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Full Length Research Paper

New pentacyclic triterpene ester and flavone glycoside from the biologically active extract of *Carduus pycnocephalus* L.

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Bioassay guided phytochemical investigation of petroleum ether, chloroform and butanol fractions obtained from biologically active total alcoholic extract of *Carduus pycnocephalus* led to isolation of two new compounds, 3-*O*-acetyl-ursolic acid-28-ethyl ester (compound 1) and diosmetin-7-*O*-*α*-L-arabinopyransyl (1```→4``)-*β*-D- glucopyranoside (compound 5) along with three known compounds, *bis* (2-ethylhexyl) benzene-1,2-dicarboxylate) (compound 2), 3α, 24 dihydroxyolean-12-en-28, 30-dioic acid dimethyl ester (compound 3) and kaempferol (compound 4). Their structures were established on the basis of spectroscopic methods (UV, MS, ^{1}H NMR, ^{13}C NMR, COSY, HSQC and HMBC) and by comparison with published data. Compounds 2 and 3 were isolated for the first time from genus *Carduus*. The cytotoxic, antioxidant, and antimicrobial activities of the ethanolic extract were evaluated and the extract showed variable degrees of activities. It showed significant cytotoxic activity against MCF-7, A-549 and HepG-2 cell lines with IC_{50}, 17.9, 17.5 and 21.8 µg, respectively. The extract displayed weak antioxidant activity by scavenging of DPPH with SC_{50} 554.2 µg. In agar diffusion assay, the extract exhibited strong antibacterial activities against Gram negative bacteria *Pseudomonas aeruginosa* and *Escherichia coli* and strong antifungal activity against *Syncephalastrum racemosum*.

Key words: *Carduus pycnocephalus* L.; new triterpene ester, flavonoidal glycoside, antioxidant, cytotoxic, antimicrobial activity.

INTRODUCTION

Genus *Carduus* which belongs to the family Asteraceae includes approximately 100 species worldwide (Chaudary, 2000) and is widely distributed around the Mediterranean. In Chinese folk medicine, the plants of genus *Carduus* are used for the treatment of various human diseases such as cold, stomach ache as well as rheumatism (Esmaieli, 2005). Genus *Carduus* was found to possess a wide range of biological activities such as liver tonic, anti-inflammatory, antispasmodic, anticancer, antiviral and antibacterial (Esmaeili, 2005; Jordon Thaden and Louda, 2003; Orhan and Ozcelik, 2009). Phytochemical studies on several *Carduus* species were

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Figure 1. *Carduus pycnocephalus* L.

carried out and revealed that this genus is rich in secondary metabolites as lignans (Fernandez et al., 1991), flavonoids, flavonoidal glycosides (Jordon - Thaden and Louda, 2003; El-Lakany et al., 1995, 1997; Amer et al., 1985; Abdallah et al., 1989; Bain and Desrochers, 1988; Abdel Salam et al., 1982; Liu, et al., 2013), coumarins (Jordon Thaden and Louda, 2003; Cardona et al., 1992), alkaloids (Zanng, 2002), sterols and triterpenes (Abdel Salam et al., 1982, 1983). Concerning the phytoconstituents of *Carduus pycnocephalus*, the literatures survey revealed the presence of flavonoids (El-Lakany et al., 1995, 1997; Amer et al., 1985), essential oils (Esmaeili, 2005; Al-Shammari, 2012), sterols and triterpenes (Gallo and Sarachine, 2009). In our previous work on Saudi plant, the isolation of seven flavonoidal compounds were reported, apigenin, kaempferol-3-*O* *β* – D-glucoside, kaempferol-3- *O* – *α* – L- rhamnoside, kaempferol-7-methoxy-3- *O* – *α* – L- rhamnoside, diosmetin-7-O-*β*-D-xylosyl-(1```→6``)-*β*-D-glucopyranoside, diosmetin- 7- *O* - *α* – L- arabinosyl (1``` →6``) – *β* – D – glucopyranoside, kaempferol-3-*O*-*α*-L-rhamnosyl—(1``` →2``) -*α*-L rhamnoside, in addition to, lupeol, *β*-sitosterol and *β*-sitosterol-3-*O*-*β*-D- glucoside were isolated from the aerial parts of *C. pycnocephalus* L. Anti-inflammatory, antispasmodic and hypotensive activities were assessed for all extracts which showed variable activities (Al-Shammari, et al., 2012). In continuation of our phytochemical and biological studies on *C. pycnocephalus* which grows in Saudi Arabia, we report here the isolation and structure elucidation of two new compounds 1, 5 and three known compounds 2 to 4. Compounds 2 and 3 were reported for the first time from genus *Carduus*. In addition, the cytotoxic, antioxidant and antimicrobial activities for the total alcoholic extract of *C. pycnocephalus* L. were evaluated.

MATERIALS AND METHODS

General experimental section

Melting points were determined on a Mettler FP 80 Central Processor supplied with a Mettler FP 81 MBC Cell Apparatus, and were uncorrected. ^{1}H and ^{13}C NMR spectra were recorded in $CDCl_3$ and DMSO-d_6 on a Bruker Avance DRX – 500 instruments (Central Lab. at the College of Pharmacy, KSU) at 500 MHz for protons and 125 MHz for carbons using the residual solvent signal as an internal standard. All 1D and 2D spectra were obtained using the standard Bruker software; EI and FAB mass spectra on a Jeol JMS, Ax 500, 5890 series II (Tokyo, Japan), Ultraviolet spectra were obtained in methanol using the shifting reagents $AlCl_3$, NaOAc, NaOMe, and H_3BO_3 for flavonoids were obtained using a Hewlett-Packard HP-845 UV-Vis spectrophotometer. The ultraviolet lamp used in visualizing TLC plates was a Mineralight® device, multiband UV-254/366 nm obtained from UVP, Inc., USA. Column chromatography was performed on silica gel (60-230 mesh, Merck) and TLC was carried out with silica gel 60 pre-coated plates F-254 (Merck).

Plant

The fresh plant (Figure 1) was collected from Al-Hada (Saudi Arabia) on March, 2008 and was kindly identified by Dr. Jakob Thomas, Professor in College of Science, KSU. A voucher specimen (#15106) was deposited at the herbarium in the College of Pharmacy at King Saud University (KSU).

