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Phytochemical Investigation and Characterization of Isolated Chemical Constituents from *Kyllinga triceps* Rottb.

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Four compounds have been isolated by column chromatography from *Kyllinga triceps* namely quercetin dihydrate (**1**), rutin (**2**), β-sitosterol (**3**) and stigmasterol (**4**). Their structures have been elucidated by, FTIR, HR-EIMS, ^{1}H NMR and ^{13}C NMR spectroscopic studies.

Keywords: Column chromatography.

INTRODUCTION

Plants make a significant contribution to health care due to their recognition in traditional medicinal systems [1]. Herbal medicine are supposed to be safer than synthetic counterparts because the phytochemicals, in the plant extract, target the biochemical pathway [2]. *Kyllinga triceps* is a herb belonging to family Cyperaceae. It is a small genus of annual or perennial herbs, distributed in the tropical and sub tropical region of the world. About eight species occur in india. *Kyllinga triceps* Rottb. Is a small tufted herb, up to 12 inches high with a short rhizome and linear leaves, one half or nearly as long as the stem, found in Kumaon at altitudes of 5000-6000 ft. and from the upper gangetic plain to West Bengal, Sundarbans and Deccan Peninsula. The plant has the same vernacular name as *Kyllinga monocephala* [3].

Traditionally the decoction of roots is used as refrigerant, demulcent and tonic. It is given to relieve thirst in fevers and in diabetes. It is also used as antidote to poisons. Oil distilled from the root is used to relieve pruritus of the skin, internally, oil is given in torpor of the liver [4]. Synonym of *Kyllinga triceps* is *Kyllinga tenuifolia* and *Cyperus triceps* (Rottb.) [5]. Fresh juice of the plant is used externally to wash the wounds. It is used in the treatment of indigestion. The oil of roots is used to promote the action of liver and relieve pruritus [6]. Decoction of roots is used in diabetes and to relieve thirst in fever. The literature reveals that no isolation work by column chromatography has been done on *Kyllinga triceps*.

In present we wish to report some of the phyto-constituents from whole plant of *Kyllinga triceps* which could be regarded as the ever identified compound.

EXPERIMENTAL

Fresh plant of *Kyllinga triceps* was collected in the month of April, 2011 from Venkateswara University, Tirupati, India. The plant was identified and authenticated by Dr. K. Madhava Chetty, Asst. Professor, Department of Botany, Sri Venkateswara University, Tirupati, Andhra Pradesh, India. A voucher specimen has been deposited at the College of Pharmacy, TMU Moradabad (TMU/Consult/2011-12/206) for future reference.

Preparation of the extract and isolation: The air-dried whole plant of *Kyllinga triceps* (1 kg) was defatted with *n*-hexane and petroleum ether followed by extraction with chloroform, ethyl acetate and *n*-butanol using Soxhlet extractor [7]. The resulting extracts were tested for the presence of flavonoids by Shinoda test [8]. Ethyl acetate fraction was found to have the highest amount of flavonoids [9]. Systematic fractionation of ethyl acetate fraction resulted in the isolation of two constituents designated as compound **1**, compound **2** and petroleum ether extract was obtained as dark green oily mass [10]. The defatted material was extracted further with solvents of increasing polarity. Systematic fractionation of petroleum ether extract resulted in isolation of two constituents designated as compounds **3** and **4** [11].

Structural determination of compound 1: Compound **1** was isolated from ethylacetate extract of *Kyllinga triceps*. It was crystallized in methanol as a yellow crystalline powder having melting point 313-316 °C. It is slightly soluble in water and diethyl ether and soluble in methanol, ethanol and acetone [12]. Chromatography was performed on precoated silica gel G plates and spots were detected by spraying it with $FeCl_3$ (2 %) in ethanol. The R_f value recorded in two solvent systems is given below.

Solvent system	R_f value
Chloroform:ethyl acetate:formic acid (5:4:1)	0.76
n-Butanol:acetic acid:water (4:1:5 upper layer)	0.82

The spots appeared blue on spraying it with $FeCl_3$ and in iodine chamber the spots appeared yellowish brown giving a clue that compound is containing phenolic group. The compound gave a positive Shinoda test thus strengthening the assumption that it is a flavonoid compound.

The UV spectrum of the compound **1** showed two major absorption band at 374.8 and 257.5 which gives an idea of its flavonol structure. The bathochromic shift in the UV was observed with sodium acetate at 391.6 and 258.4 (were related of 7 OH group of the flavonoid quercetin) and with aluminium chloride was found to be 435.4 and 270.4 (related to 5 OH group), hypsochromic shift was observed with boric acid at 342.6 and 236.8 nm, confirms the presence of 3,3',4' OH group depicted in Table-1.

TABLE-1
EFFECT OF SHIFT'S REAGENT ON
THE ABSORPTION MAXIMA OF COMPOUND **1**

Shift's reagent	Band-I	Shift in band-I	Band-II	Shift in band-II
Quercetin (Q)	374.8		257.6	
Q + MeONa	426.6	51.8	285.8	2.2
Q + $AlCl_3$	435.4	60.6	270.4	-5.4
Q + HCl	365.0	-9.8	255.2	-2.4
Q + Boric acid	342.6	-32.0	236.8	-20.8
Q + AcONa	391.6	16.8	258.4	0.8
Q + NaOH	332.6	-42.2	233.8	-23.6

The IR spectrum of the isolated compound **1** (KBr), presented various bands mentioned in the Table-2.

