Research Article

Evaluation of *In Vitro* Antioxidant and *In Vivo* Anti-inflammatory Potential of White Horehound (*Marrubium vulgare* L) Leaves

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ABSTRACT

The present study was designed to explore the anti-inflammatory effect and antioxidant potential of the methanolic extract of *Marrubium vulgare* leaves, a popular traditional medicinal herb. *In vivo* anti-inflammatory activity was evaluated by *the paw edema assay induced by Carrageenin* in rat model*,* while antioxidant activity was evaluated by two tests: *Assay of free radical-scavenging activity on DPPH and Ferric reducing antioxidant plasma (FRAP).* Total phenolic and total flavonoïd contents of the crude methanol extract were also determined by Folin–Ciocalteu's phenol reagent and by aluminium chloride method, respectively. The results showed that methanol extract had high level of polyphenols (195 ± 0.06 mg GAE/g extract) and flavonoïds (93.12 ± 0.17 mg QE/g extract). With regards to IC_{50} values (50% inhibitory concentration) of scavenging abilities of the DPPH radical, methanol extract (*MeOHE*) exhibited important antioxidant capacity with IC_{50} of 12.42 µg/ml and showing also a dose dependent manner ferric reducing capacity with a value equal 50.01±0.24 µg EAA/g of extract compared to ascorbic acid that was used as a witness in the trial. Assessment of anti-inflammatory activity showed that oral administration of MeOHE at a dose of 200 mg/kg in rats treated with carrageenin causes a significant decrease (87.3 ± 0.25%) of inflammation compared with standard diclofenac (positive control) which showed 85.52 ± 0.47% protection in this test. The analysis of C-reactive protein showed the absence of this protein in the plasma of rats treated with MeOHE of the plant. Phytochemical screening of MeOHE allowed the identification of several pharmaceutical drugs such as tannins/polyphenols, flavonoïds, terpenoïds, steroids and alkaloïds which may responsible for pharmacological properties. In addition, this study allows the identification of new flavonoïd: "Sophorin" ($C_{27}H_{30}O_{16}$) which revealed for the first time by *TL*-Chromatography on silica gel GF_{254}.

Keywords: *Marrubium vulgare L*, Lamiaceae, Pharmaceutical drugs, Antioxidant capacity, Anti-inflammatory activity.

INTRODUCTION

Nowadays, finding new therapeutic compounds from natural products for treatment and prevention of a variety of diseases is getting a great deal of attention. This approach would result in finding new drugs which are more effective and have fewer side effects than the conventional medicines. [1] The most important bioactive compounds of plants are alkaloïds, flavonoïds, tannins, glycosides and phenolic compounds. [2] These compounds possess numerous health-related effects such as antibacterial, antimutagenic, anticarcinogenic, antithrombotic and vasodilatory activities. [3]

Inflammatory diseases including different types of rheumatic diseases are very common throughout the World. Although, the rheumatism is one of the oldest known diseases of mankind and affects a large population of the world and no substantial progress has been made in achieving a permanent cure.[4] Non-steroidal anti-inflammatory drugs (NSAIDs) are used throughout the world for the treatment of inflammation, pain and fever. The use of NSAIDs, however, has not been therapeutically successful in all conditions of inflammation. Moreover, adverse effects associated with NSAIDs can lead to ulcers and hemorrhage.[4] Beside, oxidative stress has actually been described as a crucial etiological factor implicated in various human chronic diseases such as cancer, cardiovascular and neurodegenerative diseases, inflammation, diabetes *mellitus* and aging.[5] This oxidative damage is achieved through the attack of free radicals on various biomolecules, particularly proteins, lipids and DNA, resulting ultimately in cell degradation and death. Plant phenolics have been widely studied for their antioxidant properties since they are able to chelate metal ions involved in Reactive Oxygen Species (ROS) generation or scavenge free radicals and form stable intermediate structures, thus limiting free radical initiation or propagation.[6]

Marrubium vulgare (*Lamiaceae*)*,* known as horehound is a popular traditionally used herb in many countries as an antidiabetic and antihypertensive agent.[7, 8] *M. vulgare* is known for its remarkable diterpene content. Marrubiin and marrubenol are two important diterpenes from *M.*

vulgare, which have shown variety of activities. Marrubiin is reported to own analgesic, antidiabetic, antiplatelet, anticoagulant, antispasmodic, anti-hypertensive and antioedematogenic properties. [1, 9, 10] Marrubenol has shown a relaxant activity on rat-isolated aorta through blocking the *L*-type calcium channels.[8] Moreover, *M. vulgare* is characterized by the presence of a variety of compounds such as polyphenols, tannins, flavonoïds like ladanein, diterpenes, saponins and glycosidic phenylpropanoïd esters including (+) (*E*)-caffeoyl-L-malic acid, acteoside, forsythoside B, arenarioside and ballotetroside.[1, 11] The purpose of this study is to investigate *in vitro* antioxidant activity of MeOH crude extract of *M.vulgare* leaves based on their ability to scavenge non biological stable free radical (DPPH$^\bullet$), and to chelate metal ions by *FRAP assay*. *In vivo* anti-inflammatory activity was evaluated by *the paw edema assay induced by Carrageenin* in rat model.