Extraction and isolation

The air dried aerial parts of *C. pycnocephalus* L. (2.0 kg) were exhaustively extracted with 95% ethanol. The concentrated ethanol extract (210 g) was suspended in methanol: water (1:9) and fractionated by extraction with petroleum ether (40 to 60°C), chloroform, ethyl acetate and water saturated butanol, each (0.5 L ×

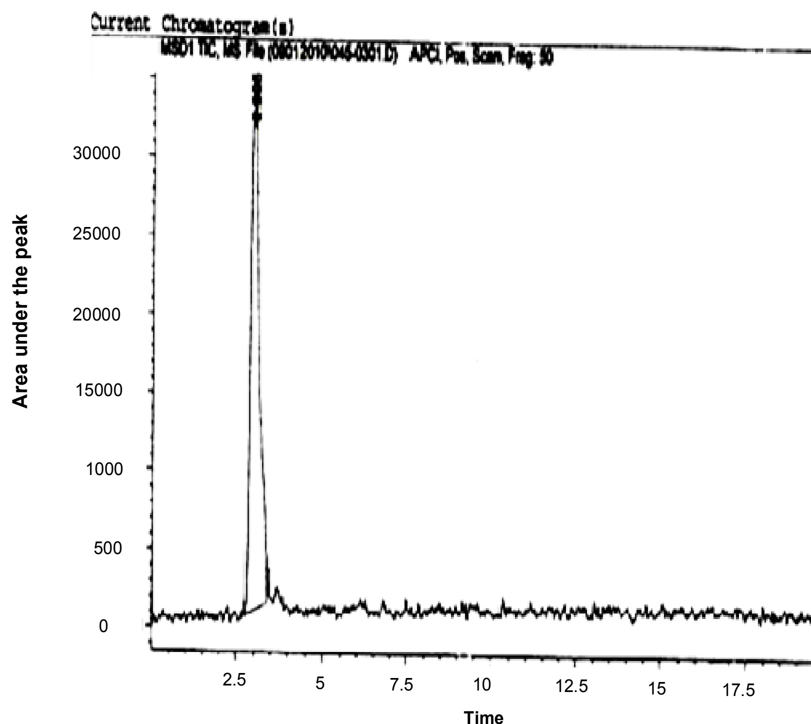

Figure 2. HPLC system used in isolation and chromatogram showing the purity of compound 5.

3), to give petroleum ether (47.3 g), chloroform (23.5 g), ethyl acetate (8.9 g), butanol (19.2 g) and aqueous (100 g) extracts.

Isolation of compounds 1 to 3 from petroleum ether extract

The petroleum ether extract (25 g) was subjected to silica gel column eluted with *n*-hexane and increasing the polarity with chloroform till 100% chloroform then with methanol, 60 fractions were collected (250 ml each), the important fractions were re-chromatographed using silica gel column eluted with *n*-hexane as eluent, then increasing the polarity by using chloroform and methanol to give compounds 1 (8 mg), 2 (1.37 g) and 3 (6 mg).

Isolation of compound 4 from chloroform extract

The chloroform fraction (2.5 g) was chromatographed on silica gel column. Elution was started with petroleum ether/chloroform (9:1) and the polarity was increased by methanol. Fifty fractions were collected, 100 ml each. The fractions were monitored by TLC and similar fractions were pooled together. From fraction 40 to 41 (120 mg) compound 4 (8 mg) was isolated by preparative TLC using chloroform: methanol (9.5:0.5) as solvent system.

Isolation of compound 5 from butanol extract

The butanol fraction (10 g) was chromatographed on silica gel column eluted with *n*-hexane/EtOAc (1:1) and increasing the polarity by ethyl acetate then by methanol. 70 Fractions were collected, 200 ml each. The fractions were monitored by TLC and similar fractions were combined together. Fractions 39 to 55 (750 mg) was subjected to preparative HPLC to yield 20 mg of highly pure compound 5 (Figure 2).

Acid hydrolysis of compound 5

Compound 5 (5 mg) was dissolved in MeOH (5 ml), to which an equal volume of 10% sulfuric acid was added. The mixture was refluxed on a boiling water bath for 3 h, and then cooled. The hydrolysate was shaken with ethyl acetate (3 × 50 ml). The combined extract was distilled off and subjected to TLC; the acidic mother liquor containing the sugar moiety (s) was neutralized with sodium carbonate, concentrated and separately spotted alongside with authentic sugars using chloroform:methanol (6:4) as solvent system and anisaldehyde sulfuric as spraying reagent. It showed the presence of *β*-D-glucose and *α*-L-arabinose as sugar moieties.

Compound 1: White powder (8 mg); R_f =0.59 [(*n*-hexane: $CHCL_3$) (2: 1)]; EI-MS *m/z* = 526 [M^+], 483 [M^+-$COCH_3$], 454 [M^+-$COCH_3$-C_2H_5], 411, 396, 382, 278, 248, 218; ^{1}H NMR and ^{13}C NMR (500 MHz, 125 MHz, $CDCl_3$, TMS) (Table 1).

Compound 2: Yellowish oily substance (1.37 g); ^{1}H NMR (500 MHz, $CDCl_3$, TMS) δ_H (ppm) 7.72 (2H, m, H-3, 6), 7.54 (2H, m, H-4, 5), 4.22 (4H, d, *J*= 6.4 Hz, H-3`, 3``), 1.70 (2H, m, H-4`, 4``), 1.35 (14H,m, H-5`, 5``, 6`, 6``, 7`, 7``), 0.88 (4H, m, H-8`, 8``), 1.35 (14 H, m, H-9`, 9``) and 0.90 (6H, t) H-10`, 10``); ^{13}C NMR (125 MHz, $CDCl_3$, TMS) δ_C 132.6 (s, C-1, 2), 129.0 (d, C-3, 6), 131.1 (d, C-4, 5), 167.9 (s, C-1`, 1``), 68.3 (t, C-3`, 3``), 38.9 (d, C-4`, 4``), 31.2 (t, C-5`, 5``), 29.6 (t, C-6`, 6``), 22.4 (t, C-7`, 7``), 14.4 (q, C-8`, 8``), 22.8 (t, C-9`, 9``) and 11.6 (q, C-10`, 10``); EI-MS: *m/z* = 390 [M^+,

Table 1. ^{1}H and ^{13}C NMR data of compound 1 in $CDCl_3$.

