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Full Length Research Paper

Cytotoxic and renoprotective flavonoid glycosides from *Horwoodia dicksoniae*

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Three flavonoid glycosides and one aglycone were isolated from the ethanol extract (EE) of *Horwoodia dicksoniae* (Brassicaceae), for the first time, and their structures were established from negative ESI-MS, ^{1}H-, ^{13}C-NMR and DEPT as luteolin 7-O-β-D-glucopyranoside (1), luteolin 6-C-β-D-galactopyranoside (2), apigenin 6-C-β-D-galactopyranoside (3) and luteolin (4). The SRB cytotoxicity assay was used to investigate the antitumor activity of the EE, Compounds 1, 2 and 4. Compound1 showed the highest cytotoxic activity against the three human cell lines, including HEP-G2, HCT-116 and MCF-7 (IC_{50} = 10.7, 9.3 and 9.9 µg/ml, respectively), compared with doxorubicin as reference drug. Compound 4 showed selective antitumor activity against the colon cell line (IC_{50} = 9.5 µg/ml). The present investigation also demonstrates the protective effect of Compounds 1, 2 and 4 with strong antioxidant potential, in glycerol-induced myoglobinuric acute renal failure in rats. Moreover, all tested compounds separately attenuated renal dysfunction, and restored the oxidant balance by decreasing renal MDA levels, increasing the activity of the depleted renal antioxidant enzymes, and the non enzymatic antioxidant GSH. They also, decreased the elevated serum inflammatory marker (TNF-α), and ameliorated apoptotic kidney damage by reduction in caspase-3 activity. Taken together, 1 was found to be the most biologically active compound.

Key words: Rhabdomyolysis, glycerol, acute renal failure, flavone glycosides, cytotoxicity.

INTRODUCTION

Horwoodia dicksoniae Turrill. belongs to the family Brassicaceae, known as mustard family that comprises about 390 genera and is represented in Saudi Arabia by 49 genera (Chaudhary, 2001). The plant is a richly branched annual desert herb, up to 30 cm high. It is found in North Hejaz, Najd, Northern and Eastern regions of Saudi Arabia. The flowers of *H. dichsoniae* are scented with a pleasant musky fragrance and are purple or rose-colored, bracts and bracteoles are absent. Theplant gives a sweet odor to the milk of the camels that feed on it in the spring. The fruit is broadly winged all around and is indehiscent. The seeds remain enclosed within and are released when there have been enough rains to soften or cause the rotting of the fruit wall. *H. dicksoniae* is known in Arabic as Khuzama. The phytoconstituents present in the aerial parts were found to be flavonoids, glucosinolates, sterols and triterpenes (Al Yahya et al., 1990; Chaudhary and Al Jowaid, 1999; Al Kahtani et al., 2000). *H. dicksoniae* contains volatile oil, which makes it one of the rare aromatic plants of Brassicaceae (Mossa et al., 1985). This paper deals with phytochemical and

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biological investigations of *H. dicksoniae* aerial parts.

MATERIALS AND METHODS

General experimental procedures

The ^{1}H- and ^{13}C-NMR, spectra were recorded on Bruker spectrometer operating at 300 and 75 MHz for ^{1}H- and ^{13}C-NMR, respectively. The chemical shift values are reported in ppm (δ) units and the coupling constants (J) in Hz. ESI-MS analyses were measured on an Agilent Triple Quadrupole 6410 QQQ LC/MS mass spectrometer with ESI ion source (gas temperature is 350°C, nebulizer pressure is 60 psi and gas flow rate is 12 L/min), operating in the negative and positive scan modes of ionization through direct infusion method using $CH_3CN–H_2O$ (1:1 v/v) at a flow rate of 0.4 ml/min. Column chromatography was carried out on various adsorbents including silica gel 230 to 400 mesh (E. Merck, Darmstadt, Germany) and Sephadex LH-20 (Pharmacia, Uppsala, Sweden). Thin layer chromatography (TLC) was performed on pre-coated silica gel F_{254} plates (E. Merck, Darmstadt, Germany); silica gel RP-18 F_{254} (E. Merck, Darmstadt, Germany), and cellulose glass plates (E. Merck, Darmstadt, Germany). Also, Whatman No. 1 sheets (Whatman Ltd., Maidstone, England) were used for paper chromatography. Solvent systems used were: S_1 [n-BuOH/HOAc/H_2O (4:1:5, v/v/v top layer)], S_2 (15% aqueous AcOH) on cellulose plates, S_3 ($CHCl_3$/MeOH 8:2) on silica gel plates and S_4 (50% aqueous MeOH) on silica gel RP-18 plates. The detection was done by UV-light at 254 and/or 365 nm. The pure compounds were visualized by spraying with freshly prepared ceric sulphate and $AlCl_3$.

