ORIGINAL ARTICLE

Cytotoxicity and antioxidant activity of new biologically active constituents from *Micromeria nervosa* grown in Egypt

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KEYWORDS
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Abstract  Chromatographic fractionation of the acetone extract of *Micromeria nervosa*, resulted in the isolation and identification of new natural furanosesquiterpene alcohol; micromeriol (1), a known sterol; β-sitosterol (2), a new natural 5-β-cholestane type; nervosane (3), two known triterpenic acids; oleanolic acid (4), and ursolic acid (5). The cytotoxicity and the antioxidant activities of the acetone extract and the isolated pure compounds (1–5) were determined.

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1. Introduction

Plants of genus *Micromeria* are well known due to a wide range of biological activities in folk medicine, such as antibacterial, heart disorders, headache, wounds, skin infections and the most usage of *Micromeria* species is in colds.1-3

*Micromeria nervosa* (Desf.) Benth. is a wild plant belonging to the family Lamiaceae, which are widespread in the Mediterranean region. The name of the genus was derived from Greek words *micro* and *meros* (meaning small and part) because they were found as dwarf fragrant of perennial herbs which usually grow in clefts of fissured rocky ground while *nervosa* with prominent nerves or veins. *Micromeria* genus was grown naturally in Egypt and in the eastern Mediterranean region.4,5

Previous research has established the genus *Micromeria* to be a rich source of polyphenolic compounds. Reviewing the current literature, nothing was reported concerning the chemical investigation of the studied plant excepting few reports concerning the occurrence of flavonoidal glycosides and volatile oil.

The present paper describes the isolation, structure elucidation and biological study of five compounds.

2. Experimental

2.1. Materials and methods

2.1.1. Plant material

The aerial parts of *M. nervosa* (2 kg) were collected from omrahams valley, Matrouh, Egypt. The plants were kindly identified by Dr. Nahaid El-Asinay, Assistant Professor of Plant Taxonomy, Faculty of Science, Cairo University. Corresponding specimens were deposited at the herbarium of the pharmacognosy department, Faculty of Pharmacy, Al-Azhar University (MV2005).
2.1.2. General procedures

The $^1$H and $^{13}$C NMR, spectra were recorded on a Bruker NM 360 spectrometer operating at 500 for $^1$H and 125 MHz for $^{13}$C NMR. All spectra were obtained in DMSO-$d_6$ and CD$_3$OD using TMS as internal standard, with the chemical shifts expressed in δ (ppm) and the coupling constants (J) in Hertz. ESI-MS analyses were measured on a TSQ700 triple quadrupole instrument (Finnigan, San Jose, CA, USA) by using the Finnigan electrospray atmospheric pressure chemical ionization source. A duo-UV lamp (254/365 nm), Desaga, Heidelberg, Germany was used to find the location of the spots in TLC and column chromatography. UV-spectra were recorded by Hitachi 340 spectrophotometer, Japan. Melting point was obtained on a Stuart SMP3 apparatus. IR spectra were carried out on a Nicolet 205 FT IR spectrometer connected to a Hewlett-Packard Color Pro. Plotter. Column chromatography was carried out on various adsorbents including silica gel 40–60 μm (E. Merck, Darmstadt, Germany) and Sephadex LH-20 (Pharmacia, Uppsala, Sweden). Thin layer chromatography (TLC) was performed on pre-coated silica gel F254 plates (E. Merck, Darmstadt, Germany). The pure compounds were visualized by spraying 1% vanillin-H$_2$SO$_4$, followed by heating at 100 °C for 5 min.

2.1.3. Extraction and isolation

The air-dried powdered aerial parts of the plant (2 kg) were subjected to extraction with acetone (82 g). The acetone extract was chromatographed on silica gel column, eluted with solvent systems of n-hexane, n-hexane: EtOAc (100:00–70:30), to give seven fractions (A–G). Fraction E (4 g) was rechromatographed on a silica gel flash column (Petroleum ether: EtOAc (90:10–75:25) and Sepralyte C18 flash column (H$_2$O: MeOH$^-$ 50:50–90:10) to afford compounds 4 (10 mg). Fr. G2 (62 mg) was subjected to silica gel column (Petroleum ether: EtOAc (85:15–75:25), silica gel flash column using n-hexane: EtOAc (90:10–75:25) and Sepralyte C18 flash column (H$_2$O: MeOH$^-$ 50:50–90:10) to afford compounds 5 (35 mg). All separated compounds were purified by Sephadex LH-20 column eluted with MeOH.

2.1.4. Material used for biological study

2.1.4.1. Acetone extracts. Air-dried aerial parts of *M. nervosa* (Desf.) Benth. (20 g), were extracted separately at room temp with acetone (3 × 100 ml). The acetone soluble portion was evaporated under *vacuo* (130 mg).