Position	δ_H	δ_C (DEPT)	Position	δ_H	δ_C (DEPT)
1	1.59, 1.0 (2H, m)	38.3 t	18	2.12 (1H, d, J=7.4 Hz)	55.3 d
2	1.57 (1H, m)	24.9 t	19	1.16 (1H, m)	40.9 d
3	4.45 (1H, t, *J* =7.0 Hz)	80.3 d	20	0.88 (1H, m)	39.8 d
4	-	37.8 s	21	1.36 (1H, m)	32.6 t
5	0.76 (1H, br)	55.3 d	22	1.26, 1.51 (2H, m)	31.9 t
6	1.32, 1.45 (2H, m)	18.3 t	23	0.80 (3H, s)	28.4 q
7	1.26, 1.46 (2H, m)	33.3 t	24	0.78 (3H, s)	16.8 q
8	-	39.8 s	25	1.25 (3H, s)	14.1 q
9	1.50 br	47.6 d	26	0.89 (3H, s)	16.8 q
10	-	37.1 s	27	1.08 (3H, s)	23.7 q
11	2.20 d, *J*= 7 Hz)	23.7 t	28	-	173.9 s
12	5.12 (1H, t, *J* =3.6 Hz)	121.7 d	29	0.90 (3H, d, *J*=5.5 Hz)	14.3 q
13	-	145.2 s	30	0.82 (3H, d, *J*=4.5 Hz)	23.5 q
14	-	41.7 s	31	-	173.7 s
15	1.62, 0.92 (2H, m)	26.1 t	32	2.20 (3H, s)	23.0 q
16	1.15, 1.50 (2H, m)	23.6 t	33	4.05 (2H, q, *J*=7.2 Hz)	60.1 t
17	-	47.2 s	34	1.20 (3H, t, *J*=5.0 Hz)	15.5 q

36%] , 149 [100%].

Compound 3: White powder (6 mg); ^{1}H NMR (500 MHz, $CDCl_3$, TMS) δ_H (ppm) 5.19 (1H, broad t, *J*= 7.6 Hz, H-12), 4.10 (1H, m, H-3), 3.50 (6H, s, two OCH_3- 31 and 32), 2.30 (1H, broad t , *J*=7.0 Hz, C-18), 1.26 (9H, s, for three methyl groups), 0.85 (6H, s, two methyl groups) ; ^{13}C NMR (125 MHz, $CDCl_3$, TMS) δ_C (ppm) 38.2 (C-1), 24.4 (C-2), 80.6 (C-3), 37.8 (C-4), 55.2 (C-5), 18.2 (C-6), 32.7 (C-7), 39.2 (C-9), 36.6 (C-10), 23.2 (C-11), 121.6 (C-12), 145.2 (C-13), 42.9 (C-14), 28.3 (C-15), 23.2 (C-16),46.7 9C-17), 37.8 (C-18), 41.8 (C-19), 47.2 (C-20), 31.1 (C-21), 34.7 (C22), 60.1 (C-23), 14.2 (C-24), 15.5 (C-25), 16.5 (C-26), 25.8 (C-27), 173.7 (CO-28), 50.3 (OCH_3-31), 29.7 (C-29), 174.0 (CO-30) and 50.3 (OCH_3-32). EI-MS: *m/z* 530 [M^+], 512, 470 and 247.

Compound 4: Pale yellow powder (8 mg); R_f =0.45 [$CHCl_3$:MeOH, 9:1]; UV λ max; MeOH (266, 338); ^{1}H NMR (500 MHz, DMSO-d_6, TMS) δ_H 6.05 (1H, *s*, H-6), 6.40 (1H, *s*, H-8), 6.85 (2H, *d*, *J* = 8.0 Hz, H-3`and 5`), 7.80 (2H, *d*, *J*= 8.0 Hz, H-2`and 6`), 5.50 (1H, s, OH-4`), 13.10 (1H, br s, OH-5); EI-MS: *m/z* = 286 [M^+], 134, 152.

Compound 5: Gray to greenish powder (20 mg); mp: 187-188 ^{0}C; R_f = 0.62 [butanol : acetic acid : water, 24:10:1]; UV λ_{max} MeOH: (nm) 269, 344; (NaOMe): 269 , 300 sh, 280; ($AlCl_3$): 273, 362, 390; ($AlCl_3$/HCl): 276, 297, 361, 383; (NaOAc): 271, 365 (NaOAc/H_3BO_3): 269, 348; ^{1}H NMR (500 MHz, DMSO-d_6, TMS); ^{13}C NMR (125 MHz, DMSO-*d*6, TMS) (Table 2); FAB-MS (Positive ion mode) *m/z* = 633 [M^++K] for $C_{27}H_{30}O_{15}$, 595 [M^++H], 463 [M^+-arab+H], 301 [M^+-glu-arab], 152.

Biological activities

All the biological activities were carried out at Regional Center for Mycology and Biotechnology (RCMB) at Al- Azhar University, Cairo, Egypt.

Cytotoxic activity

The cytotoxic effect of total alcoholic extract of *C. pycnocephalus* L. (Figure 6) was investigated at different concentrations, 50, 12.5, 6.25, 3.125, 1.56, 0.78 and 0.39 µg/ml. The tested cell lines, breast carcinoma (MCF-7), lung carcinoma (A-549) and hepatocellular carcinoma (HepG-2) were obtained from American Type Culture Collection (ATCC, Rockville, MD). The cells were grown as mono-layers in growth RPMI-1640 medium supplemented with 10% inactivated fetal calf serum and 50 µg/ml gentamycin. The monolayers of 10,000 cells adhered at the bottom of the wells in a 96-well microtitre plate incubated for 24 h at 37°C in a humidified incubator with 5% CO_2. The monolayers were then washed with sterile phosphate filtered saline (0.01 M, pH 7.2) and simultaneously the cells were treated with 100 µl from different dilutions of the test sample in fresh maintenance medium and incubated at 37°C. A control of untreated cells was made in the absence of the test sample. Six wells were used for each concentration of the test sample. Every 24 h, the observation under the inverted microscope was made. The number of the surviving cells was determined by staining the cells with crystal violet followed by cell lysing using 33% glacial acetic acid and read the absorbance at λmax 490 nm using ELISA reader (SunRise TECAN, Inc, USA) after well mixing. The absorbance values from untreated cells were considered as 100% proliferation. The number of viable cells was determined using ELISA reader as previously mentioned and the percentage of viability was calculated as:

$$[1- (ODt / ODc) \times 100\%]$$

where ODt: optical density of wells treated with the test sample. ODc: optical density of untreated cells. The IC_{50} value which reduces the cells number by 50%, was determined from dose response curve.

Antioxidant activity

Radical scavenging activity of total alcoholic extract of *C.*

Table 2. NMR data of compound 5 (500, 125 MHz, DMSO-d_6, TMS).