TABLE-2
IR SPECTRA OF COMPOUND **1**

Wavenumber (cm^{-1})	Functional groups and Bonds
3582	O-H str. free non bonded
3400, 3321	O-H str. vibrational bond in phenol
1650	C=O Aryl ketonic structure
1611, 1522, 1462	C=C aromatic ring streatching (1650-1450)
1327	C-H in-plane bending of aromatic ring
1254, 1209, 1002	C-O str. of phenol, strong band (1260-1000 cm^{-1})
1125	C-O-C str. of aryl ether (1085-1150)
839, 791, 721	Out of plane C-H bending (900-675)
1170	C-(C=O)-C str. and bend vibrations (1300-1100)

The ^{1}H NMR (Chemical shift δ in ppm, coupling constant *J* is in Hz) spectrum (300 MHz, DMSO-d_6) recorded for Cc#1 depicted following δ values 6.07 (1H, d, *J* = 2.1, C6-H), 6.29 (1H, d, *J* = 1.8, C8-H), 7.55 (1H, d, *J* = 2.1, C2'-H), 6.75-6.78 (1H, d, *J* = 8.4, C5'-H), 7.40-7.44 (1H, dd, *J* = 8.7, 2.1, C6'-H), 9.2 (1H, s, C-4'-OH), 9.1 (1H, s, C3'-OH), 9.4 (1H, s, C3-OH), 12.36 (1H, s, C-5, OH), .10.69 (1H, s, C7-OH).

^{13}C NMR (Chemical shift in ppm) represents 15 carbons. The ^{13}C NMR of compound **1** represents 10 quarternary atoms and 5-CH carbons. The multiplicity and chemical shift of different carbons of compound **1** matches with the existing literature for quercetin dihydrate is shown in Table-3.

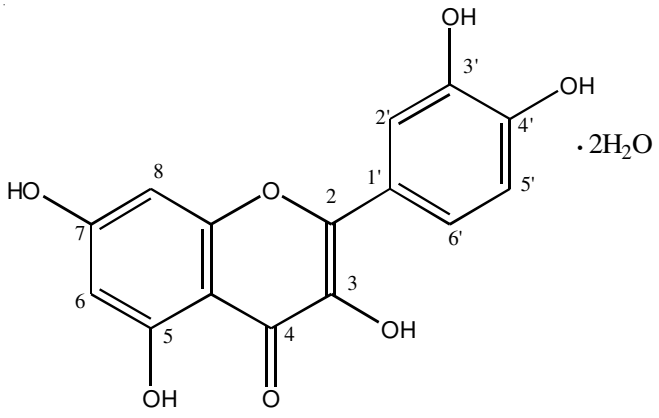
TABLE-3
^{13}C NMR SPECTRA VALUES OF COMPOUND **1**

Position	Signal	Multiplicity	Position	Signal	Multiplicity
2	146.0	-C-	10	103.2	-C-
3	135.5	-C-	1'	122.2	-C-
4	175.0	-C-	2'	115.3	-CH-
5	156.0	-C-	3'	144.9	-C-
6	92.8	-CH-	4'	147.4	-C-
7	164.8	-C-	5'	114.8	-CH-
8	93.5	-CH-	6'	120.5	-CH-
9	161.4	-C-	–	–	–

Mass spectrum of isolated compound **1** (molecular weight 302.2) recorded prominent peak at 301 ($M-H^+$), which on further fragmentation yields prominent fragments weighing 273, 178.8, 150 and 107. Which matches with the existing literature for quercetin dihydrate and thus the compound is quercetin dehydrate (Table-4).

TABLE-4
MASS SPECTRUM DATA OF COMPOUND **1**

m/z	Name of the fragment
301.1	$M-H^+$ Base peak
273.1	($M-H^+$ -CO)
178.8	(301-$C_7H_6O_2$)
150.0	(301- $C_7H_4O_4$)
107.1	$C_7H_6O_2$-CO-CO_2



Structure of quercetin dihydrate

HPLC analysis of the isolated compound **1** was performed by Shimadzu high performance liquid chromatographic system, LC 2010CHT with UV & PDA detector in combination with Class LC solution software. Kromasil C18, 5μ (250 × 4.6 mm) column was used [13]. Gradient elution was performed using anhydrous sodium dihydrogen orthophosphate buffer and acetonitrile [14]. Flow rate was maintained at 1.5 mL/min

and injection volume was 20.0 µL and detection wavelength was 370 nm [15]. The gradient flow was maintained as given below in the Table-5.

TABLE-5
GRADIENT ELUTION OF COMPOUND **1** WITH POTASSIUM DIHYDROGEN ORTHOPHOSPHATE BUFFER AND ACETONITIRILE

Time	Volume of buffer	Volume of acetonitrile
0	95	5
18	55	45
25	20	80
28	20	80
35	55	45
40	95	5
45	95	5

Retention time of compound **1** was observed at 16.568 min. with a peak area of 5546254 which occupies 99.065 %. Table-5 and chromatogram proving it a pure entity which matches with the retention time of quercetin.

