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Original Article

ANTIOXIDANT, ANTI-INFLAMMATORY AND ANTI-ACETYLCHOLINESTERASE ACTIVITIES OF LEAF, FLOWER AND SEED AQUEOUS EXTRACTS OF LAWSONIA INERMIS FROM TUNISIA

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ABSTRACT

Objective: To evaluate the antimicrobial, anti-inflammatory and anti-acetylcholinesterase capacities and ability for scavenging free radicals, and preventing lipid peroxidation of aqueous extracts obtained from different parts (leaf, flower and seed) of *Lawsonia inermis* Linn. (henna) collected in Tunisia.

Methods: The antioxidant activities of aqueous extracts obtained by decoction were evaluated through several methods: capacity for scavenging free radicals (ABTS, DPPH, hydroxyl, superoxide, nitric oxide), total antioxidant capacity by ammonium molybdate reduction method, chelating metal iond and inhibition of lipid peroxidation of lecithin liposomes. The anti-inflammatory activity was evaluated through the hyaluronidase inhibitory activity. Acetylcholinesterase inhibition was also evaluated. Antimicrobial activities were evaluated using the agar diffusion technique.

Results: Seeds had the best capacity for scavenging DPPH and ABTS free radicals, preventing lipid peroxidation and chelating iron metal ions, whereas leaves had the best capacity for scavenging hydroxyl and nitric oxide radicals. Leaf and seed extracts inhibited better hyaluronidase than flower ones. A negative correlation was found between IC_{50} values found for the capacity for scavenging superoxide, DPPH and ABTS and the concentration of phenols. A negative correlation was also observed between the IC_{50} values and inhibition of hyaluronidase. Only seed extracts showed a slight activity against *S. aureus* ATCC 6538, MRSA 12 and *P. aeruginosa*, but only at the highest volume.

Conclusion: Different parts of the plant (leaf, flowers, seeds) possessed diverse properties which may be correlated with the presence of different secondary compounds.

Keywords: Radical scavenging ability, Lipid peroxidation prevention, Acetylcholinesterase, Hyaluronidase, Henna.

INTRODUCTION

Lawsonia inermis Linn. (syn. *Lawsonia alba*, Lythraceae) known as henna is a glabrous, much branched shrub or small tree with greyish-brown bark. Leaves are opposite, sub-sessile, elliptic or broadly lanceolate, entire, acute or obtuse, 2-3 cm long and 1-2 cm wide. Flowers are small, about 1 cm across, numerous, white or rose coloured with four crumbled petals, and fragrant. Fruit is a small brown coloured round capsule opening irregularly and is many seeded. Seeds are about 3 mm across, smooth, pyramidal, hard and thick seed coat with brownish colour [1-3]. This plant is native of North Africa and South-West Asia and nowadays is widely cultivated in India, Middle East and along the African coasts of the Mediterranean Sea [2,3].

From the aerial parts of *L. inermis* have been extracted and identified naphthoquinones derivatives (e. g. lawsone), coumarines (e. g. 4-hydroxy coumarine), phenolic compounds (e.g. lawsoniaside A and B, lalioside) flavonoids (e.g. apigenin-7-glucoside, apigenin-4-glucoside, lawsochrysin), triterpenoids (e. g. lawsonic acid and lawsonin), essential oils (e. g. *Z*-2-hexenol, linalool, α-ionone, β-ionone, α-terpineol, terpinolene) [4-12]. Proteins, carbohydrates, fibres and fatty acids (behenic, arachidic, stearic, palmitic, oleic and linoleic acids), 8-octadecenoic acid methyl ester, pentadecanoic acid-6-oxo methyl ester are some of the constituents described for seeds of *L. inermis* [2,13] however; their secondary metabolites have been rarely reported [14]. Several attributes have been reported to *L. inermis* and its use depends on the region. For example, in Vietnam is used in traditional medicine to treat menstrual disorder, oedema, rheumatism, bronchitis, and haemorrhoids [11]. In Morocco, the infusion of leaves of *L. inermis* is used against diarrhoea, renal lithiase and gastric pains [15]. In India, henna leaves, flowers, seeds, stem bark and roots are used in traditional medicine to treat rheumatoid arthritis, headache, ulcers, diarrheoa, leprosy, fever, leucorrhoea, diabetes, cardiac disease, and as hepatoprotective [2]. Nevertheless and due to the presence of lawsone in leaves, henna has been used from ancient times for staining hands and as hair dye [3].

Research regarding the biological properties of henna extracts has demonstrated that they are antibacterial, antifungal, macrophage-stimulating, hepatoprotective, analgesic, anti-inflammatory, anti-complementary, cytotoxic, antioxidant, immunomodulatory, protein glycation inhibitory, inhibitor of osteoclastogenesis and moderate inhibition of urease activity [12,14,16-23].

For extracting bioactive compounds from plant material several solvents may be used: alcohols, chloroform, ethyl acetate and dimethyl sulfoxide (DMSO). However, the presence of some of these solvents even in trace amounts is harmful, and is not allowed by law [24]. In addition, the utilization of organic solvents may be enemy of the environment. The utilization of plants in folk medicine is many times under aqueous or hydro-alcoholic extracts after heating for some minutes or longer periods of time. In this way, in folk medicine the medicinal properties reported to plants must be priority attributed to the compounds with higher affinity for water. For example, the infusion of leaves of *L. inermis* is used against diarrhoea, renal lithiase and gastric pains as reported above [15].

