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Assessment of *Satureja montana* L. and *Mentha piperita* L. Antioxidant Activity, Cytotoxicity and Pattern of Apoptotic Gene Expression in Hepatoma Cells

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Abstract

This study is intended to assess the *Satureja montana* L. and *Mentha piperita* L. *var. citrata* antiradical potential and elucidate their molecular mechanisms underlying the cytotoxic induction in HepG2 cells. The total phenolic and flavonoids of the selected plants were determined. Their antioxidant activity was evaluated using 2, 2-diphenyl-1-picryl-hydrazylhydrate ($DPPH^{\cdot}$), 2,2'-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid ($ABTS^{+)}$ radicals and reducing power. The HepG2 cells were incubated with tested compounds at different concentrations ranging between 12.5 and 200µg/mL. Cytotoxicity was measured by MTT and the mRNA expression of Bcl-2, Bax and caspase-3 genes were estimated using Semi-quantitative RT-PCR. The results revealed that the *S. montana* and *M. piperita* aqueous extracts have high content of phenolic 188.2 ± 1.3 and 183.2 ± 0.9 mg GA/g and flavonoid 95.01±2.29 and 144.13±0.73 mg quercetin/g compounds. While their essential oils contain phenolic compounds 290.8 ± 1.4 and 9.2 ± 5.6 mg GA/g only. *In vitro* assays revealed a strong radical scavenging potential of the *S. montana* L. and *M. piperita* L. essential oils against stable $DPPH^{\cdot}$, $ABTS^{+}$ and reducing power showing the highest radical quenching activity compared with the aqueous extracts. In addition, the extracts and oils of the two tested plants showed a concentration-dependent increase in growth inhibition against HepG2 cell line. Semi-quantitative RT-PCR established that either *S. montana* or *M. piperita* up-regulated HepG2 Bax and caspase-3 mRNA expression and down-regulated Bcl-2. Greater variation in Bax, Bcl-2 and caspase-3 expression were visible in the oils rather than the extracts. The decrease in Bcl-2, rise in Bax and lastly, the stimulation of caspase-3 in HepG2 cells indicated the irreversible apoptotic process. Accordingly, the results confirmed that selected plants have an antioxidant potential and apoptosis ability via the activation of mitochondrial apoptotic pathways.

Keywords: *Satureja montana* L, *Mentha piperita* L, Hepatocellular carcinoma, Antioxidants activity, Total phenols and flavinoids, Cytotoxicity, Gene expression.

1. Introduction

The dominant form of primary liver tumor is Hepato-cellular carcinoma (HCC) and is a leading cause of cancer-related death (Balogh *et al*., 2016). Approximately 90 % of patients with HCC have a confirmed background of a chronic liver disease including hepatitis B and C virus chronic infections (El-Serag and Rudolph, 2007; Poon *et al*., 2009). Several treatment options are obtainable against HCC including therapeutic resection, radiofrequency ablation, liver transplantation, trans-arterial chemo-embolization and radio-embolization (Raza and Sood, 2014). The main scientific challenge confronting HCC is the limited response to chemotherapy and the resistance during the treatment due to an improved DNA repair ability and antioxidant enzymes activated in the malignant cells (Thomas, 2009). Recently, several studies have been made with folk medicine for the treatment of cancer. Traditional medicine has been utilized with chemotherapy and radiotherapy to restrain the noxious effects of them and improving overall efficiency (Ling et *al*., 2014).

The *Lamiaceae* family plants are vital ornamental aromatic plants, many of which possess essential oils that are used in medicine, food, cosmetics, and the pharmaceutical industry (Benabdallah *et al*., 2016). The medical and pharmaceutical importance of essential oils are ascribed to their protective activity against the reactive oxygen species (ROS) oxidative damage (Spiridon *et al*., 2011). The *Lamiaceae* family medicinal plants antioxidant capacity can be attributed to their polyphenolic compound content (Adaszynska and Dzieciol, 2017).

Satureja montana L. is generally known as winter savoury and is habitually used for diuretic, bronchitis, curing greasy skin and sensitivity antitussive, antidiarrheal agent stomachic, antiseptic muscle pain, wounds, urinary system infections, and gastroenteritis (Hadian *et al*., 2012; Lerebour *et al*., 2014; Jafari *et al*., 2016). *Satureja* species secondary metabolites, essential oils, phenolic compounds,

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and flavonoids are recognized for their healing properties. Plant extracts or essential oils from *Satureja* various species have revealed several biological activities (Vagionas *et al*., 2007; Grosso *et al*., 2009; Dunkic *et al*., 2010). Mediterranean basin wild species have shown a cytotoxic property against cancer cells (Chinou *et al*., 2007; Menichini *et al*., 2009; Nemati *et al*., 2018). In addition, the *S. montana* essential oil represents an appropriate agent for applications in the fortification of free radical-mediated oxidative stress biological matrices (Trifan *et al*., 2015).