MATERIALS AND METHODS

Chemicals

Gallic acid, quercetin and Folin-Ciocalteu reagent were purchased from "Sigma-Aldrich USA" and "Merck" (Germany) respectively. Potassium ferricyanide [$K_3Fe(CN)_6$], phosphate buffer, Sophorin, α-Tocopherol, ascorbic acid, aluminium chloride ($AlCl_3$), Trichloro acetic acid, 1,1-diphenyl-2-picrylhydrazyl "DPPH", Carrageenin, diclofenac, aspirin, methanol, Formaldehyde (CH_2O) and ferric chloride ($FeCl_3$) were purchased from «Sigma Aldrich CO., ST Lowis, Mo».

Plant material

The leaves of *M. vulgare* were collected from their natural habitat around "Touffana", Batna. This plant was identified previously by competent Mr. Hamchi, Park of Belezma, Department of Ecology Sciences, University Hadj Lakhdar of Batna "Algeria". After washing the leaves of the plant carefully to remove dust and sand, sample was left to dry for a period of 3 months in the shade under a cool temperature. Immediately, after the end of the drying period, leaves were crushed well to get a fine powder that put in special plastic bottles away from light and moisture to keep them from photo-oxidation until later use.

Animals

Animals used in these experiments (*Wistar* albino rats) are of both sexes male and female, weighing approximately (150-180 g). These rats are taken from the competent animal breeding Center located at the "Institute of Agricultural Sciences and Veterinary", which is supervised by Dr. Hachemi Massoud. During our measure of the anti-inflammatory effectiveness, rats were divided into homogeneous groups in terms of "weight", "sex", and placed in tightly closed cages to avoid the animals out with continuing to provide the food necessary and water as well as air under relative humidity (50-55%) and optimal temperature for life conditions (25) ° C where used for this purpose conditioners private coolers. It should be noted that all the tests applied to the rats completed in strict conditions within the limits of the laws and rules taken from the «Protection of Animal Protocols: Institutional animal ethics committee».

Preparation of methanol crude extract

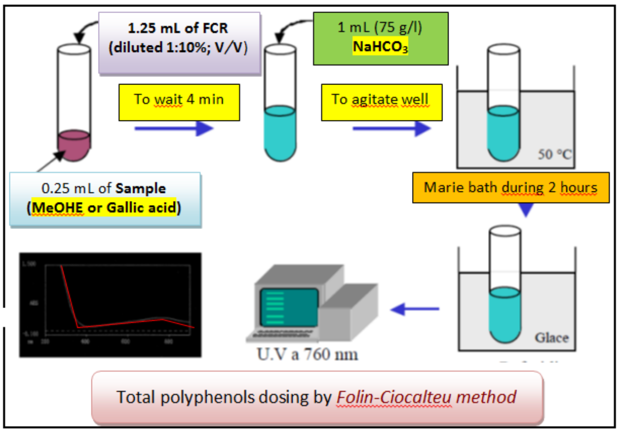
In order to obtain the methanol crude extract (MeOHE) of *M. vulgare*, amount of 500 g of plant leaves powder were macerated in a mixture component: water/methanol (20-80%; V/ V) respectively with final volume = 3L, for 72 hours at room temperature using a blender to mix reactants and accelerate the extraction procedure. Directly, after the expiry of this period filtrate obtained was concentrated and dissolved by using a special apparatus available in laboratory research called Rotavapor "Buchi type" to separate and disarm the organic solvent "methanol" under a temperature = 40° C rather than 50 ° C in order to avoid sabotage molecules effective in the filtrate, then the water layer is separated from the filtrate by lyophilization. Chlorophyll was removed by passing the filtrate through a solvent called "acetate of plumb". This step is crucial because the chlorophyll affect the results of the measurement of the antioxidant activity of this extract particular "*FRAP assay*" since chlorophyll interact with potassium ferricyanide [$K_3Fe(CN)_6$] (Hill detector), which ends returns chromate ferric trio "Fe^{3+}" to chromate ferrous Duo "Fe^{2+}". Finally, MeOHE crude extract saves in a sterile tube and placed in the refrigerator under the temperature of + 4 ° C.

Phytochemical Screening

The phytochemical screening of MeOH extract was performed using standard method. [12] Pharmaceutical drugs such as phenolic compounds, steroids and terpenoïds (*Lieberman-Burchard's test*), flavonoïds (*Shinoda test*), alkaloïds (Mayer reagent) and tannins (*$FeCl_3$ test*) were qualitatively analyzed.

Polyphenols dosing

The total polyphenols were estimated by the method described by Singleton *et al*. [13] Ranslation of the Folin-Ciocalteu reagent "FCR" causes a reduction of its colorimetric properties, thus, the total polyphenols content is determined by extrapolation on a standard curve obtained from a serial dilution in distilled water gallic acid (125 mg/L). In each test tube was added an aliquot (0.25 mL) of the test sample (extract or gallic acid), 1.25 mL of FCR (diluted 1:10%; V/V) and 1 mL (75 g/l) $NaHCO_3$. Blank was concomitantly prepared, containing 0.25 ml methanol, 1.25 ml Folin-Ciocalteu's reagent (10%) dissolved in water and 1 ml of 7.5% of $NaHCO_3$. After agitation, various solutions have been left to the dark place for 2 hours at 40°C. Absorbance was then measured at 765 nm using spectrophotometer (UV/Visible). The samples were prepared in triplicate for each analysis and the mean value of absorbance was obtained. The total phenolic content was expressed as mg of gallic acid equivalent per g of extract "mg GAE/g of extract".