No.	δ_H (δ ppm)	δ_C (DEPT)	HSQC	HMBC	HHCOSY
2	-	164.7 s	-	-	-
3	6.84 s	104.3 d	C-3	4, 10	-
4	-	182.5 s	-	-	-
5	12.90 (OH, s)	161.6 s	-	-	-
6	6.48 (1H, d, *J*=2.0 Hz)	100.1 d	C-6	8, 10	8
7	-	163.4 s	-	-	-
8	6.81 (1H, d, *J*=2.0 Hz)	95.3 d	C-8	6, 10	6
9	-	157.5 s	-	-	-
10	-	105.9 s	-	-	-
1\`	-	123.4 s	-	-	-
2\`	7.46 (1H, d, *J*=8.8 Hz)	113.7 d	C-2\`	4\`, 6\`, 2	6\`
3\`	9.24 (OH, s)	147.3 s	-	-	-
4\`	OCH_3 3.87 (3H, brs)	151.8 s; 55.8 q	OCH_3	4\`	-
5\`	7.16 (1H, d, *J*=8.8 Hz)	112.7 d	C-5\`	1\`, 3\`	6\`
6\`	7.58 (1H, dd, *J*=2.0, 8.8 Hz)	119.4 d	C-6\`	2\`, 4\`	2\`, 5\`
1\`\`	5.06 (1H, d, *J*=7.2 Hz)	100.4 d	C-1\`\`	7	2\`\`
2\`\`	3.65 (1H, m)	75.9 d	C-2\`\`	-	1\`\`, 3\`\`
3\`\`	3.25 (1H, m)	76.7 d	C-3\`\`	-	2\`\`, 4\`\`
4\`\`	3.32 (1H, m)	78.9 d	C-4\`\`	-	3\`\`, 5\`\`
5\`\`	3.10 (1H, m)	76.1 d	C-5\`\`	-	4\`\`, 6\`\`
6\`\`	3.40 , 4.10 (2H, m)	60.1 t	C-6\`\`	-	5\`\`
1\`\`\`	4.15 (1H, d, *J*= 4.8 Hz)	103.3 d	C-1\`\`\`	-	2\`\`\`
2\`\`\`	3.33 (1H, m)	70.9 d	C-2\`\`\`	-	1\`\`\`, 3\`\`\`
3\`\`\`	3.24 (1H, m)	72.9 d	C-3\`\`\`	-	2\`\`\`, 4\`\`\`
4\`\`\`	3.60 (1H, m)	67.6 d	C-4\`\`\`	-	3\`\`\`, 5\`\`\`
5\`\`\`	3.20, 3.65 (2H, m)	64.5 t	C-5\`\`\`	-	4\`\`\`

pycnocephalus L. was determined at the Regional Center for Mycology and Biotechnology (RCMB) at Al- Azhar University by the DPPH free radical scavenging assay in triplicate and average values were considered (Figure 7).

DPPH radical scavenging activity (Yen and Duh, 1994): Methanol solution (0.004% w/v) of 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical was freshly prepared and stored at 10°C in the dark. A methanol solution of the test extract was prepared. A 40 µl aliquot of the methanol solution was added to 3 ml of DPPH solution. Absorbance measurements were recorded immediately with a UV-visible spectrophotometer (Milton Roy, Spectronic 1201). The decrease in absorbance at 515 nm was determined continuously, with data being recorded at 1 min intervals until the absorbance reference compound ascorbic acid were also measured. All the determinations were performed in three replicates and averaged. The percentage inhibition (PI) of the DPPH radical was calculated according to the formula:

$$\text{DPPH scavenging activity \% [PI]} = [\{(AC - AT)/AC\} \times 100] \quad (1)$$

where AC = Absorbance of the control and AT = absorbance of the sample + DPPH.

Antimicrobial activity

Agar diffusion method (Perez et al., 1990): The total alcoholic extract of *C. pycnocephalus* L. was dissolved in DMSO and evaluated by agar diffusion method at a dose of 50 µg/ml and the results were reported as shown in Table 3. The tested fungi were *Asperagillus fumigates* (RCMB 02568), *Syncephalastrum racemosum* (RCMB 05922), *Geotricum candidum* (RCMB 05097) and *Candida albicans* (RCMB 05036); Gram positive bacteria *were Streptococcus pneumonia* (RCMB 010010) and *Bacillus subtillis* (RCMB 010067) while Gram negative bacteria were *Pseudomonas aeruginosa* (RCMB 010043) and *Escherichia coli* (RCMB 010052). Amphotericin B, ampicillin and gentamicin were used as standard antifungal and antimicrobial agents; the diameter of zone of inhibition was measured in mm. The microorganisms were obtained from Regional Centre of Mycology and Biotechnology, Al-Azhar University.

RESULTS AND DISCUSSION

Phytochemical results

Characterisation of the isolated compounds

Petroleum ether, chloroform and butanol extracts of air dried aerial parts of *C. pycnocephalus* L. were subjected to chromatographic separation followed by purification and gave five pure compounds 1 to 5. The identification

Table 3. Antimicrobial activity of total alcoholic extract of *C. pycnocephalus* L.

Microorganism	Inhibition zone diameter (mm) of	
	Total alcoholic extract	Standards
Fungi		**Amphotericin B**
Asperagillus fumigates	16.8 ± 0.58	23.7 ± 0.1
Syncephalastrum racemosum	15.2 ± 0.58	19.7 ± 0.2
Geotricum candidum	18.6 ± 0.63	28.7 ± 0.2
Candida albicans	***NA**	25.4 ± 0.1
Gram positive bacteria		**Ampicillin**
Streptococcus pneumonia	17.3 ± 0.63	23.8 ± 0.2
Bacillus subtilis	18.6 ± 0.72	32.4 ± 0.3
Gram negative bacteria		**Gentamicin**
Pseudomonas aeruginosa	15.3 ± 0.58	17.3 ± 0.1
Escherichia coli	17.2 ± 0.63	19.9 ± 0.3

*NA: No activity; Extract and used standards were tested at a dose of 50 µg.

of these compounds was carried out on the basis of spectroscopic methods (UV, IR, ^{1}H NMR, ^{13}C NMR, DEPT 135, COSY, HMBC, HSQC and MS spectroscopy).