Plant material

The aerial parts of *H. dicksoniae* (1.5 kg) were collected from Al-Qaseem region (Saudi Arabia) by Al Yahya MA, Professor of Pharmacognosy, in March 2009 and identified by M. Atique Al-Rahman, Professor of Taxonomy, College of Pharmacy, King Saud University, where a voucher specimen (No. 6) has been deposited.

Extraction and isolation

The air-dried powdered aerial parts of the plant (1.5 kg) were subjected to extraction with 80% ethanol. The total extract (100 g), obtained after evaporation of solvent in vacuum, was then suspended in water (250 ml) and successively partitioned with petroleum ether 40 to 60°C (4 × 300 ml), $CHCl_3$ (4 × 300 ml) and the remaining defatted dry residue was taken with ethanol to give dry ethanol extract (EE)of 30 g.Thereafter, it was subjected to fractionation on a Sephadex LH-20, using stepwise gradient with MeOH-H_2O (starting with 10% up to 100% MeOH) for elution. Thirty fractions were collected and grouped into (A to C) fractions by TLC analyses on silica 60 F_{254} gel-coated glass sheets developed with $CHCl_3$-MeOH (80:20), and cellulose plates with AcOH-H_2O (15:85) and n-BuOH-AcOH-H_2O (40:10:50, organic layer) for elution. Fraction A (3 g, 50% aqueous MeOH), was purified on a Sephadex LH-20 column with MeOH (eluent) to give Compound 1 (100 mg). Fraction B (1.5 g, 85% aqueous MeOH) was rechromatographed on Sephadex with MeOH to yield Compounds 2 (65 mg) and 3 (15 mg). Fraction C (1 g, 70% aqueous MeOH) was rechromatographed on a Sephadex LH-20 column using n-BuOH/iso-pr.OH/H_2O (BIW, 4:1:5, organic layer) for elution to afford compound 4 (70 mg). The homogeneity of the fractions was tested by 2D-PC using Whatman No. 1 paper sheets, and solvent systems S_1 and S_2 for elution and different spray reagents.

Luteolin 7-O-β-D-glucopyranoside (1)

A yellow amorphous powder with chromatographic properties: R_f 0.49 (S_1), 0.14 (S_2) on PC; deep yellow fluorescence ($AlCl_3$) and green color with $FeCl_3$ spray reagents. Negative ESI-MS: m/z = 447.2 $[M – H]^-$, positive ESI-MS: m/z = 449.2 $[M + H]^+$, 471.2 $[M + H + Na]^+$. ^{1}H NMR(300 MHz, DMSO-d_6): δ = 12.97 (1H, s, H-bounded OH-5), 7.44 (1H, dd, J = 8.1, 1.8 Hz, H-6′), 7.42 (1H, d, J = 1.8 Hz, H-2′), 6.90 (1H, d, J = 8.1 Hz, H-5′), 6.78 (1H, d, J = 2.1 Hz, H-8), 6.74 (1H, s, H-3), 6.44 (1H, d, J = 2.1 Hz, H-6),5.08 (1H, d, J = 7.2 Hz, H-1″), 3.72 (1H, br d, J = 9.9 Hz, H-6″a), 3.55 to 3.10 (5H, m, H-2″, 3″, H-4″, 5″, 6″b). ^{13}C NMR(75 MHz, DMSO-d_6): δ = 181.8 (C-4), 164.4 (C-2), 162.9 (C-7), 161.1 (C-5), 156.9 (C-9), 149.9 (C-4′), 145.70 (C-3′), 121.3 (C-6′), 119.1 (C-1′), 115.9 (C-5′), 113.5 (C-2′), 105.3 (C-10), 103.1 (C-3), 99.9 (C-1″), 99.5 (C-6), 94.7 (C-8), 77.1 (C-5″), 76.3 (C-3″), 73.1 (C-2″), 69.5 (C-4″), 60.6 (C-6″).