For the reasons reported above, in the present work, the extraction of secondary metabolites from leaves, flowers, and seeds of *L. inermis* was performed using water as extraction solvent and decoction as extraction method. After this extraction, the main goal of the present work was to compare the antioxidant activities of the extracts obtained from the different parts of the plant. This ability was already reported but in distinct works: leaves [3,9,12,24] and seeds [22,25]. It is well known that in the majority of cases it is difficult to compare results of antioxidant activities since the

methodologies change from laboratory to laboratory as well as the way to present results is sometimes diverse. With the present work, such does not occur, because the same methodologies and presentation of results are done for all type of extracts.

At the same time the capacity of diverse extracts for inhibiting hyaluronidase was also checked. Enzyme hyaluronidase degrades hyaluronic acid by lowering its viscosity. Hyaluronic acid not only contributes in the destruction of extracellular matrix but also plays an important role in many biological and physiological processes such as embryogenesis, angiogenesis, and inflammation, among others [26].

The enzyme acetylcholinesterase catalyses the hydrolysis of the ester bound of acetylcholine at the cholinergic synapses, ending the transmission of nerve impulses. Acetylcholinergic inhibitors enhance cholinergic function in the brain being such inhibitors used in the treatment of Alzheimer's disease because these patients often have cholinergic deficits in association with the disease. This disease is characterized by loss or decline in memory and cognitive impairment, responsible for dementia in the elderly. The benefits of cholinesterase inhibitors are also registered with patients with other types of dementia such as vascular dementia and dementia with Lewy bodies [27].

Several acetylcholinesterase inhibitors such as tacrine, donepzil, rivastigmine and galanthamine are used in the treatment of symptoms of mild to moderate Alzheimer disease. Nevertheless some adverse effects have been reported when these drugs are used such as anorexia, diarrhoea, fatigue, nausea, muscle cramps as well as gastrointestinal, cardiorespiratory, genitourinary and sleep disturbances are some of the side effects of those drugs [28]. Cheaper and safer acetylcholinesterase inhibitors with higher efficacy, bioavailability and fewer side effects, particularly from natural sources, have been extensively investigated [29].

The capacity for preventing oxidation and inflammation measured through several methods; as well as the capacity for inhibiting acetylcholinesterase of aqueous extracts of seeds, flowers and leaves of *L. inermis* was surveyed and related with the total phenols and flavonoids of the same extracts.

MATERIALS AND METHODS

Extraction of bioactive compounds

Extraction of bioactive compounds was performed by decoction of 5 g of dried plant material (leaves, flowers and seeds) for 3 h in 100 mL of distilled water. After this operation, the samples were centrifuged for 5 min, at 2000 g at 20ºC and the supernatant was removed and kept at -20ºC until determination of total phenols and antioxidant activities.

Determination of total phenols (Folin-Ciocalteau)

The total phenol content of the extracts was determined using the Folin-Ciocalteau reagent and Gallic acid as standard as described by Slinkard and Singleton (1977) [30]. The extract sample (0.5 mL) and 2 mL of sodium carbonate (75 g/L) were added to 2.5mL of 10% (v/v) Folin-Ciocalteau reagent. After 30 min of reaction at room temperature, the absorbance was measured at 765 nm in a Shimadzu 160-UVspectrophotometer. Tests were carried out in triplicate.

Determination of total flavonoids

Flavonoid content was quantified as described by Ahn et al. (2007) [31]. Briefly, 0.5 mL of 2% $AlCl_3$-ethanol solution was added to 0.5 mL of sample or standard. After 1 h at room temperature, the absorbance was measured at 420 nm. Quercetin was used as standard for the construction of calibration curve.

Reductive potential

Each sample or standard (ascorbic acid) was mixed with phosphate buffer (2.5 mL, 0.2 M, pH 6.6) and potassium ferricyanide [K3Fe(CN)6] (2.5 mL, 1%). The mixture was incubated at 50 °C for 20 min. A portion (2.5 mL) of trichloroacetic acid (10%) was added to the mixture, which was then centrifuged for 10 min at 3,000 rpm. The upper layer of solution (2.5 mL) was mixed with distilled water (2.5 mL) and FeCl3 (0.5 mL, 0.1%), and the absorbance was measured at 700 nm.

Determination of ABTS radical cation scavenging capacity

The ABTS radical cation decolorization assay was carried out using the method reported by Re et al. (1999) [32] and Ling et al. (2009) [33] with slight modifications. ABTS radical cation ($ABTS^{\cdot+}$) was produced by reacting 7 mM stock solution of ABTS with 2.45 mM potassium persulfate (final concentration) and allowing the mixture to stand in the dark at room temperature for 12–16 h before use. The $ABTS^{\cdot+}$ solution was diluted with ethanol, to an absorbance of 0.7 ± 0.02 at 734 nm.

An ethanolic solution (10 µL) of the samples at various concentrations was mixed with 990 µL diluted $ABTS^{\cdot+}$ solution. After reaction at room temperature for 6 min, the absorbance at 734 nm was measured. Lower absorbance of the reaction mixture indicates higher $ABTS^{\cdot+}$ scavenging activity. The capability to scavenge the $ABTS^{\cdot+}$ was calculated using the formula given below $ABTS^{\cdot+}$ scavenging activity (%) = $[(A_0-A_1)/ A_0] \times 100$ (%) where A_0 is the absorbance of the control (without sample) and A_1 is the absorbance in the presence of the sample. BHT was used as positive control.

