BIOLOGICAL ACTIVITY AND STANDARDIZATION OF THE ETHANOLIC EXTRACT OF THE AERIAL PARTS OF MENTHA SUAVEOLENS EHRH

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ABSTRACT

A biologically guided fractionation of the ethanolic extract of the aerial parts of Mentha suaveolens Ehrh. cultivated in Egypt revealed that the ethyl acetate fraction had the highest hepatoprotective activity as it prevented the increase caused by CCl₄ in the levels of aspartate aminotransferase (AST), alanine amino transferase (ALT) and alkaline phosphatase (ALP) enzymes by 51.6%, 57.0% and 56.7%, respectively. The same fraction also showed the highest antioxidant activity; in vivo (as it restored the glutathione level in diabetic rats by 98.0 %) and in vitro as it had the highest free radical scavenging activity (IC₅₀=31µg/ mL). It also had the highest cytotoxic activity against human liver carcinoma cell line (HEPG2) (IC₅₀ = 5.1 µg/ mL). Consequently, this fraction was purified to yield caffeic acid and rosmarinic acid. Rosmarinic acid, the major component possessed cytotoxic activity on HEPG2 (IC₅₀ = 4.5 µg/ mL) comparable to doxorubicin (IC₅₀ = 3.73 µg/mL). A rapid reversed-phase high-performance liquid chromatography (RP-HPLC) method was developed for the standardization of the active ethyl acetate fraction and the method was validated. Rosmarinic acid content in M. suaveolens was estimated by RP-HPLC, to be 1.16 and 50.17% w/w for the powdered aerial parts and ethyl acetate fraction, respectively.

Keywords: Cytotoxicity, Mentha suaveolens, rosmarinic acid, RP-HPLC, standardization.
INTRODUCTION
Despite of the tremendous advances in modern medicine, hepatic disease is still a worldwide health problem; thus the search for new medicines is ongoing. Numerous formulations of medicinal plants are used to treat liver disorders, many of them act as radical scavengers, whereas others are enzyme inhibitors or mitogens.\(^1,2\) Cancer is among the most common causes of death and morbidity worldwide. According to the International Agency for Research on Cancer (IARC), in 2002, cancer killed more than 6 million people around the world.\(^3\) Hepatocellular carcinoma is the fifth most common cancer worldwide and the third most common cause of cancer-related death with 500,000 new cases diagnosed yearly.\(^4\) A significant part of drug discovery in the last years has been focused on agents to prevent or treat cancer.\(^5\) Several chemotherapeutic, cytotoxic and immunomodulating agents are available in medicine to treat cancer. Besides being enormously expensive, these drugs are associated with serious side effects and morbidity. Therefore, the search still continues for an ideal treatment that has minimal side effects and is cost-effective.\(^3\) Natural compounds from flowering plants have played an important role in the development of several clinically useful anticancer agents.\(^5,6\)

Lack of standardized extracts of the biologically active plants hinders the incorporation of these plants in pharmaceutical preparations. The genus *Mentha*, one of the important members of the Lamiaceae family, is represented by about 19 species and 13 natural hybrids. *Mentha suaveolens* Ehrh. is native to Africa, Temperate Asia and Europe.\(^7\) To the best of our knowledge, no standardized extract was prepared from *Mentha suaveolens* Ehrh. In that view, our aim was to evaluate the potential hepatoprotective, antioxidant and cytotoxic activities of the ethanolic extract of the aerial parts of *M. suaveolens* Ehrh. cultivated in Egypt, doing a phytochemical study of the ethyl acetate fraction to isolate its major constituents caffeic acid and rosmarinic acid, and to prepare a standardized extract of the bioactive fraction using a validated HPLC method.

MATERIALS AND METHODS
Chemicals
All reagents for extraction were of analytical grade (ADWIC, Cairo, Egypt). Chromatographic grade-double distilled water, analytical grade \(O\)-phosphoric acid and HPLC grade methanol were purchased from Merck, Darmstadt, Germany. The acidic aqueous solution used for HPLC analysis was filtered through Agilent Ecno 0.45 \(\mu\)m PTFE membrane.

**Apparatus and equipment**

UV spectra were measured using a Shimadzu UV 240 (P/N 204-58000) spectrophotometer (Kyoto, Japan). Mass spectra were measured using Shimadzu QP-2010 Plus (Kyoto, Japan). NMR spectra were recorded at 300 (\(^1\)H) and 75 MHz (\(^{13}\)C) on a Varian Mercury-300 instrument (Palo Alto, CA, USA). The NMR spectra were recorded in DMSO-\(d_6\), and chemical shifts were given in \(\delta\) (ppm) relative to TMS (Tetramethylsilane) as internal standard. HPLC analysis was carried out on Agilent Technologies 1100 series HPLC system (Agilent Technologies, Palo Alto, CA), equipped with a quaternary pump and degasser G1322A, series 1200. Agilent ChemStation software was used for data acquisition and processing.

