Antioxidants, Free Radicals Scavenging and Xanthine Oxidase Inhibitory Potentials of *Ajuga iva* L. Extracts.

Abderrahmane BAGHIANI[1], Sabah BOUMERFEG[2*], Moufida ADJADJ[1], Djamila AMENI[1], Meriem DJERMOUNI[1], Fatima KHELIFI-TOUHAMI[4], Noureddine CHAREF[1], Seddik KHENNOUF[3] and Lekhmici ARRAR[1].

[1]Laboratory of Applied Biochemistry, Department of Biochemistry. Faculty of Natural and Life Sciences, University Ferhat Abbas, Setif 19000 Algeria.
[2]Department of Natural and Life Sciences, University of Bordj Bou Arreridj, 34000 Algeria
[3]Laboratory of Phytotherapy Applied to Chronic Diseases, Department of Animal Biology and Physiology, Faculty of Natural and Life Sciences, University Ferhat Abbas, Setif 19000 Algeria
[4]Department of Biology, Faculty of Natural and Life Sciences, University Mentouri, Constantine, 25000, Algeria

ABSTRACT

Introduction: Antioxidant effects of *Ajuga iva* L extracts (AIE), a traditionally used plant in Algerian folk medicine, were investigated. This plant is widely used to treat diabetes, gastric ulcer, dysuria, painful joints of the limbs and other free radicals related disorders. **Methods:** In order to determine the antioxidant activities of AIE, the shoot extracts were prepared using solvents of varying polarity. Xanthine oxidoreductase (XOR) was purified from bovine milk. Anti-radical activities were determined by enzymatic and non enzymatic methods: The enzymatic methods were realised by either production of uric acid or reduction of cytochrome c. The non enzymatic methods were conducted using in vitro techniques: NBT test, ß-carotene linoleate acid, 1,1- diphenyl-2 picrylhydrazyl (DPPH) radical-scavenging, ferric reducing /antioxidant power (FRAP) and Ferrous ion chelating activity. **Results**: All extracts showed inhibitory properties on xanthine oxidase, the IC_{50} (μM / quercetin equivalen) ranges from 3.878 ± 0.717 to 5.835 ± 0.468), with an additional superoxide scavenging capacity. These extracts showed a potent DPPH radical scavenging activity and a powerful scavenging activity of superoxide anion more than that of gallic acid using NBT test. They possess a similar inhibition ratio of the linoleic acid oxidation to that of the BHT, and gave a reduction power better than rutin in Ferric Reducing Ability of Plasma assay (FRAP). However, they were weaker than EDTA in Fe^{2+} chelating activity. **Conclusion:** *Ajuga iva* L. appears to be a valuable plant and could be used to treat conditions where inhibition and free-radicals scavenging of XO is warranted.

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Keywords: *Ajuga iva* L, reducing power, superoxide anion radicals, phenolic compounds, Antioxidants ß-carotene/linoleic acid, FRAPS.

*Corresponding author: Dr BOUMERFEG Sabah, Department of Natural and Life Sciences, Institute of Sciences and Technology University of Bordj Bou-Arreridj 34000 Algeria. **E-mail:** sabah_boumerfeg@yahoo.fr

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INTRODUCTION

Free radicals are known to be the major cause of various chronic and degenerative diseases, including aging, coronary heart disease, inflammation, stroke, diabetes mellitus and cancer[1] , and can cause cellular injuries and initiate peroxidation of polyunsaturated fatty acids in biological membranes. The tissue injury caused by reactive oxygen species (ROS) may include DNA[2] and protein[3] damage, and oxidation of important enzymes[4] in the human body. These events could consequently lead to the occurrence of various free radical- related diseases. Xanthine oxidoreductase (XOR) has long been known to be present in cows' milk, from which it is readily available, even on a gram scale. Indeed, XOR was first purified from this source over 60 years ago and is consequently one of the best studied of all enzymes[5]. It is not confined to milk, having been detected in all species examined to date, including bacteria. In mammalian tissues, the enzyme is widely distributed, particularly high levels being found in liver and intestine[6]. It is conventionally seen as a late enzyme of purine catabolism, catalysing the oxidation of hypoxanthine to xanthine and of xanthine to uric acid.

