and have antibacterial, antitumor, antifeedant and vulnerary properties.^[12] In view of the vast potentiality of aromatic

plants, the present work deals with the phytochemical analyses and antioxidant activity of these plants.

MATERIAL AND METHODS

Extraction of plant materials

Adiantum capillus-veneris, *Lavandula stoechas* and *Ajuga iva* were collected in the month of January, March and April 2014 (flowering period) from the region of Mascara (Northwest of Algeria). The plant material was dried in shade and room to get a better extraction temperature because some antioxidant compounds (flavonoids and some particularly glycosides) are unstable and can be degraded by the action of enzymes of fresh plant material. The dried plants parts were finely for use in preparing extracts. For the extraction of essential oils was used fresh plant material. 20g of powdered plant materials were separately extracted with 150 mL of different solvents (chloroform, methanol and distilled water) for 72 hours under magnetic stirring. This soaking was repeated three times by renewing the solvent every 24 hours. Maceration of each solvent are combined and concentrated to 80 mL under reduced pressure using rotary evaporator. The samples to be tested are stored in refrigerator at 4°C.^[13]

Preliminary phytochemical analysis

The summary phytochemical analysis is performed on the basis of staining characteristics tests to highlight the major chemical groups. For this purpose, several types of reagents were used. The dry matter is determined by drying 1 g of sample at 105°C to constant weight. The ash was determined by incinerating 1 g of dry sample for 5 hours at 550°C. Organic matter is the difference between the sample (dry matter) and ash resulting. The rate of lipids is determined using a Soxhlet -type apparatus. The protein content is measured by the Kjeldahl method. To assay total sugars in the samples, we applied the method of Dubois.^[14] For different dosages of secondary metabolites, 2 g of powdered plant material are defatted with 100 mL of diethyl ether for 2 hours using a soxhlet apparatus.^[15]

- Test for flavonoids

Five g of the plant material are mixed with 50 mL of 80% methanol; the extraction is done at room temperature. The mixture was quickly filtered and then re-extracted for the second and the third time with the same solvent. The filtrates obtained are evaporated to dryness obtaining a residue.^[16]

- Test for tannins

The extraction of the tannins is carried out by 70% acetone. 10 g of plant material were soaked in 100 mL of solvent for 30 minutes. The operation is repeated three times. After filtration and evaporation, the dry residue was weighed to calculate the yield of tannins.^[17]

- Test for saponins

Ten g of plant material were dispersed in 100 mL of ethanol 20%. The extraction is carried out in a water bath at 55°C under agitation. This step lasts 4 hours was repeated a second time to the residue obtained after filtration. The collected filtrates are concentrated to volume equal to 40 mL. Then performs a series of liquid-liquid extraction, the first is by 20 mL of diethyl ether by repeating this operation. After the ethereal layer was removed and the second liquid-liquid extraction is begun by the n-butanol, adding 40 mL of the latter in the aqueous phase obtained after extraction with ether. This extraction three times again. The n-butanol phase was washed twice with 20 mL of 5% NaCl and then concentrated to a dry residue which expresses the weight yield saponins.

- Test for alkaloids

Five g of the sample were weighed and 200 mL acetic acid in 10% ethanol was added, cover and let stand for 4 hours. Filter and concentrate the extract to a water bath at a quarter of the initial volume. Concentrated ammonium hydroxide was added drop wise to the extract until complete precipitation. The collected precipitate is washed with a dilute solution of ammonium hydroxide and then filtered. The residue is the alkaloid, which was dried and weighed.^[18]

Extraction of essential oils

The fresh material from three plants (100g) was subjected to hydrodistillation for 3 hours, using a Clevenger-type apparatus (ST15 OSA, Staffordshire, UK). The obtained distillate (100 mL) was extracted twice successively with 100 mL of n-hexane and methanol. The mixture was dried with anhydrous sodium sulphate. For the determination of the procedure yield, the solvent was evaporated using a rotatory vacuum evaporator. The yield of extraction is the ratio between the weight of the extracted oil and the weight of the plant to be treated. The essential oil extracted is stored at 4°C in an opaque glass bottle sealed to protect it from air and light (main agents of degradation).

Extraction of flavonoids

The aerial part of the three plants has been thoroughly cleaned and dried at room temperature and then milled using a mortar. The method Merghem *et al.*^[19] was followed for the extraction of flavonoids using organic solvents of increasing polarity. According to this method, 100 g of the powder was made up to 1 liter with methanol/distilled water (85/15 v/v). The mixture was subjected to stirring overnight at 4°C and then allowed to stand for several hours. The floating fraction was subsequently filtered to give the filtrate 1 and stored at 4°C. The extraction was repeated once and the precipitate was added to 1 liter 50% methanol to yield the filtrate which 2 was mixed with the filtrate 1. Hydro alcoholic maceration are then combined and evaporated to dryness under vacuum using a rotary evaporator. The dry residue is taken up in boiling distilled water (200 mL) which quantitatively solubilizes phenolics compounds; decantation for 12 hours and followed by filtration (or more) are used to eliminate the "sludge" (fats, resin). This will greatly facilitate purification tests chromatography. Crude extracts thus obtained were subjected to several extractions with various organic solvents: Diethyl ether (removes chlorophyll pigments, carotenoids and fat, all non-phenolic compounds), Ethyl acetate (mono-o-glucoside and partially di-o-glucosides) and Butanol (this solvent will cause the rest essentially of di-o-glycoside, tri-o-glycosides and c-glycosides). The aqueous phase and the solvent are mixed thoroughly by leaving out every time the product gases. After standing for an hour and a half, the water phase and the solvent used in charge of its specific compounds are recovered separately. For each solvent, we again two or three time this operation for optimal training separate polyphenolic groups. After several washes, it also takes the remaining aqueous phase containing flavonoids. The phases are evaporated to dryness with a rotary evaporator and taken up in methanol for assays, chromatographic diagnosis. The yield percentage of each extract was calculated as follows: (final weight of dried extract/initial weight of powder)*100.

Dosage of polyphenols

The polyphenols were determined spectrophotometrically, by following the protocol applied Miliauskas *et al.*^[20]

Dosage of flavonoids

The determination of flavonoids extracted is carried out by the colorimetric method described by Ardestani and Yazdanparast.^[21] Results are expressed in equivalent mg catechin per gram of dry vegetable matter (mg EC/g E).

