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Research Article

Phytochemical screening, total phenolic content and antioxidant activities of *Marrubium vulgare* L. collected in three development stages

Aya KHOUCHLAA[1]*, Abdessadek RAHIMI[1,2], Mostafa MIMOUNI[2], Chaimae RAIS[3], Hamid KHAMAR[4], Fatna ZAAKOUR[5]

[1]*National Agency of Medicinal and Aromatic Plants, Laboratory of Biochemistry, BP 159, Principal, Taounate, 34000, Morocco; aya.khouchlaa@gmail.com (*corresponding author)*
[2]*University Mohammed First (UMP), Faculty of Sciences Oujda (FSO), Laboratory of Applied Chemistry and Environment (LCAE), Oujda 6000, Morocco; abdessadek.rahimi@fso.ump.ac.ma; Mimouniosrn@gmail.com*
[3]*National Agency of Medicinal and Aromatic Plants, Laboratory of Botany, BP 159, Principal, Taounate, 34000, Morocco; raischaimae18@gmail.com*
[4]*University Mohammed V in Rabat, Scientific Institute, Department of Botany and Plant Ecology, B. P. 703, Rabat 10106, Morocco; khamarhamid90@gmail.com*
[5]*Chouaib Doukkali University, Department of Agricultural and Environmental Engineering, Laboratory of Sustainable Agriculture Management, Higher School of Technology Sidi Bennour, Av. des Facultés, 24123 El Haouzia, El Jadida, Morocco; zaakour-fatna@hotmail.com*

Abstract

The change of phenolic compounds and antioxidant activities of plants extracts are widely attributed to numerous conditions and parameters viz. geographical conditions, type of extraction, and type of extract. Thus, this study was the first one conducted to evaluate the phenolic composition, the content of total polyphenols, flavonoids, and proanthocyanidins, and the antioxidant activities of methanolic extract of *M. vulgare* aerial part collected in three development stages (before flowering, during flowering, and after flowering periods). Phytochemical screening was carried out using standard methods of precipitation and coloration reactions. The total polyphenol, flavonoid, and proanthocyanidins contents were determined using the Folin-Ciocalteu method, aluminum chloride colorimetric method, and vanillin method, respectively. The antioxidant activity was assessed by two methods: DPPH and ABTS scavenging activity. Preliminarily phytochemical screening revealed the presence of saponins, essential oil, cardiac glucosides, sterols and triterpenes, and the absence of alkaloids. In addition, tannins were absence in March and present in May and July. The total phenol content of *M. vulgare* extracts showed a highest flavonoid and proanthocyanidin contents during flowering period (1.644 mg QE.g^{-1} extract, and 0.195 mg CE.g^{-1} extract, respectively) while polyphenol content was highest before flowering period (0.043 mg GAE.g^{-1} extract). Afterward, *M. vulgare* methanolic extract exhibited a potent antioxidant activity during flowering period with an IC_{50} value of 2.23 mg.ml^{-1}. From the current study, this plant contains natural antioxidant substances which can be used as antioxidant agents to treat several diseases.

Keywords: antioxidant activity; *M. vulgare*; phenols contents; screening

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Introduction

Since ancient times, medicinal plants have been an integral part of human culture due to their therapeutic properties and beneficial pharmacological effects on the human body (Xavier et al., 2019). One of the noteworthy pharmacological effects of these plants is their antioxidant activity, which involves the protection of biological systems against the deleterious effects of processes or reactions that can cause excessive oxidation (Özcan *et al.*, 2009). This antioxidant activity in herbs and spices is primarily attributed to the presence of phenolic compounds, including flavonoids, phenolics, and tannins (Özcan *et al.*, 2009).

