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Integrated analysis of anatomy, phytochemistry, and biological potentials of *Marrubium vulgare* L. extracts

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Original Research

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Abstract:

This study reports on the phytochemical and bioactive characteristics of *Marrubium vulgare* L., harvested in northeast Algeria. After extraction using hydrodistillation and solvents with increasing polarity, 0.05% essential oil and 5.9% apolar crude extract were obtained. The plant presented a significant amount of phytochemicals like polyphenols (126.17 mg GAE/g), flavonoids (42.08 mg QE/g), tannins (45.07 mg TAE/g) which explained the high antioxidant power, evidenced by *in vitro* assays (FRAP, H_2O_2, $OH^{\bullet}$ and cyclic voltammetry). Furthermore, the antimicrobial test against various microorganisms (*Staphylococcus aureus*, *Salmonella sp*, *Shigella sp*. *Aspergillus niger*, *Aspergillus flavus*) showed that the methanolic extract was broadly effective against all tested strains. However, among the less polar extracts, activity varied: essential oils showed moderate activity, dichloromethanic extract was active against most bacteria and petroleum ether extract showed significant antibacterial effects. These compelling results underscore the unique and significant therapeutic potential of the different extracts derived from *Marrubium vulgare* L.

Keywords: Antimicrobial activity; Antioxidant activity; Bioactive compounds; *Marrubium vulgare* L.; Phytochemistry; Trichomes

1. Introduction

Drug resistance has reached alarming levels, posing a significant threat to human health worldwide. With a stagnation in the development of new antimicrobial agents, infectious diseases have become increasingly challenging to treat. This growing resistance has sparked renewed interest in plant-based therapeutics, as they often contain active phytochemicals with potent therapeutic properties (Zalegh et al., 2021). Medicinal plants have been widely used in traditional medicine for disease prevention and treatment, a focus that has gained increasing attention in recent research. Notably, approximately 61% of new drugs derived from natural sources have been successfully applied to treat infectious diseases (Ghaedi et al., 2015). They are a rich source of secondary metabolites, which are widely utilized in modern medicine. Several authors have mentioned that using such chemicals can stop the germination and growth of certain microorganisms. These chemicals can be found in all parts of the plant or are only concentrated in the aerial parts, usually the leaves. They are accumulated in the vacuoles or cell walls during the life of the plant (Sahraoui et al., 2013). Across various cultures, they have been used as therapeutic tools for managing inflammatory conditions, and numerous experimental studies support the effectiveness of these treatments. Additionally, many medicinal plants have therapeutic value that is closely related to their antioxidant capacity in addition to their antimicrobial effects. The pathophysiology of many chronic diseases, including infectious diseases where inflammation is a major factor, is influenced by oxidative stress, which is brought on by an imbalance between free radicals and the body's antioxidant defences (Salehi et al., 2020). Therefore, it is essential to investigate natural antioxidants derived from plants in order to create supplementary approaches for treating conditions linked to oxidative stress. This global need for effective alternatives has led researchers to investigate various medicinal plants, including *Marrubium vulgare* L., known for its

potential therapeutic properties.
The *genus Marrubium* includes approximately ten species, with *M. vulgare* L., commonly known as 'horehound', being one of the most notable species. Traditionally used in folk medicine across various cultures, *M. vulgare* is reputed for its tonic, aromatic, stimulant, expectorant, diaphoretic, and diuretic properties. It has been applied in the treatment of bronchial asthma and nonproductive cough, and it was once highly valued in managing uterine, visceral and hepatic disorders as well as in treating conditions such as phthisis (Amri et al., 2017). As an important local species, *M. vulgare* warrants extensive study to uncover the bioactive compounds responsible for its traditional medicinal uses and assess its efficacy as a natural source of antioxidants. This study, therefore, aims to identify these bioactive compounds and evaluate the antioxidant activities of petroleum ether, dichloromethane, and methanol extracts of *M. vulgare*, contributing valuable insights into its possible role in treating infection and oxidative stress-related diseases.
While *M. vulgare* has been studied in several Algerian regions (Bouterfas et al., 2016), its phytochemical and pharmacological profile in the northeast remains poorly characterized. This study addresses this gap by providing a comprehensive analysis of the species from this under-investigated area. A key aspect of our work is the evaluation of antimicrobial activity against clinically relevant strains, such as *Salmonella sp*. and *Shigella sp*., which have not been commonly included in previous pharmacological assessments of Algerian *M. vulgare*. Our objective is to elucidate the regional chemotypic variation and expanded antimicrobial profile of *M. vulgare*, offering new comparative insights that enhance the understanding of its biogeographical diversity and therapeutic potential in Algeria.

2. Experimental

2.1 Plant materials

M. vulgare, a perennial medicinal plant, is native to a vast area extending from the Mediterranean region to Central Asia. This robust species has since become widely naturalized across the globe, including the Americas, Australia, and the Canary Islands. Ecologically, the plant is highly resilient, thriving in temperate zones. It is characterized by a strong preference for alkaline and calcareous soils and is notably drought-tolerant. This adaptation allows it to colonize dry, well-drained, and disturbed areas effectively (Sagliocco et al., 2000; Aćimović et al., 2020).
In our work, aerial parts of *M. vulgare* were collected during the flowering period from the region of Setif (36°.18′41.65″N, 5°.45′74.58″E) at an altitude of 1100 m. The plant material was authenticated as *M. vulgare* by Prof. H. Laouer of the Department of Botany, University of Setif 1. To ensure botanical verification and for future reference, a voucher specimen was prepared and deposited at the herbarium of the Laboratory of Botany, Faculty of Nature and Life Sciences, University Ferhat Abbas Sétif 1, under the accession number MV253/24. The species is native to the region and is widely distributed in local uncultivated lands and steppic environments (Boutabia et al., 2015). After collection, leaves were separated from stems, cleaned of impurities and dried for a few days at ambient temperature in a shaded and ventilated place before being ground to a powder.

2.2 Chemicals

Salts and analytical-grade chemicals were sourced from BIOCHEM, while additional reagents and solvents were procured from Sigma, Prolabo, Aldrich, Organics, and Janssen Chemical. Gentamicin and amphotericin B purchased from Sigma-Aldrich and ascorbic acid from Merck.

2.3 Anatomical study

Young fresh parts containing stems and leaves were chosen, and sections were taken manually using sambucus wood and a sharp riser blade. A light microscope (Zeiss) was used to take images after double colouration of sections according to the Locquin et al. (1983) method.

2.4 Preliminary phytochemical analyses

Phytochemical screening tests consist of detecting different families of existing compounds in *M. vulgare* through qualitative reactions. The detection of these chemical compounds is based on precipitation reactions or a specific color change.

2.4.1 Test for tannins

2.4.1.1 Ferric chloride test

A 5 mL sample solution was taken in a test tube. 1 mL of a ferric chloride solution (5.0% *w/v*) was added to this sample solution. Orange precipitation appeared, which indicates the presence of tannins (Bharudin et al., 2013).

2.4.1.2 Catechin tannins Stiasny test

1 mL of each extract solution, 1 mL of Stiasny reagent was added; the resulting mixture was heated at 90 °C for 15 min. The formation of a precipitate indicates the presence of condensed tannins (Haida et al., 2021).

2.4.2 Test for alkaloids

2.4.2.1 Mayer's test

A small amount of the sample was placed on a watch glass, and a few drops of concentrated HCl were added. The mixture was then stirred, followed by the addition of one drop of Mayer's reagent. The formation of a white precipitate indicated the presence of alkaloids.

2.4.2.2 Dragendorff's test

A small amount of the sample was placed on a watch glass, and a few drops of concentrated HCl were added and stirred. Then, one drop of Dragendorff's reagent was added to the mixture. The formation of a brick-red precipitate indicates the presence of alkaloids.

2.4.2.3 Wagner's test

One mL of the test sample was placed in a test tube. Then, 0.2 mL of Wagner's reagent was added. The appearance of brown or reddish precipitate indicates the presence of alkaloids (Zumu et al., 2024).