Luteolin 6-C-β-D-galactopyranoside (2)

A yellow amorphous powder with chromatographic properties: R_f 0.40 (S_1), 0.29 (S_2) on PC; deep yellow fluorescence ($AlCl_3$) and green color with $FeCl_3$ spray reagents. Negative ESI-MS: m/z = 447.2 $[M – H]^-$, positive ESI-MS: m/z = 449.1 $[M + H]^+$, 471.1 $[M + H + Na]^+$. ^{1}H NMR(300 MHz, DMSO-d_6): δ = 13.54 (1H, s, H-bounded OH-5), 7.43 (1H, br s, H-2′), 7.37 (1H, br d, J = 8.1 Hz, H-6′), 6.92 (1H, d, J = 8.4 Hz, H-5′), 6.63 (1H, s, H-3), 6.62 (1H, s, H-8), 4.59 (1H, d, J = 9.6 Hz, H-1″), 4.10 (1H, t-like, J = 8.7 Hz, H-2″), 3.90 to 3.10 (5H, m, H-3″, H-4″, 5″, 6″a, 6″b). ^{13}C NMR(75 MHz, DMSO-d_6): δ = 181.8 (C-4), 163.6 (C-2), 163.3 (C-7), 160.5 (C-5), 156.1 (C-9), 149.6 (C-4′), 145.70 (C-3′), 121.3 (C-6′), 119.1 (C-1′), 116.1 (C-5′), 113.2 (C-2′), 108.6 (C-6), 103.3 (C-10), 102.7 (C-3), 93.6 (C-8), 81.3 (C-5″), 78.8 (C-3″), 72.9 (C-1″), 70.4 (C-2″), 70.1 (C-4″), 61.3 (C-6″).

Apigenin 6-C-β-D-galactopyranoside (3)

A pale yellow amorphous powder with chromatographic properties: R_f 0.32 (S_1), 0.45 (S_2) on PC; pale yellow fluorescence ($AlCl_3$) and green color with $FeCl_3$ spray reagents. Negative ESI-MS: m/z = 431.2 $[M – H]^-$, positive ESI-MS: m/z = 433.1 $[M + H]^+$, 455.2 $[M + H + Na]^+$. ^{1}H NMR(300 MHz, MeOH-d_4): δ = 13.27 (1H, br s, H-bounded OH-5), 7.61 (2H, br s, H-2′/6′), 6.82 (3H, br s, H-3, 3′/5′), 6.32 (1H, s, H-8), 4.70 (1H, d hidden by MeOH-d_4 signal, J = 9.5 Hz, H-1″), 4.19 (1H, br s, H-2″), 3.82 (2H, m, H-6″a/6″b), 3.46 (3H, m, H-3″, 4″, 5″).

5,7,3′,4′-Tetrahydroxyflavone (luteolin, 4)

A yellow amorphous powder with chromatographic properties: R_f 0.79 (S_1), 0.04 (S_2) on PC; deep yellow fluorescence ($AlCl_3$) and green color with $FeCl_3$ spray reagents. Negative ESI-MS: m/z = 285.1 $[M – H]^-$, positive ESI-MS: m/z = 287.1 $[M + H]^+$, 309.0 $[M + H + Na]^+$. ^{1}H NMR(300 MHz, Acetone-d_6): δ = 12.99 (1H, s, H-bounded OH-5), 7.49 (1H, d, J = 2.1 Hz, H-2′), 7.46 (1H, dd, J = 8.1, 2.1 Hz, H-6′), 6.99 (1H, d, J = 8.1 Hz, H-5′), 6.57 (1H, s, H-3), 6.52 (1H, d, J = 1.8 Hz, H-8), 6.25 (1H, d, J = 1.8 Hz, H-6).

Acid hydrolysis of Compound 1

A solution of 5 mg in 10 ml MeOH-1N HCl (1:1) was boiled under reflux for 4 h, concentrated under reduced pressure and diluted with H_2O (10 ml). It was extracted with AcOEt and the residue recovered from the organic phase yielded the aglycone. The remaining aqueous layer was neutralized with 5% aq. $NaHCO_3$ solution,

concentrated under vacuum and then the sugars were identified by comparative TLC with authentic standards.