Free radical scavenging activity (DPPH)

A methanolic stock solution (50 µL) of each sample at different concentrations was placed in a cuvette, and 2 mL of 60 µM methanolic solution of DPPH (2,2-diphenyl-1-picrylhydrazyl) was added [34]. Absorbance measurements were made at 517 nm using a Shimadzu 160-UV spectrophotometer (Tokyo, Japan) after 60 min of reaction at room temperature. The values of IC_{50} were determined as reported above. Tests were carried out in triplicate. BHT was used as positive control.

Hydroxyl radical scavenging activity

The assay of OH-scavenging activity was developed according to Chung et al. (1997) [35] with small modifications. Briefly, the reaction mixture was prepared with 10 mM $FeSO_4.7H_2O$, 10 mM EDTA, 10 mM 2-deoxyribose, 0.1 M phosphate buffer and sample at different concentrations in a test tube to give a total volume of 1.8 mL. Finally, 200 µL of H_2O_2 was added to the mixture, which was incubated at 37°C for 4 h.

After that, 1 mL trichloroacetic acid (2.8%) and 1 mL thiobarbituric acid (1%) were added to the test tube, which was boiled for 10 min to develop the pink coloured malondialdehyde–thiobarbituric acid: MDA–TBA_2 adduct. After cooling, its absorbance was measured at 520 nm in a Shimadzu 160-UV spectrophotometer. The OH-scavenging activity (%) was calculated using the following equation: Inhibition = $[(A_0-A_1)/ A_0] \times 100$ (%) where A_0 is the absorbance of the control (without sample) and A_1 is the absorbance in the presence of the sample.

Tests were carried out in triplicate. The sample concentration providing 50% inhibition (IC_{50}) was obtained by plotting the inhibition percentage against essential oils concentrations. Mannitol was used as positive control.

Superoxide anion scavenging activity (non-enzymatic method)

Measurements of superoxide anion scavenging activity of samples and positive control were based on the method described by Soares (1996) [36]. Superoxide anions were generated in a non-enzymatic phenazine methosulfatenicotinamide adenine dinucleotide (PMS-NADH) system by oxidation of NADH and assayed by reduction of nitroblue tetrazolium (NBT).

Tests were carried out in triplicate. The sample concentration providing 50% inhibition (IC_{50}) was obtained by plotting the inhibition percentage against extract concentrations. Ascorbic acid was used as positive control.

Total antioxidant capacity by ammonium molybdate reduction method

Total antioxidant capacity was measured in different concentrations of extracts [37]. These extracts were mixed with 3 mL of reagent solution (0.6M sulphuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). After 90 minutes incubation at 95ºC, the samples were

cooled at room temperature and their absorbances were measured at 695 nm in a Shimadzu 160-UV spectrophotometer. The antioxidant activity was expressed as the number of equivalents of ascorbic acid. Tests were carried out in triplicate.

Nitric oxide scavenging capacity

The nitric oxide (NO) scavenging activity of samples was measured according to the method described by Ho et al. (2010) [38]. In this method 50 µL of serially diluted samples were added to 50 µL of 10 mM sodium nitroprusside in phosphate buffer saline (PBS) into a 96-well plate and the plate was incubated at room temperature for 90 min. Finally, an equal volume of Griess reagent was added to each well and the absorbance was read at 546 nm. Several concentrations of samples were made and the percentage inhibition calculated from the formula:

$[1 - (A_{sample} - A_{sample\ blank})/(A_{control} - A_{control\ blank})] * 100$, where $(A_{sample} - A_{sample\ blank})$ is the difference in the absorbance of a sample, with or without 10 mM sodium nitroprusside, and $(A_{control} - A_{control\ blank})$ is the difference in the absorbance of the PBS control, with or without 10 mM sodium nitroprusside. The sample concentration providing 50% inhibition (IC_{50}) was obtained by plotting the inhibition percentage against sample concentrations. Rutin was used as positive control.

Chelating metal ions

The degree of chelating of ferrous ions by essential oils and their major components was evaluated according to Miguel et al. (2010) [39]. Briefly, samples were incubated with 0.05 mL of $FeCl_2$, $4H_2O$ (2 mM). The addition of 0.2 mL of 5 mM ferrozine initiated the reaction, and after 10 min, the absorbance at 562 nm was measured. An untreated sample served as the control. The percentage of chelating ability was determined according to the following formula: $[(A_0 - A_1)/A_0 \times 100]$, in which A_0 is the absorbance of the control and A_1 the absorbance of sample. The values of IC_{50} were determined as reported above. EDTA was used as positive control.

Inhibition of lipid peroxidation of lecithin liposomes

Lipid peroxidation (LP) was measured according to the method described by Mimica-Dukic et al. (2004) [40]. Liposomes were obtained from 0.4 g lecithin in 80 mL chloroform. This solution was dried under vacuum in a rotary evaporator (<50°C) to yield a thin, homogenous film and after submitted to nitrogen flux for 30 s. Liposomes were then submitted to vacuum for at least two hours until complete dryness. The film was then dispersed in eighty mL of phosphate saline buffer 0.01 M, pH 7.0. The mixture was sonicated to obtain a homogeneous suspension of liposome and kept at 4ºC until the assay.

