A NOVEL ACYCLIC DITERPENIC ALCOHOL ISOLATED FROM ANTIOXIDANT ACTIVE ETHANOL EXTRACT OF LEAVES OF CENTAUROTHAMNUS MAXIMUS GROWN IN SAUDI ARABIA

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Abstract

**Background:** Isolation and characterization of a new compound from the antioxidant active ethanol extract of leaves of an endemic plant *Centaurothamnus maximus*.

**Methods:** The air dried powdered leaves of the plant was extracted successively with n-hexane, dichloromethane, ethyl acetate and ethanol. The obtained extracts were concentrated under reduced pressure using rotary evaporator. The antioxidant activity was carried out on various concentrations (1000, 500, 100, 50 and 10 µg/ml) of all the extracts by DPPH free radical scavenging method. After screening for antioxidant potential the ethanol fraction was selected for the isolation of phytoconstituents by column chromatography using LiChrorep RP-18 as stationary phase and water, MeOH and CHCl₃ in different combinations as eluent. The chemical structures of the isolated compounds were elucidated by 1D and 2D NMR spectroscopic techniques (DEPT, COSY, HMBC and HSQC) aided by EIMS mass and IR spectra.

**Results:** The antioxidant activity of ethanol extract was highly comparable with standard ascorbic acid as compared to other extracts. A new compound along with two known compounds has been isolated from antioxidant active ethanol extract. The chemical structure of unknown compound was established as 2, 6, 10, 14-tetramethyl hexadec-12-cis-en-5α, 7α, 9α, 14α-tetraol (CM1) while the known compound isolated Luteolin-7-O-β-glucopyranosyl-6″-O-(6″→1″)-β-D-rhamno-pyranoside (CM-2) and stigmast-5,22–dien-3β–ol (CM3).

**Conclusion:** On the basis of interpretation of different spectroscopy data we concluded that the compound CM1 is an acyclic diterpenic alcohol. The authors are reporting the isolation of CM1 from plant source for the first time but CM2 and CM3 are known compounds. Ethanol extract of leaves can be recommended as potent antioxidant for ethno-medical purposes. The antioxidant properties might be due to some well-known antioxidants like (CM-2) and other falvonoidal compounds.

**Keywords:** *Centaurothamnus maximus*, Asteraceae, isolation, antioxidant, diterpenic alcohol.

**List of Abbreviations:**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>NMR</td>
<td>Nuclear magnetic resonance</td>
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<tr>
<td>BDH</td>
<td>British Drug Houses</td>
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<tr>
<td>DPPH</td>
<td>2, 2-diphenylpicrylhydrazyl</td>
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<tr>
<td>RP-18</td>
<td>Reverse phase-18</td>
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<tr>
<td>MeOH</td>
<td>Methanol</td>
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<tr>
<td>KBr</td>
<td>Potassium Bromide</td>
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<tr>
<td>CHCl₃</td>
<td>Chloroform</td>
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<tr>
<td>MeOD</td>
<td>Deteriorated Methanol</td>
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<tr>
<td>EIMS</td>
<td>Electron ionization mass spectrometer</td>
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<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
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<tr>
<td>DEPT</td>
<td>Distortion less Enhancement by Polarization Transfer</td>
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<tr>
<td>CDCl₃</td>
<td>Deuterated chloroform</td>
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<tr>
<td>COSY</td>
<td>Correlation Spectroscopy</td>
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<tr>
<td>UV</td>
<td>Ultra violet</td>
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<tr>
<td>HMBC</td>
<td>Heteronuclear Multiple Bond Correlation</td>
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<td>Infra red</td>
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<tr>
<td>HSQC</td>
<td>Heteronuclear Single Quantum Coherence</td>
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<tr>
<td>TLC</td>
<td>Thin Layer Chromatography</td>
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Introduction

*Centaurothamnus maximus* (Asteraceae) is a paleoendemic, endangered species in Saudi Arabia. It is found only within two localities in Saudi Arabia and in a few high altitude cliffs of Yemen. Compared to the populations in Yemen, the density and distribution of this species in Saudi Arabia are highly restricted, represented by not more than 200 plants (Basi et al., 2010).