Mentha piperita L. is usually known as peppermint. It is a natural hybrid of *M. aquatica* L. and *M. spicata* L. The entire herb of *M. piperita* is used in phytotherapy for their proven antibacterial, antioxidant, cytotoxic and antiallergenic activities (Capuzzo and Maffei, 2016). Furthermore, the *Mentha* species are known as good free radical scavengers that limit ROS attack on biological and food systems (Sytar *et al*., 2018). In addition, it has been found that various plants which belong to the family of *Lamiaceae*, such as *Origanum vulgare*, *O. majorana*, *Salvia fruticosa*, *S. officinalis* and *Melissa officinalis* provoke apoptosis in the tested malignant cells which plays an essential task in the elimination of injured or malignant cells (Abdel-Massih *et al*., 2010; Darzi and Amirghofran, 2016).

Therefore, the target of the current study is to inspect the *S. montana* L. and *M. piperita* L. extracts' and essential oils' phenolic and flavonoids total content, antioxidant activity and their growth inhibition ability on the HepG2 cell line. The study also presents an assessment of their effect on apoptotic gene expression pattern.

2. Materials and Methods

2.1. Chemicals

Potassium ferricyanide K_3 [Fe $(CN)_6$], ferric chloride ($FeCl_3$), trichloroacetic acid (TCA), peroxidase, hydrogen peroxide, 2, 2'-azino-bis (3-ethylbenz-thiazoline-6-sulfonic acid, diammonium salt (ABTS), 1,1-diphenyl-2-picryl-hydrazyl (DPPH); l- ascorbic acid (vitamin C), Folin & Ciocalteu's phenol reagent were purchased from Sigma-Aldrich (Schnelldorf, Germany). The acetonitrile and water were of the HPLC grade. Dimethylsulphoxide (DMSO), 5-Flurouracil and MTT (3-(4, 5- dimethyl-thiazol-2-yl)-2, 5-diphenyltetrazolium bromide) were purchased from Merck (Darmstadt, Germany). All other chemicals and reagents used in this study were of the analytical grade, and were purchased from Sigma Aldrich chemical Co. (St. Louis, MO, USA). The human liver cancer cell line HepG2 was purchased from VACSERA.

2.2. Plant Materials and Extraction

The study was carried out on *Satureja montana* L. and *Mentha piperita* L. *var. citrata* (Eau de Cologne mint) at the National Research Centre experimental field during two successive seasons (2016 and 2017). The fresh aerial parts were collected at the end of July. The aerial parts were air-dried in a room far from the sun for three weeks. A voucher specimen of the plant is deposited at the National Research Centre herbarium at Dokki, Giza, Egypt.

2.2.1. Essential Oil Isolation

The *S. Montana* L. and *M. piperita* L. *var. citrata* aerial parts were powdered (250 g) and hydro-distilled using a Clevenger type apparatus for three hours according to the method recommended in British Pharmacopoeia. The resulting essential oil was dried over anhydrous sodium sulfate, and was stored at 4°C until its use.

2.2.2. Preparation of Plant Extracts

250 g of the air-dried powdered plant aerial parts of *S. montana* L. and *M. piperita* L. *var. citrata* were extracted by maceration in 1000 mL of distilled water three successive times. The combined extracts were evaporated under reduced pressure at 45°C to the residue, and were stored at 4°C until tested.

2.3. Phenolic and Flavonoid Extracts Content

2.3.1. Total Phenolic Content

The total phenolic content (TPC) of the plants extracts and their essential oils were determined by Folin–Ciocalteu method as described by Singleton *et al*. (1999). The content of phenolics in the extracts was expressed in terms of gallic acid equivalent (mg of GA/g of extract).

2.3.2. Total Flavonoid Content

The flavonoid content of the plants' extracts was determined according to the method of Zhishen *et al.* (1999). The results were expressed as mg quercetin equivalents/g extract.

2.4. *Free Radical Scavenging Activity*

The free radical scavenging activity of the plant extracts and essential oils of *S. montana* L and *M. piperita* L. *var. citrata* was measured by 1, 1-diphenyl-2-picryl-hydrazil (DPPH) using the method of Yamaguchi *et al.* (1998). Briefly, 0.1mM solution of DPPH in ethanol was prepared, then, 1mL of this solution was added to 3 mL of the tested extracts as well as the standard solution at different concentrations (10, 25, 50, 75,100 and 200 µg/ mL). The mixture was shaken vigorously, and was allowed to stand at room temperature for thirty minutes. Then, the absorbance was measured at 517 nm in a spectrophotometer (Jasco, serial No. C317961148, Japan). The lower absorbance of the reaction mixture indicated a higher free radical scavenging activity. The DPPH radical concentration in the reaction medium was calculated via the following equation:

DPPH scavenging effect (%) = $[(A_0\text{-}A_1)/A_0) \times 100]$, Where A_0 is the absorbance of the control reaction, and A_1 is the absorbance in the presence of the sample of extracts.