The extent of LP was determined by measuring the color of the adduct produced in the reaction between TBA and malondialdehyde (MDA), as an oxidation product in the peroxidation of membrane lipids, by the TBA assay [41].

Different concentration of samples was prepared in *n*-hexane. A control with *n*-hexane instead of sample was also analyzed for system of induction of LP. The reaction mixture Fe^{2+}/ascorbate-induced LP, 60 µL of a suspension of liposomes was incubated with 20 µL of 0.01 M FeSO4, 20 µL of 0.01 M ascorbic acid, and 10 µL of samples in 2.89 mL of 0.05 M KH_2PO_4-K_2HPO_4 buffer, pH 7.4 (3 mL final solution). Samples were incubated at 37ºC for 1 h. LP was terminated using the reaction with 1.5 mL of TBA reagent and 0.2 mL of 0.1 M EDTA, heating at 100ºC for 20 min. After the solution was cooled and the precipitated proteins were centrifuged (4,000 rpm for 10 min), the content of the MDA was determined by measuring the absorbance of the adduct at 532 nm. All of the reactions were carried out in triplicate. The percentage of LP inhibition was calculated by the following equation: $I\ (\%) = (A_0-A_1)/A_0 \times 100$, where A_0 was the absorbance of the control reaction (full reaction, without the test compound) and A_1 was the absorbance in the presence of the inhibitor.

Acetylcholinesterase inhibitory activity

Acetylcholinesterase degrades the substrate acetylcholine in acetic acid and thiocholine which interacts with the 5,5'-dithiobis-2-nitrobenzoic acid (DTNB). The accumulation of 5-thio-2-nitrobenzoic acid is measured at 405 nm. The acetylcholinesterase inhibition assay was adapted from that described by Mata et al. (2007) [42]. Sample (25 µL), 425 µL Tris-HCl buffer (0.1 M, pH 8) and 25 µL enzyme (0.28 U/mL) were added before the mixture is agitated and incubated for 15 min at room temperature. After this period, 75 µL of substrate (0.005 g of iodine acetylcholine in 10 mL of buffer) and 475 µL of DTNB (0.059 g in 50 mL of buffer) were then added. The absorbance was read after 30 min and compared with that of control. The percentage inhibition of the enzyme was calculated and the IC_{50} were compared. Tests were carried out in triplicate.

Hyaluronidase inhibitory activity

Hyaluronidase inhibition was determined by measuring the amount of *N*-acetylglucosamine splited from sodium hyaluronate described by Sahasrabudhe and Deodhar (2010) [26]. Fifty microliter of bovine hyaluronidase (7900 units/mL) dissolved in acetate buffer 0.1 M (pH 3.6) was mixed with 50 µL of designated concentrations of the aqueous extracts and was incubated for 20 min at 37 ºC. After 20 min 50 µL of calcium chloride (12.5 mM) was added to the reaction mixture and again incubated for 20 min at 37 ºC. This Ca^{2+} activated hyaluronidase was treated with 250 µL sodium hyaluronate (1.2 mg/mL) and incubated for 20 min and incubated at 37 ºC for 40 min. After incubation 50 µL of 0.4 M sodium hydroxide and 100 µL 0f 0.2 M sodium borate were added to the reaction mixture and then incubated in the boiling water bath for 3 min. after cooling to room temperature 1.5 mL of PDMAB (*p*-dimethyl amino benzaldehyde) solution (4g PDMAB in dissolved in 50 mL of 10N HCl and 350 mL of glacial acetic acid) was added to the reaction mixture . The absorbance was measured at 585 nm in UV spectrophotometer. The percentage inhibition of the enzyme was calculated and the IC_{50} were compared. Tests were carried out in triplicate.

Antibacterial activity

The leaves, flower and seed extracts were tested for their antibacterial activity against *Staphylococcus aureus* ATCC 6538, methicillin resistant *S. aureus* 12 (MRSA 12) and also against the Gram negative bacterium *Pseudomonas aeruginosa* DSM 939 using the agar diffusion technique. The Brain Heart Infusion agar (Oxoid) was used to grow *S. aureus* and also to test the extracts. The Mueller-Hinton medium (Oxoid) was used to grow *P. aeruginosa* and to test the extracts. Each extract was tested using 10, 20 and 30 µL. The inoculated plates were incubated at 37ºC during 24-48h and after that the inhibition zones were measured.

Statistical analysis

Statistical analysis was performed with the SPSS 18.0 software (SPSS Inc.). Statistical comparisons were made with one-way ANOVA followed by Tukey multiple comparisons. The level of significance was set at $P<0.05$. Paired Student t test were used in some tests to determine differences at 95% significance. Correlations between phenol content and antioxidant activity were achieved by Pearson correlation coefficient (r) at a significance level of 99% ($P<0.01$).

