Available online on 15.09.2020 at <http://jddtonline.info>

Journal of Drug Delivery and Therapeutics

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Research Article

Phytochemical study and antimicrobial activity of Algerian *Marrubium vulgare* leaf and stem extracts

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ABSTRACT

Marrubium vulgare is used worldwide as a source of food flavor and for medicinal purposes. The aim of this study is to investigate polyphenol and flavonoid contents of *M. vulgare* extracts and their antimicrobial activities. Extraction was conducted using methanol and hexane. The determination of polyphenol content was realized with folin ciocalteu method and flavonoids using $AlCl_3$. Rough characterization of these compounds was done with HPLC method. Activity against bacteria and fungi was also studied. Results showed that methanolic extracts of leaves (LME) and stems SME) contain relatively high levels of polyphenols and flavonoids. Except for hexane extract, all extracts from leaves and stems possess antibacterial and antifungal effects especially against *Staphylococcus aureus* and *Candida albicans*. This finding suggests that *M. vulgare* methanolic extracts could serve as a basic material for the preparation of antimicrobial drugs.

Keywords: *Marrubium vulgare*, polyphenols, flavonoids, antibacterial, antifungal activities.

Article Info: Received 06 August 2020; Review Completed 17 August 2020; Accepted 24 August 2020; Available online 15 Sep 2020



Cite this article as:

Benzidane N, Smahi R, Zabouche B, Makrouf A, Arrar L, Phytochemical study and antimicrobial activity of Algerian *Marrubium vulgare* leaf and stem extracts, Journal of Drug Delivery and Therapeutics. 2020; 10(5) :70-74
<http://dx.doi.org/10.22270/jddt.v10i5.4353>

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INTRODUCTION

Marrubium vulgare L is a variety of perennial plant from the labiatae family, known as "horehound" in Europe, "Marrubia" in Tunisia¹ and Marriwet in Algeria². *Marrubium vulgare* is cultivated worldwide as a source of food flavor and for medicinal purposes³. Several active compounds were isolated from *Marrubium vulgare* including marrubiin⁴, Premarrubiin⁵, Marrubenol⁶, 11-oxomarrubiin⁵, Marrubiol⁷, Vulgarol⁸, Sclareol⁹, Peregrinin¹⁰, 12(S)-hydroxymarrubiin⁵ and 3-deoxy-15-methoxyvelutine C¹⁰. These metabolites including diterpenes, sesquiterpenes, flavonoids, and phenylpropanoids were identified from different parts of *M. vulgare*¹¹.

M. vulgare is traditionally used for acute and chronic bronchitis, respiratory disorders, tuberculosis, asthma, skin damage and ulcers. *M. vulgare* juice and infusion used as a gastric secretion stimulant due to the presence of bitter ingredients particularly marrubinic acid as a choleric agent. Leaves paste is applied for boils and rheumatism¹¹. Dried herb's infusion is used for debility and in high blood pressure. Leaves, flowers, and stem infusion are used as a stomachic for diabetes and in cardiac problems¹². In Mexico and Germany, it is used in traditional medicine to aid digestion and to treat stomach diseases and diabetes^{13,14}. In Brazil, it is employed against gastrointestinal disorders and inflammation¹⁵. Some of folk uses are confirmed by modern

investigations. In fact, the plant is reported to possess antihypertensive¹⁶, vasorelaxant¹⁷, hepatoprotective¹⁸ and antibacterial¹⁹ properties. We have previously demonstrated that *M. vulgare* extracts exhibit antioxidant activities by their ability to scavenge free radicals, inhibit lipid peroxidation and chelate metals²⁰. Furthermore, our results indicate that this plant has powerful effects in the inhibition of the release of inflammatory mediators anti-inflammatory cytokines such as TNF- α , IL-1 β and IL-8.

The present study aims to determine the total phenolic and flavonoid contents of various extracts from leaves and stems of *Marrubium vulgare* from Bejaia region (north Algeria), and to evaluate their antimicrobial capacity.