2.5. ABTS+ Radical Scavenging Activity

$ABTS^+$ radical scavenging activity of the plant extracts, essential oils, and standard were measured according to the method described by Thaipong *et al.* (2006). Each tested sample was prepared in five concentrations (10, 25, 50, 100 and 200µg/mL), and the ascorbic acid was used as standard material with the same concentrations. Exactly 0.2ml of peroxidase (4.4U/ mL), 0.2mL of H_2O_2 (50 µmol/l), 0.2mL of ABTS (100µmol/l), and 1mL methanol were mixed and kept in the dark for one hour to form a bluish green complex after the addition of 1mL of the tested samples and standard at different concentrations. The absorbance at 734nm was measured to represent the

total antioxidant capacity, and was then calculated as follows:

$ABTS^{+}$ radical scavenging activity % = 1- (A sample/ A control)* 100 Where A sample is the absorbance of the samples and standards, and A control is the absorbance of control.

2.6. Reduction Capability

The total ferric-reducing capacity of the plant extracts and essential oils was determined according to the method of Oyaizu (1986). The different concentrations of extracts and oils (10, 25, 50, 75, 100 and 200 µg/ mL) in 1mL of methanol were mixed with phosphate buffer (2.5 mL, 0.2M, pH 6.6) and potassium ferricyanide K_3 [Fe (CN) $_6$] (2.5 mL, 1%). The mixture was incubated at 50°C for twenty minutes. A portion (2.5 mL) of TCA (10%) was added to the mixture, which was then centrifuged for ten minutes at 1000 rpm (MSE Mistral 2000, UK, and Serial No.: S693/02/444). The upper layer of the solution (2.5mL) was mixed with methanol (2.5mL) and $FeCl_3$ (0.5mL, 0.1%), and the absorbance was measured at 700 nm in a spectrophotometer. l- ascorbic acid was used as a control. The higher absorbance of the reaction mixture indicated a greater reducing power.

2.7. Cell Culture and Treatment

Human liver cancer cell line (HepG2) cells were seeded into standard 96-well culture plates at a density of $2.0x10^4$ cells/well and cultured in RPMI-1640 supplemented with 10% fetal bovine serum (Gibco, Grand Island, NY) and 1% penicillin-streptomycin, at 37°C, in humidified air containing 5 % CO2 to ~70 % confluence. All experiments were performed while the cells were in an exponential growth phase (Lin et *al*., 2011). After the cell adherence, different concentrations of the investigated extract and essential oil ranging between 12.5 and 200µg/mL were added to the wells. The standard reference drug (5-Flurouracil) was added to the cells suspended in 0.10 ml of RPMI medium after washing the cells several times with phosphate buffered saline (PBS). All tests and analyses were run in triplicate, and the incubation continued for forty-eight hours.

2.8. Cytotoxicity Assay

Cytotoxicity was assessed by a tetrazolium-based colorimetric assay (MTT) that measures reduction of tetrazolium salt (3-[4, 5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; Sigma, St. Louis, MO) to formazan, mainly via the activity of mitochondrial enzymes according to Berridge *et al*. (2005). In brief, l0µL of MTT dye solution (5mg/mL) was added to each well, and the plate was incubated for a further four hours at 37°C. After the removal of the MTT dye solution/medium, each well received 100µL DMSO (0.1%), and the absorbance at 490nm was quantified using an ELISA plate reader. The extent of cytotoxicity induced was calculated by comparing absorbance values against those in the control wells. Cytotoxicity was expressed as the concentration of extract or oil inhibiting cell viability by 50% (IC50). The percentage of cytotoxicity was calculated as follows:

% Cytotoxicity = 1- [(OD treated-OD blank) / (OD control-OD blank)] ×100.