Compound 1, [3-*O*-acetyl ursolic acid-28-ethyl ester]: The EI-MS of compound 1 exhibited a distinct molecular ion peak at *m/z* 526 [M^{+}] corresponding molecular formula $C_{34}H_{54}O_4$. The ^{13}C NMR and DEPT data of compound exhibited 34 carbons. These carbons are attributed to nine methyls, ten methylenes, seven methines and eight quaternary carbon atoms. The ^{1}H-NMR data, (Table 1), revealed the presence of nine methyl signals, six appeared as singlet at δ_H 0.80, 0.78, 1.25, 0.89, 1.08, 2.20 and connected, via HSQC to C-23 (δ_C 28.4), C-24 (δ_C 16.8), C-25 (δ_C 14.1), C-26 (δ_C 16.8), C-27 (δ_C 23.7) and C-32 (δ_C 23.0), respectively; and two doublets at δ_H 0.90 (3H, d, *J*=5.5 Hz) and 0.82 (3H, d, *J*= 4.5 Hz) as well as one triplet at δ_H 1.20 (3H, t, *J*= 5.0 Hz) and connected to C-29 (δ_C 14.3), C-30 (δ_C 23.5) and C-34 (δ_C 15.5), respectively. In addition, the ^{1}H-NMR data showed a triplet signal at δ_H 5.12 (1H, t, *J*= 3.6 Hz) connected via HSQC to C-12 (δ_C 121.7). These information along with the carbon chemical shift of C-12 and C-13 (δ_C 121.7/145.2 and proton shift of H-12 (δ_H 5.12) suggested that compound 1 was a triterpene carrying a $(\Delta^{12})^{13}$ double bond (Ahmad and Rahman, 1994).

The presence of two douplet methyl groups at 0.90 (3H, d, *J* =5.5 Hz) and 0.82 (3H, d, *J*=4.5 Hz) for CH_3-29 and CH_3-30, respectively suggested the presence of ursolic acid nucleus. This suggestion was further confirmed by HMBC experiment (Figure 4) in which there are three bond correlations between CH_3-29 with C-18 and C-20; CH_3-30 with C-19 and C-21. Further HMBC correlations were detected between methine proton at C-12 (δ_C 121.7) and C-9 (δ_C 47.6), C-14 (δ_C 41.7), and two bonds correlations with C-11 (δ_C 23.7). The methine proton at C-3 appearing at δ_H 4.45 (1H, t, *J*=7.0 Hz) showed two bond correlation with C-2 (δ_C 24.9) and C-4 (δ_C 37.8). Also the ^{1}H NMR spectrum showed extra signals, quartet signal appear at δ_H 4.05 (2H, q, *J*= 7.2 Hz) and triplet δ_H 1.20 (3H, t, *J*=5.0 Hz) connected to C-33 (δ_C 60.1, CH_2) and C-34 (δ_C 15.5, CH_3) in HSQC spectrum, respectively which indicated the presence of ethyl group. The position of it was established from HMBC correlation of methylene group at δ_H 4.05 (2H, q, *J*= 7.2 Hz) with C=O -28 at δ_C 173.9. Further confirmation was obtained from the upfield shift of carbonyl group at 28 position to 173.9 ppm compared to that of ursolic acid 180.1 (Babalola and Shode, 2013). Other extra ^{1}H NMR signal at δ_H 2.20 (3H, s) and two ^{13}C NMR signals at δ_C 23.1 and 173.7 were observed which indicated the presence of acetyl group. The position of acetyl group was proved through HMBC correlation by three bonds of H-3 (δ_H 4.45) with carbonyl (C-31) (δ_C 173.7). The EI-MS confirmed the presence of acetyl and ethyl moieties in compound 1 where it showed fragments at *m/z* 483 [M^{+}-$COCH_3$] and 454 [M^{+}-$COCH_3$-C_2H_5]. From the aforementioned data and through comparison with reported literatures for related compounds (Ahmad and Rahman, 1994), the structure of compound 1 was established to be 3-*O*-acetyl ursolic acid-28-ethyl ester and it is the first report of this compound from nature.

Compound 2, [Bis (2-ethylhexyl) benzene-1, 2-dicarboxylate]: Compound 2 (1.37 g), was isolated as pale yellow oil, R_f 0.7 [(10% EtOAc in *n*-hexane)]. The EI/MS spectrum displayed a pseudo molecular ion [M]$^{+}$ at *m/z* 390, which is in a good agreement with the molecular formula $C_{24}H_{38}O_4$ (Figure 3). ^{1}H NMR and ^{13}C NMR data

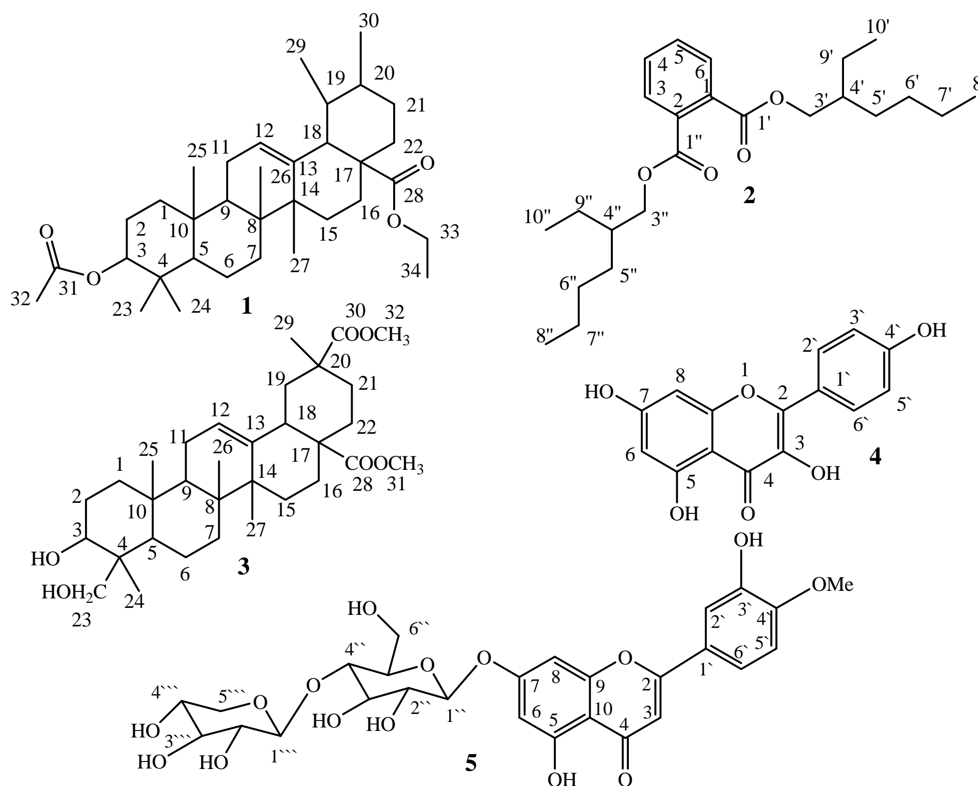

Figure 3. The chemical structures of compounds 1 to 5.