MATERIAL AND METHODS

Plant collection

Marrubium vulgare was harvested in March 2019, in the region of Kherrata, Bejaia, north Algeria and authenticated by Prof. Sabah Charvat from the Faculty of Nature and Life Sciences, Ferhat Abbas University, Setif 1. The stems and leaves were cleaned and left to dry in a dry, ventilated place in the dark. They are then ground into a powder in a blender, and then stored in glass jars protected from light.

Preparation of extracts

Powder from the leaves or stems from *Marrubium vulgare* were mixed with methanol 80 % (v/v)²¹, with magnetic stirring at 60°C for 3 h. The mixture was left to macerate for 24 hours in the dark and then filtered. The maceration is renewed, the macerate recovered. The various filtrates are combined and subjected to evaporation under reduced pressure at 40°C using a rotavapor Buchi (Germany). After evaporation, the filtrate is dried to give the leave methanolic (LME) or stems methanolic (SME) extracts. LME is oily and required hexane defatting. The hexane phase is collected to give leave hexane extract (LHE). The aqueous phase was collected, filtered and dried at 40°C in the oven to give the delipidated methanolic extract (DLME).

Determination of total polyphenols and flavonoids

The determination of the total polyphenols in the extracts of *Marrubium vulgare* (leaves and stems) was carried out according to the method of Folin Ciocalteu²². A volume of 200 µl of each extract dissolved in distilled or point-of-range water is added to 1 ml of Folin Ciocalteu's reagent (diluted 10 times in distilled water). After 4 min of incubation at room temperature, 800 µl of Na₂CO₃ (7.5%), also diluted in distilled water, were added to the mixture. The previously shaken set was incubated in the dark for 2 hours. The absorbance was then read at 765 nm by a UV / visible spectrophotometer. The concentration of total polyphenols for each sample is calculated from the regression equation of a calibration range in aqueous medium (0 to 200 µg / ml), established with gallic acid under the same operating conditions as the excerpts. Results are expressed in milligrams of gallic acid equivalent per gram of extract (mg GAE / g of extract).

Total flavonoids were quantified by the aluminum trichloride method²¹. One ml of each sample (prepared in methanol) was added to 1 ml of the AlCl₃ solution (2% in methanol). After 10 minutes of incubation, the absorbance was read at 430 nm. The concentration of flavonoids in the methanolic extract was calculated from the calibration curve established with quercetin (0 - 40 µg / ml in methanol) and expressed in milligrams of quercetin equivalent per gram of extract (mg QE / g Extract)

High performance liquid chromatography

The extracts were analyzed using high performance liquid chromatography system. Each sample was dissolved in methanol (25 mg/mL) and filtered with a 0.45 µm membrane before injection into the HPLC-system. The sample was analyzed on a VP-ODS, C18 column with a dimension (250 × 4.6 mm) and a particle diameter equal to 5 µm. The mobile phase is composed by water (A) and methanol (B). The elution was performed in isocratic gradient conditions, starting from 40 to 100 % of B in 60 min, followed by a re-equilibration step of 20 min. The flow rate was 1 mL/min and the elution was performed at room temperature. The UV detection was fixed at 254, 280 nm.

Evaluation of antibacterial and antifungal activity

One gram (+) and two gram (-) bacteria were used in this study: *Staphylococcus aureus* (ATCC 25923), *Escherichia coli* (ATCC 25922) and *Pseudomonas aeruginosa* (ATCC 27853). These strains were provided by the Microbiology Laboratory of the University Hospital Centre (CHU) of Setif, Algeria. The test of the bacteria sensitivity to different extracts was carried out in vitro using the agar medium diffusion method²². The bacterial strains were sub-cultured by the streak method on Mueller Hinton agar, and then incubated in an oven at 37 ° C for 18 to 24 hours. From these young cultures, pure colonies were isolated to prepare the bacterial inoculum. Each colony was suspended in 2.5 ml of sterile distilled water. The turbidity of the suspension was measured using a densitometer and adjusted to 0.5 Mac Farland. Sterile 6 mm diameter Wattman paper discs were impregnated with 10 µl of extract and the negative control discs are impregnated with methanol. Discs were gently placed on the agar medium seeded beforehand with a bacterial suspension. Standard Gentamicin discs (10 mg) were used as positive controls. After incubation at 37°C for 18 to 24 hours, the diameters of the clear zones of inhibition around the discs were measured. The inhibition zone (s) were recorded and the activity index (AI) was calculated by comparison with respective compounds (AI = Inhibition zone of test sample/Inhibition zone of the standard).