Cytotoxicity assay

Materials

Doxorubicin vial was a generous gift from the National Cancer Institute (Egypt) drug store. The HEP-G2 human liver cell line, MCF-7 human breast cell line and HCT-116 human colon cell lines, were obtained from the American Type Culture Collection (Rockville, MD, USA) and the tumor cell line was maintained in the National Cancer Institute, Cairo, Egypt, by serial sub-culturing. RPMI medium, Fetal Calf Serum (FCS), antibiotics for cell culture, trypsin solution and tissue culture plastic ware were purchased from Costar (Milan, Italy). All other chemicals were obtained from Sigma-Aldrich Chemical Company (St. Louis, MO, USA).

Evaluation of cellular cytotoxicity

The cytotoxic activity of EE, Compounds 1, 2 and 4 against HEP-G2 cells, MCF-7 cells and HCT-116 cells, was determined using Sulphorhodamine-B assay (Skehan et al., 1990; Daduang et al., 2011). In brief, tumor cells were seeded into 96-well micro titer plates at a concentration of 5×10^4 cells/well in fresh medium and left to attach to the plate for 24 h. Cells were then incubated for 48 h in the presence of each compound at the noted concentrations (1, 2.5, 5 and 10 µg/ml). Following 48 h exposure to the compounds, cells were fixed then stained for 30 min with 0.4% sulphorhodamine-B and then washed with 1% acetic acid. The plates were then air-dried and the optical density of each well was measured spectrophotometrically at 564 nm using the ELISA microplate reader (Meter tech© 960, USA). Surviving fraction for each cell type was performed from which IC_{50} was calculated for each compound under investigation. Worth mentioning is that the cytotoxic activity of Doxorubicin against the three cell lines was performed at the same concentrations of tested compounds.

Antioxidant and renoprotective study

Drugs and chemicals

All chemicals used in this study were analytically pure product of Sigma-Aldrich Chemical Co., St. Louis, MO, USA.

Animals

Fifty Wistar albino rats weighing 150 to 170 g were used. The rats were obtained from Experimental Animal Care Center, College of Pharmacy, King Saud University. Animals have been kept in special cages, and maintained on a constant 12-h light/12-h dark cycle with air conditioning and temperature ranging 20 to 22°C and humidity (60%). Rats were fed with standard rat pellet chow with free access to tap water *ad libitum* for one week before the experiment for acclimatization. Animal utilization protocols were performed in accordance with the guidelines provided by the Experimental Animal Laboratory and approved by the Animal Care and Use Committee of the King Saud University, College of Pharmacy.

Experimental design

In order to observe the renoprotective action of the EE and themajor isolates 1, 2 and 4 in preventing or delaying acute renal failure (ARF) in rats, the following experimental protocol was performed. Rats were randomly allocated into seven groups, each consisting of seven animals. The animals were allowed free access to food, but deprived of drinking water for 18 h before glycerol injection. Group I (Control): comprised of control group that received equivalent volume of saline. Group II (Gly): animals received an intramuscular injection of 8 ml/kg hypertonic glycerol as a divided dose into the hind limbs (Zager, 1989). Group III (Gly/EE50), and group IV (Gly/EE100) animals received the EE (50 and 100 mg/kg, p.o. route, respectively) immediately after glycerol injection. Meanwhile group V (Gly/1), VI (Gly/2), and VII (Gly/4) animals were given orally Compounds 1, 2 or 4 in a dose 50 mg/kg respectively, immediately after glycerol injection. After 24 h of the glycerol injection rats were sacrificed and the blood was collected. Freshly isolated serum was used for the assessment of renal function tests. Both the kidneys were harvested through a midline incision, rinsed in cold isotonic saline, homogenized, and frozen at -85°C for different biochemical estimations.

Assessment of renal function

Serum samples were assayed for blood urea nitrogen (BUN) and serum creatinine (Neshat et al., 2011) by using standard diagnostic kits, Audit Diagnostic (Co Cork, Ireland).