**Plant material and preparation of the extracts and fractions**

The air-dried powdered aerial parts of *M. suaveolens* Ehrh. were prepared from the plant cultivated in the Experimental Station of Medicinal and Aromatic Plants, Pharmacognosy Department, Faculty of Pharmacy, Cairo University, Giza, Egypt. The plant was kindly authenticated by Dr. Gemma L. C. Bramley, Curator of the Lamiaceae collections, Herbarium Department, Library, Art & Archives, Royal Botanic Gardens, Kew, Richmond, Surrey, United Kingdom. Voucher specimen (M-20/313) was kept at the Herbarium of the Department of Pharmacognosy, Faculty of Pharmacy, Cairo University, Giza, Egypt.

The air-dried powdered aerial parts of *M. suaveolens* Ehrh. (1.5 kg) were exhaustively extracted with 90% ethanol by cold maceration. The total extract was evaporated under reduced pressure to yield a brownish green semi-solid residue (407 g). The residue (400 g) was suspended in water (800 mL) and successively subjected to liquid-liquid fractionation with \(n\)-hexane (8 x 400 mL), chloroform (10 \(\times\) 400 mL), ethyl acetate (8 x 400 mL) and \(n\)-butanol saturated with water (8 \(\times\) 400 mL). The solvents were evaporated under reduced pressure, yielding 14.0, 7.0, 11.4 and 54.0 g from the \(n\)-hexane, chloroform, ethyl acetate and \(n\)-butanol, respectively.
Animals
Adult male rats of Sprague-Dawley strain [130 - 150 g body weight (b.wt)] and Male albino mice, 25-30 g were obtained from the animal house of the National Research Centre, Giza, Egypt. They were kept under the same hygienic conditions and were fed by the basal diet recommended by the American Institute of Nutrition.[8] All experimental procedures were performed in accordance with internationally accepted principles for laboratory animal use and were approved by the Ethics Committee of the National Research Centre (No. 9-031).

Determination of median lethal dose (LD₅₀)
The LD₅₀ of the ethanolic extract was determined following Karber's procedure (1931).[9] Male albino mice, 25-30 g were divided into groups each of 6 animals. Preliminary experiments were carried out to determine the minimal oral dose that kill all the animals (LD₁₀₀) and the maximal oral dose that fail to kill any animal. Several doses at equal logarithmic intervals were chosen in between these two doses, each dose was injected in a group of 6 animals. The number of dead animals in each group, 24 hours after injection, was recorded and the LD₅₀ was calculated.

Hepatoprotective activity
Hepatoprotective activity of the ethanolic extract and its subfractions (n-hexane, choloform, ethyl acetate and n-butanol) was evaluated by measuring of Alanine Amino Transferase (ALT), Aspartate Amino Transferase (AST) and Alkaline Phosphatase (ALP) levels in blood of CCl₄ damaged liver in rats at a dose of 100 mg/kg.b.wt. The test samples were administered daily for 7 days before induction of liver damage by intraperitoneal injection (I.P) of 5 mL/kg of 25% carbon tetrachloride (CCl₄) in liquid paraffin according to the method described by Klassen and Plaa (1969),[10] using silymarin (25 mg/kg.b.wt.) as a reference drug. The test samples as well as the reference drug were further administered to the rats for another 7 days after liver damage. Blood samples were collected of each group at zero time, 7 days after receiving the test sample, 72 hours after induction of liver damage and 7 days after treatment with the test samples. The blood samples were allowed to clot, centrifuged at 1000 xg for 40 minutes and the separated sera were used for the estimation of the levels of AST, ALT and ALP.[11,12]

In vitro antioxidant activity
The free radical scavenging activities of the ethanolic extract and its four subfractions were assessed using 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay. The method used by Takao et
al., (1994)\(^{[13]}\) and modified by Delazar et al., (2004)\(^{[14]}\) was adopted. DPPH (4 mg) was dissolved in methanol (50 mL) to obtain a concentration of 80 \(\mu g/mL\). Serial dilutions of the ethanolic extract and its subfractions were prepared in methanol (20-400 \(\mu g/mL\)). Diluted solutions (1 mL each) were mixed with equal volumes of DPPH and allowed to stand for 30 min at room temperature. The control sample was prepared by mixing 1 mL of DPPH with 1 mL methanol. The absorbance was recorded at 517 nm. The experiment was performed in triplicate, and the average absorbance for each concentration was recorded. The same procedure was followed for ascorbic acid and silymarin which were used as positive controls. The IC\(_{50}\) was calculated as the concentration (\(\mu g/mL\)) of test sample that causes 50% quenching of the UV absorption of DPPH.

**In vivo antioxidant activity**

The *in vivo* antioxidant activity of the ethanolic extract and its four subfractions was evaluated and calculated by determination of glutathione (GSH) in blood of alloxan-induced diabetic rats adopting the method of Beutler et al., (1963)\(^{[15]}\) using vitamin E as a standard drug.

