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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 LiChroprep RP-18 as stationary phase and water, MeOH and $CHCl_3$ 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-3*β* –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:
NMR- Nuclear magnetic resonance, BDH- British Drug Houses, DPPH- 2, 2-diphenylpicrylhydrazyl, RP-18- Reverse phase-18, MeOH- Methanol, KBr- Potassium Bromide, $CHCl_3$-Chloroform, MeOD- Deteriorated Methanol, EIMS-Electron ionization mass spectrometer, DMSO-Dimethyl sulfoxide, DEPT-Distortion less Enhancement by Polarization Transfer, $CDCl_3$- Deuterated chloroform, COSY- Correlation Spectroscopy, UV- Ultra violet, HMBC- Heteronuclear Multiple Bond Correlation, IR - Infra red, HSQC- Heteronuclear Single Quantum Coherence, TLC-Thin Layer Chromatography

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 (Basahi et al., 2010).

C. 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 Asteracea was reported by Guo et al., 2006. Extensive literature survey revealed that several plants belonging to the family Asteracea has excellent antioxidant property like *Chromolaena odorata* which showed excellent antioxidant property probably due to its high phenolic content (Alisi and Onyeze, 2008), *Pluchea indica* Less. which supports its dietary intake as antioxidant (Andarwulan et al., 2010) and ethanol extract of *Pluchea arabica* exhibited its antioxidant potential by inhibiting DPPH radical (Marwah et al., 2007). The methanol and

aqueous extracts of *Centaurea polypodiifolia* var. *pseudobehen* 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 Bioapex 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, 69.8g 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:

$$\% \text{ radical scavenging activity} = (A_{control} - A_{sample}) / A_{control}) \text{ X } 100$$

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.

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Table 1: Free radical-scavenging activity (DPPH-assay)

Treatment	Radical scavenging activity (%) ± SD				
	10 µg/ml	50 µg/ml	100 µg/ml	500 µg/ml	1000 µg/ml
Ethanol fraction	18.5 ± 1.42	35.4 ± 2.99	55.5 ± 3.52	95.8 ± 5.66	96.7 ± 6.07
Ethyl acetate fraction	4.69 ± 0.94	27.8 ± 3.22	42.7 ± 4.00	93.8 ± 6.14	94.9 ± 6.60
CM-1	3.54± 0.57	15.5± 0.97	30.5± 1.89	41.3± 2.42	55.5± 3.31
Ascorbic acid (STD)	41.0 ± 3.1	66.4 ± 5.56	85.5 ± 6.12	96.0 ± 6.68	97.8 ± 7.12

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 CH_3OH and $CHCl_3$ (90:10) used as mobile phase.
IR γmax (KBr): 3410, 3227, 2852, 2741, 1629, 1401, 1365, 1260, 1015 cm^{-1}. **^{1}H NMR (MeOD)** : δ 6.22 (1H, d, *J*= 6.0 Hz, H-13), 5.72 (1H, m, H-12), 3.78 (1H, brm, w1/2= 9.3 Hz, H-9β), 3.70 (1H, brm, w1/2= 12.8 Hz, H-7β), 3.21 (1H, brs, w1/2 = 8.3 Hz, H-5β), 2.59 (1H,M, H_2-11a), 2.30 (1H, m, H- 10α), 1.93 (1H, m, H_2- 4a), 1.85 (1H,m,H_2 - 4b), 1.72 (1H, m, H_2-8a), 1.65 (1H, m, H_2-2a), 1.50 (1H, m, H_2-8b), 1.47 (1H,m,H_2-2b), 1.45 (1H, m, H - 6α), 1.37 (1H, m, H_2-3a), 1.25 (1H,m, H_2-3b), 1.23 (1H, m, H_2-15a), 1.15 (1H, m, H_2 – 15b), 1.20 (3H, brs, Me-20), 1.18 (3H, d, *J*= 6.1 Hz, Me-18), 1.16 (3H, d, *J*= 6.2 Hz, Me - 19), 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). **^{13}C NMR (DMSO-d_6)**: δ 20.74 (C-1), 29.23 (C-2), 37.65 (C-3), 31.36 (C-4), 78.01 (C-5), 50.46 (C-6), 70.12 (C-7), 39.06 (C-8), 68.41 (C-9), 47.85 (C-10), 22.76 (C-11), 117.71 (C-12), 134.14 (C-13), 84.33 (C-14), 21.65 (C-15), 18.11 (C-16), 23.44 (C-17), 30.52 (C-18), 23.76 (C-19), 30.79 (C-20). **EIMS m/z (rel. int.):** 344 $[M]^+$ ($C_{20}H_{40}O_4$) (5.1), 273 (18.2), 271 (56.8), 245 (31.7), 243 (16.5), 231 (21.3), 215 (15.6), 203 (11.2), 185 (16.1), 141 (20.4).