2.9. Reverse Transcription-Polymerase Chain Reaction (RT-PCR) and Gene Expression Analysis

Semi q-PCR was performed as described previously by (Rey *et al.,* 2000). The total RNA was isolated from untreated and treated HepG2 cells with TRIzol, and oligo (dT)-primed RNA (1 µg) was reverse-transcribed using SuperScript II reverse transcriptase by RT PreMix Kit (iNtRON Biotechnology, Korea) according to the manufacturer's instructions. The obtained cDNA was used to determine the mRNA expression levels of Bcl-2, Bax and caspase-3 by PCR analysis. GAPDH was used as an internal control. The primer sequences used for the amplification of Bcl-2, Bax, caspase-3 and GAPDH were as follows: Bcl-2-F: 5'-GGATGCCTTTGTGGAACTGT-3', and Bcl-2-R: 5'-AGCCTGCAGCTTTGTTTCAT-3'.Bax–F:5'-TTTGCTTCAGGGTTTCATCC-3',Bax-R:5'-CAGTTGAGTTGCCGTCAGA3'.,caspase-3F:5'-CAGAA GATACCAGTGGAGGCC-3',caspase-3-R:,5'-TTCCG GT TAACACGAGTGAGG-3' and GAPDH- F:5′CAAG GTCATCCATGACAACTTTG-3′, GAPDH-R:5′-GTCC ACCACCCTGTTGCTG TAG -3′. All primers were synthesized by Promega Corp (Madison, WI, USA). The reaction included 1 µL cDNA, 2 µL 10X Taq Buffer, 1.2 µL 25 mM $MgCl_2$, 2 µL 10 mM dNTP, 0.1 µL 5U/µL Taq polymerase, 2 µL each primer and DEPC water up to 20 µL. The PCR conditions were as follows: 95˚C for three minutes and thirty cycles at 95˚C for thirty seconds, 56˚C for forty seconds and 72˚C for forty seconds. The samples were analyzed by 1.5 % agarose gel electrophoresis. The DNA bands were evaluated using a Gel Documentation System (Gel Doc 2000; Bio-Rad Laboratories, Inc., Hercules, CA, USA). The ethidium bromide-stained gel bands were scanned, and the signal intensities were quantified by the computerized Gel-Pro (version 3.1 for window 3). According to the amplification procedure, the relative expression of each gene was calculated using the formula: Optical density of each gene (OD)/ Optical density of GAPDH.

2.10. Statistical Analysis

The values are presented as the mean ± standard error. Results were analyzed by one-way analysis of variance. When significant treatment effects were detected, Dunken-test was performed to make pair wise comparisons between individual means. Statistical analysis software SPSS 16.0 (International Business Machines, Armonk, NY, USA) was used to conduct statistical analyses. $P \leq 0.05$ was considered a statistically significant difference.

3. Results

3.1. Total Phenolic and Flavonoid Content

The *S. montana* L. TPC, calculated from the calibration curve ($R^2 = 0.998$), equal (188.2 ± 1.3) mg gallic acid equivalents/g for the extract and (290.8 ± 1.4) mg gallic acid equivalents/g for the oil, while the TPC of *M. piperita* L. *var. citrata* was 183.2 ± 0.9 & 9.2 ± 5.6 mg gallic acid equivalents/g extract and volatile oil, respectively (Table 1). No significant variation in the TPC of the two plant extracts, while the *S. montana* L. essential oil contains a high significant TPC than the *M. piperita L. var. citrata* In regards to the *S. montana* L., the total

flavonoid content 95.01±2.29 was less than that of *M, piperita L. var. citrata* 144.13±0.73 mg quercetin/g extract. There were no flavonoids in their essential oils (Table 1).

Table 1. Total phenolic and flavonoid compounds in *Satureja montana L.* and *Mentha piperita L.* extracts and essential oils

	Satureja montana L.		*Mentha piperita L.*	
	Extract	Essential oil	Extract	Essential oil
Total Phenolic (mg eq GA/g)	188.2±1.3[a]	290.8±1.4[b]	183. 2±0.9[a]	9.2±5.6[a]
Total Flavonoids (mg eq Que/g)	95.01±2.29b	-----	144.13±0.73a	----

Data are presented as means ± SE. One-way Analysis of Variance was used for data analysis (n=3), Different superscripts within the same raw designate significant differences ($P \leq 0.05$).

3.2. Free Radical-Scavenging Activity

The plant extracts, oils, and ascorbic acid as standard free radical-scavenging activity were measured using the DPPH and ABTS assays. Both DPPH and ABTS that dissolve in methanol or ethanol and are stable free radicals, their colors show special absorptions at 517 nm or 734 nm, respectively. From the DPPH and ABTS^{+} data, the aqueous extracts and essential oils of both plant species exhibited antioxidant activity. When compared with the oxidative potential of the standard compound, remarkable activity was exerted by the essential oils of both plants as presented in Figures 1 and 2.

3.3. Reducing Capacity

The total ferric reducing capacity of an extract may be considered as a significant marker of its possible antioxidant activity. We can see that the reducing power percentage values of S. *montana* L. and *Mentha piperita* L aqueous extracts and volatile oils increased in a concentration-related manner in the range of the tested concentrations (Figure 3). Their relative reducing powers were as follows: volatile oil > aqueous extract.