2.1.4.2. Isolated compounds. 2 mg of each compound (1–5) was used in each method.

2.1.4.3. Well known antioxidant D,L-α-tocopherol and butylated hydroxytoluene (BHT) were used as reference standard. BHT has been added to foodstuffs but, because of toxicity issues, their use is being questioned.

2.1.4.4. Scavenging activities of superoxide radicals. Measurement of superoxide anion scavenging activity of *M. nervosa* acetone extract and isolated compounds

2.1.5. In vitro assays antioxidant activity of *M. nervosa* acetone extract and isolated compounds

2.1.5.1. 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay of antioxidant activity. DPPH assay was performed following the procedures of Hosny M. and Rosazza J. The lower the EC$_{50}$, the greater the antioxidant activity of the substance.

2.1.5.2. Ferrous sulfate–H$_2$O$_2$ stimulated lipid peroxidation in rat tissue homogenate. The effect of acetone extract and isolated compounds on rat tissue (brain, heart and liver) homogenate induced by ferrous sulfate–H$_2$O$_2$ and lipid peroxidation was determined by malondialdehyde (MDA)-TBA adduct according to the method described by Hosny et al.

2.1.5.3. Xanthine oxidase-induced generation of superoxide radical. The influence of acetone extract and isolated compounds was measured according to the method described by Luis Gongora et al. IC$_{50}$ values were calculated by linear regression analysis, and kinetic analysis of inhibition was determined only for those compounds with an IC$_{50}$ lower than 50 μM (see Fig. 1).

2.1.5.4. Scavenging activities of superoxide radicals. Measurement of superoxide anion scavenging activity of *M. nervosa*...
acetone extract and isolated compounds (1–5) was based on the method described by Liu et al.15

2.1.5.5. Nitric oxide radical inhibition assay. Sodium nitroprusside in aqueous solution at physiological pH spontaneously generates nitric oxide, which interacts with oxygen to produce nitrite ions, which can be estimated by the use of Griess Illosvoy reaction.16

The 50% inhibition (IC₅₀) of nitric oxide generation was estimated by comparing the absorbance values of control with those of extract or isolated compounds. d,l-α-tocopherol and Butylated hydroxytoluene (BHT), were used as positive controls (Table 1, Fig. 2).

2.1.6. Cytotoxicity assay of M. nervosa acetone extract and isolated compounds (1–5) against liver, leukemia, colon, urinary bladder, stomach, ovary, and uterus tumor cell lines

Cytotoxic activities of acetone extract of M. nervosa and isolated compounds (1–5) were tested on human liver hepatocellular carcinoma (SNU-398, Hep G2), Human Leukemia carcinoma (CCRF-CEM, HL-60 TB), Colon cancer adenocarcinoma (COLO 205, HCT-116), Urinary bladder Carcinoma

<table>
<thead>
<tr>
<th>Bioassay</th>
<th>DPPH% decoloration sample 100 µg/ml</th>
<th>Xanthine oxidase* IC₅₀ (µM)</th>
<th>PMS-NADH system inhibition (%) sample 100 µg/ml</th>
<th>Nitric oxide radical inhibition (%) sample 100 µg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetone soluble</td>
<td>68.5 ± 2.80</td>
<td>115 ± 4.18</td>
<td>70.8 ± 2.55</td>
<td>56.5 ± 2.30</td>
</tr>
<tr>
<td>M. nervosa</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Compound 1</td>
<td>55.3 ± 2.20</td>
<td>35 ± 1.15</td>
<td>67.4 ± 2.52</td>
<td>37.4 ± 1.48</td>
</tr>
<tr>
<td>Compound 2</td>
<td>30.5 ± 1.10</td>
<td>46 ± 2.28</td>
<td>10.5 ± 1.10</td>
<td>9.5 ± 0.05</td>
</tr>
<tr>
<td>Compound 3</td>
<td>48.8 ± 1.50</td>
<td>70 ± 2.73</td>
<td>24.5 ± 1.30</td>
<td>23.2 ± 1.12</td>
</tr>
<tr>
<td>Compound 4</td>
<td>31.7 ± 1.10</td>
<td>65 ± 2.39</td>
<td>17.9 ± 1.18</td>
<td>16.8 ± 1.10</td>
</tr>
<tr>
<td>Compound 5</td>
<td>35.0 ± 1.15</td>
<td>60 ± 2.25</td>
<td>13.1 ± 1.02</td>
<td>19.3 ± 1.12</td>
</tr>
<tr>
<td>d,l-α-tocopherol</td>
<td>62.8 ± 2.70</td>
<td>75 ± 2.84</td>
<td>68.5 ± 2.70</td>
<td>52.3 ± 2.25</td>
</tr>
<tr>
<td>BHT</td>
<td>50.6 ± 1.55</td>
<td>125 ± 4.60</td>
<td>42.3 ± 1.40</td>
<td>56.7 ± 27</td>
</tr>
<tr>
<td>Allopurinol</td>
<td>–</td>
<td>15 ± 0.20</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

Values are presented as mean ± SE of 3-test sample observation. P < 0.05 for all values.
* Uric acid production for controls was 58.0 ± 1.9 nmol/min.