Structural determination of compound 2: Compound **2** was isolated from ethyl acetate extract of *Kyllinga triceps*. It was crystallized in methanol as a yellow powder, soluble in water and methanol (under hot conditions) and insoluble in chloroform, acetone and ether. The melting point was found to be 242-245 °C. Chromatography was performed on pre-coated silica gel 60 F254 plates and spots were detected by spraying it with 1 % ethanolic aluminium chloride. Yellow fluorescence was observed under UV 365 nm [16]. The R_f value recorded in two solvent systems is given below.

Solvent system	R_f value
Methanol:water:acetic acid (50:50:6)	0.63
Ethyl acetate:water:formic acid (6:1:1)	0.69

The glycoside was first eluted with two dimensional TLC with ethyl acetate:water:formic acid (6:1:1) in first direction. The layer was dried at 100 °C for 15 min, the separation zone was sprayed with 5 M sulphuric acid in water:methanol (1:1) and the plate is kept at 110 °C for 40 min to hydrolyze the glycoside. After cooling elution in second direction was done with ethyl acetate:2-butanol:water (60:25:5). Plates were dried at 80 °C for 15 min and then sprayed with saturated solution of ethylenediammoniumsulphate in acetone:water (1:1). The sugar under these conditions give rise to fluorescent blue spots which may be observed under UV (365 nm) [17].

The compound in methanol gave two significant absorption band at 357.6 nm (band-I) and 257.6 (band-II) which gives an idea of flavonol structure. Significant bathochromic shift in the absorption maxima is observed with sodium methoxide, aluminium chloride and and sodium hydroxide hypsochromic shift is observed only with HCl [18]. The shift in the absorption maxima was observed on treating it with shift reagents which is given in Table-6.

The IR spectra of the isolated compound was performed with KBr disc method and various bands observed are mentioned in the Table-7.

^{1}H NMR (chemical shift δ in ppm, coupling constant *J* is in Hz) spectrum (300 MHz, DMSO-d_6) recorded as 6.0-6.08 (1H, d, *J* = 1.8, C6-H), 6.26-6.27 (1H, d, *J* = 2.1, C8-H), 7.41-7.43 (1H, d, *J* = 1.8, C2'-H), 6.71-6.73 (1H, d, *J* = 8.4, C5'-H), 7.41-7.44 (1H, dd, *J* = 8.8, 2.2, C6'-H), 9.56 (1H, s, 4'-OH), 9.06 (1H, s, 3'-OH)12.47 (1H, s, C-5, OH), 10.74 (1H, s, C-7 OH), 5.23 (1H, d, *J* = 3.9, H1-G), 5.12 (1H, d, *J* = 1.6, H1-R), 0.8 (3H, *J* = 5.8, CH_3-R).

^{13}C NMR (chemical shift in ppm) displayed the signals for various carbons of the compound **2** (DMSO-d_6) where there are One CH_3, one CH_2, 14 CH and 11 quarternary carbons, The assignment of chemical shift was facilitated on comparison with ^{13}C NMR data of rutin available in the literature. The chemical shift of various carbons is given in Table-8.

TABLE-6
EFFECT OF SHIFT'S REAGENT ON THE ABSORPTION MAXIMA OF COMPOUND **2**

Shift's reagent	Band-I (nm)	Shift in band-I (nm)	Band-II (nm)	Shift in band-II (nm)
Rutin (R)	357.6	–	257.6	–
R + MeONa	410.6	53.0	273.4	15.8
R + $AlCl_3$	428.0	70.4	275.0	17.4
R + HCl	359.6	2.0	257.2	-0.4
R + Boric acid	363.6	6.0	258.0	0.4
R + AcONa	385.4	1.8	274.2	16.6
R + NaOH	411.2	53.6	274.4	16.8

TABLE-7
IR SPECTRA OF COMPOUND **2** isolated from *Kyllinga triceps*

Wavenumber (cm^{-1})	Functional groups and bonds
3592	O-H str. (non-bonded, free) vibrational band (3700-3584)
3408, 3321	O-H str. bonded (3550-3200)
1663	C=O aryl ketonic structure (1685-1666), frequency reduced due to conjugation and intramolecular H bonding
1611, 1561, 1521	C=C aromatic ring stretching (1650-1450)
1321	C-H in-plane bending of aromatic ring
1199, 1013	C-O str. of phenol, strong band (1260-1000 cm^{-1})
1150	C-O-C str. of aryl ether (1085-1150)
865, 823, 723, 685	Out of plane C-H bending (900-675)
1264	C-(C=O)-C str. and bend vibrations (1300-1100)

TABLE-8
^{13}C NMR SPECTRAL DATA OF COMPOUND **2** ISOLATED FROM *Kyllinga triceps*

