Research Article

Phytochemical screening, antioxidant and cytotoxic activities of *M. vulgare*

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Received: 14.06.19, Revised: 14.07.19, Accepted: 14.08.19

ABSTRACT

Phytochemicals are extensively found at different levels in many medicinal plants. For that,in this study, the extracts of *Marrubium vulgare L* were studied for antioxidant and polyphenols contents as well as cytotoxicity activities.The anti-oxidative activities were determined using four methods: free radical scavenging assays (DPPH$^{\bullet}$ and ABTS$^{\bullet+}$ tests), ferrous ions (Fe^{2+}) chelating activity and reducing power assay (FRAP). Additionally, total flavonoids and phenolic contents were also determined. For all tests performed, methanolic extracts exhibited a strong antioxidant activity. The cytotoxic activity was assessed on two different breast cancer cell lines (MDA-MB-468 and MCF-7), using MTT assay. The results obtained shows the methanolic extract possesses moderate anti-proliferative activity against two human breast cancer cells lines.

Keywords: *Marrubium vulgare L.*, phenolic content, antioxidant, cytotoxicity.

INTRODUCTION

The medical field knows a very important development in the treatment of various diseases, especially the most devastating of them such as diabetes, cancer and cardiovascular diseases…etc. However, these treatments are not always accessible, present side effects and are often expensive. This requires looking for a more effective alternative which causes no adverse effects and which can be accessible for everybody. This is why scientists are more and more interested in medicinal plantsrich in secondary metabolites. The secondary metabolites such as polyphenols and flavonoids present high antioxidant activity can play a major role in scavenging free radicals and decomposing peroxides[1].Morocco is considered as a paragon of medicinal plants and contains more than 4500 species divided into 135 plant families, 940 genera and over 600 plant taxa as endemic[2]. The family of Lamiaceae is composed of about 230 genera and 7100 species worldwide. This family encloses many important species that are used in medicine, cosmetics and culinary[3]. One of the most known genus of this family is *Marrubium*. This genus contains approximately 30 species native to Europe, the Mediterranean region and Asia[4]. The species *Marrubiumvulgare L. (M. vulgare*) is found in North Africa, Europe, and Asia and growsmostlyin wasteland and on the edges of roads. This species is a perennial graying plant and can grow to reach 25 to 45 cm in height[5].*M. vulgare* L. is used in traditional medicine to treat several diseases, including gastroenterical, inflammatory, and respiratory disorders [6,7]. Other studies reported hypoglycemic, hypotensive, analgesic and antioxidant activities for this species [8]. Therefore, the main objectives of the present study were to screen the phenolic compounds and to evaluate the antioxidant activities of aqueous and methanolic extracts of *M. vulgare*. We alsoaimed to compare the effect of the methanolic plant extract on a triple-negative breast cancer cell line MDA-MB-468 and a luminal breast cancer cell line MCF7. To our knowledge, there is no study conducted on the cytotoxic effect of *M. vulgare* on the MDA-MB-468 cell line.

MATERIALS AND METHODS

Collection of plant materials

The leaves of *M. vulgare L.* were collected in January 2018, in the suburbs of Taza, Morocco and authenticated by Pr. Abdelali BOULLI, (PhD, botanist), The leaves were washed with distilled water, shade dried and powdered with the mechanical grinder. The powder was stored in an airtight container until further use.

Extraction of Plant Material

Air-dried and finely ground plant material 4.5 g was added to 45 mL of methanol 80% or dH_2O and extracted under continuous shaking (250 rpm) and in the dark for 6 hours at room temperature. The extracts were filtered with Whatman filter paper and

centrifuged at 6,000 g for 10 min. Then the solvents were evaporated in an incubator at 40ºC. The dried extracts were weighed to determine the percentage yield of the soluble constituents.

Total phenolic content

The total phenolic content was determined by the method of the Folin–Ciocalteu, following the Singleton & Rossi (1965) [9]. To 100 µl of the sample, 400 µl of Folin Ciocalteau's reagent and 1 ml of saturated Na_2CO_3 (7%) were added and the final volume was made up to 1.6 ml with distilled water. The tubes left to stand in the dark for 30 min, after which its absorbance was read at 725 nm against a blank using spectrophotometer (EPOCH, BioTek). The total phenolic content of plant extracts were expressed as milligrams of gallic acid equivalents per gram of dry weight (mg GAE/g DW) through the calibration curve with gallic acid.