Figure 2 Effects of Micromeria nervosa acetone extract and isolated compounds (1–5) and positive controls on the in vitro free radical generation.
(HT-1376), Urinary bladder transitional cell carcinoma (UM-UC-3), stomach gastric carcinoma (MKN-28, NCI-N87), ovary adenocarcinoma (NIH:OVCAR-3, SK-OV-3) and uterus uterine sarcoma (MES-SA, MES-SA/MX2) cell lines.\textsuperscript{17}

The ED\textsubscript{50} values, (concentration of test samples resulting in a 50\% reduction of absorbance compared to untreated controls) were determined.\textsuperscript{18,19}

2.1.7. Inhibitory effects of nitric oxide production from lipopolysaccharide-activated mouse peritoneal macrophages of acetone extract and isolated compounds from \textit{M. nervosa}

Inhibitory effects on NO production by mouse macrophages were evaluated using the method reported by Morikawa et al.\textsuperscript{20,21} Cytotoxicity was determined using the MTT colorimetric assay.

2.1.7.1. Statistical analysis. All cytotoxic data were expressed as mean ($\pm$ SE). Student’s \textit{t}-test\textsuperscript{22} was applied for detecting the significance of difference between each sample, \(P < 0.05\) was taken as the level of significance.

3. Results

Compound 1: a colorless oil; IR (KBr) \(\nu\) max 3365, 1668 and 1757 cm\(^{-1}\). EIS-MS \(m/z\) 279 (M + H\(^{+}\)).\textsuperscript{3}

\(^1H\) NMR (CD\textsubscript{3}OD): 7.54 (1H, brs, H-10), 4.25 (2H, dd, \(J = 11.5/5.4\) Hz, H-16), 2.28 (1H, dd, \(J = 13.5/5.4\) Hz, H-5), 1.7 (1H, d, \(J = 13.5\) Hz, H-3 \(\beta\)), 1.68 (1H, m, H-4 \(\beta\)), 1.62 (2H, ddd, \(J = 16.2/7.9/6.3\) Hz, H-8), 1.43 (1H, m, H-7), 0.98 (3H, s, H-13), 0.93 (3H, d, \(J = 7.5\) Hz, H-14), 0.89 (3H, s, H-15), 0.86 (1H, ddd, \(J = 13.5/5.5/5.3\) Hz, H-3\(\alpha\)), 0.85 (1H, ddd, \(J = 13.5/5.4\) Hz, H-4\(\alpha\)).

\(^13C\) NMR (CD\textsubscript{3}OD): 171.5 (C-12, s), 168.5 (C-11, s), 122.1 (C-1, s), 131.5 (C-9, s), 126.5 (C-10, d), 133.0 (C-2, s), 77.26 (C-3, d), 76.75 (C-6, d), 68.15 (C-12, d), 56.31 (C-17, d), 55.32 (C-14, d), 37.04 (C-9, d), 46.86 (C-13, s), 42.41 (C-7, t), 40.67 (C-4, t), 38.83 (C-10, s), 37.18 (C-1, t), 50.50 (C-5, d), 31.90 (C-25, d), 31.90 (C-2, t), 30.34 (C-22, t), 29.68 (C-16, t), 29.64 (C-8, d), 28.90 (C-20, d), 27.96 (C-23, t), 25.48 (C-15, t), 23.72 (C-11, t), 22.96 (C-26, q), 22.67 (C-27, q), 19.34 (C-19, q), 15.99 (C-21, q), 10.94 (C-18, q).

3.1. Lipid peroxidation inhibitory of different extracts and pure compounds

3.1.1. Assay for DPPH free radical scavenging activity

The antioxidant potential is measured by comparing to IC\textsubscript{50} to known antioxidants D,L-\(\alpha\)-tocopherol and BHT (Table 1, Fig. 2).

Acetone extract has higher DPPH quenching (\textit{M. nervosa}, 68.5\% activity) than D,L-\(\alpha\)-tocopherol (62.8\%) but slightly more than BHT (50.6\%) (Table 1, Fig. 2).

The new furano-sesquiterpene alcohol, micromeriol \[1\] (55.3\%), showed slightly lesser DPPH activity than D,L-\(\alpha\)-tocopherol (62.8\%), but higher than BHT (50.6\%). For the other isolated compounds 2, (30.5\%), 3 (48.8\%), 4 (31.7\%) and 5 (35.0\%) showed lesser DPPH quenching activity than D,L-\(\alpha\)-tocopherol and BHT was observed (Table 1, Fig. 2).

The results obtained with compounds (1–5) have indicated that scavenging effects are dependent on their chemical structure and thought to be due to their hydrogen donating activity.