Thin-Layer Chromatography (TLC)

TLC was used to ascertain the number of constituents present in the extract and to determine their purity. TLC was also used to determine the solvent mixture that will affect the separation of the components. The system used is: Toluene/n-butanol/methanol/petroleum ether (40/30/30/05). After the solvent had travelled some distance across the plate, the plate was dried at room temperature and examined in UV at the wavelength 256 nm and 366 nm. The retention values were calculated by making use of the distance travelled by the component/distance travelled by the solvent.

Antioxidant activity

A dilution series of each extract was prepared to evaluate the antioxidant activity of the extracts. Methanol was used to dissolve the solids, the concentrations used are: 1000, 500, 250, 125, 62.5, 31.25, 15.625, 7.81, 3.90 and 1.95 µg/mL.

DPPH• assay

The DPPH• radical-scavenging activity was determined as described by Kirby and Schmidt^[22] with some modifications. The substances to be tested for their antiradical power are solubilized in methanol at different concentrations. A volume of 50 µL of these solutions was added to 1950 µL of methanol solution of DPPH• (6.10^{-5} M) as free radical source. The mixtures were stirred vigorously for 30 seconds and then incubated for 30 min in the dark at room temperature. Scavenging capacity was measured spectrophotometrically by monitoring the decrease in absorbance at 517 nm against a blank consisting of pure methanol.^[23] Ascorbic acid and catechin were used for comparison. Finally, the scavenging activity of the test compounds was evaluated relative to a methanol solution of DPPH• radicals. In its radical form, DPPH• has an absorption band at 517 nm which disappears upon reduction by an antiradical compound. Lower absorbance of the reaction mixture indicated higher free radical-scavenging activity. DPPH• radical-scavenging activity was calculated as: $[(A_{\text{control}} - A_{\text{sample}}) / A_{\text{control}}] \times 100$.

Where: A (control) is the absorbance of the control reaction (containing all reagents except the sample) and A (sample) is the absorbance of extract or essential oil. Tests were carried out in triplicate. The results are expressed in terms of IC₅₀ (concentration of substrate that inhibits 50% of the DPPH• radicals present in the reaction medium).

Ferric-reducing activity

The reducing power of an extract was determined as described by Yildirim *et al.*^[24] Sample solutions (1 mL) with different concentrations of the each extract were mixed with 2.5 mL of 0.2 M phosphate buffer pH 6.6 and 2.5 mL of potassium ferricyanide solution ($K_3Fe(CN)_6$, 1%). The mixtures were incubated for 20 min at 50°C. After incubation, 2.5 mL of trichloroacetic acid (10% TCA) was added and the reaction mixtures were centrifuged for 10 min at 3000 rpm. An aliquot of 2.5 mL of the supernatant from each sample mixture was mixed in a test tube with 2.5 mL of distilled water and 0.5 mL of ferric chloride solution (0.1%) prepared freshly in distilled water. After a 20 min reaction time at 35°C, the absorbance was measured at 700 nm against a blank that contains all except the extract solutions and ferric chloride. The control is achieved by different concentrations of ascorbic acid and catechin. Higher absorbance of the reaction mixture indicated higher reducing power. Tests were carried out in triplicate.

Lipid peroxidation assay

The test was performed according to the protocol described by Tatiya and Saluja.^[25] The mixture contained 0.5 mL of homogenate (10%), 1 mL of KCl (0.15 M) and 0.5 mL of various concentrations of each extract. The lipid peroxidation was initiated by adding 100 μ L of ferric chloride (1 mM). After incubation for 30 min at 37°C, the reaction mixture was stopped by adding 2 mL of iced HCl (0.25N) containing 15% trichloroacetic acid (TCA) and 0.38% thiobarbituric acid (TBA) and 0.2 mL of Butylated Hydroxyl Toluene (BHT, 0.05 %). These reaction mixtures were heated for 60 min at 80°C, cooled and centrifuged at 6900 rpm for 15 min. The absorbance of the supernatant was measured at 532 nm against a blank containing all of the reagents with the exception of liver homogenate and extract. Identical experiments were performed to determine the normal (without extract and $FeCl_3$) and the level of lipid peroxidation in the tissues (with $FeCl_3$ and without extract).

Preparation of homogenate

After dissection of the rat's, the liver was quickly removed, rinsed with physiological saline and homogenized at 4°C in a solution of KCl (0.15 M) at 10%. The homogenate was centrifuged at 800 rpm for 15 min to remove cellular debris; the supernatant was recovered to examine the *in vitro* lipid anti peroxidation.^[26] The percentage effect anti lipid peroxidation (% ALP) was calculated by the following formula: $[(OD\ FeCl_3 - OD\ extract) / (OD\ FeCl_3 - OD\ normal)] * 100$.

Data analysis

All determinations were conducted in triplicates and results for each measured parameter were expressed as mean \pm SD.

RESULTS AND DISCUSSION

Preliminary phytochemical screening

Analysis of table 1 revealed that starch, anthracenosids, emodols and carotenoids are classes of chemical families completely absent in all three species. It is clear from this analysis that the alkaloid bases and salts, polyterpenes, flavones aglycones, volatile oils, fatty acids, catechin tannins, cardiac glycosides, flavonoids, reducing compounds, sterol glycosides and terpenes, amino acids and saponins are classes of chemical families present in all species studied. Several authors report the qualitative presence of these metabolites in the three plants.^[12,27] The polyuronides are present only in *Lavandula stoechas*, while coumarins, anthocyanines and gallic tannins are characterized only in *Ajuga iva*. However, sterols are present in *Lavandula stoechas*, *Ajuga iva* and absent in the *Adiantum capillus-veneris*. The variations between different substrates can be assigned to the chemical composition of these plants; the latter mainly depends on the environmental conditions in which these plants grow. This is why plants arid produce several types of secondary metabolites to defend themselves and to survive the constraints imposed by the climate and the environment. These results indicate the important medicinal value of the three plants studied.