**Cytotoxicity assay**

Human colon carcinoma cell line (HCT116), larynx carcinoma cell line (HEP2) and breast carcinoma cell line (MCF7) and liver carcinoma cell line (HEPG2), obtained from the National Cancer Institute, Cairo, Egypt, were used for screening the cytotoxic activity using the Sulpho-Rhodamine-B (SRB) colorimetric cytotoxicity assay developed by Skehan et al., (1990).\(^{[16]}\)

The ethanolic extract, subfractions and compound 2 were tested at concentrations of 0, 5, 12.5, 25 and 50 \(\mu g/mL\) in DMSO. Doxorubicin was used as a positive control at the same concentrations. Cells treated with DMSO alone served as a negative (vehicle) control. Cytotoxicity was expressed as the percent of viable cells relative to cells. The relation between the surviving fraction and the extract concentration was plotted to get the survival curve for each tumor cell line after being treated with the specified extract. Moreover, the IC\(_{50}\) value (the concentration that reduces the survival of the cancer cells to 50%) was calculated for the extract, fractions and compound 2 as well as the reference drug doxorubicin.
Purification of the bioactive ethyl acetate fraction

A weighed amount (0.5 g) of the ethyl acetate fraction was suspended in 50 mL of 5% methanol/water and then subjected to fractionation by vacuum liquid chromatography (VLC) on a 23 g RP-18 column (4 cm L × 5 cm D). Gradient elution was performed starting with 5% methanol/water mixtures. The polarity was decreased by 1 % stepwise addition of methanol till 50% methanol/water was reached. Fractions (50 mL, each) were collected and monitored by HPLC using 15 to 95% v/v methanol / 0.3% O-phosphoric acid in water at a flow rate of 1.0 mL /min for twenty minutes. Fractions with similar HPLC chromatographic pattern were pooled, evaporated under reduced pressure to yield two main subfractions (A and B). Subfraction A (eluted with 10-11% methanol/water) yielded on concentration 30 mg of compound 1. Subfraction B (eluted with 12-22% methanol/water, 200 mg) was suspended in 5 mL of 10% methanol/water and then subjected to rechromatography by vacuum liquid chromatography (VLC) on RP-18 column (10 cm L × 1 cm D). Gradient elution was performed using 10% methanol/water mixtures. The polarity was decreased by 1 % stepwise addition of methanol till 25% methanol was reached. Fractions (5 mL, each) were collected and monitored by HPLC. Fractions (3-7), eluted with 12-16% methanol/water, with similar HPLC chromatographic pattern, were pooled, evaporated under reduced pressure gave 150 mg of compound 2.

Statistical analysis

The data obtained were presented as mean ± standard error and the significance of difference between test and control groups was statistically analyzed using student's t-test. P values of 0.05 or less was considered as criteria for significance.

Chromatographic conditions for HPLC analysis

Separation was carried out on a Lichrosphere 100 RP-C18 column (250 mm L x 4 mm ID, 5 μm, Merck, Darmstadt, Germany), preceded by a C18 guard column (10 mm L x 4 mm ID, 5 μm). Column temperature was set at 25ºC. The injection volume and UV wavelength were set at 20 μL and 325 nm, respectively. Gradient elution was carried out using 15 to 95% v/v methanol/0.3% O-phosphoric acid in water at a flow rate of 1 mL/min for twenty minutes.

Sample preparation

From powdered aerial parts

Powdered aerial parts of M. suaveolens Ehrh. (2 g) were extracted with methanol (3 x 5 mL) in a stoppered conical flask (50 mL capacity) by sonication (3 x 5 min). The extract was
filtered into a volumetric flask (25 mL capacity) and the volume was adjusted with methanol. An aliquot (2 mL) was transferred to a stoppered conical flask (50 mL capacity) and evaporated under reduced pressure. The residue was dissolved in 5 mL of 30% methanol / water. The obtained solution was purified using solid phase extraction (SPE) cartridges (LiChrolut RP-18 column, Merck, Darmstadt, Germany), which was activated and pre-conditioned using methanol (3 x 1 mL), followed by distilled water (3 x 1 mL) and finally, 30% methanol/water (3 x 1 mL). Purification was then carried out by applying the dissolved extract onto the SPE cartridge, followed by elution with 30% methanol/water (3 X 5 mL). The volume of the eluate was completed to 25 mL in a volumetric flask. An aliquot (5 mL) of the purified solution was filtered through 0.45 µm GHP Acrodisc filter and 20 µL of the filtrate was injected into the HPLC system.

To confirm the suitability of the method of sample preparation, the column was washed successively with 50% and 75% methanol/water followed by methanol (5 mL, each) and the eluate, in each case, was checked for absence of rosmarinic acid by HPLC analysis.

From ethyl acetate fraction
Ethyl acetate fraction (5 mg) was dissolved in 25 mL methanol and this yielded a working solution (conc. 0.2 mg/mL). An aliquot (5 mL) of this solution was filtered through 0.45 µm GHP Acrodisc filter and 20 µL of the filtrate was injected into the HPLC system.

Construction of the standard calibration curve
Serial dilutions of compound 2 were prepared from a stock solution having a final concentration of 0.5 mg/mL, formed by weighing accurately 5 mg in a 10 mL volumetric flask and dissolving in methanol. A standard calibration curve was established using the different concentrations prepared (125, 62.5, 31.25, 15.625, 7.812 and 3.906 µg/mL). Each sample was injected in triplicates.