RESULTS AND DISCUSSION

The antioxidant properties of different plant extracts can be evaluated using diverse *in vitro* assays. Antioxidant assays in foods and biological systems can be divided in two groups: 1. Those that evaluate lipid peroxidation. 2. Those that measure free radical scavenging ability [43]. In addition, tests evaluating effectiveness against several reactive oxygen species and nitrogen reactive species ($O_2^{\bullet-}$, $HO^{\bullet}$, $NO^{\bullet}$, $ONOO^-$, H_2O_2) are also needed and generally performed [44]. For this reason, in the present work there is one assay in which the capacity for preventing lipid peroxidation was evaluated using lecithin liposomes as lipidic substrate; and the capacity for scavenging free radicals was also followed.

The antioxidant activities in the present work were presented as IC_{50} values, corresponding to the amount of extract required to scavenge 50% of free radicals present in the reaction mixture or required to prevent lecithin liposomes peroxidation. High IC_{50} values indicate low antioxidant activity. The sample seeds of *L. inermis* presented

the best capacity for scavenging the free radicals DPPH and ABTS free radicals. Nevertheless, the best capacity for scavenging the hydroxyl and nitric oxide radicals was found in leaves samples (Table 1). Significant statistical differences between seeds and leaves extracts were not found in what concerns the superoxide scavenging ability (Table 1).

Table 1: Phenol and flavonoid contents and antioxidant, anti-inflammatory and anti-acetylcholinesterase activities of aqueous extracts of *L. inermis* (henna)

Sample/ Positive control	Phenol content (mg/)+	Flavo -noid content (mg/g)+	Superoxide*	DPPH*	TEAC*	Hydroxyl*	Acetylcholinesterase	Hyaluronidase*	Total antioxidant activity°	NO*	Chelating*	Liposomes
Seeds	56.81 ±1.76[a]	10.463± 0.141[a]	0.274± 0.134[bc]	0.024± 0.002[c]	0.0185± 0.001[c]	2.362± 0.149[a]	0.893±0.018[a]	0.182±0.106[b]	11.663± 0.352[a]	1.514± 0.050[a]	0.680± 1.500[c]	0.542± 0.010
Leaves	37.90 ±1.76[b]	3.222±0.141[c]	0.430± 0.134[b]	0.094± 0.002[b]	0.036±0.001[b]	0.442± 0.149[c]	0.749±0.018[b]	0.009±0.106[b]	7.623±0.352[b]	0.810± 0.050[c]	1.560± 1.500[b]	1.964± 0.014
Flowers	18.74 ±1.76[c]	6.285±0.141[b]	1.164± 0.134[a]	0.179± 0.002[a]	0.165±0.001[a]	1.067± 0.149[b]	-	0.948±0.106[a]	2.423±0.352[c]	1.038± 0.050[b]	8.307± 1.500[a]	-
Ascorbic acid	ND	ND	0.017± 0.134[c]	ND	ND	ND	ND	ND	ND	ND	ND	ND
BHT	ND	ND	ND	0.089± 0.002[b]	0.004±0.001[d]	ND	ND	ND	ND	ND	ND	ND
Mannitol	ND	ND	ND	ND	ND	0.001± 0.149[d]	ND	ND	ND	ND	ND	ND
Rutin	ND	ND	ND	ND	ND	ND	ND	ND	ND	0.055± 0.050[d]	ND	ND
EDTA	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	0.005± 1.500[d]	ND
Gallantamine	ND	ND	ND	ND	ND	ND	0.003±0.018[c]	ND	ND			

ND: *Not Determined*

*The values are presented as IC_{50}=mg/mL, °The values are given as equivalent ascorbic acid (mg/mL), + The values are presented as concentration (mg/g, dry weight). For phenol content as gallic acid equivalent (GAE); for flavonoid as quercetin equivalent (QE),-: without activity or very poor activity, Values in the same column followed by the same letter are not significant by the Tuckey's multiple range test ($P<0.05$). Data are the mean of three replicates.

The capacity of leaf extracts for scavenging DPPH free radicals was already reported by Hsouna et al. (2011) [3]. Different fractions obtained from several solvents had diverse antioxidant activities and the component responsible for such ability was isolated and identified by the authors as being the 1,2,4-trihydroxynaphthalene-1-*O*-β-*D*-glucopyranoside. Other authors also reported the antioxidant activity of leaf extracts of henna [23] evaluated through the DPPH method, but in this case 3,3',4',7-tetrahydroxy flavanone was the component with the highest activity. Comparing antioxidant activities of henna extract and pure lawsone with concentration similar to that present in henna extracts by the DPPH method, some authors [24] reported that the antioxidant activity was not only due to the naphthoquinone derivative but also to other polyphenolic compounds not identified by the authors.

The capacity for preventing lipid peroxidation was also reported by several authors [3,45,46] using peroxide value, thiobarbituric acid and β-carotene/linoleic acid model system. Prakash et al. (2007) [46] found that fruits possessed higher capacity for preventing lipid peroxidation than leaves or twigs. In our case, seeds had the best activity after using lecithin liposomes as lipidic substrate and the formation of malondialdehyde–thiobarbituric acid: MDA–TBA_2 adduct as method of measuring the antioxidant activity of samples (Table 1). Our results also show that aqueous extracts of henna were able to scavenge superoxide anion radicals, hydroxyl and nitric oxide radicals. In contrast to the capacity for scavenging DPPH and ABTS free radicals in which seed extracts possessed the best activity, leaf aqueous extracts were significantly better ($P<0.05$) for scavenging hydroxyl and nitric oxide free radicals than the remaining samples (Table 1). Nevertheless such activities were significantly lower ($P<0.05$) to that found for the positive controls (Table 1). Seed extract was the sole sample in which its capacity for scavenging DPPH free radicals was better than the BHT (positive control).