Marrubium vulgare L., commonly known as horehound, is a perennial plant in in the mint family (Lamiaceae), native to Europe, North Africa, Southwest Africa, and Central Asia (Boutabia *et al.*, 2020; Sahpaz *et al.*, 2002). In traditional medicine, *M. vulgare* has been used to treat a variety of diseases such as respiratory, urinary, ophthalmia, and ear infections, as well as rheumatism, diabetes, migraine, diarrhea, and typhoid fever. In addition, *M. vulgare* is renowned for its vaso-relaxing, antiseptic, emmenagogue, anti-inflammatory, anti-edematous, anti-hepatotoxic, diuretic, anti-hypertensive, anti-typhoid, antioxidant, and antibacterial properties (Hayat *et al.*, 2020). Besides, numerous studies have documented the pharmacological properties of *M. vulgare*'s extract and its constituents, which exhibit pharmacological and beneficial effects such as antimicrobial, antifungal, and antioxidant activities (Hayat *et al.*, 2020; Rezgui *et al.*, 2020). In 2021, a research has evaluated the antioxidant and antimicrobial activities of *M. vulgare* (Mssillou *et al.*, 2021).

In the present study, we investigated samples of *M. vulgare* at three development stages in order to determine the total amount of phenolic compounds that were present and the level of antioxidant activity.

Materials and Methods

Plant material

M. vulgare L., commonly known as horehound, is a perennial plant in the mint family (Lamiaceae), native to Europe, North Africa, Southwest Africa, and Central Asia (Boutabia *et al.*, 2020; Sahpaz *et al.*, 2002). In continental North Africa, this species is found in Morocco, Algeria, Tunisia, Libya, and Egypt (Dobignard and Chatelain, 2012). Within Morocco, *M. vulgare* is found in Mediterranean bioclimatic zones ranging from subhumid to arid, where it occurs in forest clearings, pastures, scrublands, and plains, as well as low and medium mountainous regions (Fennane *et al.*, 2007). According to Jahandiez and Maire (1934), this species demonstrates a general indifference towards the nature of the soil. Aerial parts of *M. vulgare* were collected from El Jadida (Morocco) in the months of March, May, and July of the year 2020 when the plant was in its vegetative, pre-flowering, and during flowering stages, respectively. The plant, identified by Dr. Rais Chaimae, Laboratory of Botanic, National Agency of Medicinal and Aromatic Plant, Morocco, was ground to a fine powder and stored at room temperature. The extraction was carried out in a Soxhlet apparatus for 6h using absolute methanol. The solvent was then evaporated using rotary evaporator to obtain the crude extracts. The crude extract was stored at +4 °C until further uses. Voucher specimens have been deposited at the herbarium of the National Agency for Medicinal and Aromatic Plants and at the Scientific Institute of the National Herbarium (RAB) in Rabat.

Phytochemical screening

Preliminarily qualitative phytochemical analysis was carried out to identify phytochemical constituents of aerial part methanolic extract of *M. vulgare*. Following phytochemicals were tested tannins, saponins (Banso

and Adeyemo, 2006), anthraquinone glycoside, cardiac glycosides (Joshi *et al.*, 2013), sterols, and triterpenes (Ayoola *et al.*, 2008; Joshi *et al.*, 2013) using standard methods, as reported in the literature.

Determination of phenolic content

Determination of total phenolic

The assessment of total polyphenols was performed according to the method of (Singleton *et al.*, 1999) using the Folin-Ciocalteu as a reagent. On this base, the extracts are diluted to a concentration of 1 mg/mL. An amount of 100 µL of the diluted extract is placed in test tubes, and 500 µL of Folin-Ciocalteu reagent diluted 10 times in distilled water is added. After incubation for 1h at room temperature, 2 mL of sodium carbonate (Na_2CO_3) to 2% is added. The tubes are then shacked and placed in the dark for 30 minutes at room temperature. The same steps were followed to establish a reference range (0 to 100 µg/mL) prepared from an aqueous stock solution of gallic acid (0.5 $g.l^{-1}$). The absorbance is measured using a UV-Visible spectrophotometer at a wavelength of 760 nm (Singleton *et al*., 1999). The absorbance values of each concentration enabled us to plot the calibration curve of gallic acid. The results are expressed in mg of gallic acid equivalents per gram of dry extract (GAE $mg.g^{-1}$ of dry extract). All manipulations are performed in triplicate.