Compound 2 (CM-2): Elution of the column with chloroform- methanol (7:3) gave a yellow amorphous powder (232.5 mg) of CM-2.
IR (KBr) : 3435, 3390, 3260, 2921, 1654, 1506, 1363,1259, 1066 cm^{-1}.
1H NMR (DMSO-d_6): δ 7.49 (2H, m, H-2', H-6'), 7.24 (1H, d, *J* = 7.2 Hz, H -5'), 6.79 (1H, d, J= 1.2 Hz, H-8), 6.51 (1H, d, *J*=1.2 Hz, H-6), 6.20 (1H, s, H-3), 4.87 (1H, d, *J*=7.0 Hz, H-1"), 4.59 (1H, d, J=7.1 Hz, H-1"'), 3.90 (1H,m, H-5'), 3.73 (1H, m, H-2"), 3.56 (1H,m,H-2"'), 3.52 (1H, m, H-3"), 3.49 (1H, m, H-4"), 3.46 (1H, m,H-4"'), 3.41 (1H,m, H-5"'), 3.20 (1H, d, *J*=9.0 Hz, H_2-6"a), 3.16 (1H, d, *J*= 8.5 Hz, H_2-6" b), 1.14(3H, d, *J*=7.2 Hz, Me-6"').
13C NMR (DMSO-d_6): δ 163.10 (C-2), 103.93(C-3), 181.69 (C-4), 161.34(C-5), 98.94 (C-6), 164.42 (C-7), 94.09 (C-8), 159.30 (C-9), 103.67 (C-10), 124.78 (C-1'), 113.55 (C-2'), 146.84 (C-3'), 148.39 (C-4'), 115.92 (C-5'), 118.44 (C-6'), 101.15 (C-1"), 73 .17 (C-2"), 70.66 (C-3"), 69.68 (C-4"), 75.76 (C-5"), 66.55 (C-17.84 (C-6"').
EIMS *m/z* (rel. int.): 594 $[M]^+$ ($C_{27}H_{30}O_{15}$) (5.1), 308 (15.2), 285 (13.2), 176 (12.8), 162 (14.1), 147 (11.8), 109 (10.6).

Compound 3 (CM3):
Elution of column with petroleum ether - chloroform (7:3) furnished colourless crystalline mass (800 mg) of CM-3.
IR υ_{max} (KBr) : 3425, 2920, 2852, 1641, 1463, 1373, 1225, 1173, 801 cm^{-1}
^{1}H NMR ($CDCl_3$) : δ 5.28 (1H, m, H-6), 5.08 (1H, m, H-22), 4.95 (1H, m, H-23), 3.45 (1H, brm, $w_{1/2}$= 16.5 Hz, H-3α), 2.23 to 1.01 (25 H, m, 9 x CH_2. 7 x CH), 1.13 (3H, brs, Me-19), 0.97 (3H, d, J=6.3 Hz, Me-21), 0.84 (3H, d, *J*=6.6 Hz, Me-26), 0.75 (3H, d, J=6.0 Hz, Me-27), 0.73 (3H, d, J=6.6 Hz, Me-29), 0.62 (3H, brs, Me-18)
^{13}C NMR ($CDCl_3$): δ 36.52 (C-1), 31.90 (C-2), 71.81 (C-3), 41.90 (C-4), 140.75 (C-5), 121.72 (C-6), 31.66 (C-7), 33.94 (C-8), 51.24 (C-9), 37.26 (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 $[M]^+$ ($C_{29}H_{48}O$) (76.2), 396 (51.2), 394 (41.3), 381 (215.2), 271 (38.2), 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, peroxisomal oxidases and NADPH oxidases (Aprioku, 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

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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 other 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-rhamno-pyranoside with potential anti-oxidant effect of ethanol extract.