Validation of the RP-HPLC method
This proposed method was subjected to rudimentary validation studies according to the guidelines of the International Conference of Harmonization (ICH, 2005)\textsuperscript{17} and the United States Pharmacopoeia (USP, 2009).\textsuperscript{18} Prior to the analysis, the method was subjected to system suitability tests (SST) from typical chromatograms. It was determined by using five replicate injections of standard solution (conc. 3.906 µg/mL). The percentage relative
standard deviation (RSD), peak asymmetry factor, resolution and theoretical plates were calculated.

Linearity was determined by injecting five different concentrations of compound 2 standard solution (3.906 - 125 µg/mL). The accuracy was calculated as the percent recovery of spiked ethyl acetate fraction samples with compound 2 sample at a concentration of 110 µg/mL. Intra-day variability was estimated by injecting several concentrations of the standard solution in three replicates, for each, during a single day. Inter-day variability was determined by analyzing, in triplicate, the same solutions employed in the repeatability test on two consecutive days. Precision was expressed in both cases in terms of relative standard deviation (RSD). Stability of the ethyl acetate and standard solutions was tested at 0, 6, 36 and 96 hours. The solutions were kept at 4 °C before analysis.

Robustness of the method was determined by changing the flow rate from 1 mL/min to 0.9 and 1.1 mL/min and % RSD was calculated. Two different analysts carried out the analysis of the ethyl acetate fraction in three consecutive days to assess the ruggedness of the method. The sample solution was kept at 4 °C and its stability was tested at 0, 7 and 44 hr. Limit of quantification (LOQ) and limit of detection (LOD) were determined based on the standard deviation of the response (σ) and the slope of the calibration curve (S) following the International Conference on Harmonization (ICH) guidelines (ICH Topic Q2B 1996); LQ = 10 (σ/S), LD = 3.3 (σ/S).

RESULTS

Determination of median lethal dose (LD₅₀)

The ethanolic extracts is safe and non-toxic under the experimental condition with LD₅₀ up to 5 g/kg body weight. Thus it is considered to be safe in the range of the administered doses.[19]

Hepatoprotective activity

On comparing the increase in liver enzymes in the control group at 72 hours after induction of liver damage (by 25% CCl₄) with that of the treated groups, (Table 1), it was observed that the ethanolic extract prevented the increase in the level of AST enzyme by 65.1% followed by the ethyl acetate fraction (51.6%). This protective effect (especially the effect of the ethanolic extract) was comparable to that of silymarin which prevented the rise in the level of AST by 64.1%. The ethyl acetate fraction and ethanolic extract prevented the increase in the level of ALT enzyme by 57.0% and 51.6%, respectively as compared to silymarin (60.9%).
The ethanolic extract prevented the increase in the level of ALP enzyme by 64.4% followed by the ethyl acetate fraction (56.7%) as compared to silymarin (76%). This indicated that these extracts have hepatoprotective or antihepatotoxic activities.

**In vitro antioxidant activity**

The results of *in vitro* antioxidant activity showed that the ethyl acetate fraction had the highest free radical scavenging activity (IC$_{50}$=31 µg/mL) followed by the chloroform fraction and the ethanolic extract (IC$_{50}$=39 and 72 µg/mL, respectively). This activity was relative to silymarin (IC$_{50}$= 45 µg/mL), but less than that shown by ascorbic acid (IC$_{50}$=7.5 µg/mL). n-Hexane and n-butanol fractions were less active (IC$_{50}$=100 and 148 µg/mL, respectively).

**In vivo antioxidant activity**

According to the results shown in Table 2, the ethanolic extract and its ethyl acetate subfraction were the most active (98.6% and 98.0%, respectively relative to vitamin E) followed by the n-butanol subfraction with potency 91.1%. Choloform and n-hexane fractions were the least active (80.2% and 76%, respectively).

**Cytotoxic activity**

The ethanolic extract of *M. suaveolens* showed a significant cytotoxic activity (Table 3) on liver carcinoma cell line (HEPG2) and larynx cancer cell line (HEP2) (IC$_{50}$ = 7.28 and 7.35 µg/mL, respectively) compared to doxorubicin (IC$_{50}$ = 3.73 µg/mL for both). Consequently, the four subfractions of the ethanolic extract were also tested for their cytotoxic activity. The ethyl acetate fraction showed the highest cytotoxic activity (IC$_{50}$ = 5.1 µg/mL) against HEPG2. The chloroform fraction (IC$_{50}$ = 14.40 µg/mL) was the most potent on the colon carcinoma cell line (HCT116) compared to doxorubicin (IC$_{50}$ = 3.73 µg/mL) while n-hexane was the most active regarding the breast carcinoma cell line (MCF7) (IC$_{50}$ = 13.5 µg/mL) compared to doxorubicin (IC$_{50}$ = 2.97 µg/mL).