However, XOR is capable of much more than this and, over the last two decades, attention has been focused on other physiological roles[7]. In the early 1980s, such XOR derived agents were suggested to be involved in the pathogenesis of ischaemia-reperfusion injury[8]. In the context of the inflammatory response, XOR is believed to combat infection by generating ROS and can be seen as an agent of innate immunity[9]. Gout is a common disease which affects a substantial proportion of the adult population. Hyperuricemia leads to the accumulation of uric acid in joints and kidneys causing acute arthritis and uric acid nephrolithiasis. One therapeutic approach for gout is the use of xanthine oxidase inhibitors such as allopurinol, which blocks the synthesis of uric acid from purines[10]. However, allopurinol use can result in a number of adverse side effects[11], ranging from mild skin allergy to a concerted allopurinol hypersensitivity syndrome, which can sometimes be life threatening[12]. Thus, there is a need to develop compounds with xanthine oxidase inhibitory activities but devoid of the undesirable effects of allopurinol. Recently, natural product and food derived antioxidants such as vitamins and phenolic phytochemicals have received growing attention, because they are known to function as chemopreventive agents against oxidative damage[13]. There is abundant evidence that a great number of aromatic, spicy, medicinal and other plants contain chemical compounds exhibiting antioxidant properties. With this respect, a particular interest has been given to plant polyphenols. The natural polyphenols have an ideal structure for capturing free radicals[14], and it was found that their antioxidant activity surpasses the effect of known antioxidants, such as the vitamins A and E[15]. The antioxidant effects of plant extracts and diverse groups of plant phenolic compounds - flavonoids are well documented[16], [17].

Ajuga iva L is a common plant in North Africa, it is widely used in traditional pharmacopea to treat diabetes and other free radicals related disorders[18], [19], [20], [21], [22]. Pharmacological studies have shown that *Ajuga iva* has anti-ulcerogenic and anti-inflammatory activities. It has been used to treat dysuria and painful joints of the limbs[23]. *Ajuga iva* extract decreases plasma cholesterol and triglycerides when given as a single or repeated oral doses in normal and STZ-diabetic rats[24]. The current study was undertaken to investigate the inhibitory action on xanthine oxidase and the *in vitro* antioxidant and radical scavenging effects of the *Ajuga iva* extracts.

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MATERIALS AND METHODS

Chemicals

Bovine erythrocytes superoxide dismutase (SOD), dithiothreitol, Allopurinol, Horse heart Cytochrome c, NAD, Phenylmethylsulfonyl fluoride (PMSF), Xanthine, linoleic acid, 2,4,6- tripyridyl-s-triazine (TPTZ), sodium dodecyl sulphate (SDS), Ferrozine, Phenazine methosulfate (PMS), different polyphenols; Rutin (Rut), Quercetin, Gallic acid, Tween 40, Trichloroacetic acid (TCA), EDTA, ß-Caroten, $FeCl_3$, 2, 20-diphenylpicrylhydrazyl (DPPH), Thiobarbituric acid (TBA), Butylated hydroxytoluene (BHT) Nitroblue tetrazolium phenazine (NBT) wer purchased from Sigma (Germany), and all other reagents salts and solvents were obtained from Prolabo, Aldrich, and Fluka. All common chemicals and solvents used were of analytical grade.

Plant Material

Ajuga iva was harvested from natural resources in 2008, mainly at flowering stage from Biskra, Algeria. Botanical identification was confirmed by Pr H. Laouer and a specimen of the plant was deposited at the Laboratory of Botany. Faculty of Natural and Life Sciences, University Ferhat Abbas, Setif Algeria

Purification of milk xanthine oxidoreductase

XOR was routinely purified in our laboratory from mammalian milk (bovine), in the presence of 10 mM of dithiothreitol, by ammonium sulphate fractionation, followed by affinity chromatography on heparin-agarose[25]. XOR concentration was determined from the UV-visible spectrum by using an absorption coefficient of 36000 $M^{-1}cm^{-1}$ at 450 nm. The purity of enzyme was assessed on protein/flavin ratio (PFR = A_{280}/A_{450}) [26] and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (10%) [27]. The activity of XOR was specrophotometrically determined by measuring the production of uric acid from xanthine (100 μM, final concentration) at 295 nm using an absorption coefficient of 9600 M^{-1} cm^{-1}[28]. Assays were performed at room temperature in air-saturated 50 mM phosphate buffer, pH 7.4, supplemented with 0.1 mM EDTA.

Extraction of phenolic compounds

The extraction was carried out using various polar and non-polar solvents according to Markham[29] with slight modifications. Dried plant material was ground in warring

blender, mixed with a 10-20 volume of 85 % aqueous methanol. The slurry was placed on a shaker for 24 hours and the extract was filtered through a Buchner funnel and the methanol was removed on rotary evaporator to give crude extract (fraction labeled CE). The aqueous solution was extracted with hexane several times to eliminate lipids. The water fraction was partitioned against chloroform labeled CHE. The remaining aqueous phase was exhaustively extracted with ethyl acetate until the final ethyl acetate extract was colorless (EAE fraction), the remaining aqueous extract was labeled AE. All the solvents were removed by evaporation under reduced pressure and the extracts were stored at -20 until use.