Determination of total flavonoid contents

Flavonoid contents are measured using aluminum trichloride (AlCl3) as a reagent. 1mL of the extract of *M. vulgare* is mixed with 1 mL of the solution of aluminum trichloride (2%) and 50 µL of acetic acid. The tubes are then gently mixed and incubated in the dark for 40 minutes at room temperature. Under the same conditions, a stock solution of quercetin mass concentration 0.2 $g.l^{-1}$ was prepared in methanol. From this stock solution, a standard range of concentrations from 0 to 25 $mg.ml^{-1}$ was prepared, the absorbance measured in the same spectrophotometer at a wavelength of 415 nm (Brighente *et al.*, 2007). The obtained absorbance values enabled us to plot the calibration curve of quercetin. The results are expressed in mg of quercetin equivalent per g of dry extract (mg QE.g^{-1} of dry extract). All manipulations are performed in three trials.

Determination of proanthocyanidin content

Proanthocyanidin content were determined by the vanillin assay as described by the method of (Nakamura *et al.*, 2003). 0.5 ml of the extract of *M. vulgare* is mixed with 3 mL of the solution of vanillin (4%) and 1.5 ml of HCl. The tubes are then gently mixed and incubated for 15 minutes at room temperature. Under the same conditions, as standard range of concentrations catechin from 31.25 to 500 $mg.ml^{-1}$ was prepared and the absorbance measured in the same spectrophotometer at a wavelength of 500 nm. The results are expressed in mg of catechin equivalent per g of dry extract (mg CE.g^{-1} of dry extract). All manipulations are performed in three trials.

Assessment of antioxidant activities

DPPH radical scavenging activity

The antioxidant activity was determined according to the method (Kubola and Siriamornpun, 2008) with some modifications. Briefly, 1.8 mL of 0.1 mM DPPH solution is prepared in methanol and added into 0.2 mL tubes containing extract (methanol or ethanol) at increasing concentrations of extracts (0.039-1.25 $mg.ml^{-1}$) and are placed away from light at room temperature after agitation by a vortex. After 30 min, the absorbance is measured at 517 nm (Kubola and Siriamornpun, 2008). Ascorbic acid (vitamin C) was used as positive controls, while the methanol is used as a negative control. The scavenging activity of DPPH radical is calculated using the following formula:

$A\ \% = (Abs_{control} - Abs_{sample}) \times 100/Abs_{control}$)

$Abs_{control}$: Absorbance without antioxidant (containing all reagents except the test sample)

Abs_{sample}: absorbance with the test sample.

IC_{50} is the concentration of the test sample required to reduce 50% of DPPH radicals. IC_{50} was graphically calculated by linear regression plots graphs, percentage inhibition as a function of different concentrations of the tested fractions and standards.

ABTS radical scavenging activity

ABTS•+ can be generated directly in high yield using potassium persulphate as the oxidizing agent (Re *et al.*, 1999). Antioxidants then react only with ABTS:

ABTS + potassium persulphate → ABTS•+

The ABTS•+ radical, as described by (Re *et al.*, 1999), generated by mixing an ABTS 7 mM solution with 2.4 mM potassium persulphate in the dark for 16h, at room temperature before use. Before usage, the ABTS•+ solution was diluted to get an absorbance of 0.70 ± 0.02 at 734 nm with methanol. The spectrophotometer is preliminarily blanked with methanol. Upon adding 1 mL of the diluted ABTS•+ solution to 0.5 mL of BHT (Butylated hydroxytoluene) standard at different concentrations (0.039-1.25 $mg.ml^{-1}$) or methanolic extracts at different concentrations (0.155-5.0 $mg.ml^{-1}$). The absorbance was measured against a blank at 734 nm using spectrophotometer. Samples were prepared and measured in triplicates. The percentage of scavenging activity of each extract on ABTS•+ was calculated as % inhibition (I%) using the following equation:

$I\% = [(Abs_{control} - Abs_{sample}) / Abs_{control}] \times 100$.