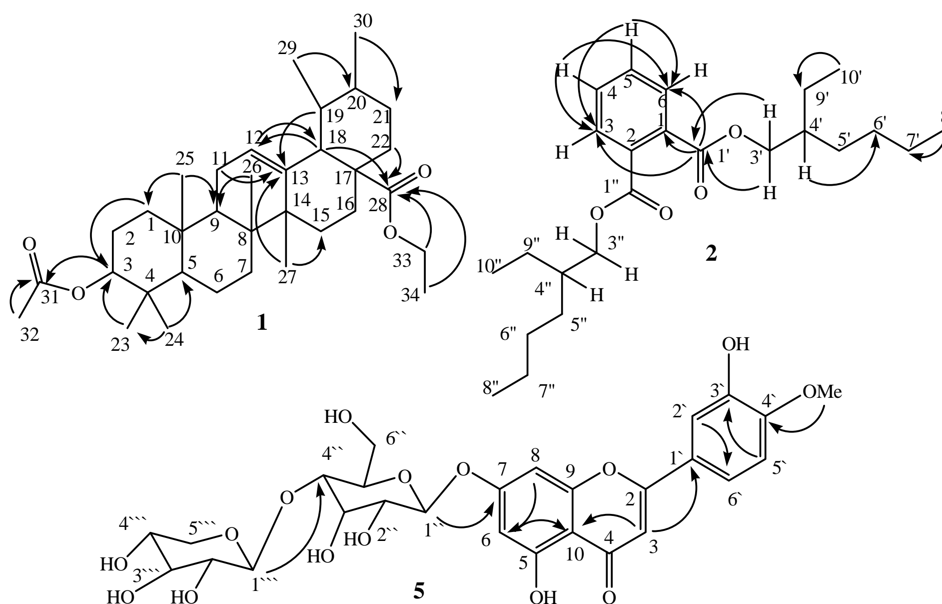

Figure 4. Important HMBC correlations of compounds **1**, **2** and **5**.

demonstrated the presence of 12 signals attributed to two methyls, five methylenes, three methines and two quaternary carbons indicated the symmetry of the compound since the number of carbons and protons measured by mass is 24 and 38, respectively. Two of the four quaternary carbons appeared at δ_C 167.9, indicated

the presence of two esters carbonyl moiety in the compound. The ^{1}H NMR data exhibited two aromatic protons appeared at δ_H 7.72 (2H, *m*, H-3, 6) and 7.54 (2H, *m*, H-4, 5), two methylene protons at δ_H 4.22 (4H, *d*, *J* = 6.4 Hz, H-3`, 3``), two methine protons at $\delta_H$ 1.70 (2H, *m*, H-4`, 4``), four methyl resonances at δ_H 0.90 (4H, 10`, 10``) and 0.88 (4H, 8`, 8``). The spectrum also showed multiplets around δ_H 1.35 accounts for eight methylenes (16 H, 5`, 5``, 6`, 6``, 7`, 7``, 9`, 9``). Combined studies of 2D NMR experiments, notable COSY, HSQC and HMBC allowed to complete assignment of the spectral data. The existence of only two aromatic peaks in ^{1}H NMR spectrum (integrated to 2 protons each) suggested that the compound must have an *ortho*-disubstituted benzene ring bearing the same substituent in both positions (Silverstein et al., 1997). The aromatic protons at δ_H 7.72 (H-3/ H-6) showed direct coupling to a carbon at δ_C 129.0 in the HSQC experiment, in addition to long range HMBC correlations with carbons at δ_C 131.1, 132.6 and the carbonyl group at δ_C 167.9. Similarly, H-4/H-5 (δ_H 7.54) revealed direct coupling to carbon at δ_C 132.6 and long range correlations to carbons at δ_C 129.0. From the aforementioned correlations, the quaternary carbon at δ_C 132.6 was assigned as C-1/C-2 and the methine carbons at δ_C 131.1 and 129.0 to C-4/C-5 and C-3/C-6, respectively. The carbonyl quaternary carbon at δ_C 167.9 (C=O) showed correlation with H-3/ H-6. The methylene protons at δ_H 4.22 showed direct coupling to an oxygenated carbon at δ_C 68.3 and long range correlation with the carbonyl group at δ_C 167.9. Therefore, these protons were assigned as H-3'/H-3''. The multiplet (integrated for 2 protons) at δ_H 1.70 exhibited coupling with H-3'/H-3'' in COSY experiment which, in turn, showed direct coupling at δ_C 38.9 (C-4'/C-4'') in the HSQC experiment and long range correlations with carbons at δ_C 68.3 (C-3'/C-3''), 31.2 (C-5'/C-5''), 29.6 (C-6'/C-6''), 22.8 (C-9'/C-9'') and 11.6 (C-10'/C-10'') in HMBC (Figure 4). The methyls at δ_H 0.90 and 0.88 exhibited direct coupling to methyl carbons at δ_C 11.6 (C-10'/C-10'') and 14.4 (C-8'/C-8''), respectively. A common long range correlations by H-4'/H-4'' and the methyls at δ_H 0.88 (H-8'/H-8'') to δ_C 29.6 confirmed their assignment as C-6'/C-6''. These methyls were also connected to C-7'/C-7'' at δc 22.4. The other two methyl groups (δ_H 0.90, H-10'/H-10'') showed long range correlations to carbons at δ_C 38.9 (C-4'/C-4''). A multiplet at δ_H 1.35 showed long range correlations to C-10'/C-10'' (δ 11.6), C-4'/C-4'' (δ_C 38.9) and C-3'/C-3'' (δ 68.3). As a result, these protons must be H-9'/H-9'', which showed direct coupling with carbons at δ_C 22.8 (C-9'/C-9''). On the basis of the aforementioned data, compound 2 was identified as bis (2-ethylhexyl) benzene-1,2-dicarboxylate). This compound is a synthetic polypropylene derivative, which is used as a plasticizer for polyvinyl chloride (PVP) (Beeler et al., 1976). Bis (2-ethylhexyl) benzene-1,2-dicarboxylate) was previously isolated and reported as natural product from *Mentha longifolia* (Ertaş et al., 2015) and *Ziziphora persica* (Nadaf et al., 2013) and it showed larvicidal activity (Katadea et al., 2006). This is the first report to isolate *bis* (2-ethylhexyl) benzene-1, 2-dicarboxylate) from genus *Carduus*.