Total flavonoïds content

Amount of 1 ml of each sample and standard (prepared in methanol) was added to 1 ml of the solution of "$AlCl_3$" (2% dissolved in methanol). After 10 minutes, the absorbance was measured at λmax = 430 nanometers against the reagent blank prepared. The concentrations of flavonoïds have been deduced from the range of the calibration curve established with quercetin (0-35 mg/ml). The results were expressed as milligrams of quercetin equivalents per g of extract "mg QE/g of extract". [14]

Antioxidant activity

DPPH radical scavenging ability

The DPPH (1, 1-diphenyl-2-picrylhydrazyl free radical) assay is an excellent *in vitro* method to investigate the free radical scavenging activity of an antioxidant. The method of Braca *et al.*[15] was used for determination of scavenging activity of DPPH free radical. Different methanolic dilutions of extract (5 µg/mL to 1 000 µg/mL) were mixed with equal volumes (1,95 ml) of freshly prepared DPPH methanol solution (0.0024%; w/v). The reaction mixture was vortexed thoroughly and then left to stand at room temperature in the dark for 30 min, the absorbance was read at λ=517 nm using a blank containing the same concentration of DPPH without extract. Gallic acid, Sophorin and Quercetin were taken as standards. Percentage of inhibition was calculated using the following equation:

$$\%\ \textbf{Inhibition of DPPH} = \frac{Absorbance\ of\ Control - Absorbance\ of\ Sample}{Absorbance\ of\ Control} * 100$$

The extract concentration providing 50% inhibition "IC_{50}" was calculated from the graph of scavenging effect percentage against extract concentration.

Ferric reducing antioxidant power (FRAP) assay

FRAP assay, is presented as a novel method for assessing ''antioxidant power.'' Ferric iron (Fe^{3+}) is initially reduced by electron-donating antioxidants present within the sample to its ferrous form (Fe^{2+}). The iron-colorimetric probe complex develops a dark blue color product upon reduction which can be measured at 540-600 nm. The FRAP of *M. vulgare* extract was determined in accordance with the protocol described in Chu *et al.* [16]. Amount of 2.5 ml of Potassium phosphate buffer (0.1 M, pH 6.6) as well as 2.5 ml of 1% (w/v) potassium ferricyanide were combined with 1.0 ml of *M. vulgare* alcoholic extract solution at various concentrations (50 - 500 µg/ml). The reaction was incubated at 50 °C for 20 min, then 2.5 ml of 10% (w/v) trichloroacetic acid was added. After that, water (2.5 ml) and 0.5 ml of 0.1% (w/v) $FeCl_3$ freshly prepared was added to 2.5 ml of the reaction mixture and incubated at 28°C for 30 min to facilitate color change. The absorbance was measured at 600 nm as a function of *M. vulgare* extract concentration (µg/ml) and compared with ascorbic acid (AA), α-tocopherol witch used as standards.

Anti-inflammatory activity

Searching for anti-inflammatory properties was conducted on the model of plantar edema induced in the rat by injection of a 1% suspension (100 µl) of carrageenin in the right leg; technical based on those described by Amezouar *et al.* [17]. The tested products were administered orally 1 hour before the injection of carrageenin. The rats were fasted for 16 hours prior to treatment and divided into four groups of five rats each. Group A witness received 0.9% NaCl (10 ml/kg bw) only, group B was treated with 200 mg/kg bw of methanol extract, rats of group C and D were treated with diclofenac (Dic) and Aspirin respectively, non-steroidal anti-inflammatory drugs of reference at a dose of 100 mg / kg bw. Evaluation of the edema was followed by recording the diameter of the inflamed paw 0, 1, 2, 3, 4 and 5 hours after injection of the phlogistic agent. For each treatment group, average diameters obtained in these surveys (Dt) were compared to that obtained before treatment (D0) and for calculating the percentage of edema (inflammation percentage) from the formula (Dt - D0) / D0 * 100. While, the percentage inhibition of edema was calculated from the formula:

$$[(Dt - D0)_{witness} - (Dt - D0)_{traited}] / (Dt - D0)_{witness} * 100$$ "Amezouar *et al.*[17]

To determine exactly whether the MeOH extract plant has an anti-inflammatory effect, rats were anesthetized immediately after the last diameter measurement using chloroform and then blood was collected from the eye in tubes containing anticoagulant (EDTA) which was centrifuged at 3000 rpm for 10 min to determine the level of C-reactive protein (CRP).

Statistical analysis

The values were expressed as "mean ± SD". Statistical analysis was performed by one way analysis of variance "ANOVA followed by "Tukey multiple comparison tests.