Total phenolic contents determination

Total polyphenols were measured using Prussian bleu assay[30]. Phenolic contents were expressed as gallic acid equivalent per gram dry weight. Flavonoids were quantified using $AlCl_3$ method described by Bahorun et al[31] and expressed as quercetin equivalents per gram dry weight.

Effects of Ajuga iva extracts (AIE) on the generation of superoxide anion radicals

Anti-radical activity was determined spectrophotometrically according to Robak and Gryglewski[32], by monitoring the effect of AIE on superoxide anion radicals produced by xanthine/xanthine oxidase system. These radicals are able to reduce cytochrome c. The reaction mixture contained xanthine (100 μM), horse heart cytochrome c (25 μM), in air-saturated sodium phosphate buffer (50 mM, pH 7.4), supplemented with 0.1mM EDTA and various concentrations of AIE. All concentrations indicated are final ones. The reactions were started by adding XOR, than reduced cytochrome c was spectrometrically determined at 550 nm against enzyme-free mixture using an absorption coefficient of 21.100 M^{-1} cm^{-1}, and the sensibility of the reaction was determined using bovine erythrocytes superoxide dismutase (SOD) (330 U/mL final concentration).

Effects of Ajuga iva extracts on XOR activity

The effect of AIE on the xanthine oxidation was examined spectrophotometrically at 295 nm following the production of uric acid using an absorption coefficient of 9600 M^{-1} cm^{-1} [28]. Assays were performed at room temperature, in the presence of final concentration of 100 μM of xanthine, and various amounts of plant extracts, The reaction was started by the addition of XOR (1176 nmol of urate /min/mg protein). Enzyme activity of the control sample was set as 100 % activity. The percent inhibition was calculated by using the following formula:

$$I\ (\%) = \frac{A_{control} - A_{sample}}{A_{control}} \times 100$$

Measurement of superoxide anion scavenging activity

The superoxide scavenging ability of AIE was assessed as described Nishikimi et al.[33] with slight modifications. Briefly, superoxide anions were generated in samples contained 100 μl each of 1.0 mM NBT, 3.0 mM NADH and 0.3 mM PMS and the final volume was adjusted to 1 ml with 0.1 M phosphate buffer (pH 7.8) at ambient temperature. The reaction mixture (NBT and NADH) was incubated with or without AIE at room temperature for 2 min and the reaction was started by adding PMS. The absorbance at 560 nm was measured against blank samples for 3 min. The decrease in absorbance in the presence of AIE indicated superoxide anion scavenging activity. The percent inhibition was calculated by using the following formula:

$$I\ (\%) = \frac{A_{blank} - A_{sample}}{A_{blank}} \times 100$$

where A_{blank} is the absorbance of the control reaction (containing all reagents except the test solutions), and A_{sample} is the absorbance of the test compound.

DPPH assay

The hydrogen atom or electron donation abilities of the corresponding extracts and some pure compounds were measured from the bleaching of the purple-coloured methanol solution of 2, 20-diphenylpicrylhydrazyl (DPPH). This spectrophotometric assay uses the stable radical DPPH as a reagent[34]. 50μl of various concentrations of the extracts in methanol were added to 5 ml of 0.004% methanol solution of DPPH. After a 30 min incubation period at room temperature, the absorbance was read against a blank at 517 nm. Inhibition of free radical DPPH in percent (I %) was calculated as following:

$$I\% = (A_{blank} - A_{sample} / A_{blank})\ X100$$

Where A_{blank} is the absorbance of the control reaction (containing all reagents except the test solutions), and A_{sample} is the absorbance of the test compound. Extract

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concentration providing 50% inhibition (IC50) was calculated from the plot of inhibition percentage against extract concentration.