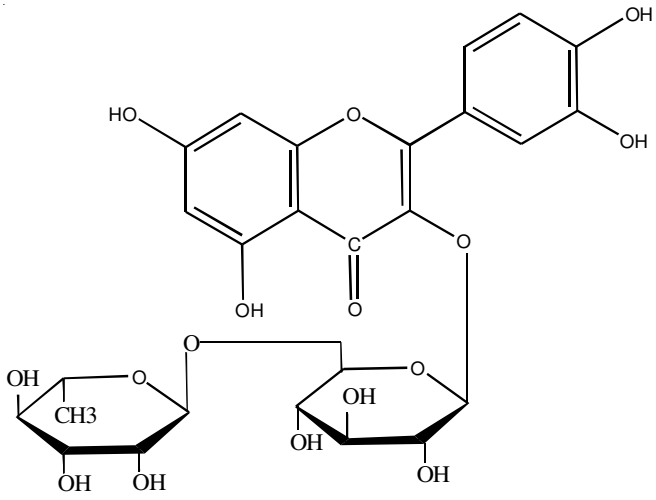
Chemical shift (δ scale)	Carbon No.	Multi-plicity	Chemical shift (δ scale)	Carbon No.	Multi-plicity
157.06	C-2	-C-	121.60	C-6'	-CH-
133.70	C-3	-C-	101.60	C1-G	-C-
177.80	C-4	-C-	74.50	C2-G	-CH-
156.80	C-5	-C-	76.90	C3-G	-CH-
99.10	C-6	-CH-	72.30	C4-G	-CH-
164.50	C-7	-C-	76.30	C5-G	-CH-
94.03	C-8	-CH-	67.40	C6-G	$-CH_2-$
161.60	C-9	-C-	101.60	C1-R	-CH-
104.40	C-10	-C-	70.80	C2-R	-CH-
122.04	C-1'	-C-	71.03	C3-R	-CH-
115.60	C-2'	-CH-	70.48	C4-R	-CH-
145.20	C-3'	-C-	68.69	C5-R	-CH-
148.70	C-4'	-C-	18.18	C6-R	$-CH_3$
116.70	C-5'	-CH-	–	–	–

The mass spectrum of the compound **2** on fragmentation gave the following fragments shown in Table-9.

TABLE-9
MASS FRAGMENTATION DATA OF COMPOUND **2**

m/z	Name of the fragment
609.15	$M-H^+$ Base peak
596.00	$M + H^+ - (CH_3)$
463.00	$M^+ - (C_6H_{10}O_4)$
325.00	$M^+ - (C_{15}H_9O_6)$
300.00	$M^+ - (C_{12}H_{21}O_9)$glycone part)

$M-H^+$ and $M+H^+$ are the most abundant and characteristic peaks in flavonols, compound **2** seems to be rutin as the mass fragmentation pattern and presence of base peak at 609.15 ($M-H^+$) on matching with the existing literature increases the probability of the compound to be rutin.



Structure of rutin

The identity of the isolated compound **2**, which may be rutin was further confirmed by HPLC using Shimadzu high performance liquid chromatographic system LC 2010CHT with UV detector. Kromasil C18 column was used. Gradient elution was conducted using potassium dihydrogen orthophosphate buffer (A) and acetonitrile (B). The buffer (A) was prepared by taking 0.140 g of anhydrous potassium dihydrogen orthophosphate (KH_2PO_4) dissolving it in 900 mL of HPLC grade water and adding 0.5 mL of orthophosphoric acid and making volume up to 1000 mL with water. The solution was filtered through 0.45 µ membrane filter and degassed in a sonicator for 3 min. HPLC grade acetonitrile was used. Flow rate was kept at 1.6 mL/min and detection wavelength was maintained at 258nm and injection volume was 20 µL. About 5 mg of the sample was weighed to a in a 25 mL volumetric flask. About 10 mL of methanol was added and sonicated cooled and made up to 25 mL with methanol. The gradient flow was maintained as given in Table-10.

The retention time obtained was 3.257 min with a peak area of 4113012 and peak area % of 98.347.

Structural determination of compound 3: Compound **3** was isolated from petroleum ether extract of the entire plant of *Kyllinga triceps*. Petroleum ether extract is first treated with alcoholic KOH to saponify the fats and washed again with petroleum ether to purify the remaining extract. This extract is further subjected to column chromatography using gradient elution with toluene and methanol, homogeneity was established by TLC studies over precoated silica gel G plates and the spots were developed by either exposing the plates to iodine vapours or spraying them with 5 % ethanolic sulphuric acid followed by heating the plates at 110 °C for 10 min. The R_f values recorded in two different solvent systems are given below.

TABLE-10
GRADIENT ELUTION OF RUTIN WITH POTASSIUM DIHYDROGEN ORTHOPHOSPHATE BUFFER AND ACETONITIRILE

Time (min)	Volume of buffer	Volume of acetonitrile
0	80	20
5	75	25
8	70	30
10	70	30
12	80	20
15	80	20
15	Stop	

Solvent system	R_f value
Benzene:chloroform (2:1)	0.64
Toluene:chloroform:methanol (1:1:0.1)	0.57

Compound **3** was found to be soluble in organic solvents like petroleum ether, benzene, dichloromethane, chloroform and methanol but insoluble in water. The melting point was found to be 160-166 °C [19].

The IR spectrum of the compound **3** gave the following peaks mentioned in Table-11.

TABLE-11
IR SPECTRA OF COMPOUND **3** ISOALTED FROM *Kyllinga triceps*

Wavenumber (cm^{-1})	Functional groups and bonds
3337.9	O-H str. vibrational band of bonded –OH
2962	CHunsymmetrical str., of steroidal ring and tertiary CH group
2872	C-H symmetrical str.
1453	$-CH_2$ cyclic bending vibration
1374	OH in plane bending vibration
1054, 965	C-H in plane bending of cycloalkenes
836	C-H out of the plane bending vibration

It is observed that with acetic anhydride and triethylamine at room temperature isolated compound afforded an acetate derivative, m.p., 143-145 °C. Thus compound **3** appeared to be stigmasterol and this assumption was confirmed by recording and analyzing ^{1}H NMR, ^{13}C NMR and mass spectrum of compound **3** [19].