*Centauras maximus* is a leafy shrub with many branches and about 1.5 m tall (Collenette, 1999). Previous phytochemical studies reported the presence of flavonoids in several genera of family Asteraceae (Valant-Vetschera and Wollenweber, 2007; Ciric et al., 2012). Sesquiterpene lactones are also prominent constituents in Asteraceae family (Ling, 1992; Iwashina et al., 1995; Tzakao et al., 1995 and Akkal et al., 1997). The aerial parts of *C. maximus* reportedly contain three sesquiterpene lactones namely guainolides chlorojanerin, cynaropicrin and janerin which are active against human cancer cell lines of malignant melanoma (SK-MEL), epidermoid (KB), ductal (BT-549) and ovarian (SK-OV-3) carcinomas with IC50 values of 2–6 µg/mL (Muhammad et al., 2003).

The presence of diterpenoids in Asteraceae was reported by Guo et al., 2006. Extensive literature survey revealed that several plants belonging to the family Asteraceae has excellent antioxidant property like *Chromolaena odorata* which showed excellent antioxidant property probably due to its high phenolic content (Alisi and Oneyeze, 2008), *Pluchea indica* Less. which supports its dietary intake as antioxidant (Andarwulan et al., 2010) and ethanol extract of *Pluchea arabaica* exhibited its antioxidant potential by inhibiting DPPH radical (Marwah et al., 2007). The methanol and
aqueous extracts of *Centaurea polypodiifolia* var. *psuedohelen* showed strong antioxidant activity (Aktumsek et al., 2013). Methanolic extract of *C. maximus* was found to possess a noteworthy growth inhibitory effect against human lung cancer (A-427), urinary bladder cancer (5637) and breast cancer (MCF-7) cell lines with IC_{50} values < 50 μg/ml and pronounced antimicrobial activity was observed only against Gram-positive bacteria among them multi resistant bacteria with inhibition zones > 15 mm and MIC values < 500 μg/ml (Mothana et al., 2009). Due to limited exploration of this plant we designed our study to investigate the antioxidant potentials of different extracts of leaves and isolation as well as characterization of new phytoconstituents (Fig. 1a) along with some known compounds (Fig. 2 and Fig.3).

**Materials and Methods**

**Plant Material**

The Leaves of *C. maximus* were collected in March 2006 from Aqabaat Al-Makhwah, after tunnel # 13, Kingdom of Saudi Arabia. The plant was identified by Dr. Mohammed Yousuf, Field Taxonomist, Department of Pharmacognosy, College of Pharmacy, KSU, Riyadh. A voucher specimen is deposited in herbarium (Voucher # 15024) of Pharmacognosy Department, College of Pharmacy, King Saud University, Riyadh.

**Apparatus and reagents**

IR spectra were recorded with an ATI Mattson genesis series Fourier transform (FT-IR) spectrophotometer. UV spectra were obtained on a Hewlett Packard 8452A diode array spectrophotometer. Optical rotations were recorded at ambient temperature using a JASCO DIP-370 digital polarimeter. Melting points were determined by using a model IA9100 melting point apparatus. 1D and 2D NMR spectra for ^1^H and ^13^C were obtained on a Bruker Avance DRX 500 spectrometer. High resolution mass spectra were obtained using a Bruker Biospectr FT-MS in ESI mode. For TLC, glass supported silica gel plates (0.25 mm layer, F250, E. Merck) were used. Silica gel (70-230 mesh) and LiChroprep RP-18 (40-63 μm; octadecyl silica (ODS) gel) from Merck was employed for column chromatography.

All chemicals were of analytical grade. n-Hexane, ethyl acetate, chloroform, methanol, ethanol, and sulphuric acid were purchased from well known International companies such as Sigma-Aldrich and BDH. Thin-layer chromatography was performed on pre-coated silica gel 60 F254 plates (Merck). Visualization of the TLC plates was performed using p-anisaldehyde as spray reagent.