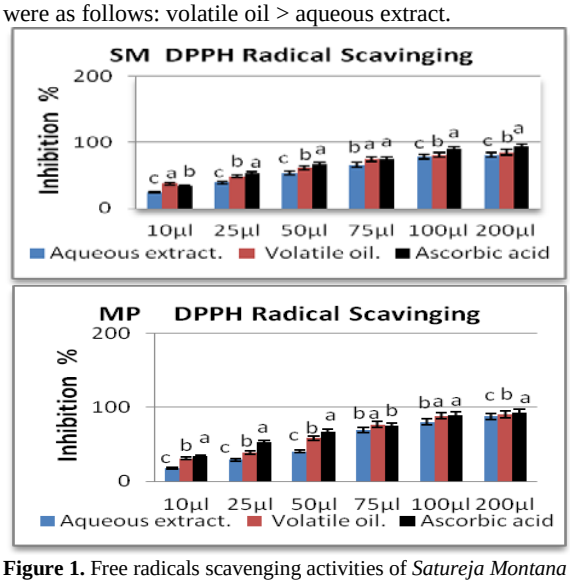

Figure 1. Free radicals scavenging activities of *Satureja Montana* L. and *Mentha piperita* L. *var. citrata* aqueous extracts and essential oils at different concentrations (10-200µg/ml) compared with vitamin C against DPPH. Data are presented as means ± SE. Different superscripts within the same raw designate significant differences ($P \leq 0.05$).

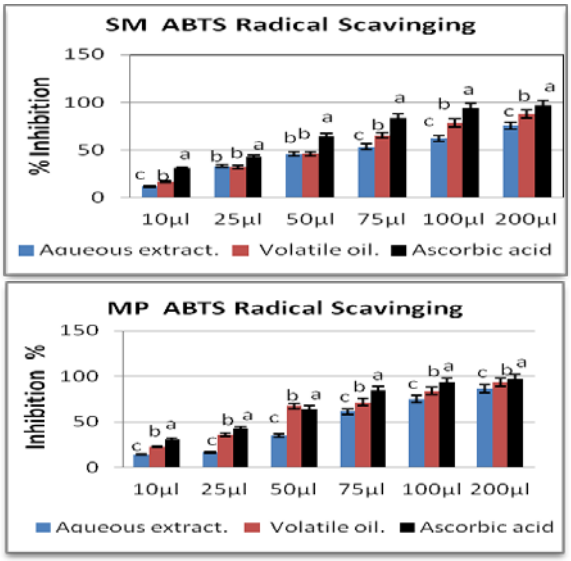

Figure 2. Free radicals scavenging activities of *Satureja Montana* L. and *Mentha piperita* L. *var. citrata* aqueous extracts and essential oils at different concentrations compared with vitamin C against ABTS. Data are presented as means ± SE. Different superscripts within the same raw designate significant differences ($P \leq 0.05$).

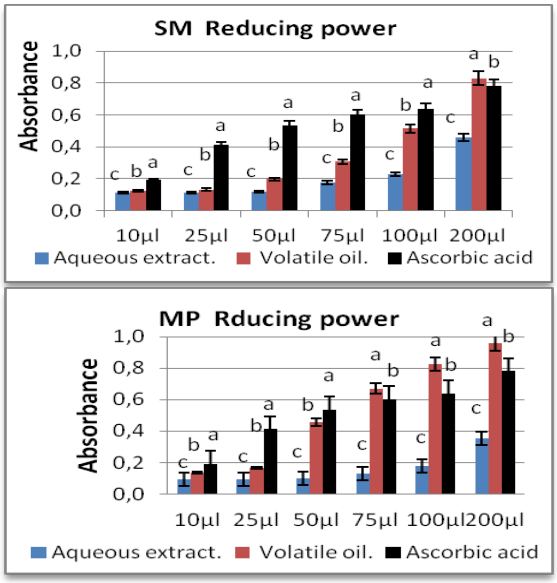

Figure 3. Total ferric reducing power (FRAP) activity of *Satureja montana* L. and *Mentha piperita* L. *var. citrata* aqueous extracts and essential oils at different concentrations compared with vitamin C. Data are presented as means ± SE. Different superscripts within the same raw designate significant differences ($P \leq 0.05$).