The ^{1}H NMR (chemical shift δ in ppm, coupling constant *J* is in Hz) spectrum (300 MHz, $CdCl_3$) recorded as 1.12 (3H, t, *J* = 7.05, C-29), 0.83 (3H, d *J* = 5.1, C-27), 0.78, (3H, d *J* = 6.3, C-26), 0.96 (3H, d, *J* = 6.3, C-21), 2.21 (1H, m, C-20), 0.62 (3H, s, C-18)1.18 (3H, s, C-19), 5.12 (1H, dd, *J* = 8.4, 8.4, C-23), 4.98 (1H, dd, *J* = 8.4, 8.4, C-22), 5.28 (1H, t, *J* = 7.5, C-6), 3.45 (1H, m, C-3), for methine and methylene protons of steroidal ring and side chain.

^{13}C NMR (300 MHz, $CDCl_3$,) apart from other signals showed the presence of one oxy carbon (δ_c, 72.1) four olefinic

carbons (δ_C, 129.2, 138.3, 121.69, 140.76) and six methyl carbon (δ_C, 12.24, 18.9, 19.39, 19.39, 12.05, 21.2) the multiplicity and chemical shift of different carbons of compound **3** are shown in Table-12.

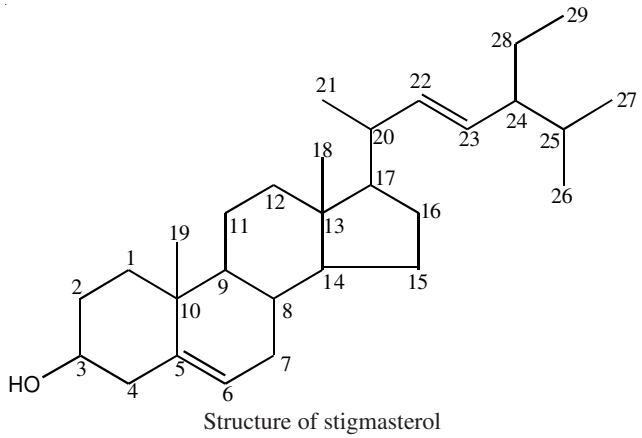
TABLE-12
^{13}C NMR SPECTRAL DATA OF COMPOUND **3**

Chemical shift (δ scale)	Carbon No.	Multi-plicity	Chemical shift (δ scale)	Carbon No.	Multi-plicity
12.05	18	CH_3	37.2	1	CH_2
12.24	29	CH_3	39.2	12	CH_2
18.98	27	CH_3	40.48	20	-CH
19.39	19	CH_3	42.32	13	-C-
21.07	11	CH_2	42.32	4	$-CH_2$
21.07	21	CH_3	50.18	9	-CH
21.2	26	CH_2	51.24	24	-CH
24.3	15	CH_2	55.97	17	-CH
25.4	28	CH_2	56.8	14	CH
28.9	16	CH_2	71.8	3	CH
31.6	2	CH_2	121.69	6	CH
31.6	7	CH_2	129.29	23	CH
31.9	8	CH	138.30	22	CH
31.9	25	CH	140.76	5	-C-
36.5	10	-C–	–	–	–

Mass spectroscopic fragmentation of the isolated compound **3** yielded M^+ at *m/z* 412, with other prominent fractions at *m/z* 395 ($C_{29}H_{47}$), 255 ($C_{19}H_{29}O$), 311 ($C_{23}H_{35}$), 297 ($C_{22}H_{33}O$) and 233 ($C_{17}H_{26}O$). Fragmentation pattern is shown in Table-13.

TABLE-13
MASS FRAGMENTATION DATA OF ISOLATED COMPOUND **3**

m/z	Name of the fragment
412	M^+ molecular ion peak
395	$(M^+$-OH)
311	$(M^+$-OH-$C_6H_{13})$
297	$(M^+$-H)- C_7H_{14}
255	M^+ - $(C_{10}H_{19})$
233	$(M^+$-H)- C_7H_{14}-C_5H_7



Structure of stigmasterol

The identity of the isolated compound **3** which may be stigmasterol was further confirmed by HPLC using Shimadzu high performance liquid chromatographic system LC 2010CHT with UV detector, Kromasil C18 column was used, gradient elution was conducted using toluene (A) and methanol (B). Flow rate was maintained at 2 mL/min and detection wavelength was maintained at 205 nm and injection volume was 20 µL. About 10 mg of the sample was weighed to a 10 mL volumetric flask.5 mL of methanol was added and sonicated cooled and volume was made up to 10 mL. Retention time of Compound **3** was found to be 6.089 min with peak area of 2581295 and peak % of 99.6 [20].

Structural determination of compound 4: Compound **4**, was isolated from petroleum ether extract of *Kyllinga triceps.* Petroleum ether extract was first treated with alcoholic potassium hydroxide to saponify the fatty acids which removed the fatty acids present in the extract. After removal of fatty acids, petroleum ether extract is subjected to gradient elution column chromatography using, toluene and methanol (95:5), 68 fractions were obtained. The fractions were concentrated and purified and subjected to identification through TLC by using various solvents. The spots of isolated compound was detected by exposing the plates to iodine vapours or observing the plates in UV light and also by spraying the plates with (5 %) sulphuric acid reagent in methanol followed by heating the plates at 110 °C for 10 min. The R_f value recorded in two different solvent system are given below.