2.4.3 Test for flavonoids

2.4.3.1 Shinoda test

A 20 mL test tube containing 0.5 g of plant sample and 5 mL of methanol was used. Three pieces of magnesium (mg) chips and a few drops of concentrated HCl were also added to the mixture. The appearance of a purple coloration served as a positive qualitative indicator for the presence of flavonoids in the sample.

2.4.3.2 Ammonia test

For the ammonia test, dilute ammonia (5 mL) was added to a portion of the aqueous filtrate of the extract. Then, concentrated sulfuric acid (1 mL) was added. The appearance of a yellow coloration that disappears upon standing indicates the presence of flavonoids (Warsi et al., 2017).

2.4.3.3 Pew test

To conduct this test, 5 mL of the aqueous extract was mixed with 0.1 g of metallic zinc and 8 mL of concentrated sulfuric acid. The reaction mixture was observed for a red color, indicative of flavonols (Shaikh et al., 2020).

2.4.4 Test for saponosides

A common and simple test for saponins is the foam test. Accordingly, a 1 mL aliquot of the aqueous sample solution was diluted with 19 mL of distilled water. The resulting mixture was then vigorously agitated for 15 minutes. The persistent formation of a stable foam layer, approximately 1 cm thick, on the surface of the liquid serves as qualitative confirmation of the presence of saponins (a positive foam test result) (Haida et al., 2021).

2.4.5 Test for cardiac glycoside (Keller-Killani test)

In this test, 0.5 g of the plant sample was placed in a 20 mL test tube and combined with 5 mL of glacial acetic acid containing a trace amount of ferric chloride. After carefully adding 1 mL of concentrated sulfuric acid down the side of the test tube, which was held at a 45° angle, the appearance of a purple ring at the interface was interpreted as the presence of cardiac glycosides (Chaudhary et al., 2023).

2.4.6 Test for steroids/terpenes

2.4.6.1 Libermann-Burchard's test

Regarding the Liebermann-Burchard test, a few milligrams of the plant material were dissolved in chloroform in a test tube. Subsequently, one to two drops of concentrated sulfuric acid were added to the solution, followed by the addition of $2-3$ drops of acetic anhydride. A light green color appeared at the interface of the two layers, confirming the presence of steroids (Adu et al., 2019).

2.4.6.2 Salkowski's test

In the Salkowski test, $1-2$ drops of concentrated sulfuric acid were added to a few milligrams of the sample dissolved in chloroform. The appearance of a red color at the interface of the two layers confirmed the presence of steroids (Faruq et al., 2024).

2.5 Extraction of crude extracts

Powdered dried plant leaves were subjected to successive solvent extraction using petroleum ether, dichloromethane and methanol applying maceration technique at a ratio of 1/10 (w/v) of plant powder to solvent volume. In each solvent, the plant was macerated for 24 hours under continuous stirring. Each time, the marc (exhausted plant material) was air-dried and later extracted with the next solvent. All the extracts were concentrated by distilling the solvent in a rotary flash evaporator (Rotavapor Büchi). The yield was then calculated using the following equation (Eq. 1):

$$\text{Yield}(\%) = \text{W}_{\text{extr}}/\text{W}_{\text{plant}} * 100 \quad (1)$$

where W_{extr} and W_{plant} respectively account for the weight of the extract and the weight of the plant material. In addition, the appearance and the color of the extracts were noted.

2.6 Quantitative phytochemical analyses

2.6.1 Determination of total Phenolic contents (TPC)

The total polyphenols content is measured using the Folin-Ciocalteu reagent in accordance as described by Gnoyke et al. (2010). In the procedure used, 20 µL of the plant extract was mixed with 100 µL of diluted FCR (1:10) and 80 µL of sodium carbonate Na_2CO_3 (7.5%) then the mixture was left in the darkness for two hours before measuring the absorbance at 765 nm. To prepare the blank, the extract was replaced with the solvent. Similarly, a calibration curve was generated under the same operating conditions using gallic acid as the standard. Absorbance measurements were taken using a microplate reader (PerkinElmer EnSpire).

2.6.2 Determination of total flavonoid contents (TFC)

The TFC is determined using a reaction with aluminum trichloride reagent. Briefly, 500 µL of the diluted extract was mixed with 2 mL of methanol and 200 µL of a aluminum trichloride ($AlCl_3$, 10% *w/v*) solution. After incubating for 3 min at room temperature, 200 µL of a 1.0 M CH_3COONa was added, and the final volume was adjusted to 5 mL with methanol. The mixture was then incubated in the dark at room temperature for 40 min. Subsequently, the absorbance was measured at 430 nm using a microplate reader (Perkin Elmer, Enspire). A calibration curve was prepared under the same conditions using quercetin, and the total flavonoid content is expressed as milligrams of quercetin equivalents per gram of dry extract (mg QE/g) (Sari et al., 2023).

2.6.3 Determination of total tannins contents (TTC)

Total tannin content was determined using the Folin-Ciocalteu method. Accordingly, a 0.1 mL aliquot of the sample extract was mixed with 7.5 mL of distilled water, followed by the addition of 0.5 mL of Folin-Ciocalteu reagent and 1 mL of sodium carbonate solution (35% *w/v*). The volume was then adjusted to 10 mL with distilled water. The mixture was shaken thoroughly, incubated at room temperature for 30 min and the absorbance was measured at 725 nm using a microplate reader (Perkin Elmer, Enspire). A blank was prepared using water instead of the sample. A series of gallic acid standard solutions were treated in the

same manner, and the results were expressed as milligrams of gallic acid equivalents per gram of extract (Tamilselvi et al., 2012).

2.6.4 Determination of total flavonols content

The total flavonoid content in *M. vulgare* was determined using the following procedure: One milliliter of plant extract (1 mg/mL) was mixed with 1 mL of aluminum trichloride solution (20 mg/mL) and a drop of acetic acid, then diluted with ethanol to a final volume of 25 mL. The absorbance at 415 nm was measured after 40 min using a microplate reader (Perkin Elmer, Enspire). Quercetin was used as the standard to establish the calibration curve, and the total flavonoid content was expressed as milligrams of quercetin equivalents per gram of dry weight. The absorbance of a standard quercetin solution (0.5 mg/mL) in ethanol was measured under the same conditions. All determinations were performed in triplicate (Kumaran et al., 2007).

2.7 Determination of the antimicrobial activity of the extracts

The antimicrobial activity of the solvent extracts of *M. vulgare* was performed *in vitro* by the technique of the diffusion method.

2.7.1 Diffusion method

The agar diffusion test, or the Kirby-Bauer disk-diffusion method, is a useful mean to measure the effect of an antimicrobial agent against bacteria or fungi grown in culture. To assess antimicrobial activity, the microorganisms were uniformly inoculated onto the surface of an agar plate. Subsequently, a sterile filter-paper disk, impregnated with the test compound, was placed onto the agar surface, allowing the compound to diffuse into the medium. The concentration of the compound will be highest next to the disk, and will decrease as distance from the disk increases. If the compound is effective against bacteria at a certain concentration, no colonies will grow where the concentration in the agar is greater than or equal to the effective concentration. The resulting zone of inhibition, observed as a clear area surrounding the disk, was measured to determine the compound's antimicrobial efficacy, with a larger diameter being directly proportional to greater effectiveness (Balouiri et al., 2016).

2.7.2 Tests of antibacterial and antifungal activity

The antimicrobial activity of the essential oils and extracts was evaluated using the standard disk diffusion method against four bacterial strains, namely *Staphylococcus aureus* ATCC 6538, *Salmonella enterica* ATCC 14028, *Shigella sonnei* ATCC 25931 and two fungal strains, namely *Aspergillus niger* ATCC 16888, *Aspergillus flavus* ATCC 16883. These strains were provided by Pasteur institute and the microbiology laboratory of the local Hospital of Setif.

Each inoculum is used to inoculate nutrient agar cast in Petri dishes to a thickness of 4 mm (corresponding to 20 mL in 90 mm diameter dishes) and then dried in an oven at 37 °C before use. Seeding is performed by swabbing the freshly prepared inoculum across the entire agar surface to form tight streaks, rotating the dish approximately 60° after each application to ensure even distribution of the inoculum (Balouiri et al., 2016).