Antioxidant activity of seeds was already reported by some authors [22], either for scavenging free radicals or for preventing lipid peroxidation. In the same work, the authors also reported the reducing capacity of seed extracts. They showed that ethanol fraction was better antioxidant than the aqueous fraction. In addition, ethanolic fraction was even better as antioxidants than the positive control used. In our case, only in one test such was verified: DPPH method. Our samples were also able to prevent lipid peroxidation, nevertheless leaf essential oils were the most adequate for preventing such oxidation than seed aqueous extracts, although with lower amounts of phenols (Table 1).

Formation of reactive oxygen species is closely related to the redox state of transition metals. For example, superoxide anion is readily produced through the one-electron reduction of oxygen by ferrous ion. Hydrogen peroxide is converted to hydroxyl radical by the Fenton reaction, which requires reduced iron or copper.

In this way, the capacity for chelating metal ions may be of high importance because will prevent the formation of reactive oxygen species, although the metal chelation has been often considered of minor importance in the antioxidant activity [47].

In spite of that opinion, in the present work the chelating capacity was performed. Seed extracts had the highest ability for chelating iron metal ions, nevertheless significantly inferior to that of positive control (EDTA). The poorest activity was found for flower aqueous samples (Table 1). Polyphenols with galloyl or catechol groups are generally the most potent antioxidants because of the large iron-binding stability constants for these groups. Compounds with the hydroxyl-keto moiety can also chelate iron contributing to the antioxidant activity [48]. The identification of phenol compounds was not performed but such results may reveal the predominance of some groups of phenols in seed extracts which are responsible for the best activity found for this sample. All samples exhibited antioxidant activity in a concentration-dependent manner (data not shown). Such was also reported for Hsouna et al. (2011) [3] for different fractions of henna from Sfax (Tunisia). The aqueous extracts also had reducing ability as depicted in Fig. 1 in a concentration-dependent manner as reported for the above assays.

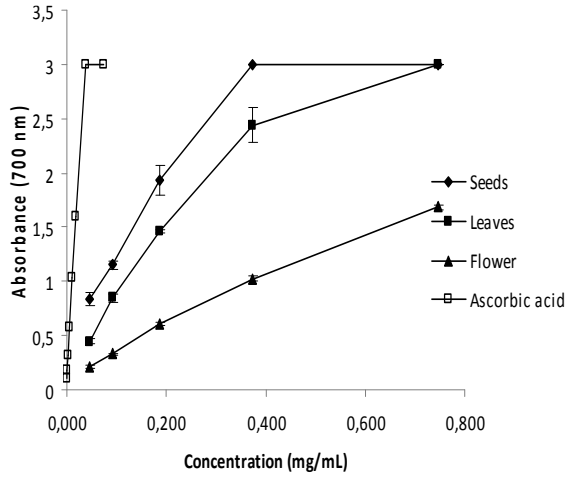

Fig. 1: Reducing power of aqueous extracts obtained from different parts of *L. inermis*.

The positive control (ascorbic acid) had significantly higher capacity, immediately followed by the seed extract, leaf and finally flower extracts (Fig. 1). Total antioxidant capacity measured through the ammonium molybdate reduction method also revealed better antioxidant activity of seeds in contrast to that of flower extracts (Fig. 2 and Table 1). Considering that the mechanism involved in this method is the reduction of the molybdate (VI) to molybdate (V) and in DPPH and TEAC methods are involved transfer of electrons to the antioxidant sample to the radicals, it was expectable a relationship between the methods as well as for the reducing power.

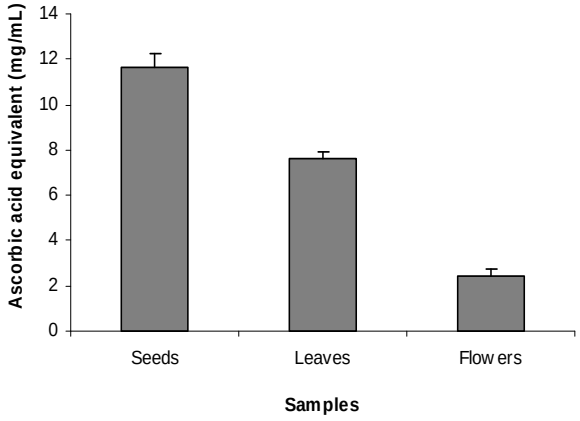

Fig. 2: Total antioxidant activity aqueous extracts obtained from different parts of *L. inermis*.