Solvent system	R_f value
Chloroform: ethanol (9.6:0.4)	0.55
Chloroform: ethyl acetate (8.5:1.5)	0.63

Compound **4** was found to be soluble in organic solvents like petroleum ether, benzene, dichloromethane, chloroform and methanol but insoluble in water. The melting point was found to be 172-174 °C [21].

The IR spectrum of the compound **4** gave the following peaks mentioned in Table-14.

TABLE-14
IR SPECTRA OF COMPOUND **4**

Wavenumber (cm^{-1})	Functional groups and bonds
3476	O-H str. vibrational band of bonded –OH
2962	CH unsymmetrical str. of *geminal* dimethyl group
2831	CH str. of steroidal ring and tertiary CH group
1653	C=C str. of unconjugated alkene showing weak absorption band
1453	-CH bending vibration due to $-CH_2$ scissoring vibration
1379	O-H bending vibration
1077	-C-O stretching (1260-1000 cm^{-1})
953, 837	C-H in plane bending of cyclo alkenes (1000-650 cm^{-1})

It is observed that with acetic anhydride and triethylamine at room temperature isolated compound afforded an acetate derivative. Thus compound **4** appeared to be a steroidal compound and this assumption was confirmed by recording and analyzing ^{1}H NMR, ^{13}C NMR and mass spectrum of compound **4**.

The ^{1}H NMR (chemical shift δ in ppm, coupling constant *J* is in Hz) spectrum (300 MHz, $CdCl_3$ recorded as 0.80 (3H, t, *J* = 7.05, C-29), 0.84 (3H, d *J* = 6.3, C-27), 0.61 (3H, d *J* = 9.3, C-26), 1.10 (3H, d *J* = 9.3, C-21), 2.24 (1H, m, C-20), 1.18 (3H, s, C-19), 0.61 (3H, s, C-18), 5.27 (1H, t *J* = 5.4, C-6), 3.41 (1H, m, C-3), for methine and methylene protons of steroidal ring and side chain.

The ^{13}C NMR of compound **4** was recorded in $CDCl_3$ and the chemical shift of different carbon is mentioned in Table-15.

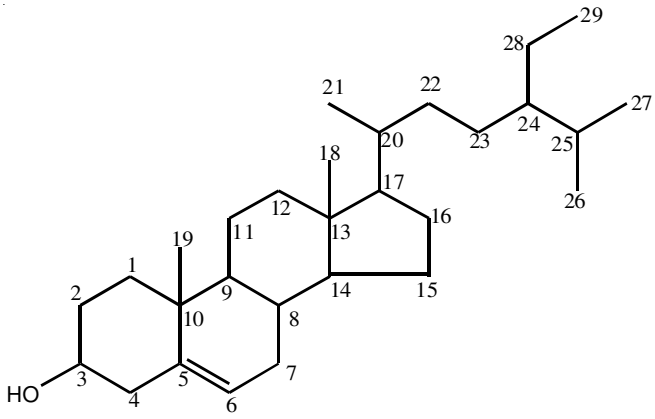
TABLE-15
^{13}C NMR SPECTRAL DATA OF COMPOUND **4**

Chemical shift (δ scale)	Carbon No.	Multi-plicity	Chemical shift (δ scale)	Carbon No.	Multi-plicity
11.84	18	CH_3	33.99	22	CH
11.97	29	CH_3	36.13	20	CH_2
18.77	21	CH_3	36.52	10	CH_2
19.04	27	CH_3	37.28	1	-CH
19.37	19	CH_2	39.81	12	-C-
19.78	26	CH_3	42.32	4	$-CH_2$
21.09	11	CH_2	42.30	13	-C-
23.11	28	CH_2	45.90	24	-CH
24.29	15	CH_2	50.19	9	-CH
26.19	23	CH_2	56.11	17	-CH
28.22	16	CH_2	56.80	14	CH
29.23	25	CH_2	71.80	3	CH
31.69	2	CH	121.68	6	CH
31.93	7	CH	140.78	5	CH
31.93	8	-CH	–	–	–

Mass spectroscopic fragmentation of the isolated compound **4** yielded M^+ at *m/z* 412, with other prominent fractions at *m/z* 395 ($C_{29}H_{47}$), 255 ($C_{19}H_{29}O$), 311 ($C_{23}H_{35}$), 297 ($C_{22}H_{33}O$), 233 ($C_{17}H_{26}O$). Fragmentation pattern is shown in Table-16.

TABLE-16
MASS FRAGMENTATION DATA OF ISOLATED COMPOUND **4**

m/z	Name of the fragment
414	M^+ molecular ion peak merged/$C_{29}H_{50}O$
397	$(M^+ - OH)/C_{29}H_{49}$ base peak
383	$(M^+ - OH - CH_3)/C_{28}H_{47}$
257	$(M^+ - OH)-C_{10}H_{21}/C_{19}H_{29}$



Structure of β-sitosterol

The identity of the isolated compound **4** which was found to be β-sitosterol was further confirmed by HPLC using Shimadzu high performance liquid chromatographic system with LC 8A pump and UV detector and polymer ELS detector in combination with class LC10A software. Kromasil RP C-8, 250 × 4.6 mm × 5 μ column was used. Isocratic elution was conducted using methanol:water (99:1). ELSD (evaporative light scattering detection) conditions includes nebulized temperature of 75 °C, evaporation temperature 90 °C and gas flow was maintained at 1.0 SLM. For sample preparation 10 mg of sample was dissolved in 5 mL of acetone, sonicated, slightly warmed at 40 °C for 0.5 min and made up to 10 mL and injected immediately.