Quantitative phytochemical

Primary metabolites

Ajuga and *Lavandula* have a high dry matter (DM): $80.09 \pm 0.66\%$ and $88.16 \pm 0.45\%$ respectively. Against *Adiantum* contains more water, its dry matter content being $55.17 \pm 0.94\%$. *Adiantum* is rich in water because it is a semi- aquatic plant with a deep root system. The three plants have a relatively high percentage of organic matter (OM). The lowest level is observed for *Adiantum* with $76.84 \pm 1.23\%$. *Ajuga* and *Lavandula* have similar values: $88.9 \pm 0.55\%$ and $90.05 \pm 0.87\%$, respectively. The percentage of mineral matter (MM) content in each plant is variable. *Adiantum* contains the highest content with $23.16 \pm 0.56\%$ followed by *Ajuga* and *Lavandula* who have average levels: $11.10 \pm 0.02\%$ and $10.95 \pm 1.09\%$, respectively. Proteins, sugars and lipids are indicators of the nutritional value of food. The results show that our plants have good potential nutrient, the percentage of the highest protein

is observed in *Ajuga* with $11.67 \pm 0.54\%$ followed by *Adiantum* and *Lavandula* with $9.81 \pm 0.98\%$ and $8.00 \pm 0.13\%$ respectively.

Table 1: Phytochemical screening of chloroform, methanol and aqueous extracts of *Ajuga iva*, *Adiantum capilus-veneris* and *Lavandula stoechas* (+: presence, -: absence).

Active ingredients		<i>A. iva</i>	<i>A. capilus-veneris</i>	<i>L. stoechas</i>
Chloroform	Alkaloid bases	+	+	+
	Sterols	+	-	+
	Polyterpenes	+	+	+
	Carotenoids	-	-	-
	Coumarins	+	-	-
	Flavones aglyones	+	+	+
	Emodols	-	-	-
	Volatile oils	+	+	+
	Fatty acids	+	+	+
	Alkaloid salts	+	+	+
Methanol	Catechin tannins	+	+	+
	Gallic tannins	+	-	-
	Cardiac glycosides	+	+	+
	Flavonoids	+	+	+
	Reducing compound	+	+	+
	Anthracenosides	-	-	-
	Anthocyanins	+	-	-
	Amino acids	+	+	+
	Sterol glycosides and terpenes	+	+	+
	Saponins	+	+	+
Aqueous	Polyuronides	-	-	+
	Starch	-	-	-

The determination of lipids and sugars shows that *Ajuga* has the lowest levels ($3.12 \pm 0.87\%$, $8.79 \pm 1.07\%$). These low values indicate that this plant is used as an anti obesity agent.^[28] The contents of sugars and lipids to *Adiantum* and *Lavandula* are very close (*Adiantum*: $11.43 \pm 1.23\%$, $6.20 \pm 0.23\%$, *Lavandula*: $10.91 \pm 0.05\%$, $6.17 \pm 0.07\%$). The percentage of primary metabolites may undergo variations could be explained by the different vegetative stages of plants, for the harvest season and especially by the drought that characterizes the regions where these plants grow. Other factors such as soil type, climate and stage of maturity vary greatly contribute to the concentration of mineral elements in plants.^[28] In addition to the use of medicinal plants in the areas, the results of primary metabolites assay reveals a good nutritional value of three plants.

Secondary metabolites

The high level of flavonoids in three plants (*Ajuga*: 69.8 ± 2.17 mg/100g, *Adiantum*: 76.98 ± 2.09 mg/100g, *Lavandula*: 59.77 ± 1.31 mg/100g) reveals that these plants are good managements of cardiovascular disease and oxidative stress. Flavonoids in combination with vitamins and enzymes can help the body to better defend against diseases related to oxidative stress.^[29] Oxidation of low density lipoproteins (LDL) plays an important role in atherosclerosis; immune cells (macrophages) accumulate one on the other contributing to the formation of a plate causing stenosis arteries. Several studies confirm that flavonoids have the ability to prevent the oxidation of LDL.^[30] The high content of alkaloids in *Ajuga* and *Lavandula* (50.56 ± 1.08 mg/100g, 37.84 ± 2.41 mg/100g) can explain the traditional therapeutic uses reported by several authors. The content of alkaloids in *Adiantum* is 1.04 ± 0.64 mg/100g. The high content of saponins in *Lavandula* (31.26 ± 1.34 mg/100g) gives this plant an anti asthmatic activity.^[31] The content of saponins in *Ajuga* and *Adiantum* are respectively 2.20 ± 0.12 mg/100g and 1.31 ± 0.34 mg/100g. Low levels of tannins in *Adiantum* (1.08 ± 1.12 mg/100g) and *Lavandula* (1.05 ± 0.56 mg/100g) indicate non-toxicity of the two plants. The tannin content in *Ajuga* (13.51 ± 0.34 mg/100g) gives it a hemostatic activity.

Yield of extraction

The yield of essential oil was widely variable, where *Lavandula* has the highest yield ($2.82 \pm 0.84\%$), $0.23 \pm 0.52\%$ for *Ajuga* and $0.11 \pm 0.45\%$ for *Adiantum*. The calculation of yields relative to the dry weight of the plant powder showed that the crude extract (CrE) represents the highest yield for the three plants (*Lavandula*: $5.41 \pm 0.43\%$, *Adiantum*: $3.63 \pm 0.062\%$, *Ajuga*: $3.37 \pm 0.53\%$), then the aqueous extract (*Lavandula*: $3.31 \pm 0.26\%$, *Adiantum*: $2.61 \pm 0.07\%$, *Ajuga*: $1.01 \pm 0.05\%$), followed by butanol extract (*Lavandula*: $1.85 \pm 0.08\%$, *Ajuga*: $1.6 \pm 0.22\%$, *Adiantum*: $0.71 \pm 0.3\%$) and finally extracted with ethyl acetate. Three plants gave dry masses acetate extracts 1g/100g ethyl below. Lavender gave an average percentage of $0.36 \pm 0.037\%$ against by the capillary is the plant that has the lowest yield at around $0.2 \pm 0.014\%$. *Ajuga* has the highest yield with $0.51 \pm 0.082\%$.

Content of total phenols and flavonoids in dry extracts

Phenolic compounds are known as powerful antioxidants.^[32] They are very important components in the extracts and their ability of scavenging of free radicals is due to their hydroxyl groups. From the results shown in table 2, the Ethyl acetate extract (EAE) is the

richest in polyphenols followed by Butanol extract (BtE) then Crude extract (CrE) and finally Aqueous extract (AqE) where *Lavandula* has the highest content (189.16 ± 2.12 mg EGA/g E, 104.67 ± 1.98 mg EGA/g E, 43.15 ± 0.51 mg EGA/g E, 36.02 ± 0.76 mg EGA/g E) followed by *Adiantum* and finally *Ajuga*.

Table 2: Average amount of polyphenols and flavonoids (EAE: Ethyl acetate extracts, BtE: Butanol extracts, CrE: Crude extract, AqE: Aqueous extract).