3.1.2. Ferrous sulfate–H\(_2\)O\(_2\) stimulated lipid peroxidation in rat tissue homogenate

MDA (malondialdehyde) was identified as the major product of lipid peroxidation, which reacts with thiobarbituric acid (TBA), and used as a marker of oxidative stress, giving a red species absorbing at 535 nm. The reaction mixture containing DMSO vehicle was identical to the control in the absence of the mixture of the extract or the compounds.

However, as shown in Table 2, adding 250–500 \(\mu g/\)ml \textit{M. nervosa} extract and 100–200 mg/ml isolated compounds (1–5) to rat tissue homogenates (brain, heart and liver), significantly reduces MDA formation in the presence of Fe\(^{2+}\)–H\(_2\)O\(_2\) in tissue homogenates, indicating anti-lipid peroxidation activities of \textit{M. nervosa} extracts and isolated compounds.

It was interesting to note that the inhibition effect produced by \textit{M. nervosa} and isolated compounds (1–5) was more pronounced for brain and liver tissue homogenates than heart tissue homogenate.

3.1.3. Xanthine oxidase-induced generation of superoxide radical

The inhibitory activity of each extract and isolated compounds is expressed in Table 1 in terms of IC\textsubscript{50} (the concentration of the extracts or isolated compounds required for 50\% inhibition of the uric acid formation).

The initial rate of uric acid formation caused by xanthine oxidase (0.05 U/ml; 50 \(\mu L\)) was 58.0 \pm 1.9 nmol/min. As shown in Table 1, the superoxide scavenging activities of acetone extract and isolated compounds (1–5) at concentration 10–200 \(\mu g/\)ml inhibit the activity of xanthine oxidase.

The acetone extracts (85 \(\mu M\) and 115 \(\mu M\)) for \textit{M. nervosa}, respectively, were expected to have the highest activities in xanthine oxidase inhibition.
Cytotoxicity and antioxidant activity of new biologically active constituents

### Table 2: Inhibition effect of *Micromeria nervosa* acetone extract and isolated compounds (1–5) on FeSO₄–H₂O₂ induced lipid peroxidation (MDA production) in rat tissue homogenate in vitro.

<table>
<thead>
<tr>
<th>Bioassay</th>
<th>Inhibition effect (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Brain</td>
</tr>
<tr>
<td>Sample</td>
<td></td>
</tr>
<tr>
<td>Normal control without FeSO₄–H₂O₂ (MDA level)</td>
<td>0.32 ± 0.03</td>
</tr>
<tr>
<td>Induction by FeSO₄–H₂O₂ (MDA level)</td>
<td>0.78 ± 0.08</td>
</tr>
<tr>
<td>Acetone extract</td>
<td>65.02 ± 2.10</td>
</tr>
<tr>
<td>Compound 1</td>
<td>67.80 ± 2.21</td>
</tr>
<tr>
<td>Compound 2</td>
<td>23.96 ± 1.10</td>
</tr>
<tr>
<td>Compound 3</td>
<td>26.05 ± 1.12</td>
</tr>
<tr>
<td>Compound 4</td>
<td>18.28 ± 1.10</td>
</tr>
<tr>
<td>Compound 5</td>
<td>21.56 ± 1.10</td>
</tr>
<tr>
<td>D,L,-tocopherol</td>
<td>67.50 ± 2.18</td>
</tr>
<tr>
<td>BHT</td>
<td>56.78 ± 1.72</td>
</tr>
</tbody>
</table>

The concentration of acetone extract and isolated compound was 100 μg/mLs.

* Represents type of sample.
** Values are presented as mean ± SE of 3-test sample observation.
**** M. nervosa extracts. P < 0.05 for all values.

### 3.1.4. Scavenging activities of superoxide radical

Table 1 shows the % inhibition of superoxide radical generation by 100 μg/mL of acetone extract and isolated compounds (1–5), and comparison with same concentrations of D,L,-tocopherol, and BHT.

### 3.1.5. Nitric oxide radical inhibition assay

The inhibition of nitric oxide generation, was estimated by comparing the absorbance values of control with those of tocopherol, and BHT.

### 4. Cytotoxicity assay of *M. nervosa* Acetone extract and isolated compounds (1–5) against liver, leukemia, colon, urinary bladder, stomach, ovary, and uterus tumor cell lines

The 50% effective dose (ED₅₀) obtained by measuring growth inhibition with MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) is shown in Table 3, Fig. 4.

Our results demonstrate that *M. nervosa* Acetone extract and isolated compounds (1–5) obtained in this work have growth inhibitory and cytotoxic effects on human liver hepato-cellular carcinoma (SNU-398, Hep G2), Human Colon cancer adenocarcinoma (COLO 205, HCT-116), stomach gastric carcinoma (MKN-28, NCI-N87), and ovary adenocarcinoma (NIH-OVCAR-3, SK-OV-3) cell lines recommended by NCI. The results depicted in Table 3, Fig. 4, summarize the cytotoxic effects of *M. nervosa* acetone extract against selected liver, leukemia, colon, urinary bladder, stomach, ovary, and uterus human tumor cell lines.