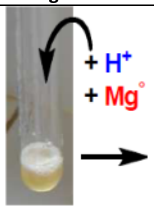
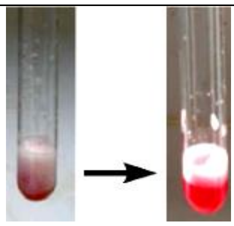
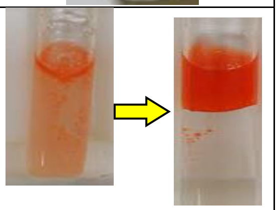
"*P* values < 0.05" were considered as significant while "*P* value < 0.0001" was considered as highly significant.

RESULTS AND DISCUSSION

Phytochemical Screening

The results of color interactions showed that methanol crude extract of *M. vulgare* contain significant amounts of biomolecules such as flavonoïds, where "*Shinoda* test" indicate the appearance of the red color caused by the oxidation-reduction reaction between the hydrogen liberated by the treatment of flavonoïds with hydrochloric acid under heating and Magnesium chips (Mg^{+}). Beside, "*Meyer reaction*" indicates the presence of alkaloïds where it appeared yellow precipitate distinctive in the tube, however, "*$Fecl_3$ test*" revealed the presence of gallic and catechic tannins with appearance of brown blackened color while, the reaction between MeOHE and acetate of sodium "*Lieberman-Burchard's test*" indicate the presence of steroids and terpenoïds with emergence of violet ring (Table 1).

To ensure that methanol crude extract of *M. vulgare* leaves has scavenging ability on free radical DPPH and to know some compounds that allow it to do this activity we have conducted a qualitative analysis based on the separation of compounds exists in the extract by Thin Layers Chromatography (TLC) on silica gel GF_{254}.

Pharmaceutical drugs & test used for Screening	Results	
	During treatment	After treatment
Flavonoïds (flavonols) « Shibata test»		
Gallic and catechic tannins "*$Fecl_3$ test*"		
Alkaloïds "*Meyer reaction*"		
Steroids and terpenoïds "*Lieberman-Burchard's test*"		

Determination of antioxidant capacity

DPPH radical scavenging activity

The detection of compounds having an activity scavenging of DPPH is performed by spraying of a methanol solution of DPPH (2 mg/ml), yellow spots revealed the presence of the active compounds. The *rate factor* (*Rf*) of the spots resulting from separation were calculated and compared with those of the witnesses (quercetin, Gallic acid and Sophorin) thus allowing the identification of the various compounds of extract (Figure 1).

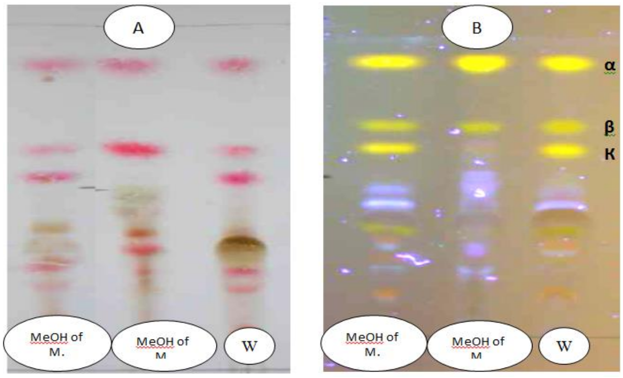
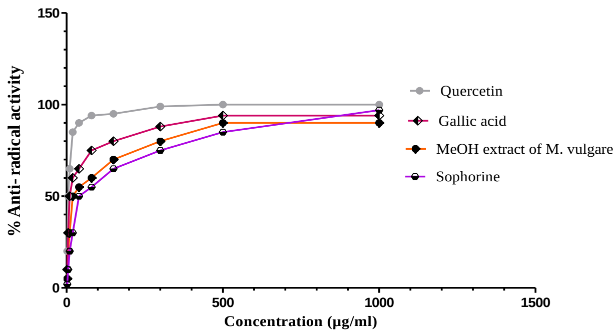


Figure 1: Thin Layers Chromatography of methanol crude extract from *M. vulgare* leaves and witnesses in Chloroform/ Methanol/ Water (65:35:5; v/ v/ v) as a solvents systems.

A) Detection with sulfuric vanillin; **B)** Detection with methanol solution of DPPH.; **α.** Gallic acid (3,4,5-trihydroxybenzoïc acid) ; **β.** Quercetin (Xanthaurin) ; **K. Sophorin (vitamin P);** W: witnesses; MeOHE of *M. deserti*: we don't care in this study.

As shown in the Figure 1, methanol extract showed the anti-radical activity (yellow spots) after the revelation with a methanol solution of DPPH at (2 mg/ml), which indicated that the compounds antioxidants included in the extract have the ability to reduce DPPH free radical.

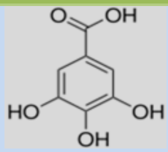
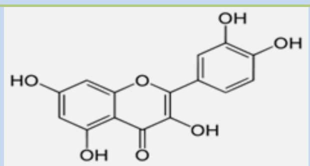
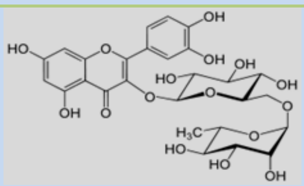
The appearance of yellow spots can be explained by the presence of an antioxidant which interact with DPPH radical by gaining one more electron or hydrogen atom from the antioxidant and convert it into yellow compound: α-α-diphenyl-β-picryl hydrazine. [18] Table 2 gives the *Rf* of the spots resulting from separation and their spatial chemical structure.