**Purification of the bioactive ethyl acetate fraction**

Purification of the ethyl acetate fraction yielded two compounds:

**Caffeic acid (1):** Pale buff powder, $R_f$ value (0.75 in Ethyl acetate-Methanol-Water-Formic acid 100:16.5:13.5:0.2 v/v), Color of the spot in UV (blue florescence) and in NP-PEG/UV (blue florescence).
IR (KBr) cm⁻¹: 3500, 2930, 2361, 1700, 1391, 1609, 1051 and 531 UV (nm): 227, 289 sh, 328. ¹H-NMR: δ (300 MHz, DMSO) 6.10 (1H, d, J=16.05 Hz, H-8), 6.77 (1H, d, J=8.5 Hz, H-5), 6.82 (1H, dd, J=2.1 Hz, H-6), 7.06 (1H, br.s, H-2), 7.26 (1H, d, J=16.05 Hz, H-7). ¹³C-NMR: δ (75 MHz, DMSO): 114.99(C-2’), 115.22(C-8’), 116.32(C-5’), 122.88(C-6’), 127.64(C-1’), 146.93(C-3’), 147.22(C-7’), 149.44(C-4’), 168.52(C-9’).

Rosmarinic acid (2): White powder, Rₚ value (0.87 in Ethyl acetate-Methanol-Water-Formic acid 100:16.5:13.5:0.2 v/v), Color of the spot in UV (sky blue) and in NP-PEG/UV (Green florescence).

EI-MS (70 ev) m/z (rel. int.): 360 (9.79%), 342 (60.8%), 198 (100%), 180 (19.73%), 123 (88.6%), 77 (22.52%).

IR (KBr) cm⁻¹: 3300, 2927, 1700, 1603

UV (nm): 330, 294 sh.

¹H-NMR: δ (300 MHz, DMSO) 2.88 (1H, dd, J=13.8, 8.8 Hz, H-7’a), 2.99 (1H, dd, J=13.8, 8.8, H-7’b), 4.99 (1H, d, J=4.8, H-8’), 6.21 (1H, d, J=16.2 Hz, H-8), 6.51 (1H, d, J=7.5, H-5’), 6.62 (dd, 1H, J=7.2, 2.1 Hz, H-6’), 6.69 (br.s, 1H, H-2’), δ 6.76 (d, 1H, J=7.8 HZ, H-5), 6.98 (1H, dd, J=7.2, 2.1 Hz, H-6) and 7.06 (1H, br.s, H-2), 7.42 (1H, d, J=16.2 Hz, H-7).

¹³C-NMR: δ (75 MHz, DMSO) 36.2 (C-7’), 73.4 (C-8’), 115.2 (C-5’), 115.7 (C-8), 116.5 (C-2), 119.8 (C-5.2’), 125.2 (C-6.6’), 127.8 (C-1.1’), 143.7 (C-3), 144.8 (C-4), 145.4 (C-3’), 145.5 (C-4’), 148.5 (C-7), 165.9 (C-9) and 171.0 (C-9’).

The structures of the isolated compounds are shown in Figure 1.

Method Development for HPLC analysis
Selection of suitable mobile phase
Gradient elution using 15 to 95% v/v methanol / 0.3% orthophosphoric acid in water at a flow rate of 1 mL/min for twenty minutes gave a rapid and satisfactory separation. A typical HPLC chromatogram illustrating the ethyl acetate profile of M. suaveolens Ehrh., obtained from 20 µl injection under the optimized chromatographic conditions, is displayed in Figure 2.

Qualitative analysis of the ethyl acetate extract
The method developed for HPLC fingerprinting provided a quick analysis of the ethyl acetate fraction. The identity of the main peak in the ethyl acetate fraction was confirmed by spiking
the sample with the standard rosmarinic acid and observing the retention time then determining the significant increase in the peak area.

**Method Validation**

Subjecting the method to system suitability tests (SST) revealed that the column efficiency was 3003 equivalent to theoretical plates. The instrument precision, determined by five replicate injections of the standard solution, exhibited a maximum RSD of 1.093%. The peak asymmetry factor was not more than 1.07. Finally, the resolution between the major peak was about 9.01. For the specificity, no interference was observed between the main peak and other peaks. All parameters were satisfactory with a good specificity for the method. The linearity of the standard curve was evaluated by analysis of six standard working solutions containing 3.906 – 125 µg/mL rosmarinic acid (Fig. 3). Peak area and concentrations were subjected to least square linear regression analysis to calculate the calibration equation and correlation coefficient. Linearity was obtained over this range with regression equation: \( y = 21.35x - 15.14 \) and a correlation coefficient \( (R^2) \) of 0.999, where \( x \) refers to the concentration of the reference compound (µg/mL) and \( y \) is the peak area (mAU). Precision, estimated through repeatability (intra-day variability), gave RSD not more than 1.008 %, while intermediate precision (inter-day variability) showed RSD not more than 0.6175%. The mean absolute recovery using the described method was 100.5%. The range of concentration used was 125-3.906 µg/mL, since the method has been shown to be precise, accurate and linear within this region. The LOD of rosmarinic acid was found to be 0.350 µg/mL. It was found that a 1.061 µg/mL solution of rosmarinic acid yielded a S/N ratio of 10 and a %RSD of 2.756 and was considered the LOQ. The ethyl acetate and standard solutions were stable showing not more than 1.30 % RSD. The chromatographic elution pattern remained unaffected by making deliberate minor variation in the flow rate employed and low values of RSD (0.575%) established the robustness of the method. The %RSD calculated after analysis of aliquots from different concentrations by different analyst was 1.015 and this proved the ruggedness. Criteria for validation are shown in Table 4.