### 5. Discussion

**Compound [1]:** was isolated as a colorless oil. Its ESIMS exhibited a pseudomolecular ion peak at m/z 279 (M + H)⁺ corresponding to the molecular formula C₁₆H₂₂O₄. The IR spectrum was found to exhibit absorptions of hydroxyl (3365 cm⁻¹), carbon–carbon double bond (1668 cm⁻¹), and lactone carbonyl groups (1757 cm⁻¹). The characteristic ¹H and ¹³C NMR signals at δH 7.54 (1H, br s) and δC 126.5 (C-10), in addition to the characteristic A₂B₂ methylene system at δH 4.13 (2H, m, H₃-17) and δC 33.5 (C-7), and δH 1.62 (2H, ddd, J = 16.2, 6.3, 7.9 Hz, H₂-8) and δC 21.5 (C-8). Furthermore, the presence of methyl signals at δH 0.89 (3H, s, H₃-15) and δC 20.5 (C-15) and three quaternary carbons at δ 131.5 (C-9), 168.5 (C-11), and 171.5 (C-12), and UV absorption at λmax 273 nm indicated the presence of ethyl group attached to furan dione moiety. ESI-MS also confirmed this observation by a fragment ion base peak at 149 (Ethyl-furan-2,5-dione + Na)⁺. The NMR spectra showed also signals for one secondary methyl group at δ 0.93 (3H, d, J = 7.5 Hz, H₃-14) and δC 19.5, one tertiary methyl group at δ 0.98 (3H, s, H₃-13) and δC 16.5, a secondary hydroxyl function at δH 4.25 (2H, dd, J = 11.5, 5.4 Hz, H₂-16) and δC 68.5, a methane proton at δ 2.28 (1H, dd, J = 13.5, 5.4, H-5), and A₂B₂ spin-system (H₂-3 and H₂-4) at δH 0.86 (1H, dd, J = 13.5, 13.5, 5.3 Hz, H-3ax), 1.70 (1H, d, J = 13.5 Hz, H-3 eq) 0.85 (1H, ddd, J = 13.5, 5.4 Hz, H-4ax), 1.68 (1H, m, H-4 eq), consistent with sesquiterpene structure with Δ[1,6] monocyclofarnesol skeleton. The gross structure was further established by the 2D NMR studies, particularly in ¹H–¹H COSY, and HMBC experiments. In fact, diagnostic ¹H–¹H COSY correlations were observed between the hydroxy methylene protons at H₂-16 and the methylene H₂-3 which was further coupled to the methine C-5 (32.5) by HMBC.

HMBC spectrum showed also many informative correlations, such as H-5 to C-3, C-4, and C-6, H-3ax to C-2, C-3, C-6 and C-7, H₃-14 to C-4, C-5 and C-6, H₂-16 to C-11, C-6 and C-7, and C-8 and C-13, carbonyl group at C-12 to H₂-8 and carbonyl group at C-11 to olefinic proton H-10 .

The relative stereochemistry of the hydroxyl group, which was suggested to be axially oriented (H-16 eq) resonates at δ 4.25 (2H, dd, J = 13.5, 5.4 Hz, H₂-16). Analysis of carbon values of the cyclohexene ring further supported this suggestion. In fact, the different shift values at C-3, C-4, and C-5 due to axial or equatorial hydroxyl substituent were in agreement with the expected calculated effects. The relative stereochemistry of the methyl groups at C-5 and C-6 was suggested...
to be cis by comparison of the $^{13}$C chemical shift of Me-14 (d 16.5) and Me-15 (d 20.5) with carbon values of natural terpenes containing cis or trans substructure. The $^{13}$C chemical shift of Me-14 has similar value (15.7–16.8 ppm) in cis and trans isomers whereas the carbon value of Me-15 is smaller in cis (20.1–21.5 ppm) than in trans (26.3–26.6 ppm) isomer due to the greater $\gamma$-type interactions between the two methyl groups in cis compounds exhibiting $H_3$-14 and $H_3$-15 cis-oriented. Finally, analysis of the coupling constants of $H-5$ ($\delta$ 2.28) that were calculated as $J_{H5ax-H4ax}$ (13.5 Hz), $J_{H5ax-H4eq}$ (5.4 Hz) by decoupling of geminal methyl $H_3$-14 (d 0.89), indicated that $H_3$-14 was axial, cis-oriented.