Compound 3, [3α, 24 -dihydroxyolean-12-en-28, 30-dioic acid dimethyl ester]: The ^{1}H NMR spectrum of compound 3 showed signals at δ (ppm) 1.26 (9H, s), 0.85 (6H, s) which in addition to the five ^{13}C NMR signals at δ_C 14.2 (C-24), 15.5 (C-25), 16.5 (C-26), 25.8 (C-27) and 29.7 (C-29) indicated the presence of five methyl groups in this compound. The other signal at δ_H 5.19 (1H, broad t, *J*= 7.6 Hz, H-12) in ^{1}H NMR spectrum with two signals at δ_C121.6 (C-12), 145.2 (C-13) in ^{13}C NMR spectrum revealed the presence of $(\Delta^{12})^{13}$ triterpenoidal compound (Ahmad and Rahman, 1994). The ^{1}H NMR signals at δ_H 4.10 (1H, *m*), 3.50 (6H, s), 2.30 (1H, broad t, *J*= 7.0 Hz) with ^{13}C NMR signals at 80.6, 50.3 and 37.8 were interpreted for CH-3, two OCH_3 groups at 31 and 32 and CH-18, respectively. The presence of CH_2OH was established from the ^{1}H NMR and ^{13}C NMR signals at δ_H 3.70 and 4.10 each for one proton with signal at δ_C 60.1 for CH_2OH at position 24 (Ahmad and Rahman, 1994). The aforementioned data with the remaining signals reported in experimental section and EI-MS spectrum at *m/z* 530, in addition to comparison with literature (Ahmad and Rahman, 1994) confirmed the structure of compound 3 as 3-*α*, 24- dihydroxyolean-12-en-28, 30-dioic acid dimethyl ester. This is the first report of this compound in genus *Carduus*.

Compound 4 [kaempferol]: The UV spectral data of compound 4 showed UV λ max; MeOH (266, 338 nm) which indicated the presence of flavonol compound. The ^{1}H NMR spectrum showed an A_2B_2 spin system for ring B at δ_H 6.85 (2H, *d*, *J*= 8.0 Hz, H-3`and 5`), 7.80 (2H, *d*, *J*= 8.0 Hz, H-2`and 6`). In addition, the presence of singlet signal at δ_H 6.40 for H-6, and other signal at δ 6.05 (1H, *s*, H-8) for H-8 indicated the presence of 3, 5, 7, 4` tetrahydroxy flavone nucleus. Also, the EI-MS spectrum showed a molecular ion peak at *m/z* 286 [M^+ , 100%] which are in a good agreement with the molecular formula $C_{15}H_{10}O_6$ from the aforementioned data and through comparison with literatures (Mabry et al., 1970; Markham et al., 1978) compound 4 was identified as kaempferol, it was previously reported in genus *Carduus*.

Compound 5 [Diosmetin- 7-*O* -*α*- L- arabinopyransyl - (1'''→ 4'') -*β* - D - glucopyranoside]: The UV spectral data of compound 5 showed absorption bands at 269 and 344 nm indicating the flavones nature of the compound. The different shifting reagents confirmed the presence of flavone nucleus with free hydroxyl group at 5 position and the absence of free hydroxyl group at 7 and 4` positions or presence of occupied ones. The FAB-MS spectrum(Figure 5), exhibited a molecular ion peak at *m/z* 633 [M^++ K] and *m/z* 595 [M^+ +H], which is in agreement

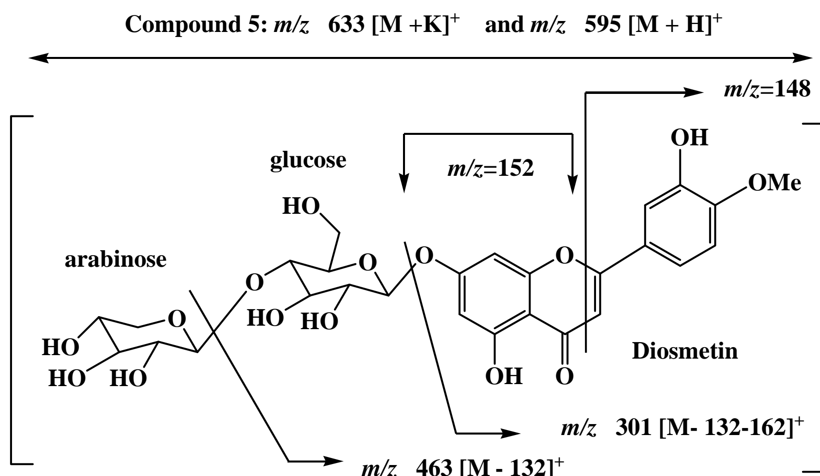

Figure 5. Fragmentation pattern of compound 5.

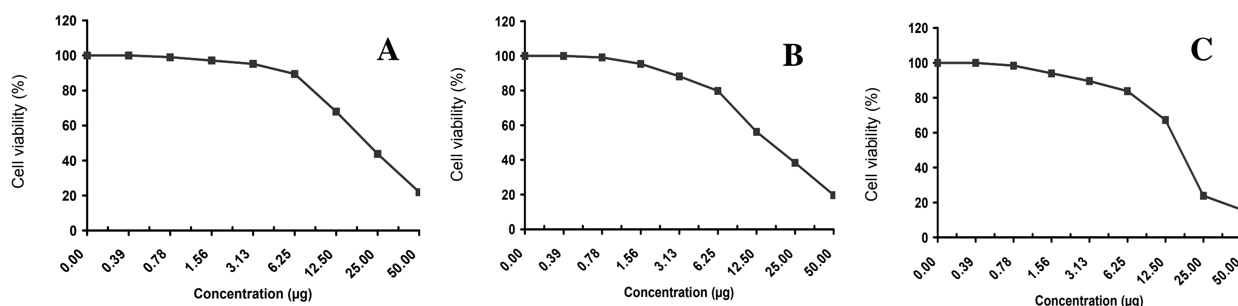

Figure 6. Cytotoxic effect of total alcoholic extract of *C. pycnocephalus* L. against HepG-2 (**A**), A-549 (**B**) and MCF-7 (**C**) cells.