3.4. Cytotoxicity

The cytotoxicity of the *S. montana* L. and *M. piperita* L. *var. citrata* aqueous extracts and essential oils was investigated using MTT assay against hepatocellular carcinoma cell lines (HepG2) in comparison with 5-Flurouracil as the reference drug. The results are expressed as the median growth inhibitory concentration (IC_{50}) which is the concentration of the tested compounds that reduces the cells, survival to the half compared with the untreated cells. The results demonstrated that the *S. montana* L. aqueous and oil extracts show cytotoxicity

against the HepG2 cells with IC_{50} value 50 & 25µg/mL, respectively. While, *M. piperita var. citrata* aqueous and oil extracts showed an anticancer effect with IC50 values of 91.5 and 20.2 µg/mL, respectively. However, the *S. montana* L. and *M. piperita* L. *var. citrata* extracts and essential oils cytotoxicity was less than that of the 5-Flurouracil IC50 = 4.5 µg/mL (Figure 4).

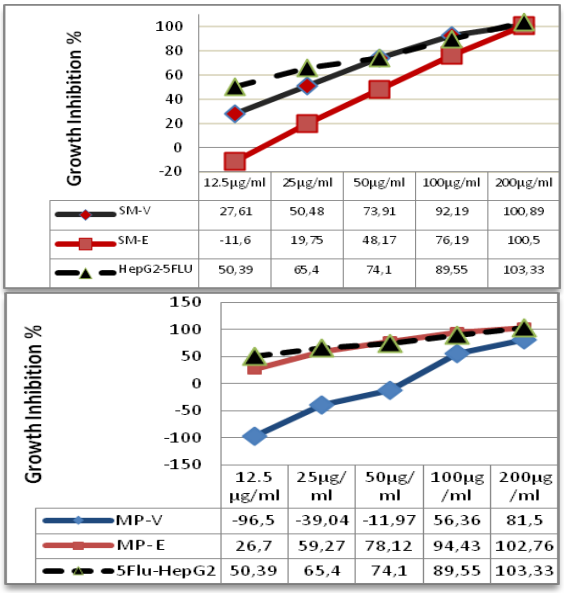

Figure 4. Growth inhibition of *Satureja montana* L. and *Mentha piperita* L. *var. citrata* aqueous extract and volatile oil concentrations against HepG2 cells.

3.5. Effects of Satureja montana L. and Mentha piperita L. Extracts on the Expression of Apoptosis-Related Genes in HepG2 Cells

To find out the molecular mechanisms of the *S. montana* L. and *M. piperita* L. *var. citrata* aqueous extracts and essential oils against the HepG2 cells, the expression of three apoptosis-related genes (caspase-3, Bax and Bcl2) expression was examined.

3.5.1. Gene Expression of Caspase-3

The semi-q PCR of caspase-3 mRNA analysis showed that the *S. Montana* L. and *M. piperita* L. *var. citrata* aqueous extracts and essential oils exhibited an increase in the expression of the caspase-3 gene in the HepG2 cells as indicted by the amount of the mRNA. Caspase-3 expressions were significantly different ($P \leq 0.05$) from those of the untreated cells and the oils have wider bands of caspase-3 mRNA than the aqueous extracts (Figures 5 and 6).

3.5.2. Gene Expression of Bcl-2 Family

The *S. Montana* L. and *M. piperita* L. *var. citrata* aqueous and oil extracts' treated the HepG2 cells for forty-eight hours, and showed stronger pro-apoptotic Bax mRNA expressions than in the untreated cells. The Bcl-2 expressions of the *S. montana* L. and *M. piperita* L. *var. citrata* incubated cells were significantly ($P \leq 0.05$) reduced compared to the control cells. Greater alterations were noticed in the volatile oil treated cells, where it significantly decreased ($P \leq 0.05$) the mRNA expression of the pro-apoptotic (Bax) and the apoptosis regulator (Bcl-2) as shown in (Figures 5 and 6).

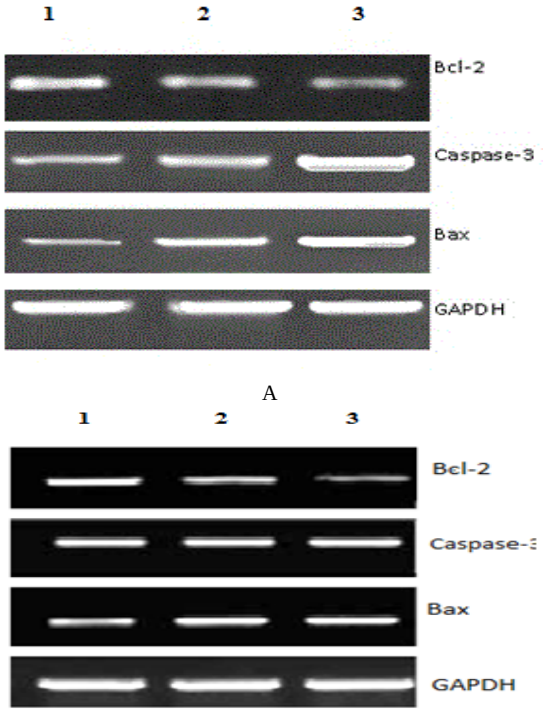

Figure 5. Effects of **(A)** *Satureja montana* L. **(B)** *Mentha piperita* L. *var. citrata* on the mRNA expression levels of caspase-3, Bcl-2 and Bax in HepG2 cells. GAPDH was used as an internal control. Where lane 1: untreated HepG2; lane 2: HepG2 treated with aqueous extract and lane 3: HepG2 treated with volatile oil.