Chromatographic paper discs, 6 mm in diameter and previously sterilized, were placed on the surface of agar seeded with microorganisms after being loaded with 30 µL of essential oils and extracts diluted in dimethyl sulfoxide (DMSO) at a concentration of 50 mg/mL. Additional discs loaded with 30 µL of DMSO served as controls (Foss et al., 2014). After incubation at 37 °C (24 hours) for bacteria and 32 °C (72 hours) for fungi, the diameter of inhibition is measured.

2.8 Determination of the antioxidant activity of *M. vulgare* extracts

To determine the antioxidant capacities of the various extracts of *M. vulgare*, four techniques were used, including hydrogen peroxide scavenging, hydroxyl radical scavenging, ferric reducing antioxidant power (FRAP) and cyclic voltammetry. Ascorbic acid was used as reference material, and the antioxidant capacities of the extracts were expressed in terms of ascorbic acid equivalents (Eq. AA). All experiments were made in triplicate.

2.8.1 Hydrogen peroxide scavenging activity

The ability of the crude extracts of *M. vulgare* to scavenge hydrogen peroxide was evaluated according to the method of Ruch et al. (1989). A solution was prepared by mixing 4 mL of the crude extract (0.5 mg/mL) with 3.4 mL of phosphate buffer (0.1 M, pH 7.4) and 0.6 mL of hydrogen peroxide (40 mM). After incubation for 10 min utes at room temperature, the absorbance of the mixture was determined at a wavelength of 230 nm using a UV-Vis spectrophotometer (SHIMADZU UV-1700) against a blank.

The scavenging percentage of hydrogen peroxide was calculated using the following formula (Eq. 2):

$$\%H_2O_2 = \left(\frac{A_{contr} - A_{sample}}{A_{contr}}\right) * 100 \quad (2)$$

2.8.2 Hydroxyl radical scavenging activity

The hydroxyl scavenging activity of crude extracts was determined according to the method of Smirnoff et al. (1989). The reaction mixture contains 1.0 mL of a solution of $FeSO_4$ (1.5 mM), 0.7 mL of a solution of hydrogen peroxide (6 mM), 0.3 mL of a solution of sodium salicylate (20 mM) and 1 mL of the extract (0.5 mg/mL). After incubation for 1 hour at 37 °C, the absorbance was measured at 532 nm with a UV-Vis spectrophotometer (Shimadzu UV-1700). The inhibition of the hydroxyl radical OH was calculated using the following formula (Eq. 3):

$$\%OH^{\bullet} = \left(1 - \frac{A_1 - A_2}{A_0}\right) * 100 \quad (3)$$

where A_0: absorbance of the blank; A_1: absorbance of the sample and A_2: absorbance without sodium salicylate.

2.8.3 Ferric reducing antioxidant power (FRAP)

The capacity of the extracts to reduce iron (Fe^{3+}) was determined according to the method described by Oyaizu (1986). The reaction mixture contains 2.5 mL of phos-

phate buffer solution (pH 6.6, 0.2 M), 2.5 mL of 1% potassium ferricyanide solution, and 1.0 mL of each extract (0.5 mg/mL). After incubation for 30 min at 50 °C, 2.5 mL of 10% trichloroacetic acid was added to the reaction mixture, which was then centrifuged at 3000 rpm for 10 min . An aliquot (2.5 mL) of the supernatant was combined with 2.5 mL of distilled water and 0.5 mL of 0.1% ferric chloride solution. The absorbance was then measured at 700 nm using a UV-Vis spectrophotometer (Shimadzu UV-1700) against a blank (without extract). The increase in absorbance of the reaction mixture indicates the reducing power of the extracts.

2.8.4 Cyclic voltammetry

The examination of the electrochemical behavior of plant extracts provides valuable insights into their antioxidant properties and redox characteristics. This analysis typically employs cyclic voltammetry (CV), a technique that enables the observation of oxidation and reduction processes of the compounds present in the extracts. Measurements were conducted at 298 K using a Voltalab PGZ 301 potentiostat. The setup consisted of a glassy carbon working electrode, a platinum counter electrode, and an Ag/AgCl reference electrode.

For each test, the scan speed was varied at 10, 20, 30, 40, 50, and 60 mV/s, with a scan potential ranging from −300 to 1200 mV. The influence of pH was assessed by preparing the extracts in three different media: 0.1 M phosphate buffer solution at pH 7.4, 0.1 M NaH_2PO_4 at pH 4.82, and 0.1 M Na_2HPO_4 at pH 9.35, all at an extract concentration of 1 mg/mL. The calibration curve was established using ascorbic acid under the same pH conditions at a scan speed of 50 mV/s across a potential range of −300 to 1200 mV (Zlatić et al., 2022).

3. Results and discussion

3.1 Morphological description of *M. vulgare*

M. vulgare (Fig. 1) is a perennial flowering plant from the Lamiaceae family, commonly referred to as white horehound. The plant typically grows to a height of 30 cm and spreads to about 75 cm in width, though under favorable conditions, it can reach up to 60 cm tall and 90 cm wide. It features numerous annual, quadrangular, and branching stems, which often become woody near the base. The leaves are opposite, petiolate, approximately 1 inch long, roundovate, with a hairy upper surface and a woolly lower surface. Each stem node bears two leaves with a wrinkled surface. The small white flowers grow in dense clusters above the nodes, located around the upper parts of the stems (Máthé et al., 2015). These morphological characteristics are consistent with those reported for *M. vulgare* populations in other Mediterranean and European regions (Aćimović et al., 2020). However, plants collected in Algerian semi-arid environments tend to exhibit a denser indumentum and slightly smaller leaves compared to temperate populations, which may reflect an adaptation to local climatic conditions (Soltani et al., 2021). Despite these min or variations, the diagnostic features of the species remain identical across regions.

3.2 Anatomical study of *M. vulgare*

The anatomical examination of *M. vulgare* uncovers notable structural characteristics in its stems and leaves. Fig. 2 and Fig. 3 that play a role in its physiological functions. This study primarily employs light microscopy to analyze young, fresh stems and leaf cross-sections. The stem of *M. vulgare* exhibits several important components: the pith, which serves as a storage area; vascular tissues, including xylem and phloem, crucial for transporting nutrients and water; the cortex, providing structural support and storage; and the epidermis, which acts as a protective outer layer (Raven et al., 1976). Notably, the cortex contains angular collenchyma tissue distributed uniformly at the stem's

Figure 1. A photograph representing the aerial parts of *Marrubium vulgare* L.

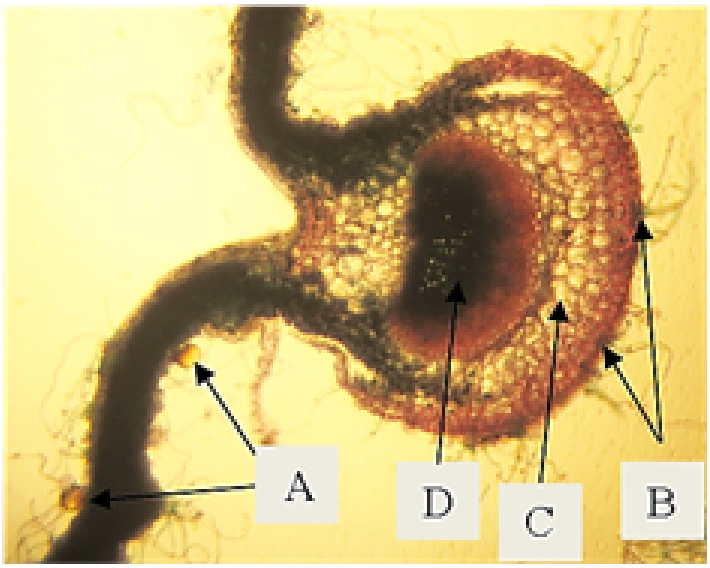

Figure 2. Cross sectional view of *M. vulgare* stem: A: Glandular trichomes, B: Stellate trichomes, C: Colenchyma tissue, D: Vascular tissue, E: Pith.

corners, enhancing flexibility and support vital for upright growth. In terms of leaf anatomy, the cross-section reveals mesophyll tissue between the upper and lower epidermis responsible for photosynthesis, along with a midrib containing vascular tissues surrounded by lacunous parenchyma for gas exchange. Both stems and leaves are covered with various trichomes that serve protective and secretory roles (Kuźniak et al., 2016). Glandular trichomes include peltate trichomes, which have a short stalk and large head that secrete essential oils, and capitate trichomes with a long stalk involved in secretion. Non-glandular types include multicellular uniserate trichomes, offering a woolly appearance, and denser multicellular branched trichomes that enhance texture. These trichomes not only help defend against herbivores but also reduce water loss through transpiration (Lauter et al., 1986).

