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# Evaluation of Algerian spontaneous species: phytochemical and biological study of aromatic and medicinal plant *Marrubium vulgare*

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**Abstract** *Marrubium vulgare L. (Lamiaceae), commonly known as "Horehound" is a widespread mediterranean plant and use in folk medicine to cure a variety of disease. The objective of this study is to determine the antibacterial activity of flavonoids extracted from leaves of M. vulgare L. towards bacterial strains pathogenic for humans. The Phytochemical screening of dried leaves of M. vulgare revealed the presence of a high amount of flavonoids. The extraction of flavonoids by the method of Charaux and Paris (1954) gave a yield equal to 5,9%. The isolated compounds were separated by TLC and their antibacterial activity against bacterial strains was determined in vitro by disc diffusion method of Bauer and al (1966), with concentrations used (Pure flavonoids, flavonoids ½, flavonoids ¼) on Mueller-Hinton and Sabouraud medium. Comparison tests with an antibiotic, Rifampin (5µg) were also included in the trials.*
*The results have demonstrated that the isolated extract is formed from two compounds with $R_f$ more or less close. From the antibacterial tests of the isolated flavonic extracts, it appears that the inhibition of growth of the strains tested varies with the nature of the bacterial species, the concentration of the extract and the culture medium used. A significant antibacterial effect was observed for some strains considered among the most resistant to antibiotics such as Pseudomonas aeruginosa 7244 and Staphylococcus aureus. In general, the areas of inhibitions are included between 4 and 12 mm for Pseudomonas aeruginosa 7244, 0 to 14 mm for Staphylococcus aureus on Mueller-Hinton medium, 8-38 mm for Pseudomonas aeruginosa 7244 and between 0 to 6 mm for Staphylococcus aureus on Sabouraud medium. The Inhibition zones far exceed those caused by the antibiotic Rifampin.*

***Key Words*:** *Antibacterial activity, Flavonoids, Marrubium vulgare L., Bacterial strains*

## 1. Introduction

Medicinal plants are widely used in the treatment of various diseases in today's world. Plant extracts and their various formulations in the treatment and/or alleviation of several disease in folk medicine have been dated back to the ancient times. Besides, some natural products also exist in vegetables, fruits and beverages [1]. The acceptance of traditional medicine as an alternative form of health care and the development of microbial resistance to the classical

antibiotics led researchers to investigate the antimicrobial activity of several medicinal plants [2]. Therefore, reports of antimicrobial activity of many plant extracts have been published from many regions in the world. It is estimated, however, that of the 250,000–500,000 species found on Earth, only 1% have been studied for their pharmaceutical potential [3].
*Marrubium vulgare* L. (Lamiaceae) commonly known as "horehound" in Europe, or "Marute" in the Mediterranean region, is naturalized the latter and Western Asia and America. As a medicinal plant, it was frequently employed in folk medicine to treat a variety of ailments, exhibits antispasmodic and antinociceptive effects in different experimental models. It possesses tonic, aromatic, stimulant, expectorant, diaphoretic and diuretic properties. It is helpful for bronchial asthma and non-productive cough. It was formerly much esteemed in various uterine, visceral and hepatic affections and in phthisis [4]. The plant is reported to possess hypoglycemic [5], antihypertensive [6], analgesic [7], anti-inflammatory [8], antioxidant activity [9], antiedematogenic activity [10], vasorelaxant [11] and many other biological activities.
Extracts coming from plants belonging to the *Marrubium* genus have shown a very complex metabolic pattern, containing, among other secondary metabolites, diterpenes [12,13], flavonoids [12,14], and a series of phenyl propanoids esters, together with their derivatives [15,16]. Particularly, the diterpene lactone marrubiin can be the molecule responsible for the majority of the biological properties.

The aim of this study was to find out the phytochemical active constituents and to determine the antibacterial activity of flavonoids extracted from leaves of *Marrubium vulgare* L. towards bacterial strains pathogenic for humans.

## 2. Materials and methods

*2.1 Plant material:*
The aerial part (leaves) of *M.vulgare* was collected during the period of flowering from Chafia village, Wilaya of El-tarf, Algeria. The leaves were washed under running tap water to eliminate dust and other foreign particles and to cleanse the leaves thoroughly and dried. The dried leaves ground into powder and stored for further use.