Generally antioxidant activities are associated to the phenol amount and composition of the sample [49]. In this way, the amount of phenols were determined and compared with the antioxidant activity. The amounts of total phenols depended greatly on the part of the plant used. Seeds had the highest concentration of phenols (56.81 mg GAE/g) in contrast to that of flower extract (18.74 mg/g) (Table 1). The amounts found are slightly inferior to those reported by Prakash et al. [46] and greatly inferior to that reported by Hsouna et al. (2011) [3], however, the type of extraction and solvent were different. The capacity for scavenging superoxide, DPPH and ABTS free radicals strongly correlated with the concentration of phenols. The Pearson correlation coefficients (*r*) showed a strong and negative relationship between the phenol content and the IC_{50} values found for the of superoxide, DPPH and ABTS scavenging ability (*r*=-0.880; *r*=-0.991 and *r*=0.912, p<0.01, respectively). Such negative correlation can also be seen in the Fig. 3-5. A negative correlation was also found between IC_{50} values of chelating activity and phenol amounts (r=-0.910, p<0.01; and Fig. 6). Nevertheless any correlation was found between the amounts of phenols and the capacity for scavenging NO and hydroxyl free radicals as well as for preventing lipid peroxidation. A positive correlation between total antioxidant activity and phenol content was observed (r=0.984, p<0.01) as can be seen in Fig. 7. Such results demonstrated the importance of phenols on this property.

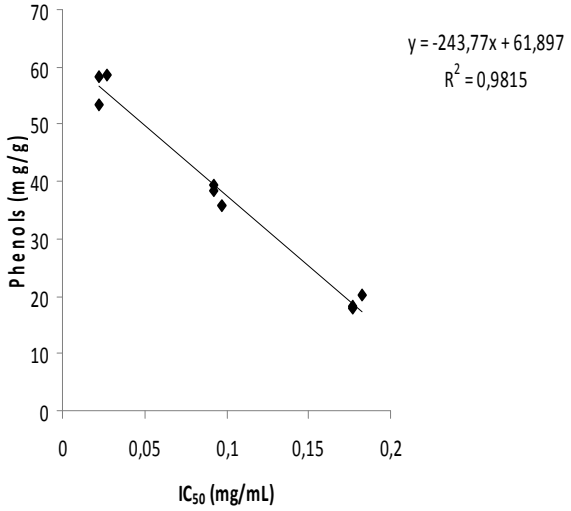

Fig. 3: Negative correlation between DPPH scavenging ability, expressed as IC_{50} and phenol content.

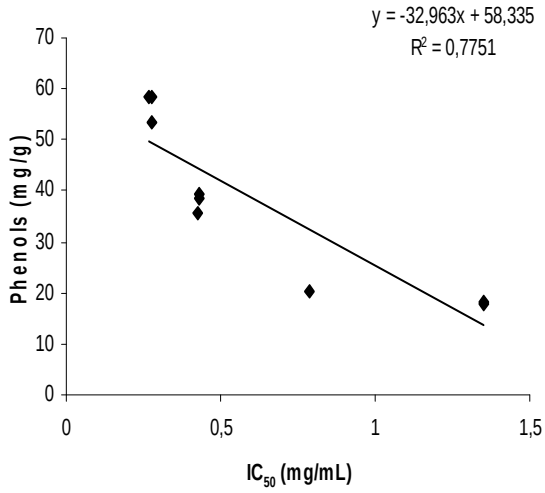

Fig. 4: Negative correlation between superoxide scavenging ability, expressed as IC_{50} and phenol content.

Flavonoids were also quantified and the highest concentration was found in seed extracts as reported for phenol content but the lowest amount was found in leaf extracts and not in flower ones as reported for phenols (Table 1). The antioxidant activity found in diverse extracts did not correlate with the flavonoid content (data not shown), as reported for total phenols. A positive correlation was only detected between NO, hydroxyl scavenging capacity and flavonoid content. Such was unexpected since it seems to reveal that the flavonoids present in the extracts are prooxidants. It has been already reported being such property widely dependent on the structure of the flavonoid [50].

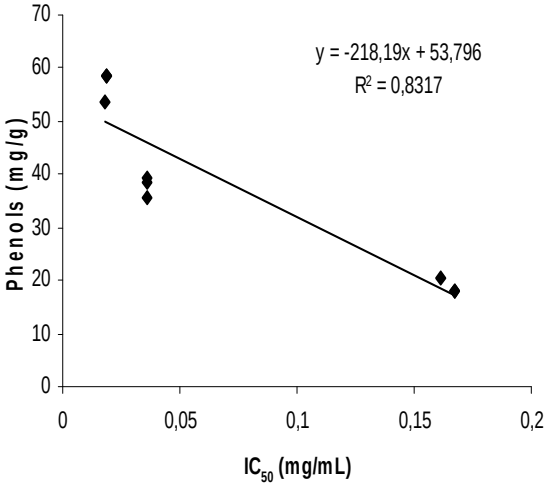

Fig. 5: Negative correlation between ABTS scavenging ability, expressed as IC_{50} and phenol content.

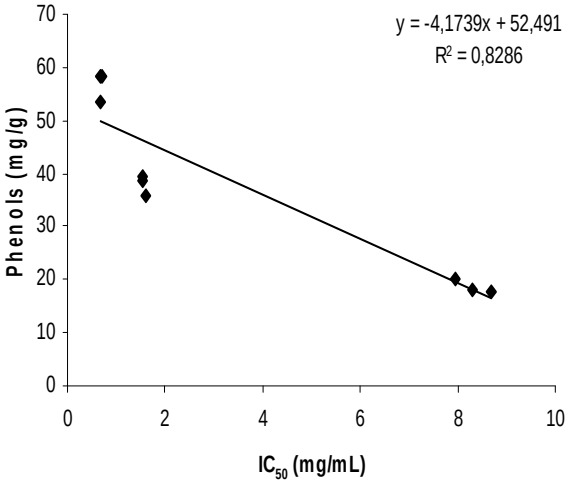

Fig. 6: Negative correlation between chelating activity, expressed as IC_{50} and phenol content.