Compound 1 (CM 1)

The IR spectrum of CM1 showed absorption bands for hydroxyl groups (3410 and 3227 cm^{-1}) and unsaturation (1629 cm^{-1}). On the basis of mass and ^{13}C NMR spectra the molecular ion peak of CM1 was determined at m/z 344 consistent with the molecular formula of a tetrahydroxy diterpene, $C_{20}H_{40}O_4$. The ion peaks arising at m/z 271 [C_{13}-C_{14} fission, M- $C_4H_9O]^+$, 245 [C_{11}-C_{12} fission, M - $C_6H_{11}O$]+ and 231 [C_{10}-C_{11} fission]$^+$ suggested the presence of one hydroxyl group at C_{14} and vinylic linkage at $C_{12(13)}$. The ion fragments generated at m/z 203 [C9-C10 fission, $C_{11}H_{23}O_3]^+$, 141[M-203, $C_9H_{17}O]^+$, 185 [C_7-C_8 fission, $C_{11}H_{21}O_2]^+$, 215 [C_6-C_7 fission, $C_{12}H_{23}O_3]^+$, 243 [C_5-C_6 fission]$^+$ and 273 [C_4-C_5 fission]$^+$ indicated the existence of hydroxyl groups at C_9, C_7 and C_5 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 ($w_{1/2}$= 9.3 Hz), 3.70 ($w_{1/2}$= 12.8 Hz) and 3.21 ($w_{1/2}$= 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 1.18 (*J*= 6.1 Hz), 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 ^{13}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, eight methine and one quaternary carbon. The ^1H-^1H COSY spectrum of CM1 exhibited interactions of H-5 with H_2- 4, H-6 and H-18, H-9 with H-7, H_2-8, H-10 and Me – 19; H-12 with H-10, H_2-11 and H-13; and Me - 2 with H-13, H_2- 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_2-11 with C-19; and H-12, H13, Me-20, H_2-15 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).

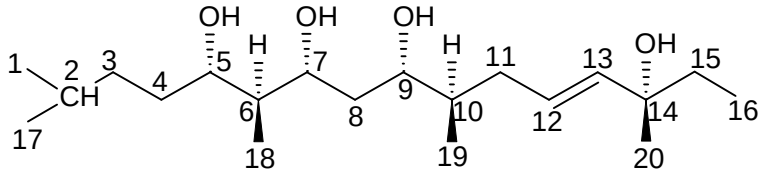

Figure 1a: (CM1): 2, 6, 10, 14-tetramethylhexadec-12-cis-en-5α, 7α, 9α, 14α-tetraol

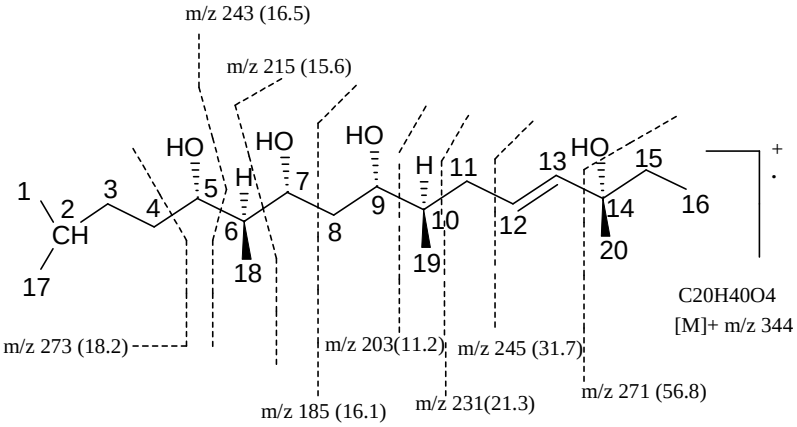

Figure 1b: Mass fragmentation pattern of CM-1

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

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cm^{-1}), conjugated carbonyl group (1654 cm^{-1}) and aromaticity (1506, 1066 cm^{-1}). A one-proton singlet at δ 6.20 and the corresponding upfield vinylic carbon signal at δ 103.93 suggested a flavone skeleton (Mabry et al., 1970; Salim et al. 2006). On the basis of mass and ^{13}C NMR spectra the molecular ion peak of CM-2 was determined at m/z 594 corresponding to molecular formula of a flavones diglycoside, $C_{27}H_{30}O_{15}$. The ion peaks arising at m/z 308 $[M - C_{12}H_{20}O_9]^+$ and m/z 285 $[M - C_{15}H_9O_6]^+$ showed the presence of two sugar moieties attached to the flavones nucleus. The ^1H NMR spectrum of **CM-2** exhibited three-one proton doublets at δ 7.24 (1H, d, J = 7.2 Hz), 6.79 (1H, d, J= 1.2 Hz), 6.51 (1H, d, J=1.2 Hz) assigned to ortho-coupled H-5′ and meta-coupled H-8 and H-6 aromatic protons, respectively, a two-proton multiplet at δ 7.49 ascribed to H-2′ and H-6′ protons 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 anomeric 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_2-6"a and H_2-6"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 ^{13}C NMR spectrum of **CM-2** revealed 27 signals, 15 of which were typical of a flavone 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, anomeric 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_2-6″ 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 ^{13}C 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-^1H 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_2-6″; 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_2-6″, 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_3-6‴ with C-1‴. The ^1H and ^{13}C 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-rhamnose, 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.**