*2.2 Microbiological material:*
Microbiological material consists of four bacterial strains. They are Gram (+) or Gram (-) *Klebsiella pneumonia, Pseudomonas aeruginosa* 7244, *Escherichia coli* 1429, *Staphylococcus aureus*. For the evaluation of antibacterial activity of flavonoids we used two culture mediums Mueller-Hinton and Sabouraud, and to compare this antibacterial effect of flavonoids with those of antibiotic (Rifampin 5µg).

*2.3 Phytochemical screening:*
The tests were done to find the presence of the active chemical constituents such as Alkaloids, Flavonoids, Terpenoids and steroids, Saponins, Anthocyans, Leucoanthocyans, Gallic tannins, Catechic tannins and Cardinolids by the following procedure:

*2.3.1 Determination of Tannins:*

- To 30 ml of the infused was added 15 ml of Stiasny reagent. After heating for 30 min in a water bath, the observation of an orange precipitate indicates the presence of Catecholic tannins.
- To 5 ml of the infused, 1 ml drops of ferric chloride 1% was added. Blue color was observed for gallic tannins [17].

*2.3.2 Determination of Alkaloids:*

Maceration of 5g dried leaves in 50 ml of hydrochloric acid (1%), the mixture was filtered and treated with a few drops of Mayer's reagent. White precipitate indicates the presence of alkaloids [18].

*2.3.3 Determination of Anthocyans:*

Search of the anthocyans based on the color change of the infused (10%) and pH: A few drops of hydrochloric acid and a few drops of ammonia. The change in color indicates the presence of *Anthocyans*.

*2.3.4 Determination of Flavonoids:*

Flavonoids research begins with a maceration of 10g of dried leaves in 150 ml of hydrochloric acid (1%) for 24 h. After filtration, 10 ml of the filtrate treated with a few drops of ammonia, after 3 hours, Formation of yellow color in the supper part of the tube, indicates the presence of flavonoids.

*2.3.5 Determination of Leucoanthocyans:*

5 ml of infused mixed with 4 ml of hydrochloric alcohol (Ethanol / hydrochloric acid pure 3/1 v/v). The mixture was heated on a boiling water bath (50°C) for a few minutes; the appearance of a cherry red color indicates the presence of leucoanthocyans [19].

*2.3.6 Determination of Saponins:*

About 2g of dried leaves was boiled with 100 ml of distilled water for 30 minutes. After filtration, adjusts the volume to 100 ml. in 10 tubes we put 1ml of the filtrate in the tube n° 1, 2ml in tube n° 2…etc. The final volume of each tube was adjusted to 10 ml with distilled water. Each tube is stirred in a vertical position, if persistent foam height in cm equal to 1cm in the tube Xe, then calculates the index of foam according to the following formula:

$$I = \frac{\text{Foam height (in cm) in the tenth tube x 5}}{0.0X}$$

The presence of saponin in the plant is confirmed with an index I superior than 100 [20].

*2.3.7 Determination of Terpenoids and steroids:*

Maceration of 5g of dried leaves in 20 ml of petroleum ether; after filtration, the organic phase is evaporated in a sand bath. The residue is dissolved in 0.5 ml of acetic acid by adding 1 ml of sulfuric acid, in the area of contact between two liquids. If there appears a circle violated, indicates the presence of terpenoids and steroids.

*2.3.8 Determination of Cardinolids:*

We realized the maceration of dried leaves 1g in distilled water 20 ml for 3 h, after filtration, 10 ml of filtrate was added to 10 ml of the mixture of solution chloroform and ethanol. The organic phase is evaporated in a sand bath. The precipitate is dissolved in 3 ml of glacial acetic acid add a few drops of ferric chloride and 1 ml sulfuric acid. The appearance of a blue green color in the acid layer indicates the presence of cardinolids.

*2.4 Extraction and separation of flavonoids:*
The method described by Charaux and Paris (1954) was used for extraction of flavonoids. It consists of taking 10g of dried leaves for 1 h in 200 ml of ethanol. After filtration, the drug is roughly pulverized and extracted with Soxhlet; 200 ml of ethanol for 4 h. After maceration for 24 h, the two ethanolic solutions were combined and evaporated under reduced pressure. The residue is taken up in 20 ml of boiling water. The liquor is exhausted in a separating funnel, several times in succession (4 × 10 ml) ether, (4 × 10 ml) ethyl acetate and (5 × 10 mL) of n-butanol. The butanol extract was evaporated in a sand bath at 30°C. The residue is then weighed and divided into two parts: the first for chromatography and the second part is reserved for biological tests.