		CrE	EAE	BtE	AqE
<i>Ajuga</i>	Polyphenols (mg EGA/g E)	9.57±0.33	159.74±4.39	48.09±0.33	3.49±0.22
	Flavonoids (mg EC/g E)	5.19±0.27	33.60±2.98	40.14±0.33	1.23±0.02
<i>Adiantum</i>	Polyphenols (mg EGA/g E)	23.44±0.14	101.85±2.84	81.90±0.42	10.24±0.69
	Flavonoids (mg EC/g E)	4.66±0.097	31.60±1.48	34.43±0.42	0.18±0.002
<i>Lavandula</i>	Polyphenols (mg EGA/g E)	43.15±0.51	189.16±2.12	104.67±1.98	36.02±0.76
	Flavonoids (mg EC/g E)	35.06±0.31	88.75±1.79	90.83±2.01	14.71±0.29

Flavonoids are phenolic compounds with well-known antioxidant activity. In various studies, the antioxidant activity was found to be quite high when the plant extracts are rich in flavonoids.^[33] The determination of flavonoids revealed that *Lavandula* has the highest content followed by *Ajuga* and *Adiantum* whose BtE represents the most flavonoid-rich fraction, second comes EAE followed by CrE and AqE. The amount of total phenols and flavonoids is related to the chemical composition of plants. In fact, the concentration of polyphenol substrates, degradability depends, is likely to be influenced by the mode of culture, the phenological stage (with the time the plants tend to harden by lignification and their nutritional value declines), the type of soil (dry soils are sandy, infertile and low in nutrients), the climate at the time of sampling (temperature and rainfall), as well as the constitution of the sample (leaf - stems proportion flowers).^[20,34] It has been proven that the levels of total phenols and flavonoids are high when the living environment of the plant is not appropriate in this case the plant promotes the synthesis of secondary metabolites to adapt and survive. The processing of these samples for the experiment (drying method, grinding and storage) is also capable of varying results.^[35] The differences can be attributed partially to genotypic factors controlling the biosynthesis and accumulation of secondary metabolites in the plant. Given that the accumulation of primary and secondary metabolites is a physiological property of the plant and it varies across species and plant families.^[36] The polyphenol content varies qualitatively and quantitatively from one plant to another, this can

be attributed to the sensitivity of the chemical method used for quantification of the phenolic compounds as well as the nature of the used standard. In fact, some chemicals used as standard tend to overestimate the concentration of the compounds in question. This is particularly the case of Quebracho tannins.^[37]

Chromatography of flavonoids fractions

The TLC showed that the various fractions are very rich in flavonoids. In *Ajuga iva*, we identified flavonol, flavone, aurone, phenolic acids and anthocyanidin 3-glycosides. Flavonic pool in this species is dominated by flavonols and phenolic acids.

Compounds identified in *Adiantum capillus-veneris* are flavonols, flavones and phenolic acids which phenolic acids are most dominant.

For *Lavandula stoechas*, we identified flavones, phenols, flavonols, phenolic acids, anthocyanidin 3-glycoside and anthocyanidin 3,5-diglycosides. The flavonic pool in this species is dominated by flavones and phenolic acids.

By comparing the observed RF and UV spots with those of standards applied in the same experimental conditions (catechin, gallic acid), catechin has been identified in *Adiantum capillus-veneris*. In our own knowledge no work has been done on the identification of flavonoids in *Ajuga iva* and *Adiantum capillus-veneris*. Regarding *Lavandula stoechas*, two compounds were identified: catechin and gallic acid.

Antioxidant activity

No single method is adequate to estimate the total antioxidant capacity of a sample, due to the variability of extract composition and the conditions of the test used. Antioxidant capacity methods can be divided into two groups depending on the following two chemical reactions: assays based on hydrogen-electron transfer (HAT) and assays based on single-electron transfer (ET). The antioxidant activity of the essential oil and flavonoids extracts was evaluated by various antioxidant assays, including 2,2-diphenyl-1-picrylhydrazyl (DPPH•) radical-scavenging activity, reducing power and lipid peroxidation assay.

DPPH free radical-scavenging activity

DPPH• is a stable free radical that shows maximum absorbance at 517 nm. When DPPH• radicals encounter a proton-donating substrate, such as an antioxidant, the radicals are scavenged and the absorbance is reduced.^[23] The decrease in absorbance is taken as a

measure of radical-scavenging activity. Phenolic acids and flavonoids are secondary metabolites that are synthesised by plants during development, which possess an array of health-promoting benefits.^[38]

Fractions flavonoids of *Ajuga iva* has an average antioxidant activity does not exceed 75% at concentration of 1000 $\mu\text{g/mL}$. At the same concentration, the ethyl acetate phase shows that it is most active with $73.23 \pm 0.67\%$ followed by the crude extract $69.78 \pm 3.11\%$, then the aqueous phase $57.74 \pm 2.61\%$ and the butanol phase $53.06 \pm 2.59\%$ (Figure 1).

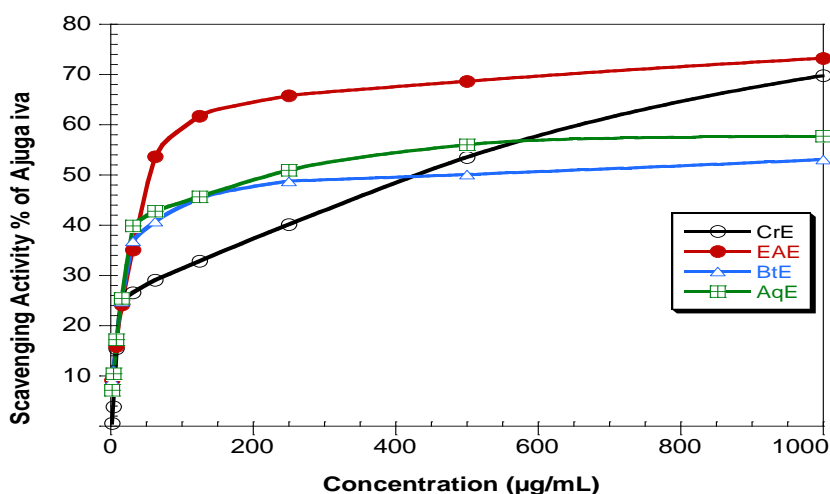


Figure 1: Free radical-scavenging capacities of flavonoids extracts of *Ajuga iva* measured by DPPH• assay. Values presented are the means of triplicate analysis. (EAE: Ethyl acetate extracts, BtE: Butanol extracts, CrE: Crude extract, AqE: Aqueous extract).