$Abs_{control}$: Absorbance without antioxidant (containing all reagents except the test sample)

Abs_{sample}: absorbance with the test sample.

The results were expressed as IC_{50}. The lower IC_{50} value is an indication of a more potent antioxidant activity.

Results

Phytochemical screening

The phytochemical screening of *M. vulgare* aerial part revealed the presence of tannins, saponins, essential oil, cardiac glucosides, alkaloid and sterols, and triterpenes, and the absence of alkaloids (Table 1). Furthermore, tannins were absence before flowering period.

Table 1. Preliminarily qualitative phytochemical analysis aerial part methanolic extract of *M. vulgare*

Plant constituents	March	May	July
Tannins	-	+++	+
Saponins	+	+++	++
Essential oil	+	+	+
Cardiac glucosides	+	+++	+
Alkaloid	-	-	-
Sterols and triterpenes	++	+++	+++

+++: highly present, ++: moderately present, +: Low, -: absent.

Total polyphenol, flavonoid, and proanthocyanidin contents

The results of total polyphenol contents are expressed in mg $GAE.g^{-1}$ dry extract by referring to the previously established calibration curve with gallic acid (polyphenol correlation: R2 = 0.95), and the results of total flavonoid contents are expressed in mg $QE.g^{-1}$ dry extract based on the established calibration curve with quercetin (flavonoid correlation: R2 = 0.999). In addition, the results of proanthocyanidin are expressed in mg $CE.g^{-1}$ dry extract based on the established calibration curve with catechin (proanthocyanidin correlation: R2

= 0.999). The results of the polyphenol, flavonoid, and proanthocyanidin contents of methanolic extracts of *M. vulgare* at three development stages are illustrated in Table 2.

From these results, we can conclude that the polyphenol, flavonoid and proanthocyanidin content varies according to the development stages. In effect, methanolic extract of *M. vulgare* collected before flowering period had higher flavonoid and proanthocyanidin contents (1.644 mg QE.g^{-1} of dry extract, and 0.1954 mg CE.g^{-1} of dry extract, respectively) than those collected during and after the flowering period. However, the polyphenol content obtained during flowering stage (0.0430 mg GAE.g^{-1} of dry extract) was higher than other phenolic stages.

Table 2. Total phenolic, flavonoid, and proanthocyanidin contents of aerial part of *M. vulgare* in three development stages

	Polyphenols (mg GAE.g^{-1} of extract)	Flavonoids (mg QE.g^{-1} of extract)	Proanthocyanidin (mg CE.g^{-1} of extract)
Methanolic extract (March)	0.0209	1.644	0.1954
Methanolic extract (May)	0.0430	0.723	0.1293
Methanolic extract (July)	0.0149	0.923	0.1174

GAE: Gallic acid equivalent, QE: Quercetin equivalent, CE: Catechin equivalent.

Assessment of antioxidant activities

DPPH radical scavenging activity

Table 3 presented the antioxidant activity was performed of *M. vulgare* methanolic extract during three development stages using *in vitro* DPPH methods. The DPPH radical scavenging activity of methanolic extract of *M. vulgare* at various concentrations are illustrated in Figure 1, and the extracts showed a scavenging activity which increases with samples concentration. A greater antioxidant activity is indicated by a lower value of IC_{50}. *M. vulgare* extract exhibited moderate antioxidant activity in DPPH radical scavenging assay with IC_{50} 2.23 ± 0.05 mg.ml^{-1} during the period of flowering (Figure 1). Before and after flowering period, methanolic extract of *M. vulgare* have an IC_{50} > 4 mg.ml^{-1} (Table 3). IC_{50} of ascorbic acid was 0.11 ± 0,002 mg.ml^{-1}. High antioxidant activity of ascorbic acid can be explained by his use as a pure molecule compares to our extract, which contained several molecules.