Rosmarinic acid content in *M. suaveolens* Ehrh. was quantified and the concentration, calculated based on calibration curve, was found to be 1.155 and 50.165% w/w for the powered aerial parts and ethyl acetate fraction, respectively.
Table 1. The effect of *M. suaveolens* Ehrh. ethanolic extract and its fractions on AST, ALT and ALP levels of liver damaged rats.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Time</th>
<th>Level</th>
<th>Control</th>
<th>Ethanol extract</th>
<th>n-Hexane fraction</th>
<th>Cholform fraction</th>
<th>Ethyl acetate fraction</th>
<th>n-Butanol fraction</th>
<th>Silymarin</th>
</tr>
</thead>
<tbody>
<tr>
<td>AST (u/L)</td>
<td>Zero¹</td>
<td>Mean± S.E.³</td>
<td>29.4± 0.9</td>
<td>32.6± 0.9</td>
<td>34.3± 1.2</td>
<td>31.8± 1.1</td>
<td>31.9± 1.1</td>
<td>33.6± 1.5</td>
<td>32.3± 1.1</td>
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<td></td>
<td>7 days¹</td>
<td>Mean± S.E.³</td>
<td>28.6± 0.4</td>
<td>32.1± 1.2</td>
<td>34.1± 0.9</td>
<td>32.2± 1.1</td>
<td>31.2± 0.8</td>
<td>31.2± 1.1</td>
<td>30.6± 0.9</td>
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<tr>
<td></td>
<td>72 hours²</td>
<td>Mean± S.E.³</td>
<td>136.9± 5.1</td>
<td>47.8± 2.3</td>
<td>114.2± 4.8</td>
<td>98.6± 4.1</td>
<td>66.3± 2.4</td>
<td>68.9± 2.3</td>
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<tr>
<td></td>
<td>% of change⁴</td>
<td>0</td>
<td>65.1</td>
<td>16.6</td>
<td>28</td>
<td>51.6</td>
<td>49.7</td>
<td>64.1</td>
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<tr>
<td></td>
<td>7 days²</td>
<td>Mean± S.E.³</td>
<td>151.7± 5.9*</td>
<td>46.3± 2.4*</td>
<td>96.3± 3.2*</td>
<td>89.4± 3.6*</td>
<td>51.6± 2.1*</td>
<td>59.2± 2.1*</td>
<td>29.7± 0.6*</td>
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<tr>
<td>ALT (u/L)</td>
<td>Zero¹</td>
<td>Mean± S.E.³</td>
<td>31.6± 1.1</td>
<td>34.2± 1.1</td>
<td>28.9± 0.7</td>
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<td>29.8± 0.7</td>
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<tr>
<td></td>
<td>7 days¹</td>
<td>Mean± S.E.³</td>
<td>30.9± 0.7</td>
<td>32.8± 1.4</td>
<td>29.2± 0.8</td>
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<td>26.8± 0.4</td>
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<td></td>
<td>% of change⁴</td>
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<td>51.6</td>
<td>18.8</td>
<td>36.3</td>
<td>57.0</td>
<td>49.8</td>
<td>60.9</td>
<td></td>
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<tr>
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<td>7 days²</td>
<td>Mean± S.E.³</td>
<td>149.2± 5.7*</td>
<td>44.2± 1.8*</td>
<td>98.2± 4.8*</td>
<td>87.4± 4.1*</td>
<td>55.3± 2.4*</td>
<td>53.9± 2.1*</td>
<td>29.2± 0.8*</td>
</tr>
<tr>
<td>ALP (KAU)</td>
<td>Zero¹</td>
<td>Mean± S.E.³</td>
<td>6.8± 0.1</td>
<td>7.2± 0.1</td>
<td>7.6± 0.1</td>
<td>7.1± 0.1</td>
<td>7.4± 0.1</td>
<td>7.3± 0.1</td>
<td>7.3± 0.1</td>
</tr>
<tr>
<td></td>
<td>7 days¹</td>
<td>Mean± S.E.³</td>
<td>7.1± 0.1</td>
<td>7.1± 0.1</td>
<td>7.5± 0.1</td>
<td>7.2± 0.1</td>
<td>7.2± 0.1</td>
<td>7.1± 0.1</td>
<td>6.9± 0.1</td>
</tr>
<tr>
<td></td>
<td>72 hours²</td>
<td>Mean± S.E.³</td>
<td>67.9± 1.8</td>
<td>24.2± 0.4</td>
<td>59.8± 1.9</td>
<td>61.4± 1.9</td>
<td>29.4± 0.7</td>
<td>33.4± 0.7</td>
<td>16.3± 0.6</td>
</tr>
<tr>
<td></td>
<td>% of change⁴</td>
<td>0</td>
<td>64.4</td>
<td>11.9</td>
<td>9.6</td>
<td>56.7</td>
<td>50.8</td>
<td>76</td>
<td></td>
</tr>
<tr>
<td></td>
<td>7 days²</td>
<td>Mean± S.E.³</td>
<td>73.4± 2.3*</td>
<td>18.8± 0.6*</td>
<td>54.2± 1.7*</td>
<td>52.3± 1.6*</td>
<td>26.6± 0.3*</td>
<td>29.4± 0.4*</td>
<td>6.9± 0.1*</td>
</tr>
</tbody>
</table>

Statistically significant difference from the control group at P< 0.01, n=6.