Total flavonoid content

The flavonoid content was assessed following the method of Huang, Chun-Der, Hsien-Jung, & Yaw-Huei (2004)[10]with some modifications. Briefly, 40 µl of the each sample was mixed with 10 µl of acetate potassium (1 M) and 10 µl of aluminum chloride (10%). Thereafter, 100 µl of methanol 50% was added and the total volume was made up to 400 µl with distilled water. The absorbance of the mixture was taken at 415 nm. Quercetin was used as standard. The flavonoid content was expressed as milligram of Quercetin equivalence (QE) per gram of extract.

DPPH radical scavenging assay

The radical scavenging ability of the extracts was monitored using the stable free radical DPPH (2,2-diphenyl-1-picrylhydrazyl) following the method described by Hatano, Kagawa, Yasuhara, & Okuda (1988) [11]with some modifications. Adequate solutions of sample were realized to obtain a final volume of 50 µL. Extract solutions (50 µL) were mixed with 150 µL of a freshly prepared DPPH solution. The mixture was shaken vigorously and left to stand in the dark and at room temperature for 30 min. The reduction of the DPPH radical was measured at 517nm. The DPPH scavenging activity was determined by calculating the percentage of DPPH discoloration using the following equation:

$$\%\text{Scavenging effect} = [(A_{DPPH} - A_S)/A_{DPPH}] \times 100$$

Where A_S is the absorbance values of the sample, A_{DPPH} is the absorbance of the DPPH solution. The extract concentration providing 50% inhibition (IC_{50}) was calculated from the graph of scavenging effect percentage against extract concentration in the solution.

Radical scavenging activity against the radical $ABTS_+$

The radical scavenging activity against the radical $ABTS_+$ was determined according to the method of Re et al., (1999)[12]. $ABTS_+$ was generated by the oxidation of ABTS with potassium persulfate. Prior to assay, the $ABTS_+$ stock solution was diluted with methanol until its reach an absorbance of 0.700±0.020 at 734 nm. Then 185 µL of a diluted $ABTS_+$ solution was mixed with 15 µL of the test sample and the absorbance was measured at 734 nm after 10 min. The radical scavenging activity was calculated using the following equation:

$$\%\text{ Scavenging effect} = [(A_{ABTS} - A_S)/A_{ABTS^+}] \times 100$$

Where A_S is the absorbance values of the sample, A_{ABTS^+} is the absorbance of the $ABTS^+$solution. The extract concentration providing 50% inhibition (IC_{50}) was calculated from the graph of scavenging effect percentage against extract concentration in the solution.

Metal chelating activity

The ferrous ion chelating potential was evaluated by Dinis, Madeira, & Almeida (1994)[13]method. The reaction mixture contained 800 µL of various concentrations of the extracts and 10 µL of $FeCl_2$ (0.6 mM).The reaction mixture was shaken vigorously and left stand at room temperature for 10 min. The reaction was initiated by the addition of 50 µL of ferrozine (5 mM), andthe final volume was made up to 1 mL with distilled water. The absorbance of the reaction mixture was measured after 10 min at 562 nm. The control contained all the reagents except sample replace with methanol. The extract concentration providing 50% inhibition (IC_{50}) was calculated from the graph of scavenging effect percentage against extract concentration in the solution.

Reducing power assay (FRAP)

The reducing power was determined by the method of Oyaizu (1986)[14]with some modifications. Briefly, 200µL of sample was mixed with 500µL of phosphate buffer (0.2 M, pH 6.6) and 500µL of potassium ferricyanide (1%). Reaction mixture was incubated at 50°C for 20 min and then 500µL of trichloroacetic acid (10%) was added and centrifuged for 10 min. From the upper layer, 500µL was mixed with 500µL of distilled water and 100µL of $FeCl_3$ (0.1%). Absorbance was measured at 700 nm. Values are presented as mg of ascorbic acid equivalent per g of dry weight (mg EA/g DW).