Fungal strain *Candida albicans* is provided by the Parasitology Laboratory of the CHU Setif. The antifungal activity was determined by the Sabouraud agar diffusion method containing. The inoculum is prepared from young cultures 2 to 3 days old. A pure colony was suspended in sterile distilled water. The turbidity of this suspension is adjusted to 0.5 Mac Farland (108 CFU / ml) and an aliquot of 0.1 ml thereof was spread on the agar. Sterile Wattman paper discs of 6 mm in diameter, impregnated with 10 µl of extract were aseptically deposited on the agar medium. The discs impregnated with DMSO and Fluconazole (10 mg / ml) were used as negative and positive controls, respectively.

Statistical analysis

Data were analyzed by means of one way Anova to determine statistically significant variance between the groups for each plant extract. Values which showed statistically significant effects were further analyzed and means were compared using Tukey's test. Differences were considered significant at a P value of less than 0.05.

RESULTS AND DISCUSSION

Polyphenol and flavonoid contents

Yields of extraction and the amounts of polyphenols and flavonoids in different extracts of *Marrubium vulgare* are presented on table 1. These results are concordant with those of *M. vulgare* harvested from Bordj Bouariridj region²⁰ except for the flavonoid content of stems extract which is higher (11.72 versus 5.21 mg QE/g).

Table 1: Yields and contents of polyphenols and flavonoids in the *Marrubium vulgare* methanolic and hexane extracts.

Extracts	Yield (%)	Polyphenols (mg GAE/ g)	Flavonoids (mg QE/ g)
LME	13.27%	60.94 ± 0.07	14.41 ± 0.15
DLME	12%	62.95 ± 0.09	8.06 ± 0.17
LHE	3.77%	49.34 ± 0.06	9.89 ± 0.06
SME	6.7%	49.46 ± 0.03	11.72 ± 0.07

It was aimed in this work to identify some of the phenolic compounds present in the methanolic extracts of *M. vulgare* using HPLC which is a high-resolution chromatographic technique probably the most widely used analytical technique for characterizing the polyphenolic compound²³.

HPLC analysis of polyphenols (Figure 1) showed that LME contains tannic acid, caffeine and ferulic acid, but when defatted gallic acid was also detected. LHE contains tannic acid, vitamin C and catechin, whereas in SME only tannic acid and catechin were detected.