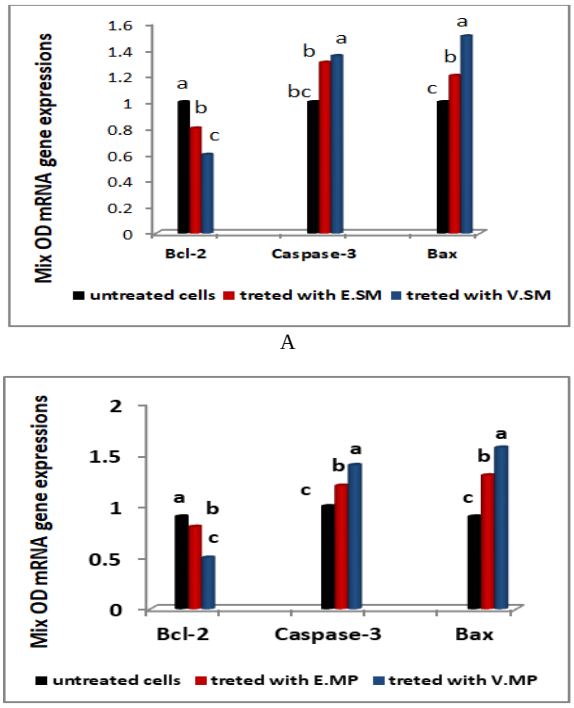

Figure 6. Effect of **(A)** *Satureja montana* L. and **(B)** *Mentha piperita* L. *var. citrata* extract and volatile on apoptotic gene expression level in HepG2 cell line4.The results illustrated are normalized to the level of GAPDH level and the data are the means of optical density for each gene divided by that for GAPDH.

4. Discussion

Most of aromatic and medicinal plants have compounds with antioxidant properties. Natural antioxidants are considered useful agents for diseases' prevention. Previous studies have shown that plant phenolic compounds display an antioxidant property because of their free radicals scavenging capacity (de Oliveira *et al*., 2011). The results of the current study revealed that the *S. montana* L. and *M. piperita* L. *var. citrata* extracts and oils contain a high phenolic content and showed high free radical-scavenging and reducing-power activities. These results are supported by the study of Pereira and Cardoso (2013), who confirmed that *M. piperita* L. plants are rich in phenolic compounds. Additionally, Hassanein *et al*. (2014) proved that *S. montana* cultivated in Egypt contains a high content of phenolic and flavonoid compounds that could be reliable for a remarkable radical scavenging and antioxidant properties. This promising efficiency suggests its potential role as an antioxidant agent, to improve the antioxidant condition and counteract the hazards of diseases related to oxidative stress.

Medicinal plants essential oils have a broad variety of biological activities including spasmolytic, hepato-protective, antiviral and anticarcinogenic effects (Lahlou, 2004). In recent years, many studies recognized the efficiency of essential oils and their chemical constituents as a source of novel bioactive natural products even against cancer (Piaru *et al*., 2012; Rasoanaivo *et al*., 2013; Zapata *et al*., 2014). Al-Okbi *et al*. (2015) revealed that *M. citrata* and *M. longifolia* own antioxidant and anticancer activities that could be referred to the occurrence of phytosterosl, phenolic compounds and specific volatile constituents. The present study's data of plant analysis revealed that the *S. montana* L. and *M. piperita* L. *var. citrata* essential oils possess stronger antioxidant properties than their extracts. This result is in agreement with Nemati *et al*. (2018) who reported that the *S. montana* L. essential oils have a prospect for reducing Fe^{+3} to Fe^{+2} and also Cu (II) to Cu (I), and possess a strong antioxidant activity.