HPLC-DAD analysis

Reversed phase HPLC method for determination of phenolic acids flavonoids and terpenoids in *M. vulgare* extracts was performed. The analytical HPLC system employed consisted of a Waters 2926 high performance liquid chromatograph equipped with a diode array detector. Software used for data acquisition and control of HPLC pumps, autosampler, and diode array system was Empower 3 (Waters Corporation, Milford, MA, USA). The separation was carried out on a reversed phase Gemini C6 – Phenyl column (250 × 4.6 mm, 3 µm)

maintained at 30 °C. The mobile phase consisted of two solvents; 0.1 % formic acid aqueous solution (A) and methanol (B) operating in gradient form (0 min, 90% A; 10 min, 90% A; 40 min, 65% A; 45 min, 65% A; 60 min, 0% A; 65 min, 0% A; 67min, 90% A; 75 min, 90% A). The flow rate of the mobile phase was 0.6 mL/min and the injection volumes for all samples and standards were 10 µL. Using different standards, the identification of eluted components was detected spectrophotometrically at 280 nm.

Table 1. Bioactive compounds obtained for the studied *M. vulgare* extracts

	Extract yield (%)	Polyphenols (mg EAG/g DW)	Flavonoids (mg EQ /g DW)
aqueous extract	20.88	32.710 ± 3.82[a]	26.023 ± 2.72[a]
methanolic extract	20.82	60.409 ± 6.63[b]	33.813 ± 2.81[a]

The values are mean± standard deviation. EAG: Gallic Acid Equivalent. DW: Dry Weight. EQ: Quercetin Equivalent. Different letters indicate significant differences between conditions ($p<0.05$)

Table 2. Values obtained in the antioxidant activity assays of the studied *M. vulgare* extract:

	Antioxidant properties (IC_{50} values; mg/mL)			Reducing power (mg EA/g DW)
	DPPH scavenging activity	ABTS	metal chelating activity	
aqueous extract	3.092 ± 1.57[a]	0.961 ± 1.31[a]	1.469 ± 1.31[a]	308.138 ± 8.49[a]
methanolic extract	2.496 ± 2.62[a]	0.874 ± 1.51[a]	0.104 ± 1.73[a]	456.930 ± 9.95[b]

All the values are mean± standard deviation. IC_{50}: The extract concentration providing 50% inhibition. DPPH: 2,2-diphenyl-1-picrylhydrazyl. ABTS: 2,2'-azino-bis(3ethylbenzothiazoline-6-sulphonic acid). EA: Ascorbic acid Equivalent. DW: Dry Weight. Different letters indicate significant differences between conditions ($p<0.05$)

Table 3. Major phenolic compounds identified in M. *vulgare* extract by HPLC.

Phenolic compounds µg/g DW	Aqueous extract	Methanolic extract
Caffeic acid	0.258	ND
Gallic acid	0.363	ND
Salicylic acid	3.709	69.222
Syringic acid	0.086	ND
P-coum acid	0.072	1.015
p-hydroxybenzoic acid	0.035	ND
Hesperidin	0.236	ND
Limonene	0.124	ND
Naringinin	0.145	1.567
Rutin	1.582	18.793
Thymoquinone	1.907	ND
Tocopherol	3.582	5.282

DW: Dry Weight. ND: not detected

Table 4. Inhibitory Concentration 50 (IC_{50}) in µL of methanolic extract of *M. Vulgare* tested against MCF-7 and MDA-MB-468 tumor cells lines.

Cells	IC_{50} µg/mL	
	methanolic extract	Doxo
MCF-7	12.05 ± 5.06	0.23 ± 0.09
MDA-MB-468	22.35 ± 4.23	0.93 ± 0.03

All the values are mean± standard deviation. IC_{50}: The extract concentration providing 50% inhibition. Doxo: Doxorubicin

Cell culture

MCF-7 (human breast adenocarcinoma) and MDA-MB-468 (Triple negative human breast carcinoma) cell line was were maintained with RPMI 1640 medium supplemented with 5% heat-inactivated fetal bovine serum, 1% penicillin G-streptomycin, and 0.2% of L-Glutamine. Incubation was performed at 37°C in humidified atmosphere containing 5% CO2.

MTT assay

The human breast carcinoma cells MDA-MB-468 and MCF-7 were harvested from starting cultures at the exponential growth phase. After PBS wash, adherent cells were harvested from sub-confluent cultures using cell scraper and suspended in RMPI.