Our microscopic observations are in agreement with previous anatomical studies on *M. vulgare* from different regions (Haratym et al., 2017), which describe the same tissue organization and trichome types. However, the Algerian material shows a relatively higher density of both glandular and non-glandular trichomes, a feature also noted in plants growing under arid conditions (Aćimović et al., 2020). Such variations likely contribute to the observed differences in essential oil yield and composition among populations.

3.3 Qualitative phytochemical screening

Chemical investigations of the plant material serve various purposes, including the determination of the substance groups (phytochemical screening), quantitative and structural analysis of active compounds, and the isolation of substances for further identification (Waksmundzka-Hajnos et al., 2008).

Some of the active constituents (or secondary metabolites) are responsible for the characteristic odors, pungencies, and colors of plants. Others impart specific culinary, medicinal, or poisonous properties to a plant or help defend it against threats (Evans et al., 2002).

Results obtained from the phytochemical investigation of *M. vulgare* leaves, shown in Table 1, revealed the existence of several compound groups known for their medicinal activities. Experiments showed a strong presence of

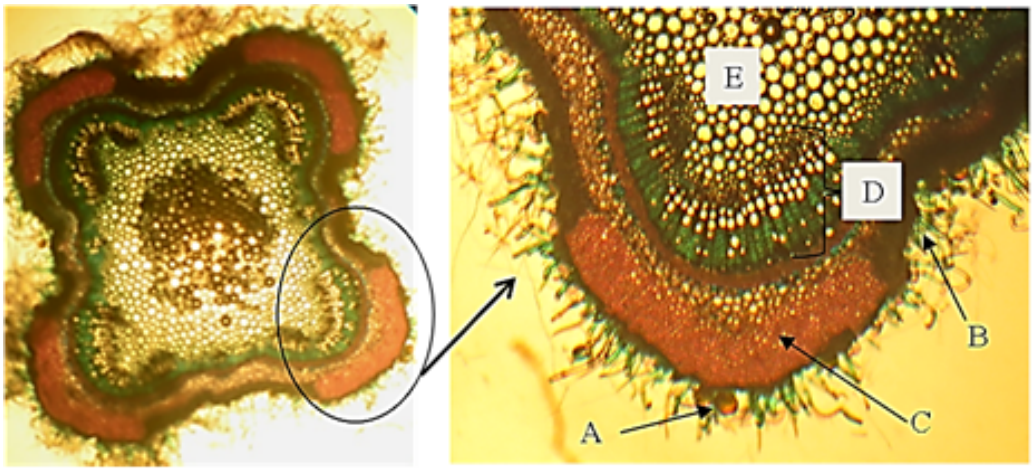

Figure 3. Cross sections of *M. vulgare* leaf. A: Glandular trichomes, B: Stellate trichomes, C: Cortex, D: Vascular tissue.

Table 1. Phytochemical screening results of *M. vulgare*.

Compounds		Result	Color in test tube
Tannins		+++	Blue-black
Catechic tannins		++	Red ring
Gallic tannins		+++	Blue-black
Alkaloids		++	Precipitates
Sterols and triterpenes		++	violet ring
Free anthraquinones		−	green
Flavonoids		++	yellow brown
Flavones		++	pink orange
Leucoanthocyanes		−	Brown
Saponosides		+	visible foam
Anthraquinones	*C*-hétérosides	++	Red
	O-hétérosides	++	Red
Mucilage		++	Flocculent precipitates

+++: Strongly positive reaction, ++: Moderate positive reaction, +: Weak positive reaction, −: Absent.

tannins, gallic tannins, flavonoids, sterols and triterpenes, linked-anthraquinones (*C*-heterosides, *O*-heterosides), and flavones. However, the table indicated a moderate presence of alkaloids, flavones, mucilage and catechic tannins and a low content of saponosides in the plant. The absence of other secondary metabolites like free anthraquinones and leucoanthocyanes was confirmed.

All phytochemicals detected in *M. vulgare* leaves used in this study have several therapeutic activities, as indicated in the literature (Pengelly et al., 2020). This may justify the various scientifically proven medicinal effects for this plant, such as antihypertensive, anti-inflammatory, antioxidant, analgesic, antispasmodic, hypoglycemic and hypolipidemic properties, expectorant, digestive stimulant, anti-inflammatory, anti-asthmatic, antihypertensive, hipolipidemic, antibacterial, antifungal, analgesic and anti-edematogenic (Meyre-Silva et al., 2010; Seca et al., 2019).

3.4 Characteristics of crude extracts

The phytochemical profile of *M. vulgare* is characterized by a low abundance of volatile constituents relative to its non-volatile fraction. The hydrodistillation of the aerial parts of *M.vulgare* collected from Setif region results in a light-yellow essential oil with a yield of 0.05% (*w/w*) which aligns with the low-end range ($0.05 - 0.17\%$) observed in populations from Poland to Romania (Zawiślak, 2013; Lodhi et al., 2017). This output is substantially lower than that of classic aromatic Lamiaceae species such as *Mentha pulegium* L. (3.25%) or *Thymus fontanesii* (3.09%) (Tomić et al., 2009), underscoring its classification as a non-essential oil-rich plant.

Conversely, solvent extraction reveals a more complex and variable picture of its non-volatile metabolites. The principle of sequential extraction, guided by "like dissolves like," is employed to fractionate compounds based on polarity (Başgel et al., 2006; Kabata-Pendias, 2010). Extraction of crude extracts from *M. vulgare* leaves using solvents with increasing polarity gave the results reported in Table 2 which showed the yields and characteristics of these crude extracts. The results indicate that the nonpolar solvent (petroleum ether) gave the highest yield 5.9% which consists of mostly lipophilic compounds (*e.g.*, alkanes, fatty acids, pigments, waxes, sterols, some terpenoids, some alkaloids, and coumarins). Medium-polarity solvent (dichloromethane) led to a yield of 4.64% which represents compounds of intermediate polarity (*e.g.*, some alkaloids, some flavonoids); however, the more polar solvent (methanol) gave a yield of 1.96%. The last extract contains the more polar compounds, such as glycosides, tannins, most of the flavonoids and some alkaloids, etc. These results indicate that there are more nonpolar compounds in *M. vulgare* than polar ones.

This pattern, where a non-polar solvent gives the highest yield, contrasts with several other studies on Algerian *M. vulgare*. For instance, comparative stufy about *M. vulgare* origating from Saida and Bechar (Algerian northwest) re-

Table 2. Characteristics of *M. vulgare* extracts using solvents with increasing polarity.

Extract / Solvent	Yield (%)	Aspect	Color
Essential oils	0.05	Liquid	Light yellow
Petroleum ether	5.9	Oily	Brown
Dichloromethane	4.64	Pasty	Black
Methanol	1.96	Pasty	Brown

ported significantly higher yields for methanolic extracts (ranging from ∼ 5 to 12.5%) compared to their hexane extracts (Bouterfas et al., 2016). This discrepancy highlights a profound chemotypic variation, suggesting that our specific plant population is remarkably rich in non-polar metabolites like waxes, fats, chlorophyll, and less polar terpenoids, while being less abundant in the highly polar phenolic and flavonoid glycosides that typically dominate methanolic extracts of this species from other regions. The very low essential oil yield is, however, consistent with reports from various Maghrebin regions (Yabrir, 2019) confirming that low volatile oil production is a shared trait among some Algerian chemotypes.