ß-carotene/linoleic acid assay

In this assay, antioxidant capacity is determined by measuring the inhibition of the volatile organic compounds and the conjugated diene hydroperoxides arising from linoleic acid oxidation[35]. A stock solution of ß-carotene-linoleic acid mixture was prepared as follows: 0.5 mg ß-carotene was dissolved in 1 ml of chloroform (HPLC grade) and 25µl linoleic acid and 200 mg Tween 40 were added. Chloroform was completely evaporated using a vacuum evaporator. Then, 100 ml distilled water saturated with oxygen (30 min 100 ml/ min) were added with vigorous shaking. 2.5 ml of this reaction mixture were dispensed into test tubes and 350 µl portions of the extracts (2 g /L) were added and the emulsion system was incubated for 48 h at room temperature. The same procedure was repeated with synthetic antioxidant, BHT, as positive control, and a blank. After this incubation period, absorbances of the mixtures were measured at 490 nm.

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Ferric reducing ability of plasma assay (FRAP)

The antioxidant capacity of each sample previously dissolved in 1mM NaOH and adjusted with distilled water, was estimated according to the procedure described by Benzie and Strain[36] with slight modifications. Briefly, 900 µL of FRAP reagent, prepared freshly and warmed at 37° C, was mixed with 90 µL of distilled water and 30 µL of test sample then read at 595 nm. Aqueous solutions of known Fe (II) concentrations in the range of 100-2000 µmol/L ($FeSO_4$, $7H_2O$) were used for calibration. The parameter Equivalent Concentration 1 or EC_1 was defined as the concentration of antioxidant having a ferric-TPTZ reducing ability equivalent to that of 1 mmol / L $FeSO_4$, 7 H_2O.

Ferrous ion chelating activity

The ferrozine test was performed according to Decker and Welch [37]. This test assesses the capability of the extracts to interact with Fe(II), preventing the formation of the complex between the reduced metal form and the specific Fe^{2+} colorimetric detector ferrozine. Briefly, different concentrations of test compounds (0.5 mL) were added to a solution of 0.6 mM of $FeCl_2$ (0.1 ml) and 0.9 ml of methanol. The reaction was initiated by the addition of ferrozine 5 mM (0.1 ml) and the mixture was shaken. Absorbance was then measured at 562 nm in a spectrophotometer. Ethylene diamine tetraacetic acid (EDTA) was used as a positive control. An EC_{50} value defined as the effective concentration of test material which produces 50 % of maximal scavenging effect.

Statical analysis

All determinations were conducted in triplicate or more and all results were calculated as mean ± standard deviation (SD). In this study, Statistical analysis was performed using Student's *t*-test for significance and analysis of variance (ANOVA) followed by Dunnet's test were done for the multiple effects comparison of the different extracts. The *p* values less than 0.05 were considered statistically significant.

RESULTS AND DISCUSSION

Xanthine oxidase purification

The freshly purified bovine milk XOR showed an ultraviolet / visible spectrum with three major peaks at 280, 325, 450 nm, with A_{280}/A_{450} (protein to flavin ratio, PFR) of 5.15 indicating a high degree of purity[26]. Run on SDS-PAGE, purified enzyme showed quite similar patterns with one major band of approximately 150 KDa.

Effects of Ajuga iva extracts On the generation of superoxide anion radicals by the xanthine/xanthine oxidase system

Ajuga iva is a traditionally recognized as folk medicine mostly used in the treatment of diabetes and other pathologies in North Africa. Our study, examined the XO-inhibitory and free radicals scavenger effects of *Ajuga iva* extracts. Cytochrome c^{3+} has been extensively used for the detection of O_2^{-} produced in biological systems due to its fast superoxide-mediated reduction to cytochrome c^{2+} [38]. The effect of three *Ajuga iva* extracts (AIE) at different concentrations were studied for their ability to scavenge superoxide anion radicals (O_2^{-}) generated by the xanthine/xanthine oxidase system. The amount of generated O_2^{-} was determined by measuring the reduction of cytochrome c. Under our experimental conditions, the activity of cytochrome c in the absence of extracts was (2135.91 nmol/min/mg protein) reduced by O_2^{-} generated from bovine milk xanthine oxydoreductase (XOR). The reduction of cytochrome c^{3+} was almost totally inhibited by superoxide dismutase

(SOD, 330 U / mL). The results showed that all the extracts were able to inhibit cytochrome c^{3+} and the superoxide scavenging effect increased with the concentration of AIE extracts. The most potent scavenger of superoxide anion radical observed was EAE with IC_{50} (μM / quercetin equivalent) of 5.095 ± 0.0321 followed by CHE 7.868 ± 0.0069 then CE with IC_{50} 13.90 ± 0 .0653 ($P \leq 0.01$), for $O_2^{\cdot-}$ generating from bovine XOR. The scavenging action of plant constituents is related to polyphenolic and flavonoids compounds[39];[40]. It is possible that the antioxidative properties of AIE are caused by the presence of polyphenols.