The fractions of *Adiantum capillus-veneris* showed a high scavenging activity of DPPH• radical, this demonstrated by the shape of the graph which plots an exponential curve with the presence of a stationary phase, which defines the almost complete reduction in its non DPPH• radical form (Figure 2). The percentage of inhibition exceeds 90% at a concentration of 1000 $\mu\text{g/mL}$ in all fractions and these results are similar results found with ascorbic acid and catechin ($97.53 \pm 4.72\%$, $94.87 \pm 1.78\%$).

The extracts of *Lavandula soechas* showed high scavenging activity of free radical DPPH•, the plotted curves have an exponential phase with presence of a stationary phase which means almost total reduction of DPPH• radical in its non form (Figure 3).

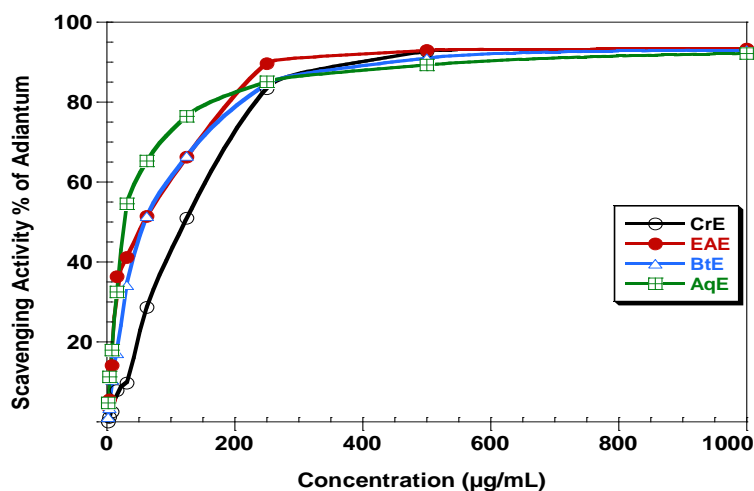


Figure 2: Free radical-scavenging capacities of flavonoids extracts of *Adiantum capillus-veneris* measured by DPPH• assay. Values presented are the means of triplicate analysis.

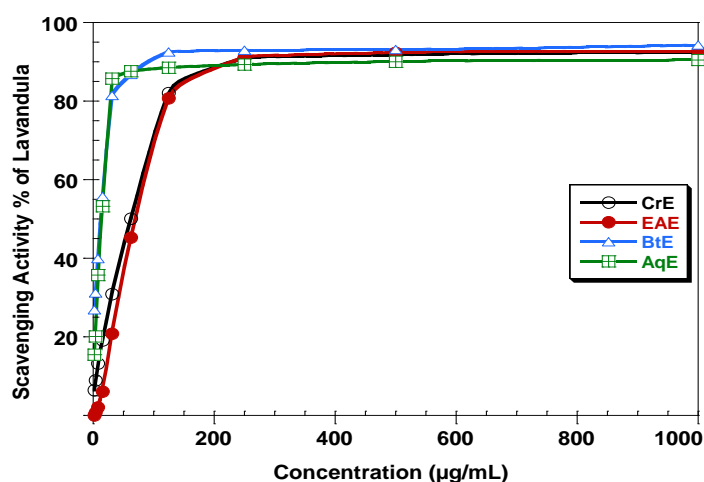


Figure 3: Free radical-scavenging capacities of flavonoids extracts of *Lavandula stoechas* measured by DPPH• assay. Values presented are the means of triplicate analysis.

In extracts of this plant, we noticed that at low concentrations, there is high percentage of inhibition, as is the case for the butanol phase which gave a percentage of inhibition equal to $81.45 \pm 4.14\%$ at concentration of $31.25 \mu\text{g/mL}$. The percentages of inhibition are very close to those of ascorbic acid and catechin. Regarding essential oils, we find that these aromatic essences have not presented an activity to the DPPH• radical, since at $1000 \mu\text{g/mL}$, we found an inhibition percentage not exceeding 18% (Figure 4).

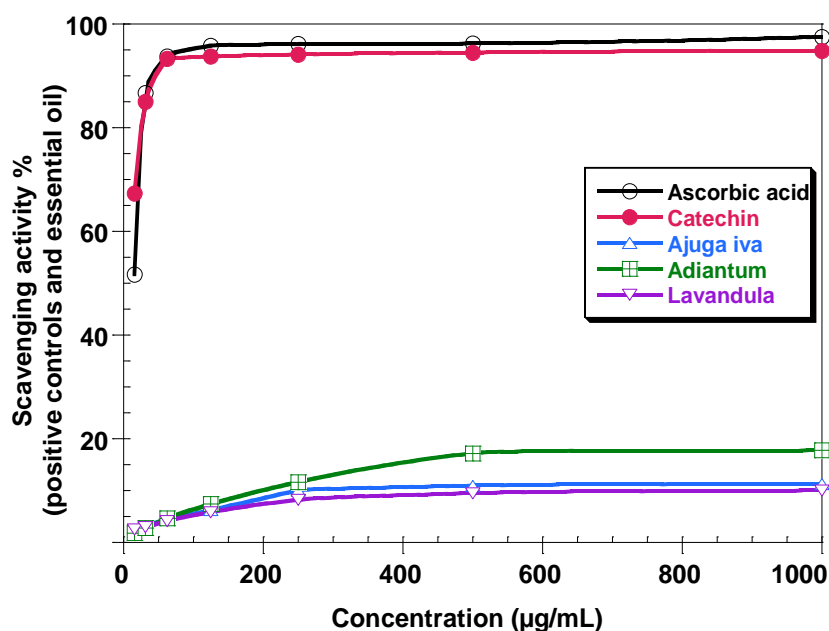


Figure 4: Free radical-scavenging capacities of positive controls (ascorbic acid and catechin) and essential oil of *Ajuga iva*, *Adiantum capillus-veneris* and *Lavandula stoechas* measured by DPPH• assay. Values presented are the means of triplicate analysis.