Table 3. Inhibitory concentration 50 (IC_{50}) values for DPPH and ABTS scavenging activities of *M. vulgare* methanolic extract during three development stages

	Before flowering (March)	During flowering (May)	After flowering (July)
IC_{50} DPPH (mg.ml^{-1})	4.32 ± 0.05	2.23 ± 0.05	4.86 ± 0.08
IC_{50} ABTS (mg.ml^{-1})	0.069 ± 0.02	0.083 ± 0.002	0.103 ± 0.006

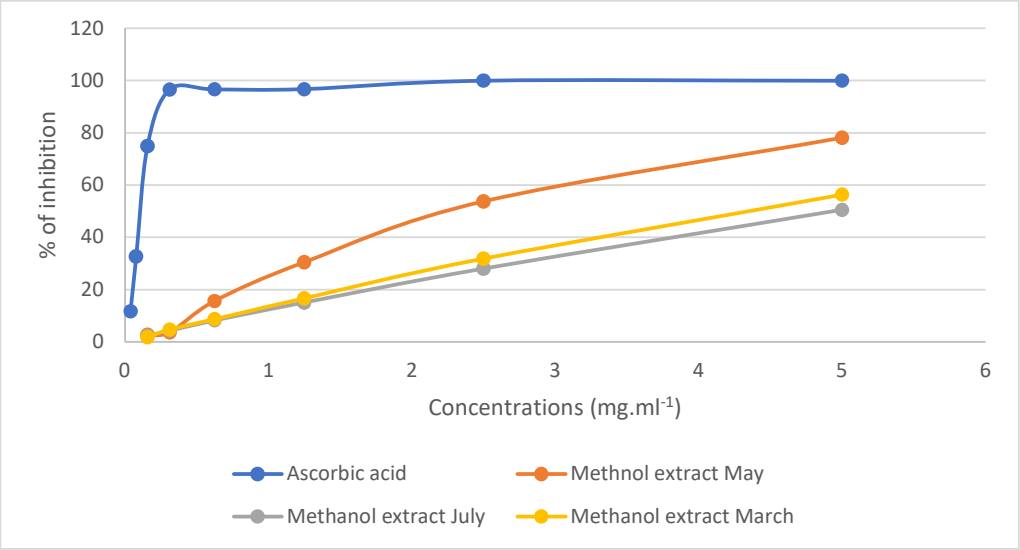

Figure 1. DPPH radical scavenging activity of *M. vulgare* methanol extract during three development stages (methanol extract in three development stages) measured at 517 nm
Data are expressed as mean ± SD. Ascorbic acid is used as standard.

ABTS radical scavenging activity

In this study, the free radical scavenging activity of methanolic extract at different concentrations (0.039-1.25 mg.ml^{-1}) of three stages of development of *M. vulgare* were evaluated by their capacity to quench ABTS+. The results obtained were shown in Table 3 and the ABTS radical scavenging activity of *M. vulgare* methanolic extract of at various concentrations is illustrated in Figure 2. From this figure, the extracts showed a scavenging activity which increases with samples concentration. The methanolic extract before the flowering period has the highest activity with an IC_{50} value of 0.069 mg.ml^{-1}. IC_{50} of ascorbic acid was 0.11 ± 0,002 mg.ml^{-1}.