Effects of Ajuga iva extracts On the xanthine oxidase activity

Since an inhibitory effect on the enzyme itself would also lead to decrease in reducing cytochrome c^{3+}, the effect of extracts on the XO activity was checked. In this regard, we evaluated the effect of these extracts on the metabolic conversion of xanthine to uric acid. In the xanthine/xanthine oxidase system, the extracts were effective in inhibiting the uric acid formation. The AIE inhibitory effects on XO were compared to allopurinol. The extracts exhibited an inhibitory effect on xanthine oxidase activity in a concentration dependent manner. The results demonstrated that EAE possessed the highiest XO inhibitory activitie ($P \leq 0.01$). The concentrations of these extract required to inhibit 50% of XO activity (IC_{50}) was 3.878 ± 0.717 μM/quercetin equivalent, followed by those of CHE and CE extracts which were to close to each other (IC_{50} = 5.36 ± 0.642, 5.835 ± 0.468 μM / quercetin equivalent respectively), meanwhile, the specific inhibitor of XO, Allopurinol gave a IC_{50} of 57.114 ± 1.093 μM higher than the AIE ($P \leq 0.01$) which indicat the potent inhibitory effects of AIE on XO. Studies have shown that xanthine oxidase inhibitors may be useful for the treatment of hepatic disease and gout, which is caused by the generation of uric acid and superoxide anion radical[41]. Xanthine oxidase-derived superoxide anion has been linked to post-ischaemic tissue injury and edema[42]; [43]. Several data has been reported in several experimental models showed that flavonoids and other phenolic compounds are considered as antioxidants not only because of they act as free radical scavengers, but also because of their ability to inhibit XO[44];[45]Total antioxidant activities of the plant extracts can not be evaluated by any single method, due to the complex nature of phytochemicals[46]. Two or more methods should always be employed in order to evaluate the total antioxidative effects of vegetables[47]. In order to correlate the observed antioxidant activity of the AIE, many other antioxidant assays were applied.

Measurement of superoxide anion scavenging activity

Superoxide anion is a reduced form of molecular oxygen, which is a highly toxic species generated by numerous biological and photochemical reactions. This radical plays an important role in the formation of other reactive oxygen species such as hydrogen peroxide, hydroxyl radical or singlet oxygen[48]. Our results show that *Ajuva iva* extracts contains scavengers of superoxide radicals and react in a dose-dependent manner which can prevent the formation of ROS. *Ajuga iva* extracts were found to be higher, as scavengers of $O_2^{\cdot-}$, than gallic acid ($P \leq 0.01$), which have an antioxidant capacity, determined by both ABTS and DPPH scavenging assays, more than that of vitamin C and other phenolic constituents such as quercetin, epicatechin, catechin, rutin and chlorogenic acid[49]. The most potent scavenger of superoxide anion radical observed was EAE with IC_{50} of 0.803 ± 0.0286 g/l which was found to be 2 times lower potent than quercetin (IC_{50} at 0.465 ± 0.05) ($P \leq 0.01$) followed by the CHE extract 2.585 ± 0.118 then the CE extract with 3.485 ± 0.033 g/l ($P \leq$ *0.01).*

DPPH assay

The DPPH radical scavenging is a sensitive antioxidant assay and is independent of substrate polarity[48]. DPPH is a stable free radical that can accept an electron or hydrogen radical to become a stable diamagnetic molecule. In this study, results showed that AIE exhibited a dose-dependent activity in scavenging DPPH radicals, and gave an effects higher that of used standards gallic acid, quercetine and rutin ($P \leq 0.01$), which indicating the effective of thees extracts (Figure.1). This suggests that

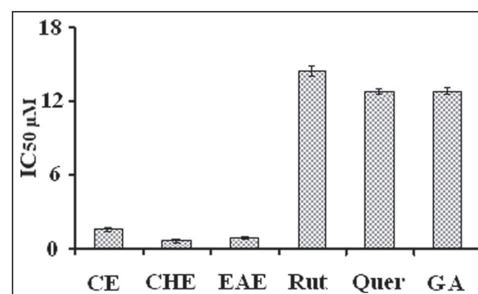

Figure 1. DPPH radical scavenging activity of different *Ajuva iva* extracts. CE: methanol extract; EAE: ethyl acetate extract; CHE: chloroform extract, Rut: rutin, quer: quercetine and GA: gallic acid. Data are presented as IC_{50} values mean ± SD (n = 3).