Values are means ± SD (n=3)

Figure 2: Anti-radical activity of methanol extract of *M. vulgare* and standards.

Table 2: The *Rf* of the compounds resulting from MeOHE of *M. vulgare and* their structure.

compounds resulting	*Rf*	Chemical structure	References
Gallic acid ($C_7H_6O_5$)	0.86		[19]
Quercetin ($C_{15}H_{10}O_7$)	0.69		[20]
Sophorin ($C_{27}H_{30}O_{16}$)	0.62		[21]

With knowledge of the author's "Sophorin" ($C_{27}H_{30}O_{16}$) was identified for the first time as a new compound from MeOH extract of *M. vulgare*. Figure 2 illustrate the anti-radical activity of methanol extract of *M. vulgare* and standards.

As shown in the Figure 2, methanol extract of *M. vulgare* has a remarkable potent radical scavenging activity with IC_{50}-value (12.42±0.23 µg/ml) which is close to that of Gallic acid and quercetin (8.64±0.51 µg/ml and 5.27±0.19 µg/ml) respectively, and slightly higher then the activity of sophorin which give IC_{50} value (22.15±0.07 µg/ml).

According to Kadri *et al.* [18] a lower value of IC_{50} (concentration of substrate that causes an inhibition of 50% of the activity of DPPH) shows higher antioxidant activity, so, our results obtained from Figure 2 revealed that quercetin has potent radical scavenging activity greater than the ability of gallic acid and both possessed inhibitory capacity higher than sophorin. These findings are consistent with the results of table 2, how so? We know that there is a close relationship between the structure of a compound and its function as the spatial structure is determined the function, our suggest in table 2 find that quercetin owns a large number of OH functions compared with those of gallic acid and sophorin which allows it to give very high inhibitory ability.

From Figure 2, we see that MeOH of *M. vulgare* and standards possessing antiradical dose-dependent activity in which the concentration increases, the radical activity increases until it reaches a plateau. Beyond this maximum, the activity remains constant. We interpret this phenomenon by the transfer of single electrons that are localized in the outer orbital of DPPH, and after reaching a given concentration, the antioxidant will react completely with the group, and when we increase the concentration, the antioxidant activity remains constant as it is accompanied by the saturation of the electron shells of the radical.

The anti-radical activity of MeOH (12.42±0.23 µg/ml) could be explained by the presence of terpenoïds have been disclosed previously in preliminary tests (see phytochemical screening tests). Several authors have reported that the antioxidant activity of *Marrubium vulgare* is due to essential oils that have a significant ability to act as donors of hydrogen atoms or electrons, hence the reductive transformation of DPPH • in DPPH-H, and therefore the formation of the yellow color was attributed to the presence of numerous bioactive molecules such as the oxygenated mono-terpenes: β-citronellol, thujones, camphor, β-bisabolene and eugenol [18, 22]. In addition, existing between the various compounds of *M. vulgare* such as flavonoïds cooperation has led to obtain these results, this cooperation is known as "synergic effect".

Ferric reducing antioxidant power (FRAP) assay

The investigated MeOH extract compared to standards ascorbic acid and α-tocopherol, is shown in Figure 3.

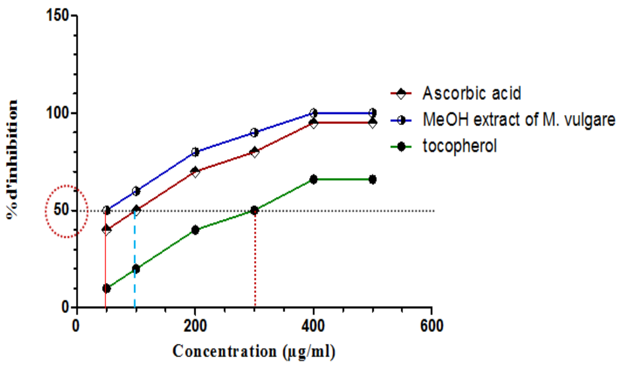


Figure 3: Ferric reducing antioxidant power of MeOH extract of M. vulgare and standards**.** Values are means ± SD (n=3)

The *Ferric reducing antioxidant power* of MeOH extract of *M. vulgare* was considerably more active than that of ascorbic acid in concentration dependent manner with the IC_{50}-values being (50.01±0.24 µg/ml and 99.87±0.55 µg/ml) respectively and the IC_{50} of α-tocopherol was found very weak to be (300.08±0.34 µg/ml) which clear in the Table 3. The chelating ability of our extract depends on the presence of reductants such as flavonoïds and polyphenols which have exhibit antioxidative potential interfered with the formation of activity and captured ferric ions (Fe^{+3}) and convert them to the ferrous ions (Fe^{+2}) .