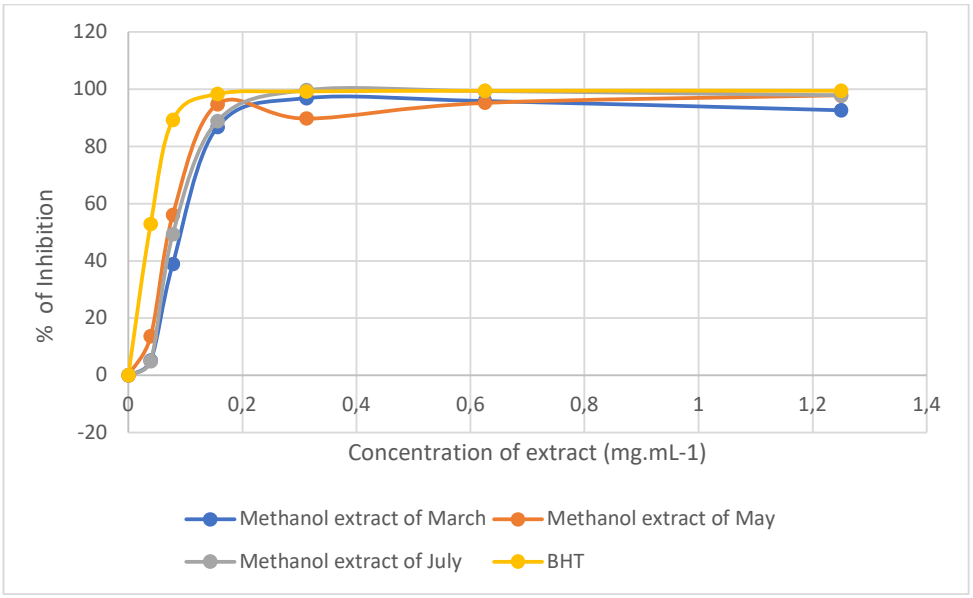

Figure 2. ABTS radical scavenging activity of *M. vulgare* methanol extract during three development stages measured at 734 nm
Data are expressed as mean ± SD. BHT is used as standard. BHT is used as standard.

Discussion

The analysis conducted in the present study enabled us to show that *M. vulgare* aerial part revealed the presence of tannins, saponins, essential oil, cardiac glucosides, alkaloid and sterols, and triterpenes, and the absence of alkaloids. These finding showed similar secondary metabolic with the study conducted by Hayat *et al.* (2020) which identified the presence of flavonoids, polyphenols, terpenoids, and catechin tannins in organic and aqueous extracts from *M. vulgare* collected from two different localities (The Cape Three Forks, and Oulad Daoud zkhanine North-Eastern Morocco). A recent study reported the presence of saponins, tannins, quinones, terpenoids, reducing compounds, and flavonoids in hydromethanolic and hydroacetonic crude extract of *M. vulgare* from Algeria (Belkacem *et al.*, 2022). The slight variation of phytochemical compounds of this plant can be explained by several parameters including altitude, temperature, humidity, rainfall, the type of soil where the plant was collected (Hayat *et al.*, 2020). Furthermore, regardless of the plant localization, the part used, and the type of extract, condensed tannins, sterols, and triterpenes were present in *M. vulgare*.

The quantitative phenol, flavonoid, and condensed tannin contents in aerial part of *M. vulgare* methanolic extract showed a variety of those contents according to the development stages. In effect, methanolic extract of *M. vulgare* collected before flowering period had higher flavonoid and proanthocyanidin contents than those collected during and after the flowering period. The concentration of polyphenol, flavonoid and tannins has been measured in different studies and presented a difference in the content. In 2017, Fathiazad *et al.* (2017) reported that methanolic extract of aerial part of *M. vulgare* collected from Iran in Jun 2013 contains 0.625 mg of GAE.g^{-1} of the dried plant of polyphenols and 1.620 mg QE.g^{-1} of the dried plant of flavonoids. While (Kabach *et al.*, 2019) investigated leaves methanolic extract of *M. vulgare* collected in January 2018 (Taza, Morocco) and showed a total polyphenol and flavonoid contents with a value of 60.409 mg GAE.g^{-1} of dry weight, and 33.813 mg EQ.g^{-1} of dry weight, respectively. A recent research conducted by (Hayat *et al.*, 2020) showed that leaves methanolic extract of *M. vulgare* (Morocco) collected in June 2019 contains 33.82 µg QE.mg^{-1} of polyphenol, 86,91 µg GAE.mg^{-1} of flavonoid, and 108.95 µg CE.mg^{-1} of tannins. The different contents in polyphenol, flavonoid and proanthocyanidin can be explicated by the influence of several parameters such as climatic condition, geographical condition, and method used but essentially by the development stage (El Idrissi *et al.*, 2020; Hayat *et al.*, 2020). In addition, the accumulation and biosynthesis of phenolic compounds can be influenced by genetic and climatic factors, and the collection period (Mssillou *et al.*, 2021). Indeed, the highest flavonoid and proanthocyanidin contents are obtained before the flowering period.