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AIE are a good free radical scavenger or hydrogen donor and contributes significantly to the antioxidant capacity of Ajuva iva extracts. Phenolic compounds are effective hydrogen donors, which make them good antioxidant[50]. The relationship between the molecular structure of a series of structurally related flavonoids (flavones, flavonols and flavanones) and their ability to scavenge 1,1-diphenyl-2-picrylhydrazyl (DPPH•) free radicals was studied, it was demonstrated that the free radical scavenger potential of polyphenolic compounds closely depends on the particular substitution pattern of free hydroxyl groups on the flavonoid skeleton, the highly active flavonoids possess a 3', 4'-dihydroxy occupied B ring and/or 3-OH group[51].

Effects of Ajuva iva extracts ß-carotene/ linoleic acid

ß-carotene/linoleic acid assay determines the inhibition ratios of the oxidation of linoleic acid as amethod to confirm the antilipoperoxidation of AIE extracts. Lower absorbance indicates a higher level of antioxidant activity. Figure 2 shows the changes in the percentage of the inhibition ratios of linoleic acid oxidation under the influence of AIE (2mg/ml) compared to that of synthetic antioxidant BHT as a positive control during 24 h under the same condition. The inhibition extent of lipid oxidation by AIE when compared to BHT, which had 96,773 % at the same concentration (2mg/ml), showed a marked activity effects ($p \leq 0.01$). The high inhibition ratios of linoleic acid oxidation were showed for CHE (95.90± 3.125%), similar to that of BHT, followed by that of EAE (65.91% ± 1.546) ($p \leq 0.01$), however CE exhibited the lowest ratio (49.37% ± 2.24) ($p \leq 0.01$).

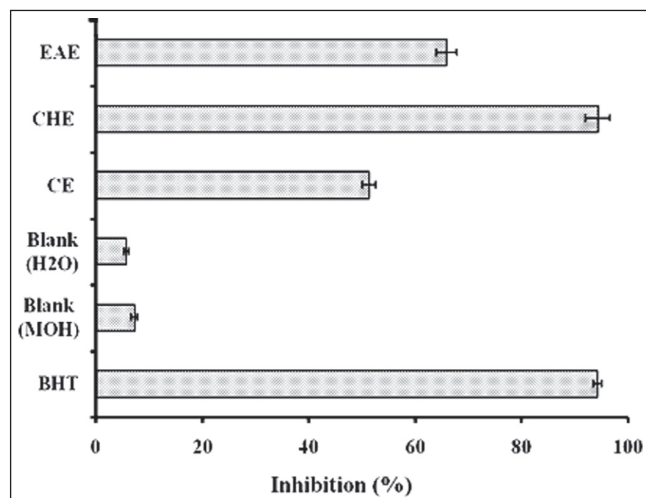

Figure 2. Percentage inhibition of the linoleic acid oxidation by the *A. iva* extracts (CE: methanol extract; CHE: chloroform extract; EAE: ethyl acetate extract), BHT and blanks (H_2O and Methanol) after 24h. Results are means of three different experiments.

The antioxidant activity of extract is based on the radical adducts of extracts with free radicals from linoleic acid. The linoleate-free radical attacks the highly unsaturated ß-carotene models. The presence of extracts shows a decrease of the free radical concentration. We can summarize the descending order of extracts tested in terms of polarity activity the apolar extracts or moderately apolar (CHE, EAE) are more active than the polar extract (CE). On the other hand, the richest extracts in phenolic compounds are more assets (CHE and EAE) so there is a good correlation between antioxidants activity and polyphenols contents in the same plant ($r^2 = 0.84$, $p \leq 0.05$). In multiphase systems antioxidants localization depends on their solubility and polarity. According to Frankel and Meyer [52] have suggested that antioxidants which exhibit apolar properties are most important because they are concentrated in the lipid-water interface, thereby preventing the formation of lipid radicals and β-carotene oxidation. While polar antioxidants are diluted in the aqueous phase and are thus less effective in protecting lipids. It was found that ethanol extracts constituting of higher amount of lipophilic fraction showed higher antioxidative activity than aqueous extracts, constituting of higher amount of hydrophilic fraction[52]

Ferric reducing ability of plasma assay (FRAP)

Compounds with reducing power indicate that they are electron donors and can reduce the oxidized intermediates of lipid peroxidation processes, so that they can act as primary and secondary antioxidants[53]. The FRAP assay was developed to determine the ferric reducing ability of biological fluids and aqueous solutions of pure compounds. The FRAP values calculated using the respective Fe (II) calibration curves. gallic acid, quercetin, rutin and ascorbic acid were used for comparison of ferric reducing ability of extracts used in the present study. The ferric reducing ability of antioxidants in the original method established at 4 min interval as suitable for such measurements, since the absorbance of the reduced ferrous-TPTZ complex was stable at this time[36]. When these conditions were used in the present study, we observed that the reduction of the ferric-TPTZ complex was not established after 4 min reaction time and most of the compounds showed a steady increase in the absorbance with time, therefore the reaction time