The current study showed that the aromatic plants, *S. montana* L. and *M. piperita* L. *var. citrate*, affected the HepG2 cells viability. Here, the *in vitro* cytotoxic study reported that the extracts and oils significantly suppressed the growth of hepatocellular carcinoma human cells (HepG2) in a dose-dependent manner with the IC50 value of *S. montana* L. = 50 and 25 µg/mL and *M. piperita* L. *var. citrata* = 91.5 & 20.2 µg/mL, respectively. It can be concluded that the essential oils of *S. montana* L. and *M. piperita* L. *var. citrata.* are two and four times more cytotoxic than the aqueous extracts. This result is in accordance with Elgndi *et al*.(2017) who demonstrated that the essential oil and CO_2 extract of *S. montana* showed a high anti-proliferative activity (IC50 = 59.85–91.05 µg/mL) against HeLa cells line, where the essential oil is 1.5 times more cytotoxic than CO_2 extracts. Fitsiou *et al.* (2016) suggested that the essential oils of *S. thymbra* and *S. parnassica* exhibited a diverse anti-proliferative activity against A549 non-small cell lung adenocarcinoma and HepG2 and Hep3B liver hepatocellular carcinoma cells. Sharifi-Rad *et al.* (2015) reported that the *S. intermedia* essential oils showed a concentration-dependent decrease in the HepG2 and MCF-7 human cancer cell lines viabilities. Arunasree (2010) confirmed that the *S. montana* cytotoxicity may be attributed to the relatively high phenolic compounds concentrations, particularly carvacrol. They demonstrated the carvacrol antitumor effects on MCF-7, MDA-MB and suggested that it has a therapeutic potential significance in cancer treatment. At the same time, Hussain *et al.* (2010) reported that the *M. piperita* L. *var. citrata* essential oil showed prominent cytotoxicity against breast cancer (MCF-7) and prostate cancer (LNCaP) cell lines with IC50 ranging from (43.5 ± 2.1 to 95.7 ± 4.5 µg/mL). Abirami and Nirmala (2014) showed that the *M. piperita* ethanolic extract revealed a cytotoxicity against human laryngeal epidermoid carcinoma with IC50 = 94 µg/mL. In addition, Ogaly *et al.* (2018) reported that the *M. piperita* L. essential oil exhibited a protective property against hepatotoxicity.

Furthermore, the results illustrated that the mRNA expression of key apoptotic regulators Bax, Bcl-2 and caspase-3 was altered following the *S. montana* L. and *M. piperita* L. *var. citrata* cell lines treatment. This is supported by Abd El Tawab *et al.* (2014) who showed that the *S. montana* extract protects the rat testis against cyclophosphamide-induced damage via anti-oxidative and anti-apoptotic mechanisms. Yfanti *et al.* (2015) concluded that the *S. horvatii* showed anticancer activity through A549 cell apoptosis. In addition, Jain *et al.* (2011) showed that *M. piperita* induced significant dose and time-dependent anticarcinogenic activity, leading to cell cycle arrest and mitochondrial-mediated apoptosis, and upregulation of Bax, p53 and p21 genes in the treated cells. Bax expression is upregulated by the tumor suppressor protein (p53), and it was shown to be involved in the p53-mediated apoptosis. The results of the present work demonstrated that the *S. montana* L. and *M. piperita* L. *var. citrata* treatment increased Bax expression in the HepG2 cells.

Caspase-3-mediated proteolysis is an important element of the mitochondria mediated apoptotic process. Active caspase-3 comprises a heterodimer of 17- and 112-kDa subunits that in turn are resultant from a 32-kDa proenzyme, a marker for apoptotic cells. Active caspase-3 proteolytically cleaves and activates other caspases and relevant targets in the cytoplasm (Jain *et al*., 2011). The same signaling pathway was identified in the HepG2 cells and evaluated in the present study. Caspase-3 activation was previously confirmed to increase following the treatment of the cells with *Satureja montana* L. (Bhattacharjee & Chatterjee, 2013). Ferreira *et al.* (2014) found that the *M. piperita* L. essential oil fatal cytotoxicity is linked with the increased levels of intracellular ROS, mitochondrial fragmentation, and chromatin condensation, without loss of the plasma membrane integrity, indicative of an apoptotic process. The *S. montana* L. constituents interact with mitochondrial membranes and alter their permeability by opening transition pores decreasing the potential of the mitochondrial membrane (Abd El Tawab *et al.*, 2014). Therefore, *S. montana* L. induces structural changes and increases the mitochondrial membrane unsaturation. This mitochondrial alteration may affect the activity of pro-and anti-apoptotic proteins of the Bcl-2 family. It was previously demonstrated that *S. montana* L. contributes to the downregulation of the anti-apoptotic

molecule Bcl-2 expression, and blocks lipid peroxidation that inhibits the apoptosis induction (Cetkovic *et al*., 2007).

5. Conclusion

Based on earlier literature and the data of the present study, it was hypothesized that the *S. montana* L. and *M. piperita* L. *var. citrata* aqueous extracts or essential oils induce human hepatocellular carcinoma cell apoptosis. The signal transduction occurs via the caspase-3 signaling pathway. Extra investigation on this signaling pathway is necessary to clarify their function in tumor invasion and survival.

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