The antioxidant activity showed that *M. vulgare* methanol extract exhibited moderate antioxidant activity in radical scavenging assay during the period of flowering. Various investigations studied the effect of *M. vulgare* organic extract to scavenge free radical using DPPH scavenging activity. In 2017, a study conducted by Fathiazad *et al.* (2017) showed that *M. vulgare* methanol extract, collected from Iran during flowering stage (June, 2013), exhibited DPPH radical scavenging activity with IC_{50} values of 177µg.ml^{-1} for the extract compared with and IC_{50} values of quercetin (3.8 µg.ml^{-1}). In Morocco, an *in vitro* study conducted by Kabach *et al.* (2019) reported that leaves methanol extract of *M. vulgare* (from Taza in January 2018) showed an IC_{50} value of 2.49 mg.ml^{-1}. This value was similar to our DPPH scavenging activity during flowering period. Recently, the leaves hydroethanolic extract of *M. vulgare* (from Fes region collected in September 2020) showed a better antioxidant activity with a value of IC_{50}=52.04 µg.ml^{-1} (Mssillou *et al.*, 2021). The important content of phenolic compounds has been related to the antioxidant activity of these extracts. In addition, numerous in vitro investigations showed the correlation between phenolic compounds and antioxidant effects (Bouyahya *et al.*, 2017; El Idrissi *et al.*, 2020). Chedia *et al.* (2014) investigated the antioxidant activities of *M. vulgare* collected from the northwestern part of Tunisia (Bousalem) in November 2012 using in vitro ABTS radicals scavenging activities. From this study, the authors reported that *M. vulgare* methanolic extract has an

IC50 = 25 ± 0.2 µg.ml^{-1}. Recently, Kabach *et al.* (2019) reported that *M. vulgare* leaves methanol extract (from Taza, Morocco) has the highest activity with an IC_{50} = 0.874 mg.ml^{-1}. The authors confirmed that this activity is due to the presence of phenolic compounds. Furthermore, numerous study showed a correlation between antioxidant activity of extract from plant samples and phenolic compounds (Bouyahya *et al.*, 2017; El Idrissi et *al.*, 2020).

Conclusions

To conclude, *M. vulgare* samples were found to have a diversity of phytochemical compounds in three stages of the plant development (pre-flowering, during flowering, and post-flowering periods) resulting for their antioxidant effects. The results of this study valorize *M. vulgare* as a medicinal plant that can be used as a source of biologically active compounds. However, further investigations are needed to evaluate the potential effect of this extract in *in vitro* and *in vivo* assays to confirm its use as a natural additive in food, cosmetic and pharmaceutical industries.

Authors' Contributions

Conceptualization: AK, FZ; Data curation: CR; Formal analysis: AR; Investigation: AK, AR; Methodology: AK, FZ; Resources: AK; Supervision: AK, MM, HK, FZ; Validation: MM, HK; Writing - original draft: AK, AR; Writing - review and editing: AK. All authors read and approved the final manuscript.

Ethical approval (for researches involving animals or humans)

Not applicable.

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Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

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