The IC₅₀ parameter commonly used to measure the antioxidant activity is necessary for each extract to reduce 50% of DPPH• radical concentration in a defined period of time, a low IC₅₀ value corresponds to a higher antioxidant activity extract. The results show that the antioxidant activity of different extracts studied directly depends on the concentrations. For each sample studied, we analyzed a dilution series to determine the concentration of each extract required to reduce 50% of the free radical (IC₅₀). By comparing the IC₅₀ of various extracts compared to the control, we observed a high antioxidant activity of the ethyl acetate fraction of three plants where the ethyl acetate phase of *Lavandula stoechas* has an IC₅₀ less than that of the ascorbic acid and catechin with 13.70µg/mL (Table 3). It is the same for the butanol fraction with 15.45µg/mL. Our results confirm those reported by Tian et al.^[39] and Fabri et al.^[40] Essential oils are very high and exceed IC₅₀ 1000 µg/mL. The antiradical activity of extract is relatively dependent on the content of total polyphenols and flavonoids.

Table 3: IC 50 values ($\mu\text{g/mL}$) found for the various extracts of the three plants studied (EAE: Ethyl acetate extracts, BtE: Butanol extracts, CrE: Crude extract, AqE: Aqueous extract, EO: Essential oil).

	EO	CrE	EAE	BtE	AqE
<i>Ajuga iva</i>	1472.2	55.04	39.53	44.17	512.25
<i>Adiantum capillus-veneris</i>	1173.36	65.85	27.66	57.42	140.61
<i>Lavandula stoechas</i>	1967.93	68.09	13.70	15.45	71.19
Ascorbic acid: 17.21	Catechin: 14.26				

For example, The Ethyle acetate extract, which is the richest in polyphenols and flavonoids fraction, has the most powerful scavenger effect compared to other extracts. Reverse against, the aqueous extract which is the poorest in polyphenols and flavonoids, shows the lowest effect scavenger. The mechanism of the reaction between the antioxidant and the $\text{DPPH}\cdot$ depends on structural conformation of the antioxidant.^[40,41] Some compounds react very rapidly with the $\text{DPPH}\cdot$ reducing a number of molecules of $\text{DPPH}\cdot$ equal to that of the hydroxyl groups of the antioxidant.^[42] The scavenger effect of flavonoids on free radicals depend on the presence of free OH groups, in particular 3-OH, with a configuration 3',4'-rthodihydroxy.^[43] The obtained herein were found to be in agreement with the findings of several authors who reported that the efficiency of an antioxidant component to reduce $\text{DPPH}\cdot$ essentially depends on its hydrogen donating ability, which is directly related to the presence of phenolic compounds^[44] and the abundance of monoterpenes hydrocarbons^[45] and oxygenated monoterpenes.^[46]

Reducing power

The reducing power assay is often used to evaluate the ability of natural antioxidant to donate an electron or hydrogen.^[23] The determination of the ferric reducing/antioxidant was based on the reduction of Fe^{3+} /ferricyanide complex to the ferrous form in presence of reductants (antioxidants) in the tested samples. The Fe^{2+} was then monitored by measuring the formation of Perl's Prussian blue at 700 nm. In fact, it is widely accepted that higher absorbance at 700 nm is correlated to power reducing.^[47]

The reducing capacity of the flavonoids extracts and the essential oil from three plants increased in a concentration-dependant manner. For *Ajuga iva* (Figure 5), we note that all flavonoids fractions showed a very low activity for reducing iron, values observed by optical density not exceeding 0.5, while ascorbic acid and catechin provide an OD of 2.069 ± 0.03 and 2.66 ± 0.016 in the same concentration ($1000 \mu\text{g/mL}$).

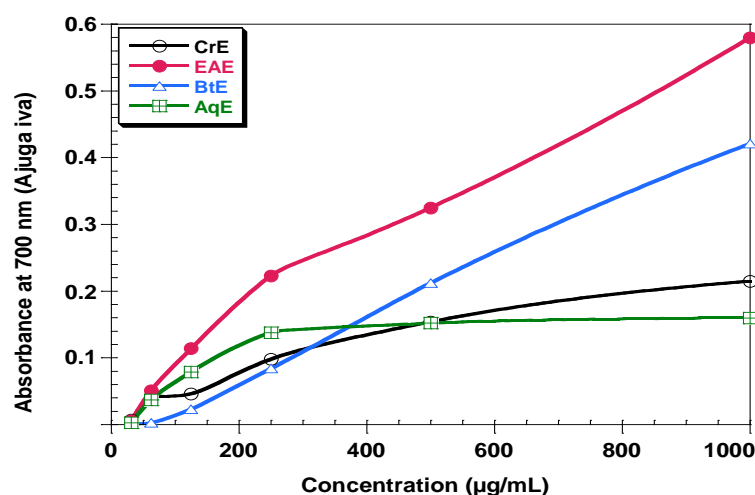


Figure 5: Antioxidant capacities of *Ajuga iva* using ferric reducing power method. Values presented are the means of triplicate analysis.

All fractions of *Adiantum capillus-veneris* except the aqueous extract showed significant capacities to the reduction of iron (Figure 6).

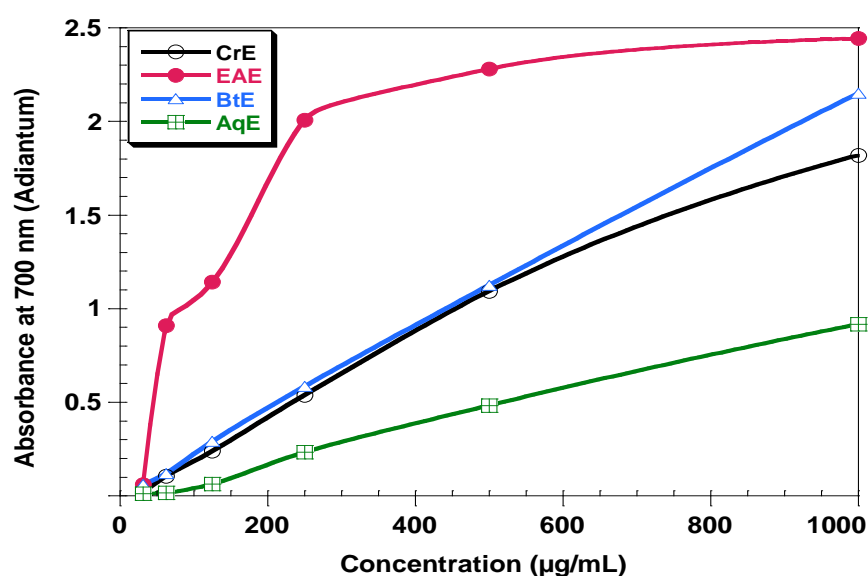


Figure 6: Antioxidant capacities of *Adiantum capillus-veneris*, using ferric reducing power method. Values presented are the means of triplicate analysis.