In the present work, only the aqueous extracts of leaves and seeds have the capacity for inhibiting acetylcholinesterase, nevertheless much lower when compared to gallantamine (Table 1). Gholamhoseinian et al. (2009) [29] found that the percentage for inhibiting acetylcholinesterase of methanolic leaf extracts of henna was also only 8.6%. In contrast to that observed for antioxidant activity in which higher amounts of phenols conducted to better antioxidants activity, in what concerns the anti-acetylcholinesterase activity, such was not observed.

A positive correlation between the IC_{50} values and pehenol concentration was found (r=0.879; p<0.01) (Fig. 8). Higher amounts of phenols reduced the capacity for inhibiting the enzyme. Other components may be responsible for such activity. The type of phenols in each part of plant may also be different and therefore responsible for the activities found. Different phenols show distinct activities [51]. For example, these authors found that among several phenol acids and flavonoids, quercetin presented the best capacity for inhibiting the activity of acetylcholinesterase.

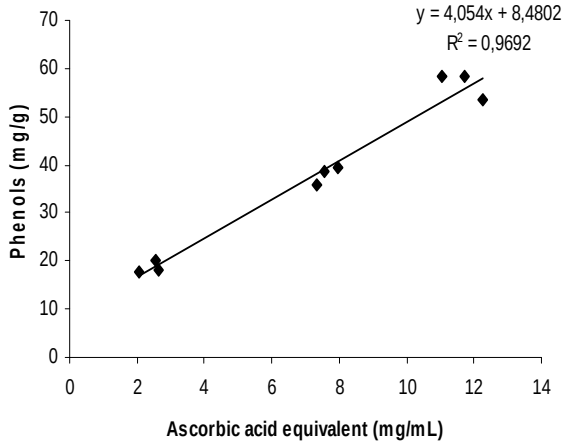

Fig. 7: Positive correlation between total antioxidant activity, expressed as equivalent ascorbic acid and phenol content.

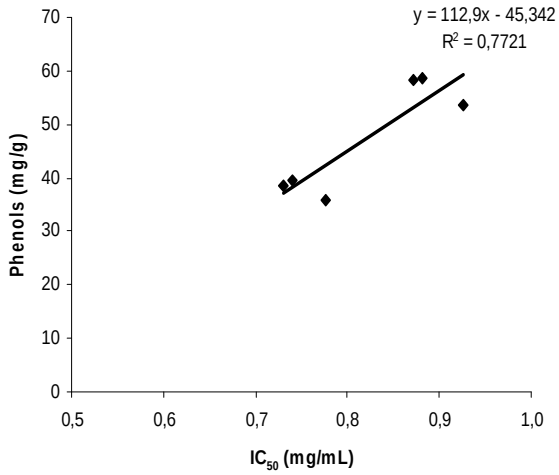

Fig. 8: Positive correlation between inhibition of acetylcholinesterase, expressed as IC_{50} and phenol content.

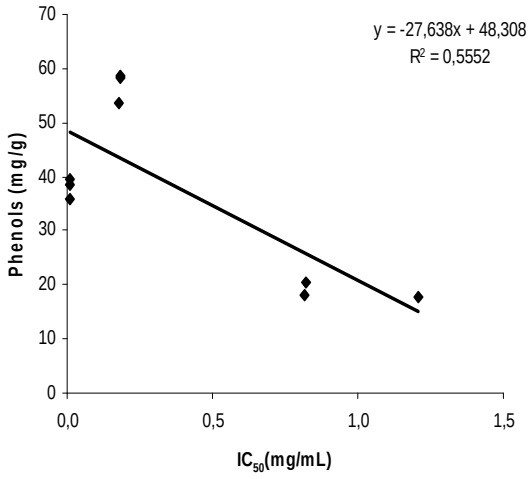

Fig. 9: Negative correlation between inhibition of hyaluronidase, expressed as IC_{50} and phenol content.

Leaf and seed extracts inhibited better hyaluronidase than flower ones (Table 1). A negative correlation (r=-0.745; $p<0.01$) was observed between the IC_{50} values and inhibition of hyaluronidase activity (Fig. 9), which indicates the importance of these compounds in the inhibition. Phenol and particularly flavonoids have been reported as good inhibitors of hyaluronidase [52-54].

The extract samples showed different antibacterial activities, namely the extracts from leaves and flowers showed no activity against the tested bacteria, even at the highest volume tested (30 µL). In contrast the extracts from seeds showed a slight activity but only at the highest volume, namely the inhibitory zones for *S. aureus* ATCC 6538, MRSA 12 and *P. aeruginosa* were 12.00±0.00 mm, 9.00±0.0 mm and 10.50±0.70 mm. These findings are in accordance with the previous reported by Kathem et al. (2008) [55] where water extracts of leaves of *L. inermis* did not showed antibacterial activity. The antibacterial activity observed in the seed extracts may be associated with the higher phenol and flavonoid content.

CONCLUSION

Seed and leaf extract showed higher capacity for scavenging free radicals and preventing lipid peroxidation. The best capacity for chelating iron ions was observed for seed extracts. Leaf extracts possessed the best capacity for inhibiting hyaluronidase and acetylcholinesterase.

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