The harvested cells were plated at a density of (7 x 10^4 cells per well) for MDA-MB-468 or (10^5 cells per well) for MCF-7 in flat-bottomed 96-well microplates containing 100 µl of complete medium and were allowed to adhere overnight before treatment. The cells were treated with several concentrations of *M. Vulgare*extract from 0.78 to 100 µg/ml, and with cisplatin at concentrations from 0.01 to 25 µg/ml. Control cells were treated with DMSO alone. Extracts dissolved in DMSO completed with medium. The final concentration of DMSO was not exceeded 0.1%. The cells were allowed to grow for 48 h in humidified atmosphere at 37°C and 5% CO2, then100 µL of medium was carefully removed from each well and replaced with 20 µL MTT solution (5 mg/mL PBS). After 4 h incubation under the same conditions, the cleavage of MTT to formazan by metabolically active cells, which were dissolved in DMSO, was quantified by scanning the plates at 570 nm using a Multiskan EX (Finland) apparatus. Three independent sets of experiments performed in duplicate were evaluated. The % of cell viability was calculated by the following formula:

% Cell Viability = (A/A_0) *100

Where A_0 and A are the absorbance of negative control and test culture, respectively. The cytotoxic effects of pyridazin-3(2H)-one derivatives against the cell line were compared using their IC_{50} values (concentration of tested molecules leading to 50% inhibition of cell viability).

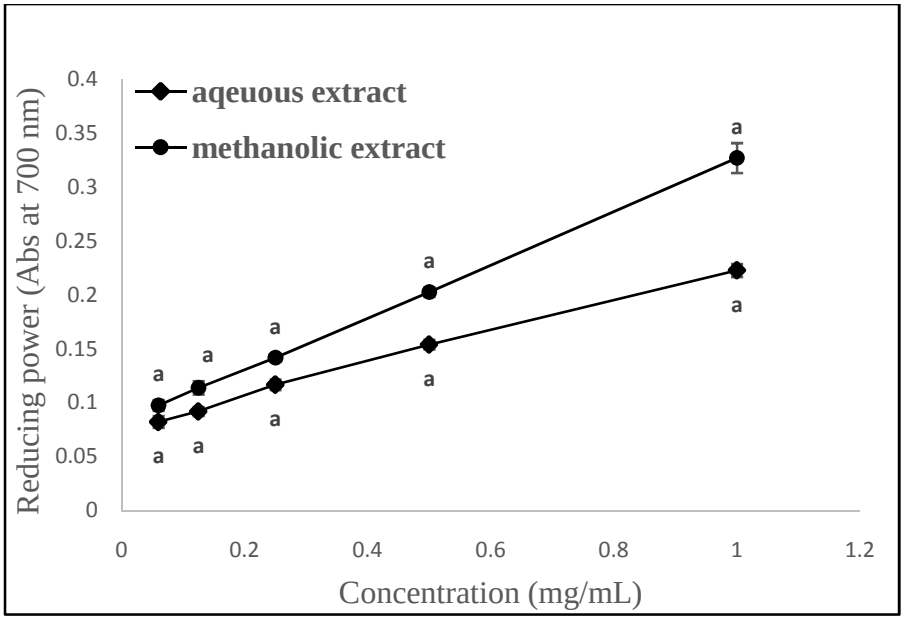
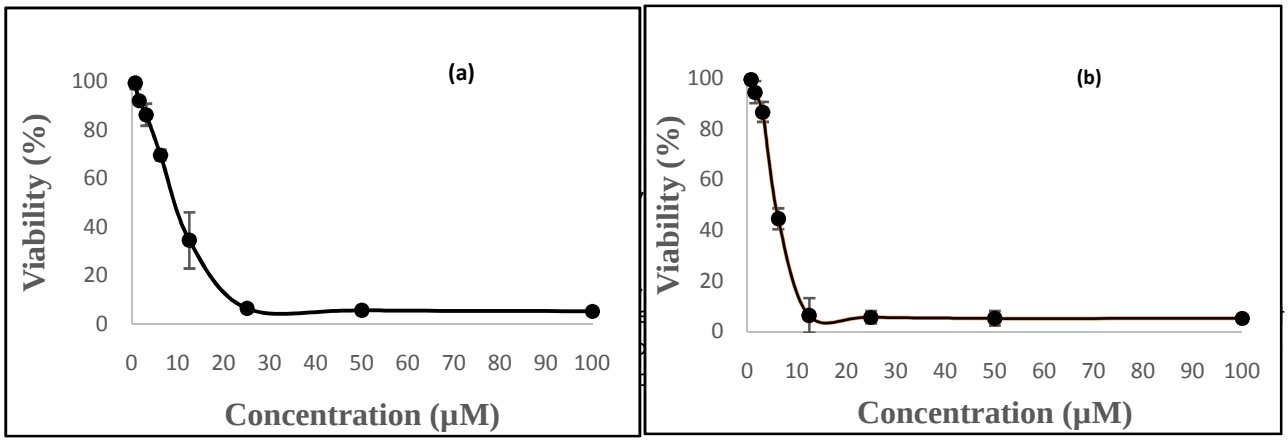