In fact, the ethyl acetate and butanol fractions showed very near to that of the ascorbic acid with significantly higher optical densities (2.44 ± 0.04 and 2.15 ± 0.15 respectively at 1000 µg/mL). While, crude and aqueous extracts made except by mean optical densities 1.82 ± 0.002 and 0.917 ± 0.056 respectively. We note for *Lavandula stoechas*, the ethyl acetate phase has the greatest activity to reduce iron compared to other fractions and even compared to

positive controls where the optical density reached 3 ± 0.11 at $1000 \mu\text{g/mL}$ (Figure 7). In fact, the butanol extract and crude extract were showed a power compared to positive controls (2.081 ± 0.31 and 1.87 ± 0.38 respectively at $1000 \mu\text{g/mL}$). The aqueous extract has a low capacity which does not exceed 0.8 at the same concentration. However, the reducing power of essential oil from three plants remained significantly lower than that of controls (Figure 8). This difference may be explained by lower content of electron donor compounds in the chemical composition of this oil.

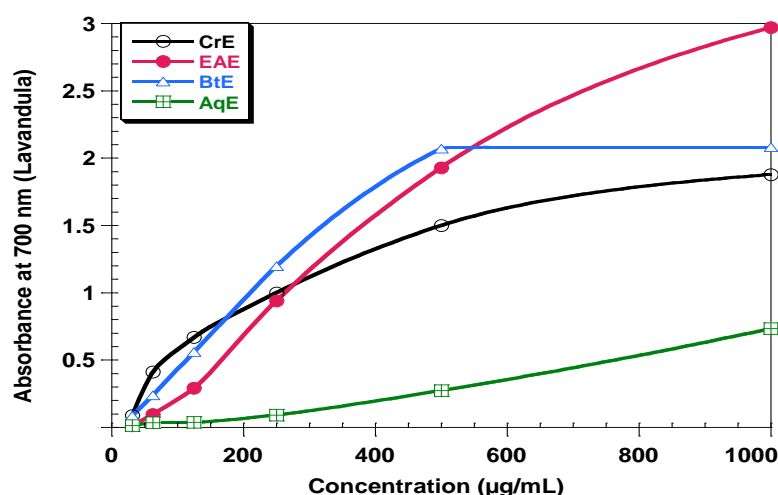


Figure 7: Antioxidant capacities of *Lavandula stoechas*, using ferric reducing power method. Values presented are the means of triplicate analysis.

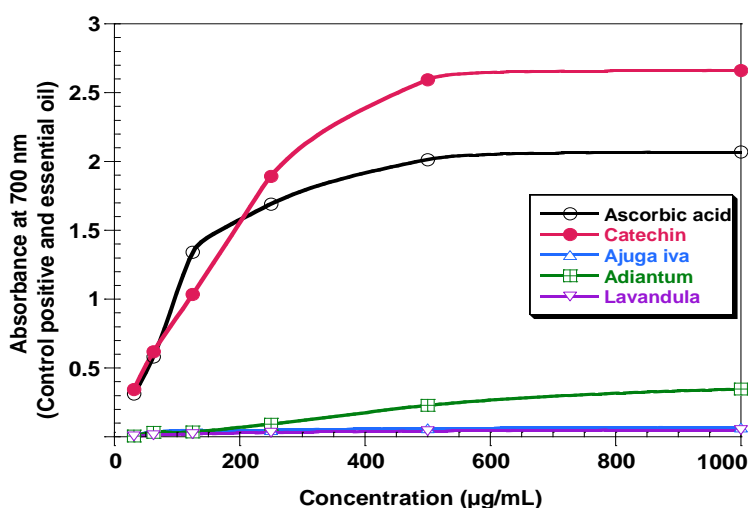


Figure 8: Antioxidant capacities of positive control (ascorbic acid, catechin) and essential oil of three plants, *Lavandula stoechas*, *Ajuga iva* and *Adiantum capillus-veneris*, using ferric reducing power method. Values presented are the means of triplicate analysis.

We can classify power reduction of iron by different fractions from three different plants studied as follows: Catechin, ascorbic acid, ethyl acetate phase, butanol phase, crude extract, aqueous extract and essential oils. In accordance of our data, Shimada *et al.*^[23] reported that the reductive potential may be related to the presence of phenolic compounds, such as isothymol and carvacrol, due to hydroxyl substitutions in the aromatic ring, which possesses potent hydrogen-bonding abilities.

Lipid peroxidation

Lipid peroxidation is associated with a loss of membrane fluidity and an increase of membrane permeability, causing a decrease in physiological performance.^[48] The inhibitory effect of the flavonoids extract and essential oil of three plants on Fe^{2+} induced lipid peroxidation in rat's homogenates is shown in figures 9, 10, 11 and 12.

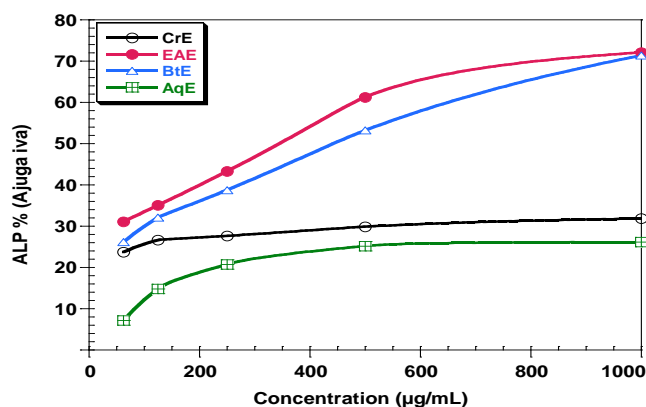


Figure 9: Antioxidant capacities of the flavonoids extract of *Ajuga iva*. Values presented are the means of triplicate analysis.