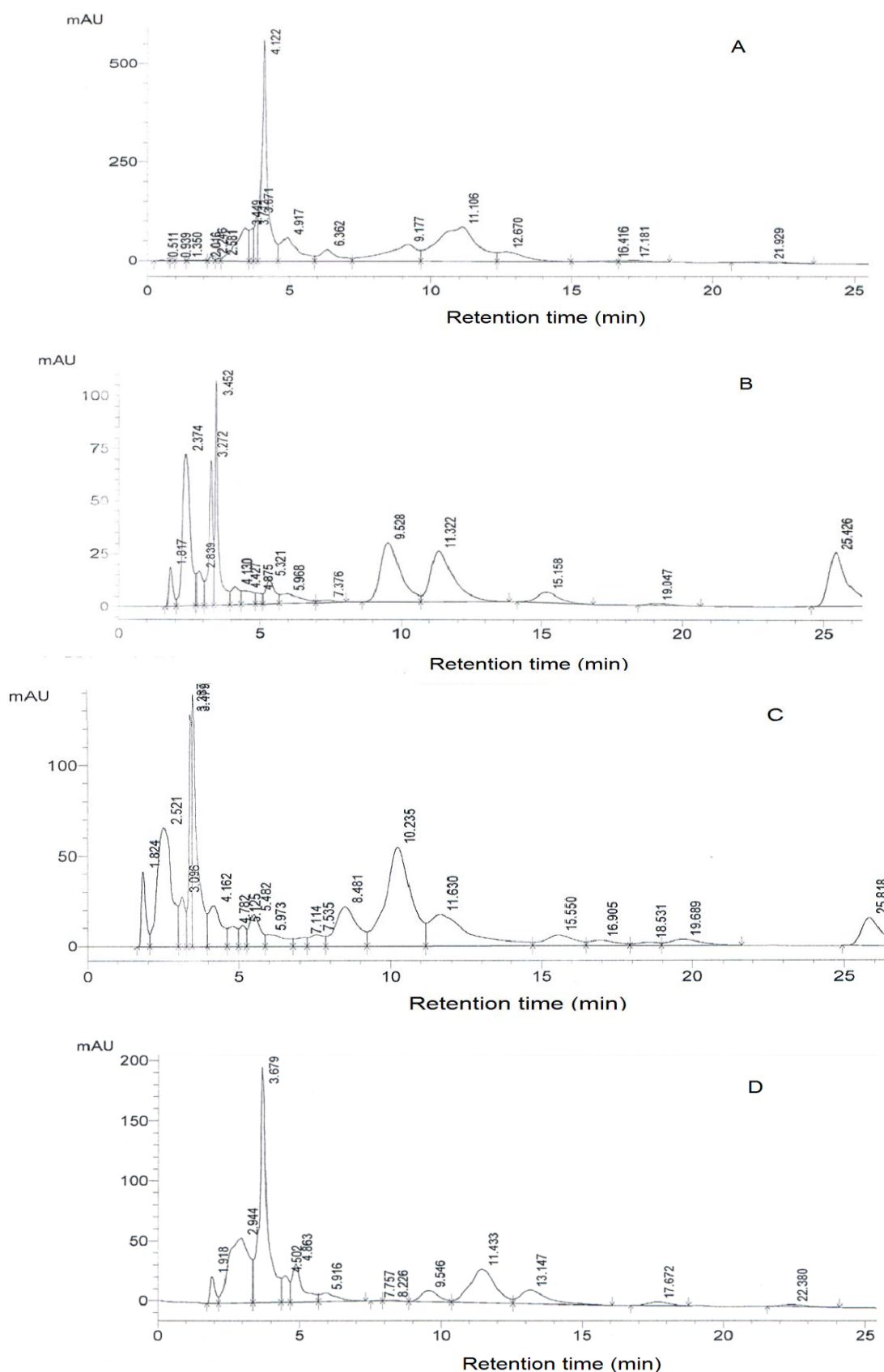


Figure 1: HPLC Chromatograms of *Marrubium vulgare* extracts. A: leaves methanolic, B: defatted leaves methanolic, C: Leaves Hexane and D: Stem methanolic extracts.

Antimicrobial activity

To evaluate the antimicrobial activity of *Marrubium vulgare*, the activity index (AI) was calculated on the basis of

comparison with standard (gentamycin for bacteria and Fluconazole for fungi) and extracts (Figure 2).