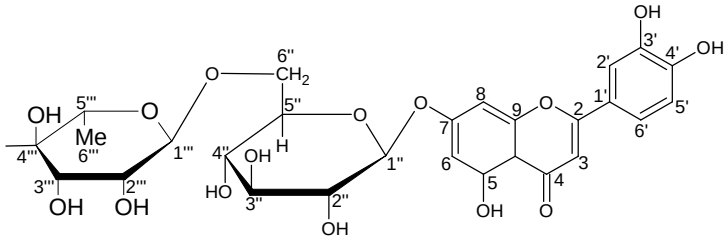

Figure 2: (CM2): Luteolin-7-O-β-glucopyranosyl-6"-O-(6"→1"')-β-D-rhamno-pyranoside

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 ^{13}C NMR spectra, its molecular weight was established as 412 consistent with the molecular formula of a sterol, $C_{29}H_{48}$ O. The prominent ion peaks generated at *m/z* 396 $[412- Me]^+$, 394 $[412-HOH]^+$, 271 $[412-C_{10}H_{19}$,side chain$]^+$, 255 $[271-Me]^{+\cdot}$ 240 $[255-Me]^+$, 213 [255-ring D fission]$^+$ and 198 $[213-Me]^+$ suggested that was a stigmastene-type sterol containing a C_{10} 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 vinylic 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 methylene and methine protons resonated between δ 2.23-1.01. The ^{13}C 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 and methine carbons between δ 56.87-11.87. The ^1H NMR and ^{13}C 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-^1H COSY spectrum of CM3 showed correlations of H-3 with H_2-2 and H_2-4; H-6 with H_2-4, H_2-7 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_2-4 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 spectral data analysis the structure of CM3 has been established stigmast-5,22–dien-3*β* –ol. Authors are reporting the presence of all these compounds in *C. maximus* for the first time.