Retention time of compound **4** was found to be 3.025 min with peak area of 946261 with peak % of 99.62.

RESULTS AND DISCUSSION

The four compounds were isolated (two from ethyl acetate fraction and two from petroleum ether extract) of *Kyllinga triceps*. Both the compounds isolated from ethyl acetate fraction gave positive Shinoda test for flavonoids and in TLC also. The compound gave blue spots on spraying it with ferric chloride thus confirming the phenolic nature of both the compound. Compound **1** was crystallized in methanol as a yellow crystalline powder having melting point 313-316 °C. It is slightly soluble in water and diethyl ether and soluble in methanol, ethanol and acetone. Compound **2** was crystallized in methanol as a yellow powder, soluble in water and methanol (under hot conditions) and insoluble in chloroform, acetone and ether. It was found to have melting point 242-245 °C.

Both the compounds when treated with shift reagent depicted bathochromic and hypsochromic shift which is the characteristic feature of flavonol glycosides.

IR spectroscopic analysis of compound **1** showed absorption band at 3582 cm^1 that is characteristic of free OH stretching, absorption bands at 3400, 3321 cm^1 are due to bonded OH group, absorption band at 1650 cm^{-1} is due to aryl ketone and the decrease in the absorption frequency is due to intramolecular hydrogen bonding between OH group of 3C, C=O group of 4C and OH group of 5 carbon. Absorption at 1611, 1522, 1462 is due to C=C aromatic stretching. Absorption at 839, 791, 721 cm^{-1} is quiet prominent due to out of plane (oops) C-H bending in aromatic ring.

Compound **2** gave various absorption bands which are 3592 cm^1 (free OH groups), 3408, 3321 cm^1 (due to bonded OH groups), 1663 cm^1 (aryl ketonic group representing intra molecular hydrogen bond), 1611, 1561, 1521 cm^1 (C=C aromatic ring stretching), 865, 823, 723, 685, cm^1 due to out of plane (oops) C-H bending in aromatic ring. The absorption bands were found in the same range so possibilities are that in both the compounds some of the bonds and functional groups are common. For further confirmation both the compounds were subjected to ^1H NMR, ^{13}C NMR and Mass spectroscopic analysis.

The ^1H NMR and data of compound **1** resembles with that of quercetin with singlets at δ value 9.2, 9.1, 9.4, 12.36, 10.69 for OH at C4', C3', C3, C5 and C7 respectively. Doublet were observed at δ value 6.07, 6.29, 7.55, 6.78 for protons at C6, C8, C2' and C5' and double doublet at 7.40-7.44 for, C-6'H, which matches with the existing literature for quercetin.

The ^1H NMR and data of compound **2** resembles with that of quercetin but some extra peaks were observed at at δ value 5.23 (for H1-G, glucose moiety), 5.12 (for H1-R, rhamnose moiety) and 0.8 for CH_3-R, which confirms that compound **2** is a glycoside of quercetin with glucose and rhamnose moiety attached with it. Other peaks of aglycone moiety resembling quercetin were observed as singlets at δ value 9.56, 9.06, 12.47, 10.74 for OH at C4', C3', C5 and C7

respectively. Doublet were observed at δ value 6.08, 6.27, 7.44 and 6.73 for protons at C6, C8, C2', C5'.and double doublet was also observed at 7.41-7.44 for, C-6'H, some more peak doublet which matches with the existing literature for rutin.

The value of ^{13}C NMR of compound **1** and **2** resembles closely with each other and with that of existing literature. The δ value of compound **2**, differs from that of compound **1** due the presence of carbons in the glucose and rhamnose moiety (Tables 3 and 8).

The retention time of isolated compound **1** was found to be 16.568 min and that of compound **2** was 3.257 min.

Mass spectrum of isolated compound **1** (molecular weight 302.2) recorded prominent peak at 301 ($M-H^+$), which on further fragmentation yields prominent fragments weighing 273, 178.8, 150 and 107. Which matches with the existing literature for quercetin dihydrate and thus the compound is quercetin dihydrate. Mass spectrum of isolated compound **2** ($M-H^+$ and $M+H^+$) are most abundant and characteristic peaks in flavonols. Compound **2** seems to be rutin as the mass fragmentation pattern and presence of base peak at 609.15 ($M-H^+$) on matching with the existing literature increases the probability of the compound to be rutin.

Compounds **3** and **4** were isolated from the petroleum ether fraction of *Kyllinga triceps* from which the fatty acids were saponified using alcoholic KOH and the remaining extract was further purified with petroleum ether and subjected to column chromatography using gradient elution technique. Both compounds **3** and **4** gave positive tests for steroids and alcohols so it was assumed that compound contains steroid nucleus along with a alcoholic functional group attached to it. Both the compounds were found soluble in organic solvents like petroleum ether, benzene, dichloromethane, chloroform and methanol but insoluble in water.