The obtained results from the present work showed that the MeOH extract of *M. vulgare* had the highest content of polyphenols and flavonoïds (195 ± 0.06 mg GAE/g extract and 93.12 ± 0.17 mg QE/g extract) respectively (Table 3).

Table 3: Total polyphenols & flavonoïds contents and IC_{50}-values of antioxidant activities of MeOH extract of *M. vulgare* leaves.

Extract & witnesses	IC_{50} (µg/ml)		Total Polyphenols mg GAE/g extract	Flavonoïds content mg QE/g extract
	FRAP assay	DPPH test		
MeOH extract	**50.01±0.24 ^{a}**	**12.42±0.23^{a}**	**195 ± 0.06**	**93.12 ± 0.17**
Quercetin	No tested	5.27±0.19^{a*}	/	+++
Gallic acid	No tested	8.64±0.51^{a*}	/	/
Sophorin	No tested	22.15±0.07^{b*}	/	+++
Ascorbic acid	99.87±0,55 ^{b}	No tested	/	/
α-tocopherol	300,08±0,34^{c}	No tested	/	/

Each value represents a means ± standard deviations (n=3).
Significant differences in the same row are shown by letters $^{a-b*}$ varieties (P<0.05); highly significant differences in the same row are shown by letters $^{a-c}$ (P<0.0001). (GAE)/g extract: mg gallic acid equivalents/g extract, (QE)/g extract: mg quercetin equivalents/g extract.

Table 4: Effect of MeOH extract of *M. vulgare* & standards on the plantar edema induced by carrageenin in rats.

Groups & Doses (mg/Kg)	Time (hours) and plantar diameter (mm)				
	1h	2h	3h	4h	5h
Control group	6.40 ± 0.5	6.87 ± 0.7	6.99 ± 0.60 ^{a}	6.87 ± 0.41 ^{a}	5.62 ± 0.73 ^{a}
MeOH extract	6.39 ± 0.2	6.42 ± 0.5	4.80 ± 0.11 ^{b}	4.41 ± 0.30 ^{b}	4.36 ± 0.22
Diclofenac	6.37 ± 0.9	5.95 ± 0.5	4.73 ± 0.53 ^{b}	4.71 ± 0.62	4.38 ± 0.31
Salycilic acid	6.41 ± 0.00	5.23 ± 0.71	4.16 ± 0.47 ^{b}	3.98 ± 0.86 ^{b}	3.79 ± 0.59 ^{b}

Values represented as mean ± SD (n = 3). Significant differences in the same row are shown by letters $^{a-b}$ varieties (P<0.05);

According to the results of the Table 3, it is clear that the inhibitory ability of α-tocopherol has very weak compared to those of ascorbic acid although α-tocopherol is a compound with a high functional antioxidant ability, so the question is why in this experiment we obtained a weak inhibitory activity IC_{50} value? In a previous study of the antioxidant activity of extracts of *M. vulgare,* Ghedadba (2014) [22] conducted a test using the same witness (α-tocopherol) where it was measuring by β-carotene bleaching test, the results obtained showed that tocopherol terminate the activity of free radicals chain reactions 100%, we can deduce new information that the type of test used in the measurement of antioxidant activity affect the results, but how? The answer is simple as it is in the normal course of experiments carried out in aqueous solution such as DPPH and FRAP assay antioxidant compounds that have the capability of dissolution in water, such as ascorbic acid and quercetin be the effective unlike other compounds that don't dissolve in water like α-tocopherol which explains our results acquired in table 3, while, the experiments carried out in the fatty circles such as β-carotene bleaching test using arachidonic acid, fat-soluble antioxidants such as α-tocopherol become very effective which is consistent with the findings of the researcher Ghedadba *et al.* [22].

On the other hand, we know that methanol is characterized by a very high polarity such as water, which allows it to attracting compounds such as flavonoïds, phenols and Alkaloïds which are considered good and powerful anti-oxidants agents, which is consistent with the results obtained in this study. If one refers to the biochemical composition of *Marrubium vulgare* where it is found that the species also includes glycosidic phenylpropanoïd esters which are potent antioxidants [11]. The most important are the forsythoside, ballotetroside, arenarioside and acteoside. According Sahpaz *et al.* [11], *ortho*-diphenol groups of phenylpropanoïd confer high antioxidant activity much greater than that of the flavones. This may be due to a transfer between the two OH radical intramolecularly, which allows a strong stabilization and prevents intermolecular transfer. The result is in accordance with the previous published data showing the high antioxidant activity of *M.vulgare* [18, 22, 23].

Also, Vander-Jagt *et al.* (2002) [24] analyzed the total antioxidant capacity of aqueous extracts of *M. vulgare* using a two-stage Trolox based assay. The antioxidant content of the aqueous extracts was 560 µmol/g Trolox equivalent/g dry weight.

Anti-inflammatory activity

The evolution of inflammation for different groups is shown in Table 4 and Figure 4. Anti-inflammatory potential of MeOH extract and standards was assessed in terms of inhibition of plantar diameter. The results illustrated in Table 4 demonstrated that the administration of *M. vulgare* methanol extract at a dose of 200 mg/kg b.w prevents significantly (P <0.05) the plantar edema in rats from the third hour of treatment which is close to that of diclofenac and aspirin. The highest value of inhibition estimated by 87.3 ± 0.25% compared to diclofenac and aspirin (85.52 ± 0.47% and 90%) respectively (Figure 4).