3.5 Quantitative phytochemical analyses

The quantitative phytochemical profile shown in Table 3 revealed a significant concentration of bioactive compounds, including a high total phenolic content about 126.2 mg GAE/g and total tannins content about 45.1 mg TAE/g, which is the primary indicator of the *M. vulgare* extract's strong antioxidant, antibacterial and astringent properties. Furthermore, notable amounts of flavonoids (42.08 mg QE/g) and flavonols (14.81 mg QE/g), subclasses recognised for their elevated free-radical scavenging activity and association with anti-inflammatory and cardiovascular protective effects, are also present (Lodhi et al., 2017).

Table 3. Total Phenolic, flavonoids, flavonols and tannins content of *M. vulgare* extract.

Phytochemicals	Content (mg/g DW)
Total phenolic content	126.17 ± 1.7 (mg GAE /g)
Total flavonoids content	42.08 ± 0.6 (mg QE /g)
Total flavonols content	14.81 ± 0.3 (mg QE /g)
Total tannins content	45.07 ± 0.8 (mg TAE /g)

*Values were expressed as means ± SD

Calibration curves used for the quantification of TPC, TFC, TTC and total flavonols in *M. vulgare* extract have been shown in Fig. 4. This rich blend of phenolics, flavonoids, and tannins supports the documented traditional uses of *Marrubium* species in herbal medicine and confirms the plant's potential as a natural antioxidant source.

3.6 Antimicrobial activity of *M. vulgare*

The antimicrobial activity of the extracts is performed by the technique of the diffusion method. The obtained results are presented in the Table 4.

The sensitivity of the strains is classified based on the diameter of the inhibition zones. A diameter of less than or equal to 8 mm indicates no sensitivity, those between 8 and 14 mm suggest limited sensitivity, while diameters between 14 and 20 mm are considered to reflect average sensitivity. If the diameter is greater than or equal to 20 mm, the strain is considered highly sensitive (Sharma et al., 2017).

The findings revealed that various extracts of *M. vulgare* exhibited differing degrees of antimicrobial activity against all tested bacterial and fungal strains. While the essential oil, dichloromethane, and petroleum ether extracts showed low to moderate activity, the methanolic extract demonstrated consistent, broad-spectrum inhibition. Among the extracts, the methanolic extract exhibited the most pronounced antimicrobial efficacy, showing significant inhibition against both bacterial and fungal species. Notably, the methanolic extract displayed comparable activity against fungi such as *Aspergillus flavus* relative to the other extracts. The dichloromethane extract, although generally effective, showed somewhat reduced activity against *Salmonella sp.* and *Aspergillus flavus*, indicating that these strains were less sensitive to it compared to others. Nonetheless, the dichloromethane extract still exhibited acceptable antimicrobial performance. On the other hand, the petroleum ether extract demonstrated notable antimicrobial activity, especially against *Staphylococcus aureus* and *Shigella sp.*, with slightly less effectiveness against other strains. However, all extracts were significantly less effective than the positive controls (Gentamicin and Amphotericin B). These findings are consistent with previous studies on Algerian *M. vulgare*, where methanolic extracts consistently showed superior antimicrobial efficacy compared to non-polar fractions such as essential oils (Benzidane et al., 2020; Mssillou et al., 2021).

3.7 Determination of the antioxidant activities of the extracts

3.7.1 Hydrogen peroxide scavenging activity

Fig. 5 illustrates the percentage of inhibition of hydrogen peroxide (H_2O_2) by *M. vulgare* extracts obtained using three different solvents: Petroleum ether (PE), dichloromethane (DCM), and methanol (MeOH).

The inhibition values demonstrate a specific pattern that is associated with the solvents' polarity. The strongest inhibition (91.91%) was obtained by the non-polar PE extract, significantly surpassing the more polar DCM (71.67%) and MeOH (70.55%) extracts. This potent neutralization of hydrogen peroxide by the non-polar extract is particularly notable when compared to the standard antioxidant, ascorbic acid, which exhibited an inhibition percentage of 78.8% at the same concentration. This finding suggests that the compounds primarily responsible for neutralizing hydrogen peroxide are non-polar or lipophilic, consistent with the hypothesis that the H_2O_2 scavenging mechanism is specifically mediated by the plant's characteristic lipophilic diterpenes, such as marrubiin, which are preferentially extracted by non-polar solvents like PE (Michalak et al., 2024).

3.7.2 Hydroxyl radical scavenging activity

The hydroxyl radical ($OH^{\bullet}$) scavenging activity of various extracts of *M. vulgare* is shown in Fig. 6, revealing a complex, non-linear relationship between solvent polarity and antioxidant efficacy. The apolar extract (petroleum ether) demonstrates the highest inhibition, approximately 72%, indicating strong antioxidant activity. The polar extract (methanol) also exhibits significant inhibition at 66.47%, likely due to its content of effective antioxidant compounds. In contrast, the medium-polarity extract (dichloromethane) shows the lowest inhibition at 37.75%, indicating weaker antioxidant properties. This pattern suggests that phytochemicals of varying polarities contribute to this activity.

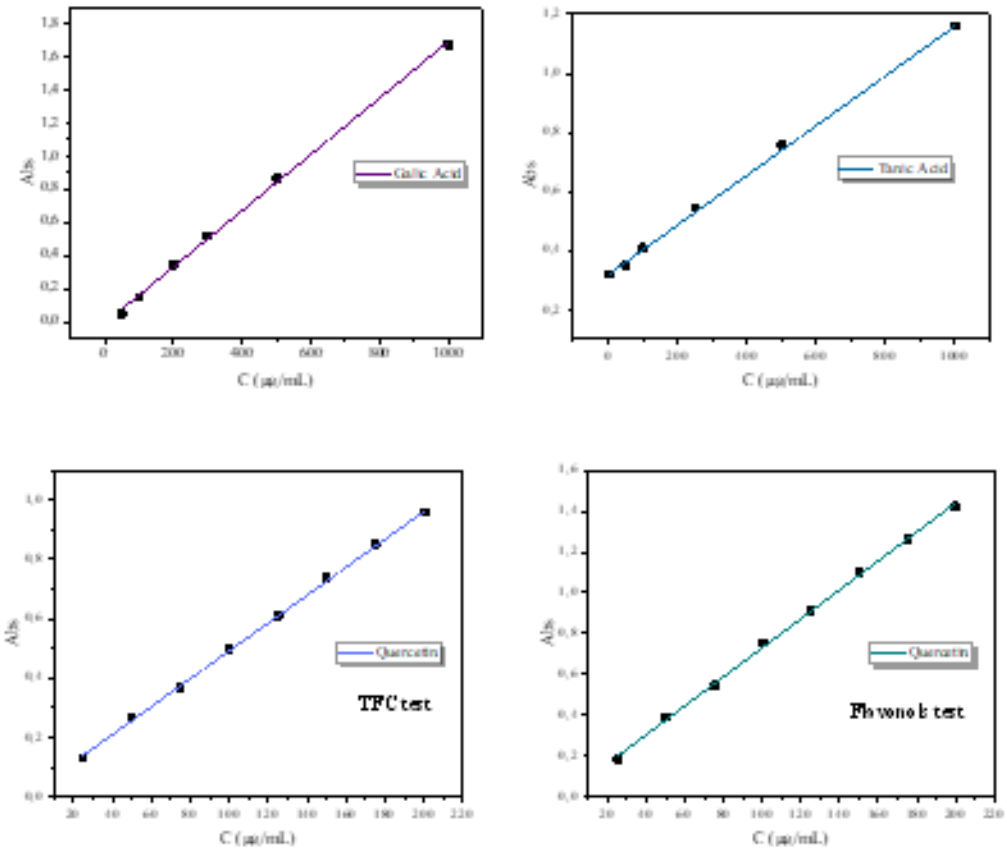

Figure 4. Calibration curves for the quantification of TPC, TFC, TTC and total flavonols in *M. vulgare* extract.

The high effectiveness of the methanol extract is attributed to its richness in polar phenolic compounds and flavonoids. Conversely, the strong performance of the petroleum ether extract indicates that lipophilic compounds, such as the diterpene marrubiin, also play a crucial role.