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was prolonged for several hours and the continuous increment of absorbance at 595 nm was determined in interval time. However 30 min was maintained as one of the best time showing the antioxidant efficiency of the studied samples, this was in agreement with Pulido et al.[54] studding the antioxidant effect of many pure polyphenols. The 4 min absorbance recording established in the original method was also keept for comparison results showed that some antioxidants even doubled their initial absorbance after 30 min of reaction, as was the case with the most standards and samples. The equivalent concentration 1 or EC_1, is the concentration of antioxidant having a ferric-TPTZ reducing ability equivalent to that of a 1 mmol / L concentration of $FeSO_4$, $7H_2O$ was measured . The higher EC1 value, expressed as μmol / L, the lower antioxidant activity. Figure.3 shows the EC_1 values at 4 and 30 min of the studied samples (AIE) and standards. EC_1 values, and therefore ferric reducing ability, were lower at longer reaction times as expected from the kinetic behaviour of the compounds at 4min. Either at 4min and 30 min the AIE exhibit a reducing power higher than all the used standards gallic acid , quercetin and ascorbic acid ($p \leq 0.01$)

It is probable that the antioxidant activity increased proportionally with the polyphenol content (a linear relationship between FRAP values and total polyphenol contents ($r^2 = 0.87$, $p \leq 0.05$).) agreeing with results obtained by Maksimovic´et al.[55] worket on various polyphenol classes in the silks of fifteen maize hybrids. These results in a different classification in terms of antioxidant efficiency of the studied compounds at short versus long reaction times, the quercetin showed the lowest EC1 values, followed by gallic and ascorbic acids, Ascorbic acid had a ferric reducing ability lower than those of most polyphenols[54]. In general, the classification of antioxidants according to their ferric reducing ability reported in this study agreed with results reported by other authors using different methods to estimate antioxidant power. Thus, Wang and Goodman[56] reported a decreased effect of polyphenols inhibiting peroxidation of LDL in the order quercetin > rutin > gallic acid.

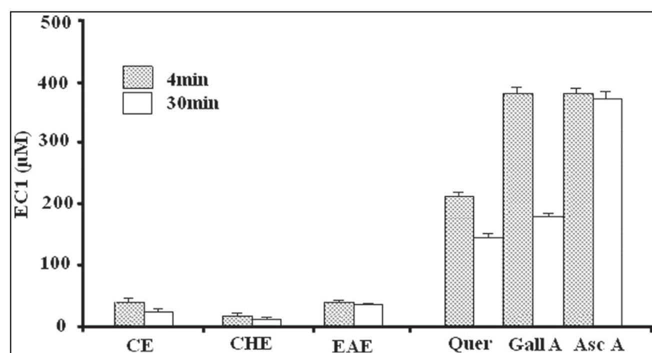

Figure 3. Comparison the EC_1 values of *Ajuga iva* extracts (CE: methanol extract; CHE: chloroform extract; EAE: ethyl acetate extract), with standards; quercetin, gallic acid, and ascorbic acid. Data are the mean values ± SD, n=3. EC_1: concentration of antioxidant having a ferric reducing ability equivalent to that of 1 mmol/L $FeSO_4$,$7H_2O$.