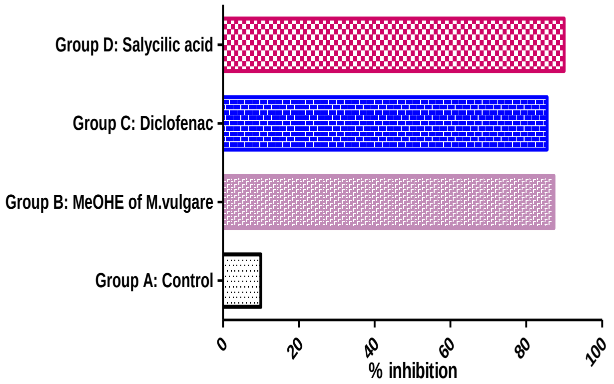


Figure 4: The percentage inhibition of edema induced by carrageenin.

This suggests the significant anti-inflammatory effect of the extract of the plant, it could be due to the richness of the methanol extract of *M. vulgare* in bioactifs compounds, mostly polyphenols, flavonoïds and phenylpropanoïds glycosilated. The compounds including (+) (*E*)-caffeoyl-L-malic acid, acteoside, forsythoside B, arenarioside, and ballotetroside are identified as the principally bioactive constituents related to the anti-inflammatory activity. In addition, glycosidic phenylpropanoïd esters from the aerial parts were shown to inhibit the cyclooxigenase enzyme (COX) and three of them, acteoside, forsythoside B and arenarioside, displayed higher inhibitory potencies on Cox-2 than on Cox-1[1, 11]. Beside, verbascoside has been reported like a phenylenthanoïd glycoside with different activities, *i.e.*, strong anti-leukaemic and cytotoxic activity against a murine cell line and anti-inflammatory activity[25]. Verbascoside also has antioxidant activity and reduces NF-κβ activation and nuclear translocation and thus may modulate inflammatory reactions [25].

On the other hand, Stulzer *et al.* [10] analyzed marrubiin in a model of micro-vascular leakage in mice ears. The results obtained for ID_{50} values (mg/kg) and maximal inhibition (%), for the different phlogistic agents used, were: histamine 13.84 mg/kg and 73.7%; bradykinin 18.82 mg/kg and 70.0%; carrageenin 13.61 mg/kg and 63.0%. In addition, marrubiin (100 mg/kg) significantly inhibited the ovalbumin-induced allergic œdema in actively sensitised animals. Previous published data showing that marrubiin was more potent than some known anti-inflammatory drugs, as it had lower IC_{50} compared with aspirin and diclofenac. [10] The results obtained in this study are consistent with those found by Kanyonga *et al.* [26]. Also, other studies have shown that many specie of the family Lamiaceae such as *Thymus vulgaris* L., *Rosmarinus officinalis* develop an anti-inflammatory activity *in vivo* [10].

CONCLUSION

In the current study, methanolic extract of *M. vulgare* leaves has both anti-inflammatory and antioxidant activities which could be due to the richness of the methanol extract in polyphenols and flavonoïds particularly quercetin and sophorin. Many other compounds such as glycosidic phenylpropanoïd esters, terpenoïds and alkaloïds were previously identified may be participating in these activities. The result obtained justifies the use of the plant species by traditional medicine practitioners in Algeria. However, more studies are needed to further elucidate the mechanism of the anti-inflammatory and antioxidant actions of *Marrubium vulgare*. It is important to remember that these results were obtained in rats. It is therefore essential to carry out experiments at first in another animal model, and then in a second time in humans, to obtain confirmation of the potential of this plant.

REFERENCES

1. Yousefi K, Fathiazad F, Soraya H, Rameshrad M, Maleki-Dizaji N, Garjani A, *Marrubium vulgare* L. methanolic extract inhibits inflammatory response and prevents cardiomyocyte fibrosis in isoproterenol-induced acute myocardial infarction in rats, BioImpacts, 4(1), 2014, 21-27.

2. Akinmoladun AC, Obuotor EM, Farombi EO, Evaluation of antioxidant and free radical scavenging capacities of some Nigerian indigenous medicinal plants, J Med Food, 13, 2010, 444-451.

3. Sathees SL, Murugan K, Antimicrobial activity of protease inhibitor from leaves of *Coccinia grandis* (L.) Voigt, I. J Exp Biol, 49, 2011, 366-374.

4. Parvin MS, Das N, Jahan N, Akhter MA, Nahar L, Ekramul-Islam M, Evaluation of *in vitro* anti-inflammatory and antibacterial potential of *Crescentia cujete* leaves and stem bark, BMC Res Notes, 8, 2015, 412.

5. Uttara B, Singh AV, Zamboni P, Mahajan RT, Oxidative stress and neurodegenerative diseases: a review of upstream and downstream antioxidant therapeutic options, Curr. Neuropharmacol, 7, 2009, 65-74.