Determination of lipid peroxides (MDA) and glutathione (GSH) level in kidney tissue

The degree of lipid peroxidation in kidney tissues was determined by measuring thiobarbituric acid reactive substances (TBARS) in the supernatant tissue from kidney homogenate (Uchiyama and Mihara, 1978). The absorbance was measured spectrophotometrically at 532 nm and quantified as nmols of malondialdehyde (MDA)/g wet tissue). Kidney tissue levels of acid-soluble thiols, mainly reduced glutathione (GSH) were determined colorimetrically at 412 nm (Ellman, 1959). Homogenates were precipitated with trichloroacetic acid, and after centrifugation, supernatants were used for the estimation of protein thiols (Protein-SH) expressed as µmol/gm wet tissue.

Determination of superoxide dismutase (SOD)

Superoxide dismutase activity was assayed spectrophotometrically as described by Marklund (1985).

Determination of catalase (CAT) activity

Catalase activity was determined as described by Aebi (1974).

Determination of glutathione peroxidase (GPX) activity

The glutathione peroxidase (GPX) activity was measured by the spectrophotometric assay of Paglia and Valentine (1967) and Deplanque et al. (2003).

Determination of glutathione-S-transferase (GST) activity

The GST activity was determined using the spectrophotometric assay of Habig et al. (1974).

Measurement of caspase-3 activity

In order to monitor apoptosis in kidney homogenates, caspase-3

Table 1. *Invitro* antitumor activities of EE and isolates 1, 2 and 4.

Compound no.	IC_{50} (µg/ml)[a]		
	HEP-G2	HCT-116	MCF-7
EE	18.1	11.9	23.6
1	10.7	9.3	9.9
2	15.8	11.2	19.5
4	12.3	9.5	12.7
Dox.	4.3	4.5	4.5

[a]IC_{50} sample concentration required to inhibit tumor cell proliferation by 50%.

activity was measured. Kidneys were homogenized in 400 µl of ice cold 50 mM HEPES (4-(2-hydroxyethyl)-1-piperazine ethane sulfonic acid) buffer, pH 7.4, 1 mM DTT (Dithiothreitol), 0.1 mM EDTA, 0.1% CHAPS (3-[(3-cholamidopropyl) dimethylammonio]-1-propane sulfonate). Non-soluble material was removed by centrifugation for 10 min. The activity was measured by using a caspase-3 cellular activity assay kit (catalog number 235419, CalBiochem, San Diego, CA, USA). As the acetyl-DEVD-p-nitroanilide substrate was cleaved by activated caspase-3, liberated p-nitroaniline was detected at 405 nm in a spectrophotometer equipped for 96 well micro-titer plates (model EL 312e, Bio-Tek Instruments Inc., Winooski, VT, USA). Casp-3 activities are reported as units of casp-3/ mg protein and 1 unit of casp-3 is defined as the release of 1 pmol/min of p-nitroaniline from the 200 µM AcDEVD p- nitroanilide.

Determination of tumor necrosis factor-α (TNF-α) concentration

The concentration of inflammatory cytokines (TNF-α) in serum was determined using commercially available ELISA assays following the instructions supplied by the manufacturer (DuoSet kits, R & D Systems; Minneapolis, MN, USA). The results are shown as pg/ml of cytokine.

Determination of tissue protein content

Protein was determined using Bovine Serum Albumin (BSA) as a standard, and measured at 660 nm (Lowery et al., 1951).

Statistical analyses

Data were expressed as mean ± S.D. For multi-variable comparisons, one-way ANOVA was conducted, followed by Bonferroni testing using the GRAPHPAD INSTAT (ISI Software) computer program. Differences were considered significant at p values of less than 0.05.

RESULTS

Chemistry

The EE extract of *H. dicksoniae* was subjected to a series of column chromatographic separations with convenient isolate compounds 1 to 4. Their accurate structures were elucidated by comparative thin layer chromatography (CoTLC) with authentic samples, chemical degradation, and comparison of their spectroscopic data (ESI-MS, NMR) with published data of structural related compounds (Mabry et al., 1970; Agrawal and Bansal, 1989; Williams and Harborne, 1994).