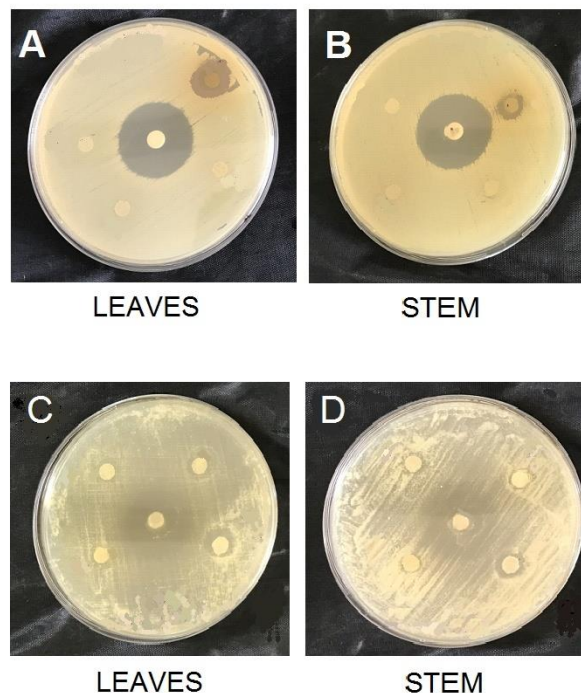


Figure 2: Inhibition of the growth of *Staphylococcus aureus* (A, B) and *Candida albicans* (C, D) by *Marrubium vulgare* methanolic extracts using the disc diffusion method.

The results indicated that the methanolic extract of leaves showed higher activity against *Staphylococcus aureus* (IZ-11.00 mm; AI-0.53) and *Escherichia coli* (IZ-7.5 mm; AI-0.33) at the dose of 500 mg/ml but was less active against *Pseudomonas aeruginosa*. When defatted with hexane, this methanolic extract showed lower activity (IZ-9.5 mm; AI-

0.41). Stem methanolic extract has good activity against *S. aureus* (IZ-9.0; AI-0.47). In contrast, hexane extract does not have any antibacterial activity (Table 2).

Concerning the antifungal effects against *Candida albicans*, LME even defatted (DLME) possessed the best activity with IZ-13 & 10 mm, and AI-0.52 & 0.45, respectively (Table 2).

Table 2: Antimicrobial activities of *Marrubium vulgare* extracts (500 mg/ml). IZ: inhibition zone, AI: Activity index

Extracts	<i>Staphylococcus aureus</i>		<i>Escherichia coli</i>		<i>Pseudomonas aeruginosa</i>		<i>Candida albicans</i>	
	IZ	AI	IZ	AI	IZ	AI	IZ	AI
LME	11 ± 1.5	0.53	7.5 ± 0.5	0.33	-	-	13 ± 1.5	0.52
DLME	9.5 ± 0.5	0.41	-	-	-	-	10 ± 1.5	0.45
LHE	-	-	-	-	-	-	-	-
SME	9 ± 1.5	0.47	-	-	-	-	9 ± 1.5	0.36

Staphylococcus aureus species is currently one of the major causes of nosocomial infections²⁴. Recent studies have shown inhibition zone for *S. aureus* in the range of 0.0-18.0 mm²⁵⁻²⁷. Cavanagh & Wilkinson²⁸ and Serban et al.²⁷ investigations showed that the antimicrobial properties depend on the composition of the extract and the species of microorganism. Phytochemical analysis of plant extracts indicates that the presence of one or more groups of phytoconstituents like flavonoids, tannins, glycoside,

phenols, etc. is responsible for antibacterial activity alone or in combinations²⁹.

Candidiasis is more frequent in human immunodeficiency virus (HIV)-infected patients and knowledge about the distribution and antifungal susceptibility of oral *Candida* species is important for effective management of candidiasis³⁰. The prolonged management of oral candidiasis in HIV patients might cause the development of drug resistance candidiasis³¹.

CONCLUSION

In conclusion, the studied pathogens were more sensitive to methanolic extracts than to the hexane extract, although the polyphenol and flavonoid contents are close. This finding suggests that some of the active compounds in the methanolic extracts are polar, whereas the hexane extract may have dissolved out nonpolar compounds that possess less antimicrobial activity. By their antimicrobial activity, *Marrubium vulgare* extracts could serve as a basic material for the preparation of drugs for the treatment of nosocomial infections and candidiasis.

ACKNOWLEDGEMENTS

The authors acknowledge the Algerian Ministry of Higher Education and Scientific Research (MESRS) and the General Directorate of Scientific Research and Technological Development (DGRSDT) for their financial support.

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