This structural hypothesis was further supported by comparison of NMR values with those of literature sesquiterpene
Compound [3] was obtained as colorless needles, and gave a positive Liebermann-Burchard test for steroid/or triterpenoid. Its IR (KBr cm⁻¹) spectrum showed absorption bands assigned for hydroxyl groups (3435 cm⁻¹), a terminal methylene group (1638 and 884 cm⁻¹) and CH-stretching vibrations (2925 cm⁻¹). The UV spectrum of [3] exhibited a maximum at 228 nm. The positive ion mode ESI-MS of compound [3] showed quasimolecular ion peaks at m/z 433 (M+H)⁺, 455 (M+Na)⁺ and 887 (2M+Na)⁺ indicating its (M)⁺ to be 432 which was compatible with molecular formula C₂₈H₄₈O₃. Careful examination of the ¹H and ¹³C NMR data revealed that compound [3] was a steroid possessing the 5β-cholestan skeleton. The ¹H and ¹³C NMR spectroscopic data of [3] showed the existence of five typical steroidal methyl groups at δH 0.91 (3H, s) and δC 10.94 (C-18), δH 1.27 (3H, s) and δC 19.34 (C-19), 1.32 (d, J = 6.2 Hz) and δC 15.99 (C-21), δH 0.95 (6H, d, J = 7.2 Hz) and δC 22.27 (C-26 and 27). The latter two signals, together with allyl methine proton at δH 2.34 (1H, septet, J = 7.4 Hz) and δC 31.90 (C-25), showed the presence of an isopropyl group, characteristic of all cholesterol skeleton, a pair of broad singlets (δ 4.71 and 4.58, CH2-24), which was confirmed by a methylene carbon in the ¹³C NMR spectrum at δC 109.67 (t), characteristic of a double bond in the side chain. Signals of three carbinolic protons (CHOH) at δH 3.18 (dd, J = 11.4/4.8 Hz) and δC 77.26 (C-3), δH 4.27 (dd, J = 10.9, 10.9, 5.1 Hz) and δC 76.75 (C-6) and δH 4.19 (dt, J = 10.8, 4.8 Hz) and δC 68.15 (C-12), assignable to three oxymethylene signals at C-3, C-6 and C-12, respectively.

The combined analysis of the 1D NMR spectra suggested that compound [3] was a cholestane skeleton with the disappearance of olefinic proton on carbon C-6, and the presence of a signal at δH 2.20 (brd, J = 11.9 Hz, H-5) and δC 50.50 (C-5). The chemical shift of C-1 to C-13 and H-3, H-4ax, H-4 eq, H-5, H-7ax and H-7 eq coincided with those previously described of 5β-steroid substitution. The four membered rings of a steroid unit in compound [3] are in a chair conformation, the geminal and axial-axial vicinal protons typically have larger coupling constants (8–14 Hz) than those of axial-equatorial vicinal protons and equatorial-equatorial vicinal protons (0–5 Hz). Therefore, in addition to the connectivities, it became very important to determine the coupling constant values if the α and β-configurations of the protons are to be assigned. The DQF-COSY spectrum of compound [3] provided not only the connectivities, but also positive and negative contour lines corresponding to the magnitudes of the coupling constants (J values), thereby affording the possibility of distinguishing axial and equatorial protons.

In compound [3], the H-3α resonance at δH 3.18 (dd, J = 11.4/4.8 Hz), was taken as a starting point, assuming a β-oxygen group as an axial proton with a geminal oxygen functionality, its connected with the C-2 methylene carbon group. From the H-3α proton, the H-2 protons were found by their respective connectivities. The DQF-COSY spectrum showed that the H-3α and the H-2β protons of the cholestane skeleton unit at δH 1.08, δd had a very strong contour with a coupling constant of 12.9 Hz, but H-3α and H-2α (δH 1.25, dt), had a light contour with smaller coupling constant (5.1 Hz). From H-2β, H-1 α (δH 1.46, m) and H-1β (δH 1.50, dt, J = 12.9/5.1 Hz) were assigned. In the same way, starting from H-5β signal (δH 2.20, d, J = 13.9 Hz), the H-6β signal (δH 4.27, ddd, J = 10.5, 10.5, 5.1 Hz), was easily found, which permitted H-7α (δH 1.40, d, J = 10.5 Hz), and H-7β (δH 2.34, 2.52, m), H-8β (δH 3.00, m), H-9α (δH 2.34, 2.02, m) and H-9β (δH 1.56, 2.00, m) to be assigned.
and J = 4.5 Hz), to be assigned. Methine protons H-9 (δH 1.65, dd, J = 13.2/2.5 Hz), and H-12 (δH 4.19 (dt, J = 10.8, 4.8 Hz)), were recognized by their carbon chemical shifts at δC 55.32 (C-9) and 68.15 (C-12), respectively, in the 13C NMR spectrum. The H-11 signals (δH 2.17, m, 11α, 1.95, m, 11β), were distinguished by their DQF-COSY contours. The H2-16 signals (δH 1.68, ddd, J = 14.5, 9.6 4.5 Hz, H-16δ) and (δH 2.29, td, 14.5, 7.9 Hz, H-16β), were also revealed by the chemical shift of its carbon (δC 32.12). This proton (H-16α) showed small coupling constant with the H2-15 (δH 1.28, m, 15α, 1.49, d, J = 12.2 Hz, 15β) proton signals.