**Extraction and Isolation**

The air dried powdered leaves (1000 g) of the plant extracted exhaustively with n-hexane with a Soxhlet apparatus. This process was repeated, until the complete exhaustion of the plant material. The extract was concentrated in vacuo using rotary evaporator at 40°C. Remaining marc was dried and extracted with the same apparatus till exhaustion of the drug material using dichloromethane and concentrated with similar procedure. The same procedure was followed for extraction with ethyl acetate and ethanol (90%). The quantities of dried extracts were 46.8 g, 62.5 g, 69.8 g and 81.6 g with the solvents n-hexane, dichloromethane, ethyl acetate and ethanol respectively. Extracts were investigated for their antioxidant potential. Being most potent antioxidant, ethanol fraction of extract was subjected for isolation by reverse phase column chromatography using LiChroprep RP-18 as stationary phase and water, MeOH, CHCl$_3$ and petroleum ether used in different combinations as eluent.

**Antioxidant activity**

The antioxidant activity of all the extracts was carried out by scavenging of DPPH free radical as described by Brand et al. (1995). The DPPH free radical was reduced to corresponding hydrazine when reacted with hydrogen donors. The DPPH radical was purple in color and upon reaction with hydrogen donor changed to yellow. It was a discoloration assay, which was evaluated by the addition of the antioxidant to a DPPH solution in methanol or ethanol and decrease in absorbance was measured at 517nm. Various concentrations (1000, 500, 100, 50 and 10 μg/ml) of all the extracts of leaves of *C. maximus* were used. The assay mixtures contained in total volume of 1000, 500, 100, 50 and 10 μL of the extract, 125 μL prepared DPPH and 375 μL solvent. Ascorbic acid was used as the positive control. After 30 min incubation at 25 °C, the decrease in absorbance was measured at λ=517 nm. The radical scavenging activity was calculated from the equation:

% radical scavenging activity = (A$_{control}$-A$_{sample}$) / A$_{control}$) X 100

**Results**

**Antioxidant activity**

The plant was endemic to Saudi Arabia and only limited literature is available but in future these studies might be effective in promoting the use of the plant as ethnomedicine. n-Hexane and dichloromethane extracts of leaves of *C. maximus* showed no antioxidant activity while ethanol and ethyl acetate extracts of leaves of *C. maximus* exhibited a highly effective free radical scavenging activity in DPPH assay at somewhat low to high concentrations. Ethanol fraction showed better antioxidant potential than ethyl acetate fraction at similar concentrations (Table 1). The antioxidant activity of ethanol extract was highly comparable with standard ascorbic acid as compared to the ethyl acetate extract. The high antioxidant potential of ethanol extract of leaves of *C. maximus* encouraged the authors on the isolation and characterization of some new phytoconstituents by using reverse phase column chromatography method.
Table 1: Free radical-scavenging activity (DPPH-assay)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Radial scavenging activity (%) ± SD</th>
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<tbody>
<tr>
<td></td>
<td>10 µg/ml</td>
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<tr>
<td>Ethanol fraction</td>
<td>18.5 ± 1.42</td>
</tr>
<tr>
<td>Ethyl acetate fraction</td>
<td>4.69 ± 0.94</td>
</tr>
<tr>
<td>CM-1</td>
<td>3.5±0.57</td>
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<tr>
<td>Ascorbic acid (STD)</td>
<td>41.0±3.1</td>
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</table>

Compound 1 (CM1)

CM1 was obtained as yellowish white crystalline solid (389 mg) having m.p. 201-202°C. CM 1 was eluted from gradients of CH₃OH and CHCl₃ (90:10) used as mobile phase.