Biological study

In vitro antitumor evaluation

The antitumor activity of tested compounds against the three cell lines was determined using Sulphorhodamine-B assay and Doxorubicin (Dox.) as a reference drug. The response parameter (IC_{50}) was calculated for each cell line (Table 1). The antitumor drug discovery screen has been designed to distinguish between broad-spectrum antitumor compounds and tumor selective agents.From the results shown in Table 1, it could be seen that isolate 1 shows the highest broad-spectrum antitumor activity, out of the tested compounds, against HCT-116, MCF-7 and HEP-G2 carcinoma cell lines (IC_{50} = 9.3, 9.9 and 10.7 µg/ml, respectively), compared with the reference drug (IC_{50} = 4.5, 4.5 and 4.3 µg/ml).

Overall, all tested compounds and EE exhibited significant antitumor activity against the three cell lines in the order that could be put as 1>4>2> EE. Compound 4 showed selective high activity against colon cell line (IC_{50} = 9.5 µg/ml).

Kidney function

The kidney function data are shown in Figures 1 and 2. As expected, at 24 h following glycerol injection, serum creatinine and BUN were significantly increased. The glycerol group values of blood urea were significantly increased in comparison to the control group (65.78 ± 9.84 mg/dl vs. 17.905 ± 1.266 mg/dl, at $p < 0.001$).

Moreover, serum creatinine levels were significantly increased ($p < 0.001$) in the glycerol group vs. control mobile and stationary phases (previously aforementioned

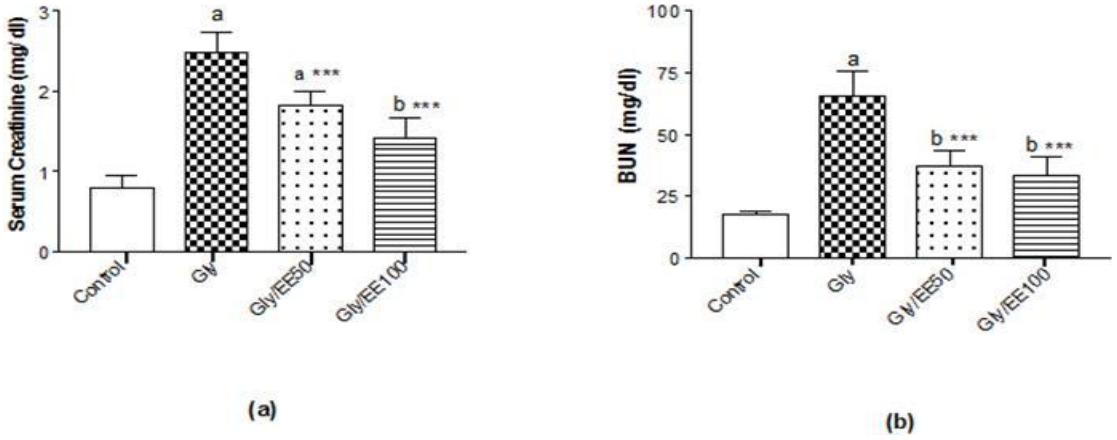


Figure 1.(a) Effect of EE (50 and 100 mg/kg) on serum creatinine; **(b)** blood urea nitrogen (BUN) in glycerol treated rats. The values are expressed as mean ± S.D. [a], p< 0.001, [b], p< 0.01compared to normal control group, ***, p< 0.001 compared to glycerol treated group,respectively, using ANOVA followed by Bonferroni as a post-ANOVA test.