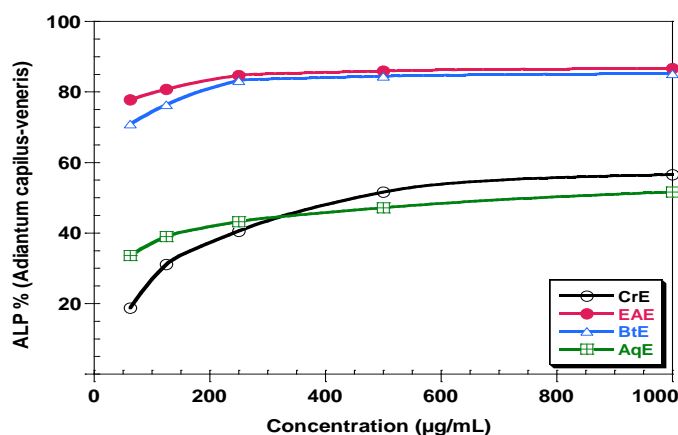


Figure 10: Antioxidant capacities of the flavonoids extract of *Adiantum capillus-veneris*. Values presented are the means of triplicate analysis.

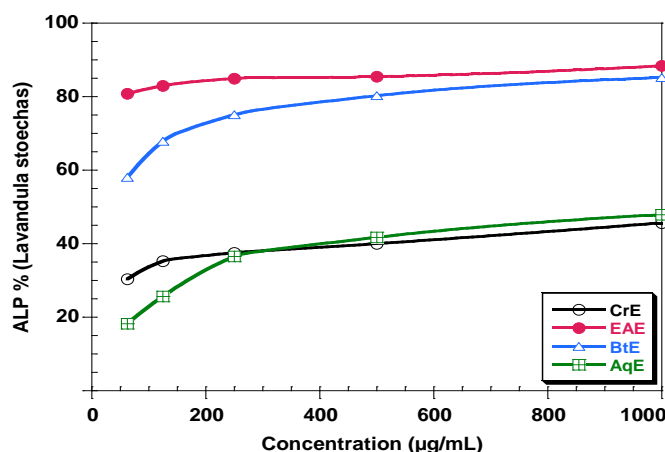


Figure 11: Antioxidant capacities of the flavonoids extract of *Lavandula stoechas*. Values presented are the means of triplicate analysis.

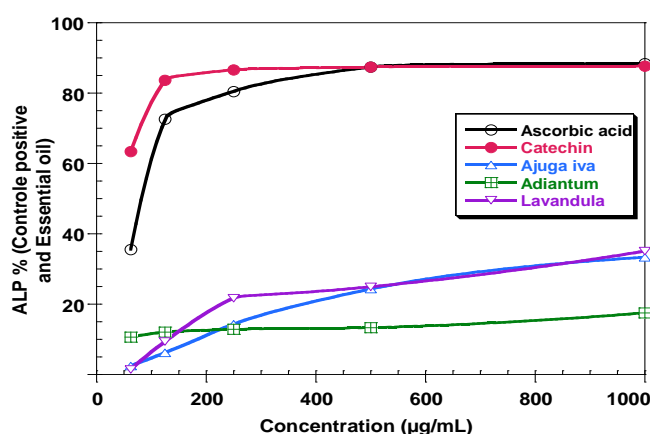


Figure 12: Antioxidant capacities of positive control (ascorbic acid, catechin) and essential oil. Values presented are the means of triplicate analysis.

The chemical structure of iron, and its capacity to drive one electron reactions, makes iron a key factor in the formation of free radicals.^[49] The butanol and ethyl acetate extracts of all plants protects against lipid peroxidation induced by Fe^{2+} , considerably increase percentage of anti lipid peroxidation in a dose-dependent manner and are close to those of ascorbic acid and catechin with a percentage more than 80% at 1000 $\mu\text{g/mL}$. These extracts are effective in inhibiting the lipid peroxidation induced by the system Fe^{2+} ascorbate in rat's homogenate. The generation of malondialdehyde (MDA) and related substances which react with the thiobabaturique acid are inhibited by extracts.^[25] This indicates significant activity of inhibiting lipid peroxidation of extracts. The preventative effects demonstrated by the extract could be due to the presence of antioxidant compounds.^[50] The aqueous extract, crude extract and essential oils show a low power anti lipid peroxidation.

CONCLUSION

This study is focused to phytochemical analyses and antioxidant activity of the flavonoids extracts and essential oils from *Adiantum capillus-veneris*, *Ajuga iva* and *Lavandula stoechas*. Preliminary phytochemical screening show that the alkaloid bases and salts, polyterpenes, flavones aglycones, volatile oils, fatty acids, catechin tannins, cardiac glycosides, flavonoids, reducing compounds, sterol glycosides and terpene, amino acids and saponins are classes of chemical families present in all species studied. The polyuronides are present only in *Lavandula stoechas*, while coumarins, anthocyanins and gallic tannins are characterized only in *Ajuga iva*. Sterols are present in *Lavandula stoechas*, *Ajuga iva* and absent in the *Adiantum capillus-veneris*. Quantitative phytochemical study shows that our plants have good potential nutrient. The high level of flavonoids reveals that these plants are good managements of cardiovascular disease and oxidative stress. Alkaloids and flavonoids are the source of antimicrobial activities. The high content of alkaloids in *A. iva* and *L. stoechas* can explain the traditional therapeutic uses. The high content of saponins in *L. stoechas* gives this plant an anti asthmatic activity. Low levels of tannins in *A. capillus-veneris* and *L. stoechas* indicate non-toxicity of the two plants since the lethal dose is 5%. The tannin content in *A. iva* (13.51 ± 0.34 mg/100 g) gives it a hemostatic activity. Phenolic compounds are the important source for antimicrobial, antioxidants and insecticidal activities. The ethyl acetate extract (EAE) is the richest in polyphenols followed by Butanol extract (BtE) then crude extracts (CrE) and finally aqueous extract (AqE) where *L. stoechas* has the highest content followed by *A. capillus veneris* and finally *A. iva*. Flavonoids are phenolic compounds with well-known antioxidant activity. The determination of flavonoids revealed that *L. stoechas* has the highest content followed by *A. iva* and *A. capillus-veneris* whose butanol extract represents the most flavonoid-rich fraction, second comes ethyl acetate extract followed by crude extracts and aqueous extract. The TLC showed that the various fractions are very rich in flavonoids. The present study demonstrated that the flavonoids extract of all plants possesses a strong antioxidant capacity through different mechanisms, which could be derived from compounds like phenolic acids and flavonoids. These results provide evident indicators that the free radical scavenging and strong reducing power of this extract may contribute to the prevention of Fe²⁺-induced lipid peroxidation. Moreover, the results of this study suggested the possibility of using the flavonoids extracts or some of their components as natural food preservatives, because the different extracts of all plants possesses strong antioxidant activity. Further research is needed in order to obtain information regarding the practical effectiveness of theses extracts to prevent the oxidation of food under specific application conditions.

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