The C-6α equatorial orientation of the hydroxyl group was also confirmed by the coupling constant between the H-6β signal (δH 4.27, ddd, J = 10.5, 10.5, 5.1 Hz) and its adjacent protons H2-7 (JH6-7 (ax) = 10.5 Hz), and (JH6-7 (eq) = 4.5 Hz). Thus the orientation of H-6 was decided to be β, and in conclusion this hydroxyl group has α-orientation.

The location of the third hydroxyl group was based on the 13C NMR spectrum, where a signal at δ 10.94 (Me) was attributed to C-18, requiring the hydroxyl attached to C-12. Moreover, the hydroxyl should also occupy a β-position, being cis to Me-18. The 1H-1H-COSY experiment showed cross-peaks between a doublet (Me, δ 0.89) and a multiplet (α 1.4), two methyl signals (d, α 1.03) with an allyl proton (α 2.34, hepatis, J = 7.4 Hz, H-25). These observations led to propose a 24-double bond; the multiplets at δ 1.95, 1.20 in compound [2] (β-sitosterol) was shifted to δH 4.19 (dt, J = 10.8, 4.8 Hz) and δC 68.15 (C-12), which confirms the β-configuration of the 12-OH. This was corroborated by the presence of a γ-gauche effect between the hydroxyl group on C-12 and the methyl group (C-18, δ 10.94).

Thus, on the basis of analysis of the above 1D and 2D NMR spectra of compound [3], the structure has been determined to be: 24-methylene-3β, 6α, 12β-trihydroxy-5β-cholestane, a new natural 5β-cholestan type and was named Nervosane.

Other compounds were identified as β-sitosterol (2), oleanolic acid (4) and ursolic acid (5) by analysis of the spectroscopic data and comparison of their data with those in the literature.

### 5.1. Lipid peroxidation inhibitory of different extracts and pure compounds

#### 5.1.1. Assay for DPPH free radical scavenging activity

The DPPH radical is considered to be a model of a lipophilic radical. A chain reaction in lipophilic radicals was initiated by the lipid autoxidation. The scavenging effects of acetone extracts and isolated compounds (1–5), and positive controls, were determined. The scavenging activities of d,L-α-tocopherol and BHT on DPPH radical are compared and shown in Table 1, Fig. 3. According to the results shown in Table 1, Fig. 3, M. nervosa had significant scavenging effects on the DPPH radical and the effects increased with increasing concentration in the range 200–800 μg/ml. There were no significant differences (P > 0.05) in the scavenging effects between 800 μg/ml and 1000 μg/ml. Compared with that of BHT, the scavenging effect of M. nervosa was higher. Several studies reported that the activity of antioxidants of isolated compounds corresponds to the number of hydrogens available for donation by hydroxyl groups. The radical scavenging effects of M. nervosa might be due to the hydroxyl groups in the isolated antioxidant compounds.

#### 5.1.2. Ferrous sulfate–H2O2 stimulated lipid peroxidation in rat tissue homogenate

Our results clearly showed that lipid peroxidation in rat tissue homogenates (brain, heart and liver) induced by ferrous ion/ H2O2, as measured by MDA formation, was inhibited by M. nervosa extracts (acetone and n-BuOH), and isolated compounds (1–5). Since α-tocopherol is thought to be associated with lipid-rich membranes, it is anti-oxidative is highly effective in protecting membranes against lipid peroxidation, as peroxyl and alkoxy radicals. The data obtained from the present study indicate that extracts and isolated compounds (1–5), have an anti- lipid peroxidative character with similar reaction mechanisms to those of d,L-α-tocopherol and BHT.

These compounds may suppress lipid peroxidation through different chemical mechanisms, including free radical quenching, electron transfer, radical addition, or radical recombination. Further composition analysis is necessary to better understand the relationships between chemical structures/composition and antioxidant properties. The findings above show the presence of natural antioxidant compounds in these plants, with better performance than BHT known as a very efficient synthetic antioxidant agent and widely used in food technology.

#### 5.1.3. Xanthine oxidase-induced generation of superoxide radical

The accumulation of uric acid causes inflammation through production of leukotriene B4 and stimulation of a respiratory burst in neutrophils, in which O−2 (superoxide anion) contributes to the exacerbation of the defense response.

On the basis of the results obtained (Table 1), it can be suggested that the compounds isolated from M. nervosa extracts would be effective as natural antioxidants, through their double ability to inhibit xanthine oxidase activity, and be potentially useful for the treatment of some free radical-induced disorders. Moreover, because of this, inhibition of superoxide anion production of these compounds could be an effective strategy in the treatment of inflammation.