The hydroxyl radical ($OH^{\bullet}$) scavenging capacity of the tested extracts was assessed by generating $OH^{\bullet}$ radicals using ascorbate and detecting them through their ability to hydroxylate salicylic acid. The inhibition of this hydroxylation process served as the measure of antioxidant activity (Sanna et al., 2022). This method revealed a pattern indicating that phytochemicals of varying polarities contribute to the observed effects. The high efficacy of the polar methanol extract is attributed to its richness in phenolic compounds and flavonoids, which are potent antioxidants known to scavenge radicals by donating hydrogen atoms. Conversely, the strong performance of the non-polar petroleum ether extract suggests that lipophilic compounds, such as the diterpene marrubiin, also play a crucial role, as these compounds are preferentially extracted by non-polar solvents and signifi-

Table 4. The antimicrobial activity of *M. vulgare* extracts and essential oils.

Strains	Inhibition zone (mm)						DMSO
	Essential oils	Methanolic extract	Dichloromethane extract	Petroleum ether extract	Positive control		
Staphylococcus aureus	13 ± 0.07	10 ± 0.09	11 ± 0.1	13 ± 0.07	Gentamicin	21 ± 0.1	8
Salmonella sp	11 ± 0.1	10 ± 0.08	8 ± 0.07	11 ± 0.08		18 ± 0.1	
Shigella sp.	13 ± 0.12	10 ± 0.1	11 ± 0.1	12 ± 0.09		17 ± 0.09	
Aspergillus niger	12 ± 0.18	12 ± 0.08	11 ± 0.09	10 ± 0.08	Amphotericin B	16 ± 0.08	
Aspergillus flavus	10 ± 0.09	12 ± 0.2	9 ± 0.08	11 ± 0.09		19 ± 0.2	

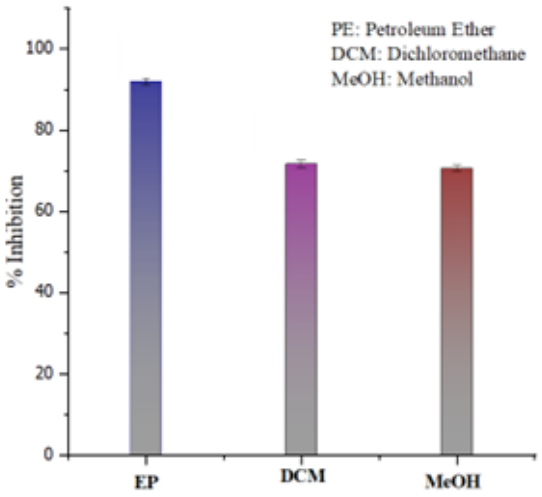

Figure 5. Inhibition Percentage of *M. vulgare* extracts against hydrogen peroxide (H_2O_2).

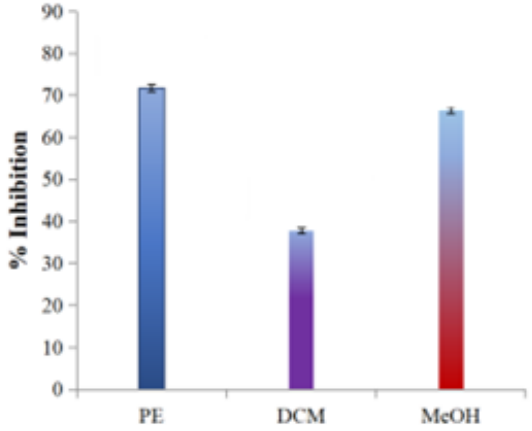

Figure 6. Inhibition percentage of the $OH^{\bullet}$ radical by *M. vulgare* extract.

cantly contribute to the plant's antioxidant capacity (Gavarić et al., 2021).

3.7.3 Ferric reducing antioxidant power (FRAP)

The antioxidant activity results for *M. vulgare* extracts (Fig. 7) indicate that PE extract demonstrated the highest antioxidant potential (75.94%). This suggests that the compounds extracted by petroleum ether, possibly non-polar or lipid-soluble antioxidants, play a significant role in reducing ferric ions, indicating a high electron-donating ability, this higher activity in PE may be due to the extraction of specific antioxidant compounds that are less polar, such as certain flavonoids or terpenoids. However, regarding the experimental results, both the DCM and MeOH extracts showed comparatively lower antioxidant activity at 44.88%, as for ascorbic acid it exhibited at the same concentration inhibition percentage of 68.3%.

The extracts exhibit moderate antioxidant potential compared to the standard, except for the PE extract, which demonstrated the highest antioxidant activity. The variation in antioxidant activity among different solvents suggests that *M. vulgare* contains a diverse array of antioxidant compounds with varying solubilities. The higher activity observed in PE highlights the potential of non-polar extracts from *Marrubium* to serve as potent antioxidants, making them valuable for applications requiring strong, lipid-soluble antioxidant agents. Conversely, the moderate results for DCM and MeOH suggest that while *Marrubium* contains polar antioxidants, their effects may be less pronounced in the FRAP assay.

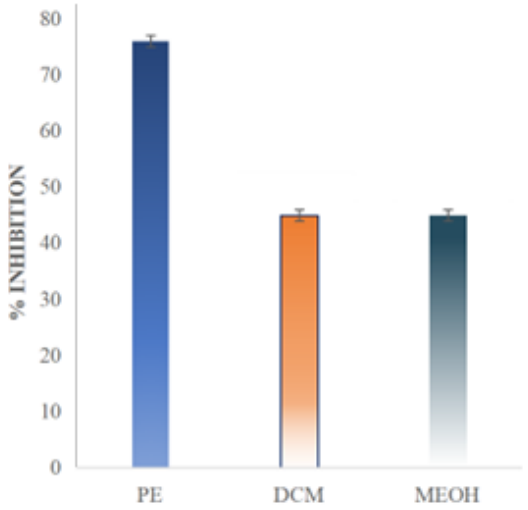

Figure 7. Inhibition percentage of the FRAP radical by *M. vulgare* extract.

3.8 Cyclic voltammetry

The study of the electrochemical behavior of *M. vulgare* extracts by cyclic voltammetry reveals interesting information on their antioxidant activity, which is highly dependent on the pH of the medium and the solvent used, as well as experimental conditions such as scanning speed and extract concentration.

The results show that the antioxidant activity of *M. vulgare* extracts is particularly marked in acidic and neutral media, as evidenced by the well-defined anodic oxidation peaks in the voltammograms (Fig. 8). These peaks indicate that the extracts are capable of undergoing oxidation, which is a typical feature of antioxidant compounds. The absence of an electrochemical response under basic conditions (pH = 9.35) suggests that high pH conditions may inhibit or modify oxidation processes, thus reducing the ability of compounds present in extracts to act as antioxidants.

In acidic conditions (pH = 4.82) (Fig. 9), the anodic oxidation peaks are well pronounced, depending on the scan speed, with maximum intensity observed at a scan speed of 60 mV/s. This indicates greater stability and efficiency of antioxidant compounds at this pH, which may be linked to the greater availability or reactivity of the functional groups responsible for antioxidant activity under acidic conditions. On the other hand, in a neutral medium (pH = 7.4) (Fig. 10), although antioxidant activity is still present, it is optimal at a scan speed of 50 mV/s. This difference from the acidic environment could be due to a variation in the reactivity of antioxidant compounds or to a change in their chemical structure, influencing their ability to be oxidized.