Ferrous ion chelating activity

The xanthine/xanthine oxidase system and other system are known to generate $O_2^{\bullet-}$ and H_2O_2. There is some evidence that $O_2^{\bullet-}$ alone formed extracellularly does not cause cell death. However, its contribution to a metal-ion catalysed Haber-Weiss reaction, by reducing transition metal ions and thereby accelerating the cycle of Fenton-type reaction in which the reduced ions are oxidized by H_2O_2 generating $HO^{\cdot}$ radicals the most powerful radical involved cytotoxic reactions which can directly oxidize macromolecules including DNA, protein, and lipids[57], so the chelating of metal-ions lead to stop the Fenton-type reaction thereby the reduction of $HO^{\cdot}$ production. Further, extracts or compounds with chelating activity are believed to inhibit lipid peroxidation by stabilizing transition metals[58]. Ferrozine can quantitatively form complexes with metal-ion Fe^{2+}. In the presence of other chelating agents, the complex formation is disrupted and as a result that the red color of the complex is decreased. Measurement of the rate of color reduction therefore allows the estimation of the chelating activity of the coexisting chelator[37]. In this study AIE and EDTA showed chelating activity as demonstrated by their effectiveness in inhibiting the formation of Fe^{2+}-ferrozine complex , the absorbance of this complex was dose dependently decreased[59]. In this study, both AIE extracts and EDTA showed chelating activity as demonstrated by their effectiveness in inhibiting the formation of ferrous and ferrozine complex. The absorbance of Fe^{2+}-ferrozine complex was dose dependently decreased. In general the iron chelating ability of AIE was not far from EDTA. The CE showed an excellent chelating ability with IC_{50} of 6.171± 0,053 µM quercetin equivalent, lower than that of EDTA (IC_{50} = 0,563 ± 0,053µM) with approximately10 folds ($p \leq 0.01$). However CHE and CE were less effective than CE by 7 and 9 folds respectively ($p \leq 0.01$)

Results revealed a poor correlation between Metal chelating capacity and phenolic content in different extracts ($P > 0.05$), indicating that polyphenols might not be the main ion chelators suggested that partly responsible for the antioxidant activities. These results

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Table 1. Total polyphenols and flavonoid contents of *Ajuga iva* root extracts Value are expressed as mean ± SEM, n = 5

Extracts	% yield (W/W)	Total polyphenol and Flavonoids	
		mg Equivalent gallic acid/glyophilisate	mg Eqwuivalent quercetin/glyophilisate
Crud	12.163±1.471	20.125±2.257	10.248±1.079
Chloroform	1.253±0.370	30.46±3.724	18.42±1.936
Ethyl acetat	0.897±0.117	42±1.679	12.380±0.873

were comparable to those of Zhao et al[60] working on typical malting barley varieties.

The results reveal that CE proved to be better as metal-chelating ability than CHE and EAE. This may be explained by the interaction of the different flavonoids[61], and/or other compound present in the extracts which are in transition metals[62]. It is reported that the terpenoids and flavonoids having glycosidic linkage are likely to be extracted into aqueous extracts[63]. The compounds containing nitrogen are generally more powerful chelating than phenolic compounds[64]. Moreover, a sample rich in compounds phenolic could not chelate transition metals if the polyphenols don't have the necessary functional groups for chelating activity[62].

In conclusion the total antioxidant activities of AIE were measured by many different methods. Results showed a good correlation between values obtained from different assays and methods. In general a positive and significant correlation existed between antioxidant activity and total phenolic content revealed that phenolic compounds were the dominant antioxidant. Previous reports noted that polyphenolics are associated with antioxidant activity and play an important role in stabilizing lipid peroxidantion. The present study confirms that extract from *Ajuga iva* represent a significant source of phenolic compounds (Table 1); value varied from 20.125 ± 2.257 to 42. ± 1.679 mg equivalent gallic acid/g lyophilisate and from 10.248 ± 1.079 to 18.42 ± 1.936 mg equivalent quercetin/g lyophilisate for polyphenols and flavonoids, respectively. The highest level of polyphenols and flavonoids were recorded in CHE followed by EAE.

Flavonoids stabilize the reactive oxygen species by reacting with the reactive compound of the radical. Because of the high reactivity of the hydroxyl group of the flavonoids, radicals are made inactive[65]. Brand-Williams et al.[66] found that gallic acid was the most effective polyphenol scavenging the DPPH radical, followed by vitamin C. Finally, Rice-Evans et al.[50] using the TEAC method observed a decreased efficiency of polyphenols toward the scavenging of the $ABTS^+$ radical in the order quercetin > rutin > ascorbic acid. The observed activities could be due to one or more of the compounds found in *Ajuga iva* L. such as 8-O-acetyl harpagide, ajugarine, apigenin-7-O neohesperidoside, barpagide, caffeine, clorogenes, cyasterone, diglycerides, 14,15-dihydroajugapitin, ecdysones, ecdysterones, flavonoids, iridoides, makisterone A, naringin, neohesperidoside, phenylcarboxylic acids, tannin polyphenols [66];[67]. It was reported that the *Ajuga iva* extract decreases plasma cholesterol and triglycerides rats[24]. In addition, no apparent toxicity was observed for this plant[21]. In the light of pharmacological and toxicological studies, *Ajuga iva* L. appears to be a valuable plant and could be used to treat conditions where inhibition and free-radicals scavenging of XO is warranted. Finally, our results further support the view that some medicinal plants are promising sources of natural antioxidants.

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