**IR (KBr):** 3410, 2952, 2872, 1629, 1401, 1365, 1260, 1015 cm⁻¹. **H NMR (MeOD):** δ 6.22 (1H, d, J= 6.0 Hz, H-13), 5.72 (1H, m, H-12), 3.78 (1H, brs, w= 9.3 Hz, H-9b), 3.70 (1H, brs, w= 12.8 Hz, H-7b), 3.21 (1H, brs, w= 8.3 Hz, H-5b), 2.59 (1H, m, H-11a), 2.30 (1H, m, H-10a), 1.93 (1H, m, H-2a), 1.85 (1H,m,H-4b), 1.72 (1H, m, H-8a), 1.65 (1H, m, H-2a), 1.50 (1H, m, H-8b), 1.47 (1H,m,H-2b), 1.45 (1H, m, H-6a), 1.37 (1H, m, H-3a), 1.25 (1H, m, H-15a), 1.23 (1H, m, H-13a). 1.15 (1H, m, H-15b), 1.20 (3H, brs, Me-20), 0.80 (3H, t, J= 6.3 Hz, Me-19), 1.16 (3H, d, J= 6.2 Hz, Me-18), 0.95 (1H, m, H-10), 0.86 (3H, d, J=6.7 Hz, Me-1), 0.84 (3H, d, J= 6.3 Hz, Me-17), 0.80 (3H, t, J= 6.5 Hz, Me-16). **13C NMR:** δ 100.91 (C-10), 21.07 (C-11), 39.76 (C-12), 42.30 (C-13), 56.87 (C-14), 24.17 (C-15), 28.67 (C-16), 55.96 (C-17), 11.99 (C-18), 19.41 (C-19), 36.68 (C-20), 18.79 (C-21), 138.33 (C-22), 129.27 (C-23), 45.83 (C-24), 27.28 (C-25), 19.83 (C-26), 18.99 (C-27), 23.11 (C-28), 11.87 (C-29). **EIMS m/z (rel. int.):** 412 [M]+ (C₂₉H₄₈O₄) (76.2), 396 (51.2), 394 (41.3), 381 (215.2), 271 (328.2), 255 (66.6), 240 (27.2), 213 (36.5), 198 (23.3), 192 (11.6), 183 (16.2), 178 (32.8), 164 (23.7), 159 (83.6), 145 (84.2), 133 (65.3), 122 (31.9), 108 (69.3), 107 (73.8), 95 (97.3), 91 (70.2), 57 (100).

**Discussion**

Reactive oxygen species (ROS) were produced by mitochondria and endoplasmic reticulum during normal cell functions (Alfadda & Sallam, 2012). Also produced by phagocytes When attacked, the invading pathogens as part of the inflammatory response produced from enzyme such as xanthine oxidase, cytochrome P450 enzymes, peroxosimal oxidases and NADPH oxidases (Apriku, 2013). ROS such as Superoxide anion (the precursor of most ROS), hydrogen peroxide, hydroxyl radical and nitric oxide (Turrens, 2003), acts on lipids, proteins and DNA leading to diseases such as brain dysfunction, cancer, heart disease, age related degenerative conditions, imbalance in the immune system and DNA damage. Most of the
antioxidants found in diet. Many fruits like berries, cherries, olives, grapes, apples and vegetables like cacao beans, tomatoes, spinach and garlic are rich source of antioxidants. Green and black tea, coffee and chocolate are also potential sources of natural antioxidants (Masood et al., 2013). The use of ethnomedicines by local population as anti-oxidant can enhance the percentage of healthy people in that area because oxidative stress is one of the major causes of many diseases.

Though the anti-oxidant effect of isolated new acyclic diterpenic alcohol was insignificant yet the anti-oxidant effect of ethanol extract at 500 µg/ml concentration was significantly comparable with the standard ascorbic acid and this dose can be recommended as ethno medical remedies. There may be some other falvonoids and/or flavonoidal compounds like Luteolin-7-O-β-glucopyranosyl-6''-O-(6''→1'')-β-D-rihamno-pyranoside with potential anti-oxidant effect of ethanol extract.