The separation of flavonoids was carried out by Thin-Layer Chromatography TLC (Silica gel 60 F254, aluminium plates, Merck) as follows: TLC was developed in ascending direction with ethyl acetate, Methanol and water (5V/2V/1V), then spots were visualized with aluminium chloride solution $AlCl_3$ (1%) under ultraviolet light (UV) at λ=366 nm.

*2.5 Antibacterial assay:*
An antibacterial activity of the flavonoids was screened against four human pathogenic bacteria. The inhibitory effect on bacterial growth was determined using agar disc diffusion assay [22]. A sterile filter disc (Whatman paper) having 6 mm of diameter containing the test products (Pure flavonoids, flavonoids ½ and flavonoids ¼) was placed at the surface of culture medium. The treated Petri dishes were incubated at 37°C for 18 to 24 h. The antibacterial activity was evaluated by measuring the clear zone surrounding the Whatman paper. Standard discs of the antibiotic rifampin 5µg and sterile distilled water were applied as a positive antibacterial controls.

## 3. Results

*3.1 phytochemical screening:*
Phytochemical screening (Table I) was carried out to highlight the existing chemical groups in the studied plant, in order to have an idea of the chemical nature of the active ingredients responsible for its biological effects.

*3.2 Extraction of flavonoids:*
The extraction of flavonoids from leaves of the studied species gave a yield of about 0,59g which corresponds to a percentage equal to 5,5%.

*3.3 Separation of flavonoids:*
Separation by TLC of flavonoids under long UV-365 nm, showed the presence of two spots of different $R_f$ and brown color. After a spray of aluminium chloride solution $AlCl_3$ over the paper chromatography showed a distinct color spots (Table II).

Table I: Results of phytochemical screening of studied plant.

| Chemical constituents | Leaves of *M. vulgare* |
|---|---|
| Saponins | (+) |
| Catecholic tannins | (+) |
| Gallic tannins | (-) |
| Anthocyans | (+) |
| Leucoanthocyans | (-) |
| Flavonoids | (+) |
| Alkaloids | (-) |
| Terpenoids and steroids | (+) |
| Cardinolids | (-) |

+ = Detected - = Not detected

Table II: $R_f$ and spots color before and after addition of aluminium chloride $AlCl_3$.

| Spot | $R_f$ | Color | |
|---|---|---|---|
| | | Before addition of $AlCl_3$ | After addition of $AlCl_3$ |
| Spot 1 | 0,64 | Brown | Yellow |
| Spot 2 | 0,88 | Brown | Ocher |

*3.4 Antimicrobial activity:*

The antimicrobial activity of the tested *M. vulgare* flavonoids was evaluated against four bacterial strains pathogenic. It appears that flavonoids from leaves of this plant exhibited various levels of antibacterial effect against the tested bacterial strains. The inhibition of the growth of bacteria varies with the nature of the bacterial species, the concentration of the flavonoids and the culture medium used.The results of antibacterial activities of the flavonoids extract of *M. vulgare* on culture mediums (Mueller-Hinton and Sabouraud) are grouped in Tables III and IV.

Table III: Antibacterial activity of flavonoids of *M. vulgare* using agar disc diffusion on Mueller-Hinton.

| Strains | Mueller-Hinton | | | | |
|---|---|---|---|---|---|
| | Flv | Flv ½ | Flv ¼ | Water | Rif 5µg |
| *P. aeruginosa* 7244 | 04 ±0,721 | 12 ±1,527 | 06 ±0,721 | 00 ±00 | 00 ±00 |
| *E.Coli* 1429 | 34 ±0,568 | 40 ±1,154 | 32 ±0,808 | 00 ±00 | 42 ±0,519 |
| *S.aureus* | 00 ±00 | 12 ±1,985 | 14 ±2,076 | 00 ±00 | 12 ±0,776 |
| *K. pneumonia* | 38 ±1,014 | 34 ±0,550 | 32 ±0,901 | 00 ±00 | 38 ±1,154 |