6. Moon JK, Shibamoto T, Antioxidant assays for plant and food components, J. Agr. Food Chem., 57, 2009, 1655-1666.

7. Elberry AA, Harraz FM, Ghareib SA, Gabr SA, Nagy AA, Abdel-Sattar E, Methanolic extract of *Marrubium vulgare* ameliorates hyperglycemia and dyslipidemia in Streptozotocin-induced diabetic rats, Int J Diabetes Mellit, 3, 2015, 37-44.

8. El Bardai S, Hamaide MC, Lyoussi B, Quetin-Leclercq J, Morel N, Wibo M, Marrubenol interacts with the phenylalkylamine binding site of the L-type calcium channel, Eur J Pharmacol, 492, 2004, 69-72.

9. Mnonopi N, Levendal RA, Davies-Coleman MT, Frost CL, The cardioprotective effects of marrubiin, a diterpenoïd found in *Leonotis leonurus* extracts, J Ethnopharmacol, 138, 2011, 67-75.

10. Stulzer HK, Tagliari MP, Zampirolo JA, Cechinel-Filho V, Schlemper V, Antioedematogenic effect of marrubiin obtained from *Marrubium vulgare*, J Ethnopharmacol, 108, 2006, 379-84.

11. Sahpaz S, Garbacki N, Tits M, Bailleul F, Isolation and pharmacological activity of phenylpropanoïd esters from *Marrubium vulgare*, J Ethnopharmacol, 79, 2002, 389-392.

12. Aliyu AB, Musa AM, Abdullahi MS, Ibrahim H, Oyewale AO, Phytochemical screening and antibacterial activities of *Vernonia ambigua*, *Vernonia blumeoides* and *Vernonia oocephala* (Asteraceae), Acta Poloniae Pharmaceut Drug Res, 68, 2011, 67–73.

13. Singleton VL, Rossi JA, Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents, Am J Enol Vitic, 16(3), 1965, 144-153.

14. Ramful D, Aumjaud B, Neergheen VS, Soobrattee MA, Googoolye K, Aruoma OI, Polyphenolic content and antioxidant activity of *Eugenia pollicina* leaf extract *in vitro* and in model emulsion systems, Food Res Int, 44(5), 2011, 1190-1196.

15. Braca A, Sortino C, Politi M, Morelli I, Mendez J, Antioxidant activity of flavonoïds from *Licania licaniaeflora*, J Ethnopharmacol, 79(3), 2002, 379-381.

16. Chu YH, Chang CL, Hsu HF, Flavonoïd content of several vegetables and their antioxidant activity, J Sci Food Agric, 80, 2000, 561-566.

17. Amezouar F, Badri W, Hsaine M, Bourhim N, Fougrach H, Antioxidant and anti-inflammatory activities of Moroccan *Erica arborea* L, Pathologie Biologie, http://dx.doi.org/10.1016/j.patbio.2013.03.005.

18. Kadri A, Zarai Z, Békir A, Gharsallah N, Damak M, Gdoura R, Chemical composition and antioxidant activity of *Marrubium vulgare* L essential oil from Tunisia, Afr J Biotechnol 10(19), 2013, 3908–3914.

19. Nayeem N, Asdaq SMB, Salem H, AHEl-Alfqy S, Gallic Acid: A Promising Lead Molecule for Drug Development, J App Pharm, 8: 213, 2016.doi:10.4172/1920-4159.1000213.

20. Wojdylo A, Oszmianski J, Czemerys R, Antioxidant activity and phenolic compounds in 32 selected herbs, Food Chem, 105, 2007, 940–949.

21. Ashok PK, Saini B, HPLC Analysis and Isolation of Rutin (sophorin) from Stem Bark of *Ginkgo biloba* L., Journal of Pharmacognosy and Phytochemistry, 2(4), 2013, 68-71.

22. Ghedadba N, Bousselsela H, Hambaba L, Benbia S, Mouloud Y, Évaluation de l'activité antioxydante et antimicrobienne des feuilles et des sommités fleuries de *Marrubium vulgare* L. Phytothérapie, 12, 2014, 15–24.

23. Berrougui H, Maxim I, Cherki M, Khalil A, *Marrubium vulgare* extract inhibits human-LDL oxidation and enhances HDL-mediated cholesterol efflux in THP macrophage, Life Sciences, 80, 2006, 105–112.

24. VanderJagt TJ, Ghattas R, VanderJagt DJ, Crossey M, Glew RH, Comparison of the total antioxidant content of 30 widely used medicinal plants of New Mexico, Life Sciences, 70, 2002, 1035-1040.

25. Carrillo-Ocampo D, Bazaldúa-Gómez S, Bonilla-Barbosa JR, Aburto-Amar R, Rodríguez-López V, Anti-Inflammatory Activity of Iridoids and Verbascoside Isolated from *Castilleja tenuiflora*, Molecules, 18, 2013, 12109-12118.

26. Kanyonga PM, Faouzi MA, Meddah B, Mpona M, Essassi EM, Cherrah Y, Assessment of methanolic extract of *Marrubium vulgare* for anti-inflammatory, analgesic and anti-microbiologic activities, J Chem & Pharm Res, 3(1), 2011, 199-204.

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