**Compound 1 (CM1)**

The IR spectrum of CM1 showed absorption bands for hydroxyl groups (3410 and 3227 cm<sup>-1</sup>) and unsaturation (1629 cm<sup>-1</sup>). On the basis of mass and <sup>1</sup>C NMR spectra the molecular ion peak of CM1 was determined at m/z 344 consistent with the molecular formula of a tetrahydroxy diterpene, C<sub>20</sub>H<sub>40</sub>O<sub>4</sub>. The ion peaks arising at m/z 271 [C<sub>17</sub>-C<sub>14</sub> fission, M- C<sub>14</sub>H<sub>10</sub>O<sup>+</sup>], 245 [C<sub>13</sub>-C<sub>12</sub> fission, M - C<sub>12</sub>H<sub>7</sub>O<sup>+</sup>] and 231 [C<sub>10</sub>-C<sub>11</sub> fission] suggested the presence of one hydroxyl group at C<sub>14</sub> and vinylic linkage at C<sub>12</sub>(13). The ion fragments generated at m/z 203 [C<sub>9</sub>-C<sub>10</sub> fission, C<sub>1</sub>_3H<sub>5</sub>O<sub>3</sub>], 141[M-203, C<sub>6</sub>H<sub>4</sub>O<sub>3</sub>], 185 [C<sub>14</sub>-C<sub>13</sub> fission, C<sub>10</sub>H<sub>6</sub>O<sub>3</sub>], 215 [C<sub>12</sub>-C<sub>11</sub> fission, C<sub>8</sub>H<sub>5</sub>O<sub>3</sub>], 243 [C<sub>17</sub>-C<sub>16</sub> fission] and 273 [C<sub>12</sub>-C<sub>11</sub> fission] indicated the existence of hydroxyl groups at C<sub>6</sub>, C<sub>13</sub> and C<sub>11</sub> carbons. The 1H NMR spectrum of CM1 exhibited the presence of a one-proton doublet at δ 6.22 (J= 6.0 Hz) and a one-proton multiplet at δ 5.72 assigned to cis-oriented vinylic H-13 and H-12 protons, respectively. Three one-proton multiplets at δ 3.78 (J= 9.3 Hz), 3.70 (ω<sub>1</sub>= 12.8 Hz) and 3.21 (ω<sub>1</sub>= 8.3 Hz) ascribed correspondingly β-oriented carbinol H-9, H-7, and H-5 proton. A three-proton broad singlet at δ 1.20 due to tertiary C-20 methyl protons, four-three-proton doublets at δ 1.16 (J= 6.2 Hz), 0.86 (J= 6.7 Hz) and 0.84 (J= 6.3 Hz) attributed to secondary C-18, C-19, C-1 and C-17 methyl protons respectively, a three-proton triplet at δ 0.80 (J= 6.5 Hz) accounted primary C-16 methyl protons and the remaining methine and methylene protons between δ 2.59- 1.15 and at δ 0.95. The <sup>13</sup>C NMR spectrum of CM1 displayed signals for vinylic carbons at δ 117.71 (C-12) and 134.14 (C-13), carbinol carbons at δ 78.01 (C-5), 70.12 (C-7), 68.41 (C-9) and 84.33 (C-14) and methyl carbons at δ 20.74 (C-1), 18.11 (C-16), 23.44 (C-17), 30.52 (C-18), 23.76 (C-19) and 30.79 (C-20). The DEPT spectrum of CM1 showed the presence of six methyl, five methylene, five methine and one quaternary carbon. The 1H-1H COSY spectrum of CM1 exhibited interactions of H-5, H-6 and H-18, H-9 with H-7, H-11 with H-13 and Me – 19; H-12 with H-10, H<sub>2</sub>- 15 and Me – 16. The HMBC spectrum of CM1 showed correlations of H-5, H-6, H-7 with C-18; H-9, H-10 and H<sub>2</sub>-11 with C-19; and H-12, H13, Me-20, H<sub>2</sub>-15 and Me-16 with C-14. The 1H NMR values of the methyl, carbinol and vinylic carbons were correlated with the respective carbons in the HSQC spectrum. On the basis of these evidences the chemical structure of CM1 has been elucidated as 2, 6, 10, 14-tetramethylhexadec-12-cis-en-5α, 7α, 9α, 14α-tetraol This is a new acyclic diterpenic alcohol (Fig. 1a and 1b).