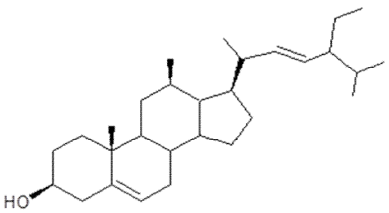

Figure 3: (CM3): **stigmast-5, 22–dien-3*β* –ol**

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

1. Ahmad, A., Kim, S.H., Ali, M., Park, I., Kim, J.S., Kim, J.S., Kim, E.H., Lim, J.J., Kim, S.K. and Chung, I.M. (2013). New chemical constituents from *Oryza sativa* straw and their algicidal activities against blue-green algae. Agric. Food Chem., 61, 8039-8048.
2. Akkal, S., Benayache, F., Benayache, S. and Jay, M. (1997). Flavoniods from *Centaurea incana* (Asteraceae). Biochem. Syst. & Eco., 25(4), 61-362.
3. Alfadda, A.A., and Sallam, R.M. (2012). Reactive oxygen species in health and disease. J. biomed. & biotech. 2012, Article ID 936486.
4. Alisi, C. S. and Onyeze, G.O. (2008). Nitric oxide scavenging ability of ethyl acetate fraction of methanolic leaf extracts of *Chromolaena odorata* (Linn.). African Journal of Biochemistry Research, 2 (7), 145-150.
5. Andarwulan, N.R., Batari, D.A., Sandrasari, B., Bolling, H. and Wijaya, A. (2010). Total phenolics and Antioxidant activity of *Pluchea indica*. Food Chem., 12, 34.
6. Aprioku, J.S. (2013). Pharmacology of free radicals and the impact of reactive oxygen species on the testis. J. reproduction & infertility 14(4), 158-72.
7. Basahi, M. A., Thomas, J., Al-Turki, T. A., Alfarhan, A. H., Al-Atar, A. A., Sivadasan, M. (2010). Current status and conservation efforts of *Centaurothamnus maximus* Wagenitz & Dittrich (Asteraceae), an endemic, endangered species from Saudi Arabia. Plant Archives, 10(2), 807-814.
8. Brand-Williams, W., Cuvelier, M.E. and Berset, C. (1995). Use of free radical method to evaluate antioxidant activity. Lebenson Wiss Technol, 28, 25-30.
9. Ciric, A., Karioti, A., Koukoulitsa, C., Sokovic, M. and Skaltsa, H. (2012). Sesquiterpene lactones from *Centaurea zuccariniana* and their antimicrobial activity. Chem. Biodivers., 9(12), 2843-53.
10. Collenette, S. (1999). Wildflowers of Saudi Arabia. The National Commission for Wildlife Conservation and Development (NCWCD), Riyadh, Saudi Arabia.
11. Guo, Y., Li, Y., Xu, J., Watanabe, R., Oshima, Y., Yamakuni, T. and Ohizumi, Y. (2006). Preliminary Phytochemical and Antimicrobial screening of *Bucharrisgaudichaudiana*. J. Nat. Prod., 69, 274.
12. Iwashina, T., Kadota, Y., Ueno T. and Ootani S. (1995). Foliar favonoid composition in Japanese *Circium* species (Compositae) and their chaemotaxonomic significance. J. Jap. Bot., 70, 280-289.
13. Jung, W.S., Chung, I.M., Ali, M. and Ahmad A. (2012). New steroidal glycoside ester and aliphatic acid from the fruits of *Lycium chinensis*. J. Asian Nat. Prod. Res. 14 (4), 301-307.
14. Ling, Y.R. (1992). Chemotaxonomy of *Artemisia L*. Comp. Newsletter, 22, 18-23.
15. Mabry, T.J., Markham, K.R. and Thomas, M.B. (1970). The systematic Identification of flavonoids, Springer-Verlag, New York, pp 140147, 267-268.
16. Markham, K.R. (1982). Techniques in Flavonoid Identification, Academic Press, London, UK, Pp 36-45.
17. Marwah, R.G., Fatope, M.O., Mahrooqi, R.A., Varma, G.B. and Abadi, H. (2007). Total phenolics and Antioxidant activity of *Pluchea arabica*. Food Chem., 101, 465.
18. Masood, S.R.A., Iqbal, Z., Mansoor, M.K., Sindhu, Z.U.D., Zia, M.A. and Khan, J.A. (2013). Role of natural antioxidants for the control of coccidiosis in poultry. Pak. Veterinary J. 33(4), 401-407.
19. Mothana, R.A.A., Gruenert, R., Bednarski, P. J. and Lindequist, U. (2009). Evaluation of the *in vitro* anticancer, antimicrobial and antioxidant activities of some Yemeni plants used in folk medicine. Pharmazie, 64(4), 260-268.
20. Muhammad, I., Takamatsu, S., Mossa, J.S., El-Feraly, F.S., Walker, L.A. and Clark, A.M. (2003). Cytotoxic Sesquiterpene Lactones from *Centaurothamnus maximus* and *Vicoa pentanema*. Phytother. Res., 17(2), 168-73.

http://dx.doi.org/10.4314/ajtcam.v12i3.5

21. Mustafa, A. and Ali, M. (2011). New steroidal lactones and homomonoterpenic glucoside from fruits of *Malva sylvestris* L. Acta Poloniae Pharmac. Drug Res., 68 (3), 393-401.
22. Salim, M., Kim, H.J., Han, C.K., Jin, C. and Lee, Y.S. (2006). Phytochemistry, 67, 1390-1394, 2006.
23. Tzakou, O., Couladis, M., Verykokidou E. and Loukis, A. (1995). Leaf Flavonoids of *Achillea ligustica* and *Achillea holosericea*. Biochem. Syst. & Eco., 23(5), 569-570.
24. Valant-Vetscheraa, K. M. and Wollenweberb, E. (2007). Chemodiversity of Exudate Flavonoids in Seven Tribes of Cichorioideae and Asteroideae (Asteraceae). Z. Naturforsch., 62c, 155-163.
25. Waffo, A.F.K, Coombes, P.H., Mulholland, D.C., Nkengfaek, A.E. and Fomum, Z.T. (2006). Flavones and isoflavones from the west African Fabaceae *Erythrina vogelii*. Phytochemistry, 67, 459-463