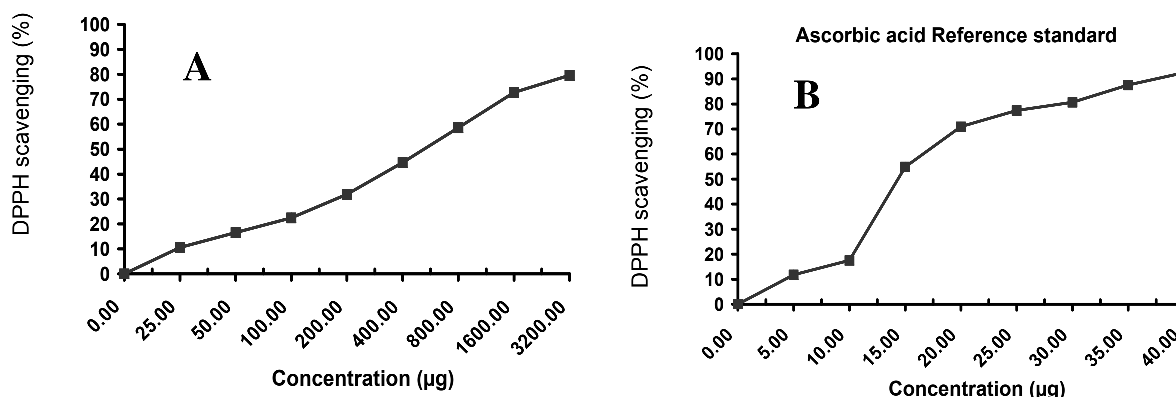

Figure 7. Antioxidant activity of total alcoholic extract of *C. pycnocephalus* (**A**) using DPPH free radical scavenging test and ascorbic acid as a reference compound (**B**).

with molecular formula $C_{27}H_{30}O_{15}$. The ^{1}H and ^{13}C NMR spectral data of compound 5 (Table 2) were close to that of compounds reported (El-Lakany et al., 1997; Saeidnia et al., 2011) suggesting that 5 had diosmetin as an aglycon. This was confirmed from the ^{13}C NMR spectral data of compound 5 which showed 27 carbons, 16 of which were very similar to those for diosmetin. Further confirmation for the presence of diosmetin was the ^{1}H NMR which showed the presence of methoxy group appeared as singlet signal at δ_H 3.88.

This methoxy group connected via HMBC correlation to C-4` at δ_C 151.8 (Figure 4). Furthermore, the ^{1}H NMR

and H-HCOSY data showed ABX spin system for ring B appearing at δ_H 7.58 (1H, dd, *J* = 2.0, 8.8 Hz, H-6`), 7.46 (1H, d, *J* = 2.0 Hz, H-2`), 7.16 (1H, d, *J* = 8.8 Hz, H-5`). In addition to the presence of two meta coupled signals at δ_H 6.48 and 6.81 (*J* = 2.0 Hz) suggested the 5, 7-disubstituted A ring of flavonoid and assigned to H-6 and H-8, respectively. This was confirmed from the HMBC experiment that exhibited correlations of H-2' (δ_H 7.46) with C-2 (δ_C 164.7), H-6' at δ_H 7.58 with C-2 (δ_C 164.7) and C-4' (δ_C 151.8) and H-5' (δ_H 7.16) with C-1` (δ_C 123.4) and C-3' (δ_C 147.3). Further HMBC correlations of H-6 (δ_H 6.48) with C-10 (δ_C 105.9) and H-8 (δ_H 6.81) with C-6 (δ_C 100.1) and C-10 (δ_C 105.9) were detected. These data are in a good agreement with diosmetin (El-Lakany et al., 1997; Saeidnia et al., 2011). The remaining ^{1}H NMR signals and the other eleven ^{13}C NMR signals, with ^{1}H ^{1}H COSY spectrum (Table 2) of compound 5 suggested the presence of two sugar moieties (hexose and pentose), the sugar moieties were identified by acid hydroplysis of compound 5 as *β*-D-glucose and *α*-L-arabinose. The FABMS fragments at *m/z* 463 [M$^+$-ara] and *m/z* 301 [M$^+$- glu - ara + H] confirmed the presence of glucose and arabinose. The linkage of glucose moiety was found to be at C-7 from HMBC correlations (Figure 4), as the anomeric proton of glucose at δ_H 5.09 showed three bond correlation with C-7 (δ_C 163.4). The position of arabinose was deduced from the downfield shift of C-4`` ($\delta_C$ 78.9) of glucose and this confirmed the connection of arabinose moiety at C-4`` of glucose. From the earlier discussed NMR data, compound 5 was identified as diosmetin- 7-*O*-*α*-L- arabinopyransyl - (1'''→4'') -*β* - D - glucopyranoside. This is the first report of this compound in nature.

Cytotoxic activity

The cytotoxic effect of total alcoholic extract of *C. pycnocephalus* was assessed against breast carcinoma (MCF-7), lung carcinoma (A-549) and hepatocellular carcinoma (HepG-2) cell lines at different concentrations, 50, 12.5, 6.25, 3.125, 1.56, 0.78 and 0.39 µg/ml, and the extract showed strong activity against the tested cell lines with IC_{50} 16.9, 17.5 and 21.8 µg/ml, respectively as shown in Figure 6.

Antioxidant activity

The antioxidant effect of total alcoholic extract of *C. pycnocephalaus* using DPPH free radical scavenging method was measured at eight different concentrations and the extract showed weak antioxidant activity in comparison with ascorbic acid with SC_{50} 554.2 and 14.2 µg/ml, respectively as shown in Figure 7.

Antimicrobial activity

The antimicrobial activity against fungi, *A. fumigates* (RCMB 02568), *S. racemosum* (RCMB 05922), *G. candidum* (RCMB 05097), *C. albicans* (RCMB 05036); Gram positive bacteria, *S. pneumonia* (RCMB 010010), *B. subtillis* (RCMB 010067) and Gram negative bacteria *P. aeruginosa* (RCMB 010043) and *E. coli* (RCMB 010052) was evaluated using agar diffusion technique. The extract exhibited strong antifungal activity against *S. racemosum* with inhibition zone diameter of 15.2 mm compared to that of amphotericin B 19.7 mm. In addition, a wide range of inhibitory activity against Gram-negative bacteria was observed. The extract showed strong anti-bacterial activity against *P. aeruginosa* and *E. coli* with inhibition zone diameter of 15.3 and 17.2 mm compared to that of gentamicin 17.3 and 19.9 mm, respectively. The extract showed also variable activities against other tested microorganisms as shown in Table 3.

Conclusion

Conclusively, this work represented the isolation of two new compounds 1 and 5 from *C. pycnocephalus* extract with three known compounds 2 to 4. In addition, the plant extract showed highly significant anticancer and antimicrobial activity; so it is considered as a good source for strong anticancer and antimicrobial principles.

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Conflict of interest

Authors declare that there are no conflicts of interests

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