![Figure 1a: (CM1): 2, 6, 10, 14-tetramethylhexadec-12-cis-en-5α, 7α, 9α, 14α-tetraol](http://dx.doi.org/10.4314/ajtcam.v12i3.5)

**Compound 2 (CM-2)** was obtained as a yellow amorphous product from chloroform- methanol (7:3) eluents. It responded positively to flavones and phenolic test with ferric chloride. It is a known compound isolated from the leaves of *C. maximus*. The UV absorption maxima at 263, 305 and 341 nm were typical of a flavone (Markham, 1982; Ahmad et al. 2013). Its IR spectrum exhibited absorption bands for hydroxyl groups (3435, 3390 and 3260 cm<sup>-1</sup>), and is a new acyclic diterpenic alcohol (Fig. 1a and 1b).
C. maximus revealed 27 signals, O. The prominent ion peaks –ol. Authors are reporting CM-2ʹ, H
The ion peaks exhibited the presence of one methyl, one methylene, sixteen methine and nine quaternary carbons. The 5.28, 5.08 and 4.95 assigned to vinylic H-6, H-22 and H-23 protons, respectively. A one-proton broad multiplet at δ 3.45 with CM-2 was elucidated as m/z showed the presence of two sugar moieties attached to the flavones nucleus. The and 198 15 -1 (CM2): exhibited interactions of H-3 with C-4; H-6 and H-8 with C-7; H-2 with C-18 and C-19 tertiary methyl protons, respectively. Four doublets at δ 0.97 (J=6.3 Hz), 0.84 (J=6.6Hz), 0.75 (J = 6.0 Hz) and 0.73 (J = 3.6 Hz), all half width of 16.5 Hz was attributed to proton multiplets at δ 3.20 and a one-proton singlet at δ 6.20 attributed to H-3 proton. Two one-proton doublets at δ 4.87 (J=7.0) and δ 4.59 (J=7.1) were due to anomic H-1" and H-1‴ protons, respectively. The other sugar oxygenated methine protons appeared between δ 3.90 -3.41. Two one-proton doublets at δ 3.20 (J=9.0 Hz) and 3.16 (J= 8.5 Hz) were ascribed to oxygenated methylene H-6"a and H-6"b, respectively. The presence of rhamnose was determined by the presence of a three-proton doublet at δ 1.14 (J= 7.2) accounted to C-6‴′′ methyl protons. The 13C NMR spectrum of CM-2 revealed 27 signals, 15 of which were typical of a flavonoid skeleton and other 12 carbons were assigned to sugar moiety. The C-4 flavone carbonyl carbon appeared at δ 181.69, other flavone carbons resonated between δ 164.42 and 103.67, anomic carbons at δ 101.15 (C-1‘’) and 100.62 (C-1‴′), methyl carbon at δ 17.84 (C-6‴″) and other sugar carbons from δ 75.76 to 66.55. The presence of the oxygenated methylene H-2″′ proton signals in the 1H NMR spectrum in the deshielded region at δ 3.20 and 3.16 and the respective carbon signal in the 13C NMR spectrum at δ 66.55 suggested (6‴″→1‴′′) linkage of the sugar units. The DEPT spectrum of CM-2 exhibited the presence of one methyl, one methylene, sixteen methine and nine quaternary carbons. The 1H-13C COSY spectrum of CM-2 showed correlations of H-6 with H-8; H-6″ with H-2′ and H-5″; H-1″ with H-2″′, H-3″ and H-5″; H-1‴′ with H-2″”, H-5‴′ and H-2′; and H-5‴′ with H-4‴″ and Me-6‴″. The HMBC spectrum of CM-2 exhibited interactions of H-3 with C-4; H-6 and H-8 with C-7; H-2′, H-5″ and H-6′ with C-1′; H-1″ with C-7; H-6″, H-2″ and H-3‴′ with C-1″; and H-4″ and H-5‴′ with C-6″′. The HSQC correlations were used to assign all the protons and carbons in the molecule and some important correlations were H-1′ with C-1′, H-1″ with C-1‴′ and H-1‴′ with C-1‴″. The 1H and 13C NMR spectroscopic data of the flavone unit were compared with the reported values of these compounds (Waffo et al., 2006; Ahmad et al. 2013). Acid hydrolysis of CM-2 yielded luteolin, D-glucose and D-xylose, confirmed by comparison of co-TLC. On the basis of the analysis of the spectroscopic data and chemical reactions, the structure of CM-2 was elucidated as Luteolin-7-O-β-glucopyranosyl-6″″-O-(6‴′→1‴′″)-β-D-rhamno-pyranoside.