1: Enzyme level before induction of liver damage; 2: Enzyme level after induction of liver damage; 3: Enzyme level expressed as Mean ± S.E.;
4: % of change is calculated as regards to the control group; * Statistically significant from the control group at p< 0.01; † Statistically significant from 72 hours after injection of carbon tetrachloride at p<0.01. KAU, King-Armstrong unit, n=6.
Table 2. *In vivo* study of the antioxidant activity of *M. suaveolens* Ehrh. ethanolic extract and its fractions.

<table>
<thead>
<tr>
<th>Animal group</th>
<th>Dose</th>
<th>Blood glutathione (mg%)(Mean ± S.E.)</th>
<th>% of inhibition as compared to the control</th>
<th>% of potency as compared to the standard</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control non-diabetic</td>
<td>1 ml saline</td>
<td>36.2 ± 1.4</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Diabetic untreated</td>
<td>1 ml saline</td>
<td>21.8 ± 0.6*</td>
<td>39.8</td>
<td>0</td>
</tr>
<tr>
<td>Diabetic + Ethanolic extract</td>
<td>100</td>
<td>35.3 ± 1.2</td>
<td>2.5</td>
<td>98.6</td>
</tr>
<tr>
<td>Diabetic + n-Hexane fraction</td>
<td>100</td>
<td>27.2 ± 0.8*</td>
<td>24.9</td>
<td>76.0</td>
</tr>
<tr>
<td>Diabetic + Choloform fraction</td>
<td>100</td>
<td>28.7 ± 0.6*</td>
<td>20.7</td>
<td>80.2</td>
</tr>
<tr>
<td>Diabetic + Ethyl acetate</td>
<td>100</td>
<td>35.1 ± 0.9</td>
<td>3.0</td>
<td>98.0</td>
</tr>
<tr>
<td>Diabetic + n-Butanol fraction</td>
<td>100</td>
<td>32.9 ± 1.3</td>
<td>9.1</td>
<td>91.1</td>
</tr>
<tr>
<td>Diabetic + Vitamin E</td>
<td>7.5</td>
<td>35.8 ± 1.1</td>
<td>1.1</td>
<td>100</td>
</tr>
</tbody>
</table>

Table 3. IC$_{50}$ of *M. suaveolens* Ehrh. ethanolic extract and its fractions on the different cell lines.

<table>
<thead>
<tr>
<th>Extract</th>
<th>IC$_{50}$ (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HEPG2</td>
</tr>
<tr>
<td>Ethanolic extract</td>
<td>7.28</td>
</tr>
<tr>
<td>n-Hexane fraction</td>
<td>13.70</td>
</tr>
<tr>
<td>Choloformic fraction</td>
<td>18.80</td>
</tr>
<tr>
<td>Ethyl acetate fraction</td>
<td>5.10</td>
</tr>
<tr>
<td>n-Butanol fraction</td>
<td>16.40</td>
</tr>
<tr>
<td>Doxorubicin</td>
<td>3.73</td>
</tr>
</tbody>
</table>

Table 4. Criteria for validation of HPLC-based analytical procedure

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Acceptance criteria</th>
<th>Result obtained</th>
</tr>
</thead>
<tbody>
<tr>
<td>% RSD of standard</td>
<td>&lt; 2</td>
<td>1.093</td>
</tr>
<tr>
<td>Peak asymmetry factor</td>
<td>&lt; 2</td>
<td>1.07</td>
</tr>
<tr>
<td>Theoretical plates</td>
<td>&gt; 1800</td>
<td>3003</td>
</tr>
<tr>
<td>Resolution</td>
<td>&gt; 2</td>
<td>9.01</td>
</tr>
<tr>
<td>Specificity</td>
<td>No interference with other peaks</td>
<td>Passes</td>
</tr>
<tr>
<td>Linearity</td>
<td>$R^2 = 0.995$ to 1.0</td>
<td>0.999</td>
</tr>
<tr>
<td>Repeatability</td>
<td>RSD &lt; 2%</td>
<td>1.008</td>
</tr>
<tr>
<td>Intermediate precision</td>
<td>RSD &lt; 2%</td>
<td>0.6175</td>
</tr>
<tr>
<td>Accuracy</td>
<td>Recovery: 98-102%</td>
<td>100.5%</td>
</tr>
<tr>
<td>Limit of detection (LOD)</td>
<td>Conc. with a signal-to-noise (S/N) ratio of 3:1</td>
<td>0.35 µg/ml</td>
</tr>
</tbody>
</table>
Limit of quantitation (LOQ) | Conc. with a signal-to-noise (S/N) ratio of 10:1 | 1.061 µg/ml  
---|---|---  
Stability of solutions | RSD < 20% | 1.30  
Robustness (Flow rate) | RSD < 3% | 0.575  
Ruggedness | RSD < 2% | 1.015  
RSD, relative standard deviation