Fig 1. Ferric reducing power of *M. vulgare* extracts.
All the values are mean± standard deviation. Different letters indicate significant differences between conditions (p<0.05).



STATISTICAL ANALYSIS

The used data are mean values ± SD(standard deviation). Results were subjected to a one-way analysis of variance (ANOVA) followed by the Tukey

test using SPSS statistics (version 18). The differences were considered to be significant when $P < 0.05$.Several reports indicated that the most convenient solvent for phenolic compounds extraction from Lamiaceae is methanol [17]. In fact the use of water, methanol, chloroform and petroleum ether to extract phenolic substances from *T.polium,* showed that methanol is the best solvent that gave the highest extraction yield [18]. In the present study, the highest extraction yield was also achieved with methanolic extraction (20.82%) (Table 1). On the other hand, the highest concentrations of polyphenols (60.409 mg EAG/g DW) were recorded for the methanolic extractcompared to the aqueous extracts (32.710 mg EAG/g DW). These results suggest that methanol is a good solvent for extracting phenolic compounds of *M. vulgare*. Our results are in agreement with those reported by previous studies[19,20].

Total flavonoid content

Flavonoids are secondary metabolites of plant which involved in protect against human diseases that are associated with oxidative stress[21].In fact, multiple reports conducted on flavonoids from different plant species have reported free-radical scavenging ability and protection from oxidative stress for these metabolites [22]. Total flavonoid of the M. vulgare extracts was analyzed and the results are presented in Table 1. The flavonoid content was different between the aqueous and the methanolic extracts. In fact, the methanolic extract shows a higher content (33.813 mg EQ/g DW) compared to the aqueous extracts (26.023 mg EQ/g DW). Our results are different from those obtained by Khodja et al., (2014)[8]who found that thetotal flavonoid of methanolic extract of *M. vulgare* was 7.03 mg EQ/g DW.In the same context,Chediaaet al.,(2014)[23]obtained 0.61 mg catechin equivalents per mLfor methanolic extracts of *M. vulgare* leaves.

Free radical scavenging activities on DPPH and ABTS

Free radicals produced in the cell are generally considered to be relatively associated with the etiology of different diseases such as cancers, diabetes[24]. Dietary antioxidants which are able to scavengefree radicals, candecrease the risk of these diseases. Therefore, it was interesting to determine the radical scavenging effect of the antioxidants in *M. vulgare*leaves. Thus, in the present study, the free radical scavenging potentials of the aqueous and methanolic extracts at different concentrations were tested and the concentration of extract necessary to decrease the initial concentration of DPPH by 50% (IC_{50}) was determined. The results obtained were shown in table 2. For our sample, the methanolic extract exhibited the highest DPPH free radical scavenging with an IC_{50}equal to 2.496 mg/mL compared to the aqueous extract with an IC50 equal to 3.092 mg/mL. In other results, Khaled-Khodja et al. (2014)[8] who obtained IC_{50}(0.52 mg/mL) for the methanolic extracts of *M. vulgare*. Moreover, another study of *M. vulgare* exhibited an IC_{50} value of 36.69 μg/mL [25].The capacity of *M. vulgare* extracts to scavenge free radicals was also examined by their capacity to quench $ABTS^{+}$. As can be seen from the Table 2in ABTS assay, themethanolic extract has the highest activity with an IC_{50}equal to 0.874 mg/mL, while for the aqueous extracts it was about 0.961 mg/mL.Those results are in accordance with a previous report showing higher antioxidant activity of *M. vulgare* methanolic extract [26]. The high ability in scavenging ABTS radicals may be assigned to the presence of some phenolic compounds. In fact, previous studies showed an excellent linear correlation between phenolic contents and antioxidant capacity[27,28].