Table IV: Antibacterial activity of flavonoids of *M. vulgare* using agar disc diffusion on Sabouraud.

| Strains | Sabouraud | | | | |
|---|---|---|---|---|---|
| | Flv | Flv ½ | Flv ¼ | Water | Rif 5µg |
| *P. aeruginosa* 7244 | 38 ±0,763 | 08 ±1,184 | 08 ±0,288 | 00 ±00 | 08 ±0,513 |
| *E.Coli* 1429 | 34 ±0,568 | 40 ±1,154 | 42 ±0,808 | 00 ±00 | 26 ±0,950 |
| *S.aureus* | 00 ±00 | 04 ±0,152 | 06 ±2,083 | 00 ±00 | 44 ±0,776 |
| *K. pneumonia* | 38 ±1,193 | 38 ±0,503 | 38 ±351 | 00 ±00 | 54 ±0,577 |

*Flv: flavonoids. E.Coli*1429 : *Escherichia coli*1429. *P. aeruginosa* 7244 : *Pseudomonas aeruginosa* 7244. *S. aureus* : *Staphylococcus aureus. K.pneumonia: Klebsiella pneumonia.* Rif 5µg : Rifampin 5µg**.**

## 4. Discussion

The preliminary phytochemical tests have demonstrated that the leaves of *M. vulgare*. contained many bioactive chemical constituents including saponins, catecholic tannins, anthocyans, flavonoids, terpenoids and steroids. The extraction of flavonoids gave a yield equal to 5,9%, This result is comparable with a yield of flavonoids extracted from *Marrubium peregrinum* species [23]. The work conducted by [19] for the separation of flavonoids reveals that the flavonoids extracted from the leaves of *Buxus madagascaria* Lease. have a same $R_f$ and Spots color of the *M. vulgare.* flavonoids.

The results confirm once more the effectiveness of extracts of medicinal plants and their antiseptic power. Many studies emphasize the antibacterial effect of natural products. Indeed, Mubashir et al. [25] reported that the aqueous extract of the leaves of the species *M. vulgare* exerts a strong inhibitory activity on strains: *Staphylococcus aureus* MTCC 740, *Staphylococcus epidermidis* MTCC 435 and a lesser degree of activity for *Escherichia coli* MTCC 443.

Strains *Klebsiella pneumonia*, and *Escherichia coli* 1429, are most susceptible against the flavonoids on Sabouraud and Mueller-Hinton. Indeed, *Escherichia coli* 1429 strain, which was moderately sensitive to flavonoids extracted from *M. vulgare* species special in Mueller-Hinton, was also the subject of another work showed that it was resistant to petroleum ether extract of *M. alyssum* leaves [26]. For *Pseudomonas aeruginosa* 7244 the effect of the pure flavonoids, flavonoids ½ and flavonoids ¼ is often greater than that recorded in the presence of the antibiotic rifampin 5µg (4 to 12 mm on Mueller-Hinton and 8 to 38 mm on Sabouraud).

The antibiotic, which has been the subject of our study (rifampin 5µg), gave satisfactory results against the strains studied, particularly *Staphylococcus aureus and Klebsiella pneumonia* in Sabouraud, *Escherichia coli* 1429 and *Klebsiella pneumonia* in Mueller-Hinton.

This result is consistent with previous work that used rifampin 30µg resulted in a significant inhibitory effect of most bacterial species tested [27].
According to this results and some current research, there is an association between the flavonoids compounds and antibacterial activity [28, 29], the flavonoids extracts of *M.vulgare* have an excellent antimicrobial activity against the strains.

## 5. Conclusion

Results of our study suggest the great value of the species *M. vulgare* for use in pharmacy and phytotherapy. Based on this information, it could be concluded that this plant is natural sources of antibacterial substances of high importance.

On the whole, these compounds appear to be effective at all concentrations used. In all strains tested, the strain *Escherichia coli* 1429 was highly sensible. The zones of inhibition recorded of most strains are often similar to those caused by the antibiotic rifampin 5µg, and particularly those of *Escherichia coli* 1429 and *Pseudomonas aeruginosa* 7244.

It will be important in future studies to identify the active constituents responsible for the observed activities of *M. vulgare*, and should be directed to realize *in vivo* studies of its medicinal active components in order to prepare a natural pharmaceutical product of high value.

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