Fig. 1. Structures of the isolated compounds

**Fig. 2.** HPLC profile of the ethyl acetate fraction under final optimized conditions.

**Fig. 3.** Calibration curve for Rosmarinic Acid using the proposed quantitative HPLC method.
DISCUSSION

Increased lipid peroxidation induced by free radical derivatives is one of the main factors involved in CCl₄-intoxication. As a result of this oxidative damage to the liver a marked increase in the serum transaminase levels are observed. One of the principal cellular defense molecules is glutathione (GSH). GSH in the liver is involved in many cellular processes including the detoxification of endogenous and exogenous compounds.¹²⁰ Thus, in order to evaluate the antihepatotoxic activity in the present study, effects of the test samples on elevated serum levels of hepatospecific enzymes, AST, ALT and ALP, in CCl₄-damaged liver, in addition to their ability to restore glutathione levels and their free radical scavenging activity were evaluated. Moreover, the cytotoxic activity of the extracts were also evaluated. The rise in the serum level of liver enzymes (AST, ALT and ALP) due to CCl₄-induced liver damage was significantly reduced in the experimental animals pre-treated (for 7 days) with the ethanol extract and its ethyl acetate and n-butanol subfractions, this proved the hepatoprotective action of these extracts. Furthermore, administration of the ethanol extract and ethyl acetate fraction for another 7 days after induction of liver damage led to a significant decrease in the enzyme levels regarding their respective normal values which indicated stabilization of the hepatocyte cell membrane as well as repairing of hepatic tissue damage caused by CCl₄.¹²¹ This also suggested the possible hepatocurative activity of the ethanol extract and its ethyl acetate subfraction. The mechanism by which the ethyl acetate subfraction exerts its hepatoprotective activity may be through free radical scavenging activity (IC₅₀=31μg/mL in DPPH assay) or through its potent ability to restore the reduced
glutathione levels (98.0 %). Glutathione is the principal cellular defense molecule capable of protecting the cells from the toxic effects of free radicals.

As established by the American National Cancer Institute (NCI), a crude extract is said to exert a cytotoxic effect when its IC$_{50}$ is less than 30 µg/mL when preliminary assayed.$^{[22]}$ In this respect, the preliminary screening of the ethanolic extract of $M$. suaveolens showed a significant cytotoxic activity on liver and larynx carcinoma cell lines when compared to doxorubicin. Consequently, the four subfractions of the ethanolic extract were also tested for their cytotoxic activity. The ethyl acetate fraction showed the highest activity against liver cancer cell line, while the n-hexane, choloform and n-butanol fractions were less active.

The investigation of the ethyl acetate fraction showed the presence of one minor compound together with a major compound, these compounds were isolated and identified as caffeic acid (1) and rosmarinic acid (2), respectively. The identification of the two compounds depends on their UV, IR, MS, $^1$H and $^{13}$C NMR spectra and comparison with the literature data.$^{[23-25]}$ To the best of our knowledge, this is the first report on the isolation of these compounds from $M$. suaveolens Ehrh.

The cytotoxic activity of rosmarinic acid was evaluated against HEPG2 against which the parent ethyl acetate fraction demonstrated its highest activity. Rosmarinic acid proved high cytotoxic activity (IC$_{50}$ = 4.5 µg/mL) as regard to doxorubicin (IC$_{50}$ = 3.73 µg/mL) on HEPG2 (Fig. 2). Rosmarinic acid was also reported to possesses antioxidant activities.$^{[26]}$

These properties directed us to choose rosmarinic acid as a marker for standardization of the bioactive ethyl acetate fraction.

To obtain the best overall chromatographic conditions, the mobile phase was optimized by examining the effect of organic modifier (acetonitrile and methanol). Methanol was suitable for optimal resolution between peaks, rather than acetonitrile. Consequently, methanol was used for further method development. Choice of the proper gradient elution was determined through trial and error experiments to obtain optimal parameters. Gradient elution using 15 to 95% v/v methanol / 0.3% orthophosphoric acid in water gave a feasible, reproducible HPLC fingerprint of the ethyl acetate fraction, with a relatively short analysis time, allowed the authentication and identification of the sample.

A method for the detection was developed using reversed-phase HPLC with an UV detector. The results of analysis were calculated by statistical method and guided by ICH guidelines.
The method satisfied all the criteria required for validation of the HPLC-based analytical procedure.

The extraction method for rosmarinic acid from the powdered aerial parts was optimized by HPLC analysis of the extract. The residue did not contain rosmarinic acid, indicating the extraction was complete. For quantitative analysis, the conditions were optimized using trial and error experiments to achieve a simple and accurate method.

CONCLUSION
Here we report the hepatoprotective, antioxidant and cytotoxic activity of the ethanolic extract of *M. suaveolens* Ehrh. cultivated in Egypt as well as its ethyl acetate subfraction. In addition, a simple, precise, accurate, reproducible, selective, robust, stability-indicating, linear and time saving RP-HPLC analytical method for the characterization and determination of rosmarinic acid from the active ethyl acetate fraction of the ethanolic extract was described.

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Conflict of interest
The authors declare no conflict of interest

REFERENCES