Figure 2: (CM2): Luteolin-7-O-β-glucopyranosyl-6″″-O-(6‴′→1‴′″)-β-D-rhamno-pyranoside

Compound 3 (CM-3) was obtained as colorless crystalline mass from petroleum ether - chloroform (7:3) eluants. It responded positively to steroidal tests. Its IR spectrum showed characteristic absorption bands for hydroxyl group (3425 cm−1) and unsaturation (1641 cm−1). On the basis of mass and 13C NMR spectra, its molecular weight was established as 412 consistent with the molecular formula of a sterol, C30H4O. The prominent ion peaks generated at m/z 396 [412- Me]−, 394 [ 412-HO]H], 271 [412-C12H10,side chain]−, 255 [271-Me]−, 240 [255-Me]−, 213 [255-ring D fission ]− and 198 [213-Me]− suggested that was a stigmastene-type sterol containing a C30 unsaturated side chain. The 1H NMR spectrum of CM3 displayed three one-proton multiplets at δ 5.28, 5.08 and 4.95 assigned to vinyl H-6, H-22 and H-23 protons, respectively. A one-proton broad multiplet at δ 3.45 with half width of 16.5 Hz was attributed to α-oriented oxygenated methane H-3 proton. Two three-proton broad singlets at δ 0.62 and 1.13 were assigned to C-18 and C-19 tertiary methyl protons, respectively. Four doublets at δ 0.97 (J=6.3 Hz), 0.84 (J=6.6Hz), 0.75 (J = 6.0 Hz) and 0.73 (J = 3.6 Hz), all integrating for three protons each, were accounted to secondary C-21, C-26 and C-27 methyl and primary C-29 methyl protons, respectively, all attached to the saturated carbons. The remaining methine and methylene protons resonated between δ 2.23-1.01. The 13C NMR spectrum of CM3 showed important signals for vinylic carbons at δ 141.75 (C-5), 121.72 (C-6), 138.33 (C-22) and 129.27 (C-23), carbinol methine carbon at δ 71.81 (C-3) and the remaining methyl, methylene methine carbons between δ 56.87-11.87. The 1H NMR and 13C NMR spectral data of the steroidal nucleus were compared with other stigmastene-type molecules (Jung et al., 2012; Mustafa and Ali, 2011). The number of methyl, methylene and methine carbon was determined by analysis of DEPT spectrum. The 1H-H COSY spectrum of CM3 showed correlations of H-3 with H-2 and H-4; H-6 with H-2, H-7 and H-8; and H-22 with H-20, Me-21, H-23 and H-24. The HMBC spectrum of CM3 exhibited interactions of C-5 with H-3, H-4 and H-6; and C-22 with H-20, Me-21, H-17, H-22 and H-24. The positions of the carbon with their respective hydrogens were determined by analysis of HSQC spectrum. On the basis of the spectroscopic data analysis the structure of CM3 has been established stigmast-5,22-dien-3β-o1. Authors are reporting the presence of all these compounds in C. maximus for the first time.
The isolation of the above two compounds (CM1 and CM2) probably revealed the reason behind the antioxidant potential of ethanol extract of leaves of *C. maximus*. Usually the molecules having flavanoidal (C$_6$-C$_3$-C$_6$) nucleus possess good antioxidant potential which may also be supported by a molecule with vinylic linkage having hydroxyl groups. The results of antioxidant activity of ethanol extract were promising and strongly recommend the plant as a free radical scavenger. The plant can be used to relieve the oxidative stress which was already proved to be a major reason for many severe diseases such as cardiac ailments, diabetes and hypertension etc.

**Conclusion**

In this study the maiden report on one new compound (CM1) along with two known compounds (CM2 and CM3) in *C. maximus* has been presented with significant anti-oxidant effect of ethanol extract of the plant. On the basis of the above findings the ethanol extract of the plant can be recommended as useful anti-oxidant for ethno-medical purposes.

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**References**