Ferric reducing/antioxidant power (FRAP) assay

FRAP assay was used for the assessment of antioxidant activity of different food product samples [29,30,31].The assay of reducing activity was based on the reduction of Fe^{3+}/ferricyanide complex to the ferrous form in presence of reductants (antioxidants) in the tested samples [32]. Results of reducing power assay, expressed as equivalent ascorbic acid, are shown in Table 2. The reducing power of methanolic extracts was higher (456.930 mg EA/g DW) compared to the aqueous extracts (308,138 mg EA/g DW). Previous studies have shown that extracts of species belonging to the genus Marrubium (*Marrubiumglobosum Montbr*. Subsp. *Globo-sum*) have strong reducing power [33]. Fig.1 shows the reducing power of extracts versus their concentration. The reducing power of the extracts increased with increasing in concentration which is in line with other previous studies [34,35].

Metal chelating activity

Antioxidants have the ability to chelate/deactivate the transition metals implicated in the decomposition of hydrogen peroxide and Fenton-type reactions[36]. Therefore, it was considered important to screen the iron (II) chelation ability of*M. vulgare* extracts. The obtained results in Table 2 showed thatIC_{50} of methanolic extract showed extremely higher metal chelating activity (0.104 mg/mL) compared to IC_{50} ofaqueous extracts (1.469 mg/mL). In fact, this can also be attributed to the phenolic compounds which were reported to exhibit considerable metal ion-chelating propertiespreventing metal-induced free radical formation [37]. In addition, phenols contain a hydroxyl group that is fixed directly to the aromatic ring and the Hatom of the hydroxyl group and can therefore trap peroxyl radicals which may contribute to the prevention of other compounds to be oxidized [38].

High performance liquid chromatographic (HPLC) separation and determination of main phenolic compounds in *M. vulgare* extracts

The components presented in Table 3 were identified by high performance liquid chromatographic (HPLC)

of *M. vulgare*extract by comparisons to the retention time and UV spectra of authentic standards. The quantitive data were calculated from the calibration curves. Salicylic Acid was the dominant phenolic compound in aqueous and methanolic extract, since it constituted 31% and 72% of the total extracted compounds respectively. Furthermore, differences in the composition of the secondary metabolites between the aqueous and methanolic extracts were noted, in fact some compounds in the aqueous extract were absent in the methanolic extract such as caffeic acid, gallic acid, syringic acid, p-hydroxybenzoic acid, hesperidin, limonene and thymoquinone. This variability in the results could be related to the climatic conditions of the biotope of the species or to the different methods followed during the extraction.

Cytotoxic activity

To determine the cytotoxicity of methanolic extract to human cancer cells lines (MCF-7 and MDA-MB-468), the cancer cells lines were treated with increasing concentrations of methanolic extract and the cytotoxicity were determined using MTT assay. The results are shown in Figure 2. We observe that both cell lines were sensitive to methanolic extract and inhibited the growth of cell lines in a dose-dependent manner. Cytotoxicity of methanolic extract was compared with that of doxorubicin, one of the drugs currently used for treatment cancer cells lines. The IC_{50} values for methanolic extract and doxorubicin calculated are shown in Table 4. The toxicity of the methanolic extract on MCF-7 cells was higher than MDA-MB-468 cells. Several studies in this filed have shown that genus Marrubium contain potent antiproliferative agents[39,40,41]. Yamaguchi et al., (2006)[39]reported an antiproliferative activity of *M.vulgare* leaves in human colorectal cancer cells through inhibition of cell growth and induction of apoptosis. Elsewhere, *M. cylleneum* and *M. velutinum* were evaluated for their cytotoxic effects against different cancer cell linesby Karioti et al., (2007)[40], showing strong tumor regression in a large range of tumor cells.Moreover, another study highlighted the antiproliferative activity of the methanolic extract of *M. persicum*against MCF-7 cells in a dose dependent manner[41].

CONCLUSION

Overall, this study suggests the antioxidant and cytotoxic activity of *M. vulgare* extract might be helpful in preventing or slowing the progress of various oxidative stress related diseases such as breast cancer. For that, we study antioxidant activity and the cytotoxic impact of *M. vulgare* extract on 2 cancer cell lines (MDA-MB-468 and MCF-7).Our results have Shown high antioxidant capacities, and manifested strong anticancer potential in MCF-7 cell line with an IC_{50} of 12.05±5.06 µg/mL.Further studies are required to elucidate the precise molecular or molecules involved in cell growth inhibition by fractionation studies.

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