#### 5.1.4. Scavenging activities of superoxide radicals

The acetone extracts of M. nervosa had strong superoxide radical scavenging activity and exhibited higher superoxide radical scavenging activity than d,L-α-tocopherol and BHT. The results were found statistically significant (P < 0.05). As seen (Table 1), the percentage inhibition of superoxide generation by 100 μg/ml concentration of d,L-α-tocopherol, BHT, acetone extracts of M. nervosa was found as 88.8%, and 70.8% and greater than that of same doses of α-tocopherol and BHT (68.5% and 42.3%, respectively). The isolated compounds (1–5) were screened for their superoxide-scavenging activity in the PMS/NADH–NBT system, and the results are shown in Table 1. In the PMS/NADH–NBT system, superoxide anion derived from dissolved oxygen by PMS/NADH coupling reaction reduces NBT. The decrease of absorbance at 560 nm with antioxidants thus indicates the consumption of superoxide anion in the reaction mixture.
Cytotoxicity and antioxidant activity of new biologically active constituents

5.1.5. Nitric oxide radical inhibition assay

The acetone extract obtained from M. nervosa was expected to have milder nitric oxide radical scavenging activity (56.5%) than D.L-α-tocopherol (52.3%). The n-BuOH extract (15.8%) showed quite lesser nitric oxide radical activity than D.L-α-tocopherol (52.3%) and BHT (56.7%) at concentration 100 μg/mL for test samples and controls (Table 1, Fig. 2).

The results obtained with compounds 1–5, 1 (37.4%), 2 (9.5%), 3 (23.2%), 4 (16.8%) and 5 (19.3%) exhibited a lower nitric oxide radical scavenging activity than D.L-α-tocopherol and BHT (Table 2, Fig. 2).

5.2. Cytotoxic assay of M. nervosa extracts and isolated compounds (1–5) against liver, leukemia, colon, urinary bladder, stomach, ovary, and uterus tumor cell lines

The precise mechanism responsible for the cytotoxic activity is not thoroughly understood. Damage to the DNA topoisomerase-mediated activation and livability of cytochrome P450 of carcinogens in human cells in vitro and improve the activities of conjugating enzymes involved in carcinogen detoxification pathways Live seems to be a candidate mechanism, by which some flavonoids and diterpenes may exert their cytotoxic potential. Thus, there is now preliminary scientific validation for the use of some of these plants for anticancer. Cell lines Hep G2, COLO 205, MKN-28, NCI-N87, and NIH:OVCAR-3, were the most sensitive of all lines examined for the activities of these extracts with ED₅₀ = 5.98–32.88 μg/mL. M. nervosa extracts also showed moderate cytotoxic activities when tested against liver hepatocellular carcinoma (SNU-398), Colon cancer adenocarcinoma (HCT-116) and ovary adenocarcinoma (SK-OV-3) (ED₅₀ = 36.70 to 83.60 μg/mL). However, neither extract, showed any cytotoxic activity (>100 μg/mL) against human Leukemia carcinoma (CCRF-CEM, HL-60 TB), urinary bladder Carcinoma (HT-1376), Urinary bladder transitional cell carcinoma (UM-U-3), and uterus uterine sarcoma (MES-SA, MES-SA/MX2) cells in a concentration-dependent manner at the recommended NCI (USA) doses. The activities observed with crude extracts was surprised while non-give activities surprising those of some isolated compounds, these extracts contain mixtures of compounds that could function together synergistically in displaying cytotoxicity.

Table 3, Fig. 4 summarizes the cytotoxic activity of the active compounds isolated from M. nervosa. Furano-sesquiterpene alcohol (micromeriol) showed the most potent cytotoxic activities of the active compounds isolated and identified, showing significant activity (ED₅₀ = 2.24–13.57 μg/mL), with human liver hepatocellular carcinoma (Hep G2), Colon cancer adenocarcinoma (COLO 205), stomach gastric carcinoma (MKN-28, NCI-N87), and ovary adenocarcinoma (NIH:OVCAR-3) cell lines. 5β-cholestane [nervosane, 3] and triterpenic acids [oleanolic acid, 4 and ursolic acid, 5] were more cytotoxic vs. both human liver hepatocellular carcinoma (SNU-398) and ovary adenocarcinoma (NIH:OVCAR-3) cell lines.

Relatively limited information exists on the structure activity relationships for the cytotoxicities of these classes of compounds.

Our results provide some insights as to structural moieties that modulate cytotoxic activity, and add to existing knowledge in the cytotoxicities of these classes of compounds.

The search for anticancer agents from natural sources has been successful worldwide. Active constituents have been isolated and nowadays are used to treat human tumours. The ethnopharmacological knowledge is helpful to lead the search for plants with potential cytotoxic activity. Although such studies are highly suggestive of the beneficial effects of M. nervosa. Furano-sesquiterpene alcohol (micromeriol, 1) with regard to cancer prevention, definitive intervention trials haven’t been completed and there is not any scientific validation. One goal of this evaluation is to understand better its use.

6. Conflict of interest

None declared.

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