These observations highlight the importance of the chemical environment on the effectiveness of *M. vulgare* extracts as

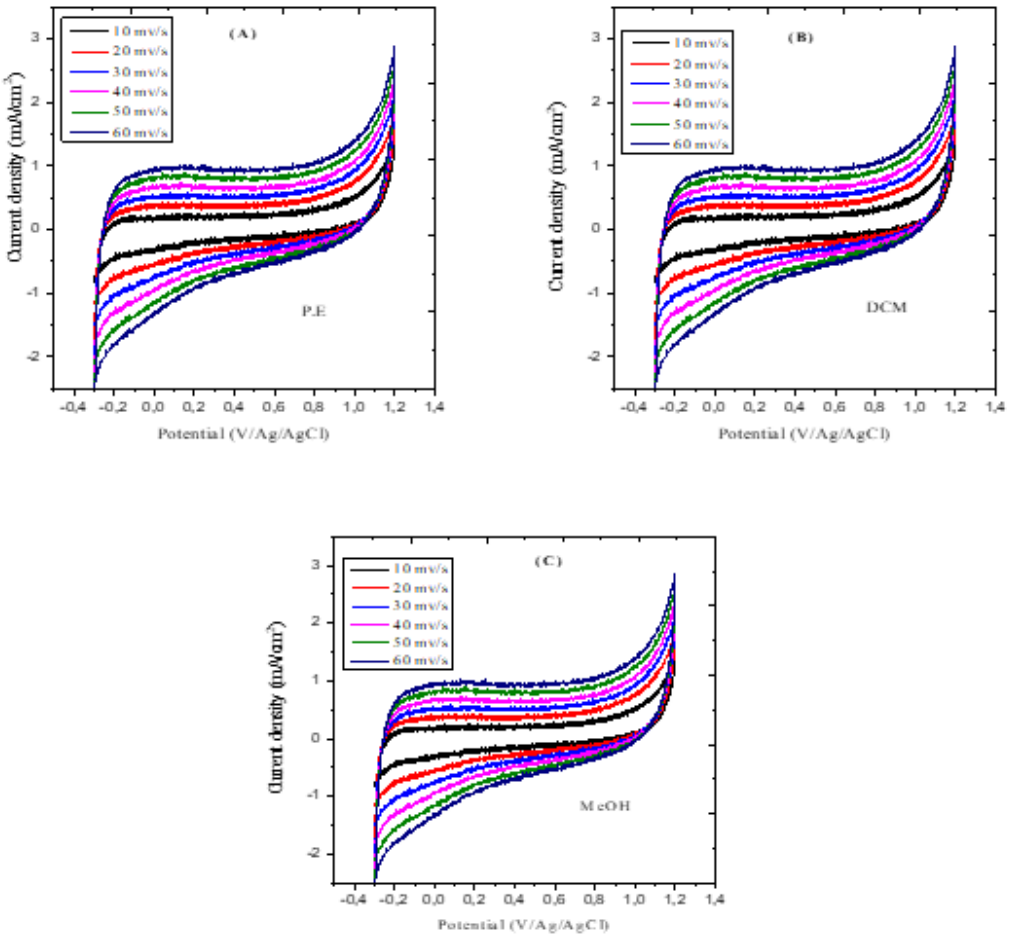

Figure 8. Voltammograms of *M. vulgare* extract in different solvents in basic conditions pH = 9.35.

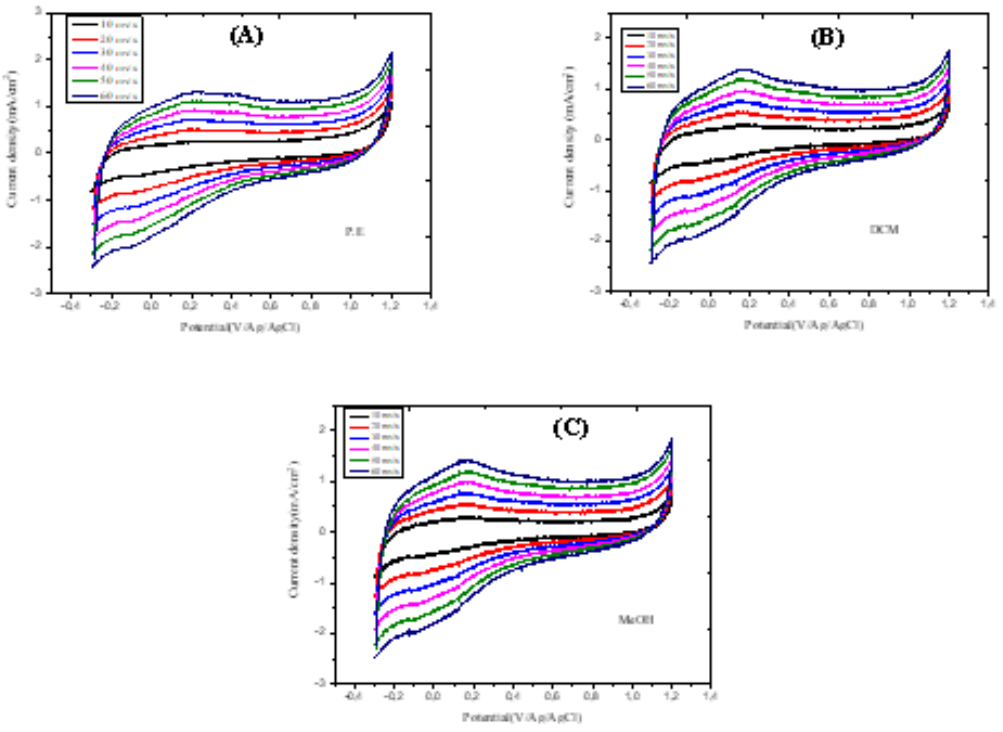

Figure 9. Voltammograms of *M. vulgare* extract in different solvents at pH = 4.82.

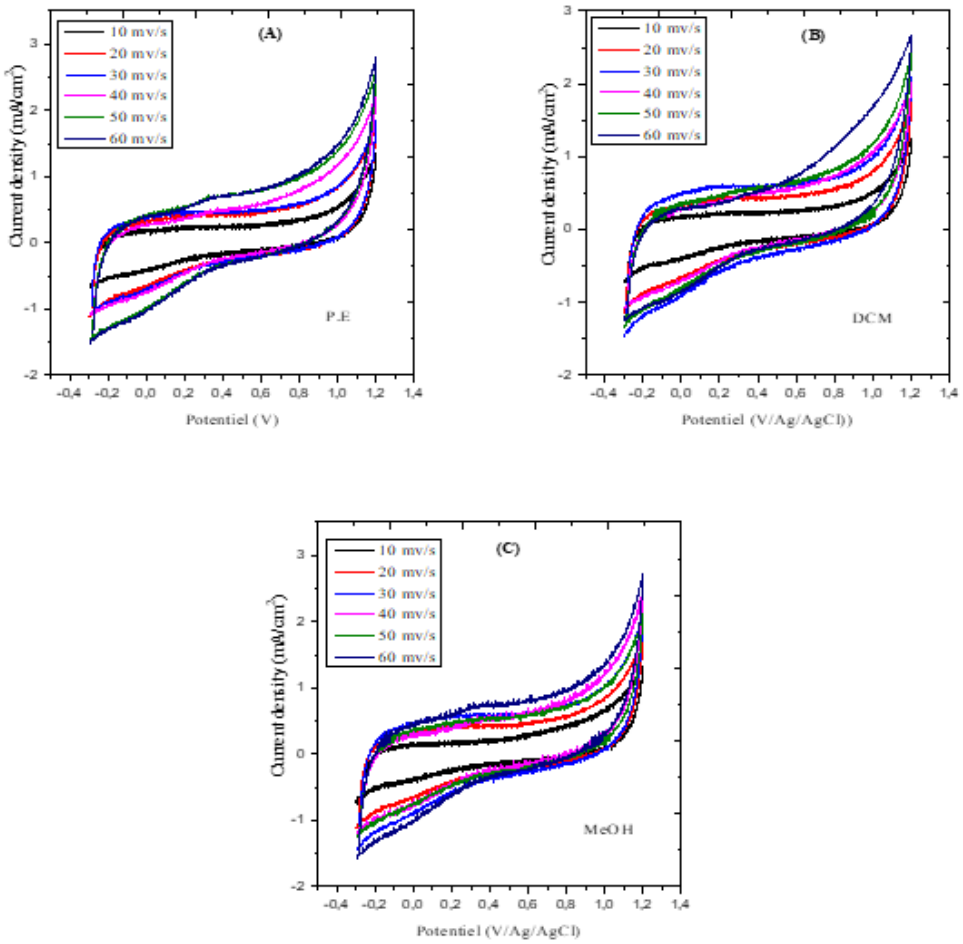

Figure 10. Voltammograms of *M. vulgare* extract in different solvents at pH = 7.4.

antioxidants. The choice of solvent, pH and experimental conditions plays a crucial role in determining their antioxidant potential, which could have important implications for their use in therapeutic or food applications.