Compound **3** is a white crystalline substance with melting point 160-166 °C. The IR spectroscopic analysis showed the absorption band at 3337.9 cm^{-1}, which is characteristic of OH stretching. Absorptions at 2962 cm^1 and 2872 cm^1 is due aliphatic CH stretching. Other absorption frequencies include 1453 cm^1 as a result of bending frequency for cyclic $(CH_2)_n$ and 1374 cm^1 for OH in plane bending vibration. The absorption frequency at 1054, 965 cm^1 signifies –CH out of the plane bending of cycloalkene. The out of plane CH vibration of unsaturated part was observed at 881 cm^1. These absorption frequencies resemble the absorption frequencies observed for stigmasterol.

Compound **4** is a white crystalline substance with melting point 172-174 °C. On subjection to IR spectroscopic analysis, the observed absorption bands are 3476 cm^1 that is characteristic of OH stretching. Absorption at 2962 cm^1 and 2831 cm^1 is due aliphatic unsymmetrical and symmetrical CH stretching. Other absorption frequencies include 1653 cm^1 as a result of CH=CH stretching of unconjugated alkene showing a weak absorption band, 1453 cm^1 bending vibration due to $–CH_2$ scissoring vibrations, 1379 cm^1 is due to OH in plane bending vibration. The absorption frequency at 1077 is due to –C-O vibration of –C-OH group, 953, 837 cm^1 signifies –CH in plane cyclo alkenes. These absorption frequencies resemble the absorption frequencies observed for β-sitosterol. In case of compound **3** the proton NMR showed the proton at C-3 appeared as a multiplet at δ 3.41 and revealed the existence of signals for olefinic proton at C-6 (δ 5.28), C-22 (δ 4.98), C-23 (δ 5.12), angular methyl proton at C-18 (δ 0.62), C-19 (δ 1.18), were observed. In case of compound **4** the proton NMR showed the proton at C-3 appeared as a multiplet at δ 3.41 and revealed the existence of signals for olefinic proton at C-6 (δ 5.27), C=C is not present in C-22 and C-23, so it can be easily distinguished from another compound **3** which have prominent peaks downfield due to olefinic protons in this position. Angular methyl proton at C-18 (δ 0.61), C-19 (δ 1.18), were observed.

The ^{13}C NMR signals of compound **3** has shown recognizable signals 140.76 and 121.69 ppm, which are assigned C5 and C6 double bonds respectively as in Δ5 spirostene. The value at 19.39 ppm corresponds to angular carbon atom (C19). Spectra show twenty nine carbon signal including six methyls, nine methylenes, eleven methane and three quaternary carbons. The other alkene carbons appeared at C-22 (δ 138.3), C-23 (δ 129.29). The ^{13}C NMR signal of compound **4** has shown recognizable signals 140.78 and 121.68 ppm, which are assigned C5 and C6 double bonds respectively as in Δ5 spirostene which closely resembles compound **1**. The value at 19.37 ppm corresponds to angular carbon atom (C19). The values of C-22 (δ 33.99), C-23 (δ 26.19) differed from the compound **3** and thus proves that there is no double bond in the C-22 and C-23 position this is further strengthened by the ^1H NMR values at these carbons.

The weak molecular ions of compound **3** were given at *m/z* 412 and the characteristic peaks were given at *m/z* 395 that corresponds to (M^+-OH). Other ion peaks are *m/z* 311 due to (M^+-OH-C_6H_{13}), peaks at 297, 255 due to the formation of carbocation by β bond cleavage of side chain leading to the loss ofC_7H_{14} and $C_{10}H_{19}$ from molecular ion M^+, that corresponds to the M^+115 and M^+157. The molecular weight and fragmentation pattern indicate that the compound presenting this information is stigmasterol. The compound **4** on mass fragmentation gives a weak molecular ion peak M^+ at 414 which appear merged with 411, other prominent peaks appears at *m/z* 297 which is the base peak, most abundant fragment and is due to (M^+OH), other peak at *m/z* 383 and 257 are due to (M^+-OH-CH_3) and (M^+-OH)-$C_{10}H_{21}$. The data of this compound sindicates that the compound is β-sitosterol.

Further confirmation of both the compounds **3** and **4** was achieved by conducting the HPLC. For the compounds **3** gradient elution was done with toluene and methanol using UV detector and the retention time of 6.08 min was obtained. For compound **4** isocratic elution was done using methanol: water (99:1), ELS detector was used due to weak chromophoric group in this compound ELSD (evaporative light scattering detection) conditions includes nebulized temp of 75 °C, evaporation temperature 90 °C and gas flow was maintained at 1.0 SLM (standard liters per min).The retention time of 3.02 min was obtained which was found to be reproducible.

From the findings of IR, ^1H NMR, ^{13}C NMR and MS spectral data and their comparison with those described in the literatures showed that, compound **3** was stigmasterol and compound **4** was β-sitosterol both of them were isolated from petroleum ether extract of the *Kyllinga triceps* and chemical

structures elucidated respectively. It was carried out by means of various physical (solvent extraction, TLC, Column chromatography) and spectral techniques. The only difference found between the two compounds is the presence of C22=C23 double bond in Stigmasterol and C22-C23 single bond in β-sitosterol.

Conclusion

The study concluded the traditional use of the plant drug in the treatment of diabetes probably due to the isolated flavonoids *i.e.* quercetin, rutin and such other compounds.

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