Table 5 and Table 6 present the antioxidant activity of various *M. vulgare* extracts in two distinct pH environments: neutral (pH 7.4) and acidic (pH 4.82). The antioxidant activity of *M. vulgare* extracts differs significantly between these environments, with slightly higher activity observed in the acidic medium. Across both pH conditions, MeOH and DCM extracts exhibited greater antioxidant activity than petroleum ether, indicating that extract polarity enhances antioxidant potential. The activity, measured by anodic peak current density (Ipa), generally increased with the scan rate for all extracts, reaching maximum values at 60 mV/s. In the neutral medium, the PE extract had a peak Ipa of 0.806 mA/cm^2, while DCM and MeOH extracts reached 1.22 and 0.832 mA/cm^2, respectively. In the acidic medium, Ipa values were significantly higher: PE and DCM both reached 1.315 mA/cm^2, and MeOH exhibited the highest value at 1.405 mA/cm^2. These results demonstrate that an acidic environment enhances the antioxidant capacity of *M. vulgare* extracts, with polar extracts (MeOH and DCM) showing the most pronounced activity. This suggests that both pH and extract polarity are critical factors influencing antioxidant effectiveness.

The electrochemical characterization from cyclic voltammetry gives direct evidence of the redox-active compounds that drive traditional estimates of antioxidant capacity. The well-defined anodic peaks align with published studies describing the oxidation of flavonoid aglycones, supporting the inference that these compounds are significant contributors to the high electron-donation capacity of the extract (Nikolić et al., 2019).

3.9 Phytochemical basis of biological activity

The biological activities of plant extracts, particularly their antioxidant and antimicrobial properties, are fundamentally dictated by their complex phytochemical composition. In general, the antioxidant capacity of a plant is largely attributed to its content of phenolic compounds, including flavonoids, phenolic acids, and tannins (Wojdylo et al., 2007). These molecules are structurally primed to act as reducing agents, hydrogen donors, and free radical scavengers. Their efficacy stems from the reactivity of the hydroxyl (-OH) groups on their aromatic rings, which can readily donate a hydrogen atom to stabilize highly reactive species like hydroxyl radicals (OH$^\bullet$). This mechanism effectively terminates the oxidative chain reactions that can lead to cellular damage. The synergistic interaction between different classes of compounds, such as flavonoids and terpenoids, can further enhance this activity, creating a more potent effect than any single compound could achieve alone (Mimica-Dukić et al., 2007).

Table 5. Antioxidant activity of *M. vulgare* extracts in an acidic medium (pH = 4.82).

Apolar Extract	Scan rate (mV/s)	Ipa (mA/cm^2)	C (A.A eq)
Petrolum ether	10	0.2096	-
	20	0.4716	-
	30	0.6864	0.0179
	40	0.9004	0.0378
	50	1.086	0.0550
	60	1.315	0.076
Slightly polar extract	Scan rate (mV/s)	Ipa (mA/cm^2)	C (A.A eq)
DCM	10	0.2694	-
	20	0.522	-
	30	0.7533	0.0242
	40	0.9389	0.0414
	50	1.086	0.0632
	60	1.355	0.0823
Polar extract	Scan rate (mV/s)	Ipa (mA/cm^2)	C (A.A eq)
MeOH	10	0.2815	-
	20	0.5336	-
	30	0.774	0.0261
	40	0.9981	0.046
	50	1.109	0.0628
	60	1.405	0.0846

From an antimicrobial perspective, the explanation is similarly rooted in phytochemistry but involves a broader range of mechanisms of action. The ability of an extract to inhibit microbial growth depends on the specific chemical nature of its constituents and their capacity to disrupt essential microbial structures or metabolic pathways (Cowan, 1999). For example, terpenoids and essential oils, which are rich in lipophilic compounds, can compromise the integrity of bacterial cell membranes. They intercalate into the lipid bilayer, increasing its permeability and causing leakage of vital intracellular components, ultimately leading to cell death (Nazzaro et al., 2013). This mechanism helps explain why non-polar extracts of *M. vulgare*, rich in diterpenes such as marrubiin, exhibit significant antibacterial activity. On the other hand, polar compounds like flavonoids and tannins target different targets. They can inhibit microbial enzymes, chelate essential metal ions crucial for bacterial growth, or bind to the cell wall to disrupt its function (Daglia, 2012). The broad-spectrum efficacy often observed in methanolic extracts of Lamiaceae species likely arises from a high concentration of these polar compounds, which attack microbes through multiple simultaneous pathways, making it difficult for resistance to develop (Khaled-Khodja et al., 2014).

Table 6. Antioxidant activity of *M. vulgare* extracts in a neutral medium (pH = 7.4).

Extract	Scan rate (mV/s)	Ipa (mA/cm^2)	C (A.A eq)
Petroleum ether extract	10	-	-
	20	0.2308	-
	30	0.3324	-
	40	0.4245	-
	50	0.6405	-
	60	0.8059	0.0148
Slightly polar extract	Scan rate (mV/s)	Ipa (mA/cm^2)	C (A.A eq)
DCM	10	-	-
	20	0.4149	-
	30	0.5559	-
	40	0.4612	-
	50	0.5673	-
	60	1.222	0.151
Polar extract	Scan rate (mV/s)	Ipa (mA/cm^2)	C (A.A eq)
MeOH	10	-	-
	20	0.3882	-
	30	0.4757	-
	40	0.4841	-
	50	0.5028	-
	60	0.8319	0.0185

4. Concluding remarks

This study presents a comprehensive pharmacognostic evaluation of *M. vulgare* collected from the northeastern region of Algeria, systematically validating its traditional medicinal properties through anatomical, phytochemical, and bioactivity analyses. Anatomical examination revealed distinctive features, including various types of trichomes, which are often key sites for the biosynthesis of secondary metabolites involved in the plant's defense mechanisms and therapeutic effects. Phytochemical screening confirmed that the leaves of *M. vulgare* are a rich source of bioactive compounds, notably alkaloids, tannins, sterols, triterpenes, and particularly high levels of flavonoids. Bioactivity assays demonstrated a clear correlation between the phytochemical profile and the potent pharmacological effects of the extracts. The methanolic extract exhibited the highest activity, showing broad-spectrum antimicrobial effects against all tested bacterial strains (*Staphylococcus aureus*, *Salmonella sp*., *Shigella sp*.) and fungal strains (*Aspergillus niger*, *Aspergillus flavus*). Additionally, the extracts displayed strong antioxidant capacity across multiple complementary assays (FRAP, H_2O_2 scavenging, $OH^{\bullet}$ scavenging), confirming their robust ability to neutralize reactive oxygen species and function as reducing agents.

The combination of these findings-abundant phytochemistry, impressive antimicrobial efficacy, and significant antioxidant activity-offers new opportunities for potential applications. For example, the broad-spectrum efficacy of the methanolic extract suggests its potential as a natural preservative in the food and cosmetics industries to control spoilage organisms and pathogenic bacteria, such as *Staphylococcus aureus*. Furthermore, the high levels of polar antioxidants, particularly flavonoids, indicate potential uses in nutraceuticals and functional foods aimed at reducing chronic diseases associated with oxidative stress. From a therapeutic perspective, the combined antimicrobial and antioxidant activities suggest that *M. vulgare* could serve as a source of lead compounds for phytopharmaceutical development. Specific applications might include topical treatments for skin infections or wound healing, where managing microbial load and oxidative damage is critical for improved healing outcomes.

Authors contributions
Souad Djellali supervised and conceptualized the study, drafted the initial manuscript provided critical revision, and approved the final version. Rachid Sahraoui co-supervised the work, contributed to data validation, and drafted the initial manuscript. Yasmine Abdelouahed analyzed the data, drafted the initial manuscript, revised the manuscript. Mohamed Amine Benyahia performed the experiments, data interpretation, and manuscript writing. Mira Chribet performed the experiments, data interpretation, and manuscript writing. All authors reviewed and approved the final manuscript.

Availability of data and materials
The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflict of interests
The authors declare that there is no conflict of interest.

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