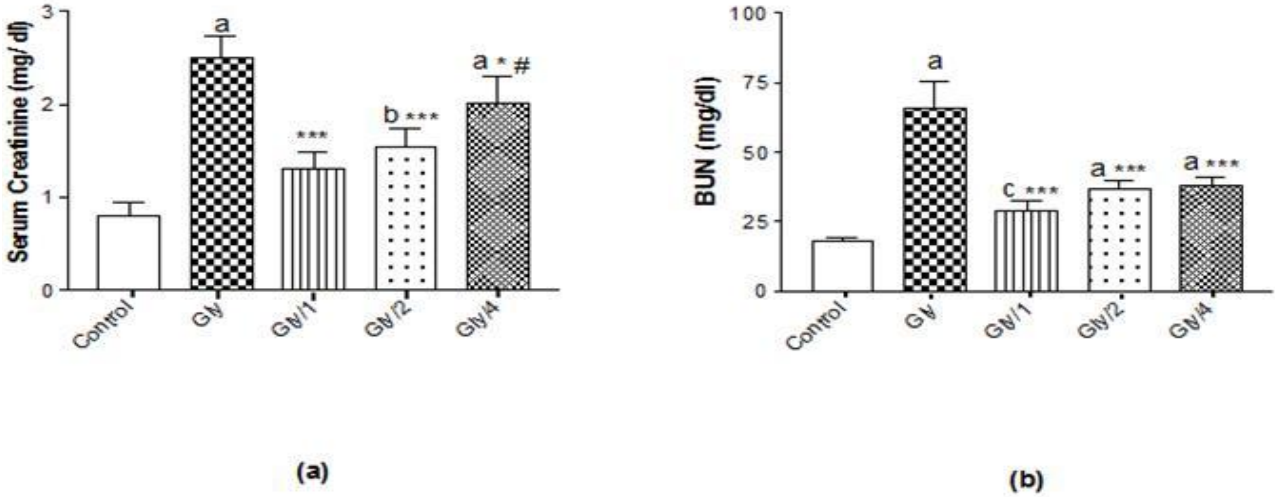


Figure 2.(a) Effect of the pure isolates 1, 2, 4 (50 mg/kg) on serum creatinine; **(b)** Blood urea nitrogen (BUN) (b) in glycerol treated rats. The values are expressed as mean ± S.D. [a], p< 0.001, [b], p< 0.01, [c], p < 0.005, compared to normal control group, *,p< 0.05, ***, p< 0.001 compared to glycerol treated group, [#], p< 0.01 compared to Gly/1-treated grouprespectively, using ANOVA followed by Bonferroni as a post-ANOVA test.

in experimental) to (2.493 ± 0.253 mg/dl vs. 0.788 ±0.157 mg/dl). Meanwhile, the levels of blood urea and serum creatinine were significantly reduced in all treated groups (III to VII) as compared with gly-treated animals (p < 0.01). However, treatment with 1 diminished these increases in BUN and creatinine level nearly to 50%. This indicates the strong renoprotective effect of Compound 1.

Lipid peroxidation and glutathione content

Following glycerol injection, TBARS levels were significantly increased by 100% as compared to the control group. Treatment with either EE (either 50 or 100 mg/ kg) or the major isolates 1, 2 4, nearly inhibited lipid peroxidation as compared with normal control group, and produced a significant reduction (p < 0.001) in TBARS content as compared with glycerol-treated rats. On the other hand, the levels of reduced glutathione in kidney tissues were significantly decreased after treatment with glycerol. However, treatment with each of the tested compounds significantly inhibited (p < 0.05) the glycerol-induced reduction in renal reduced glutathione content as compared to glycerol intoxicated rats (Tables 2 and 3).

Table 2. Effects of treatment with EE on lipid peroxidation, non-enzymatic, and enzymatic antioxidants in kidney tissues of glycerol treated rats.

Group	TBARS (n mol/g tissue)	GSH (µ mol/g tissue)	SOD (U/mg protein)	CAT (U/mg protein)	GPX (n mol/min/mg protein)	GST (µmol/min/g tissue)
control	151.31 ± 6.14	4.22 ± 0.78	2.53 ± 0.095	33.45 ± 1.64	40.57 ± 1.25	21.45 ± 3.85
Gly	303.23 ± 41.12^{a}	1.036 ± 0.04^{a}	1.37 ± 0.035^{a}	22.15 ± 2.08^{a}	23.97 ± 0.64^{a}	5.42 ± 1.13^{a}
Gly/EE 50	200.24 ± 32.67***	1.75 ± 0.12 ^{a}	1.71 ± 0.081^{a}**	25.02 ± 1.19^{a}	26.33 ± 0.59^{a}	10.77 ± 0.90 ^{a}*
Gly/EE 100	174.98 ± 9.316***	1.89 ± 0.07 ^{a}	1.72 ± 0.103^{a}**	26.12 ± 0.66^{b}*	35.17 ± 1.35 ^{a}***$^{\Omega}$	15.71 ± 0.86 ^{c}***

Data (n = 7) are presented as mean ± S.D. ^{a}, p< 0.001, ^{b,} p< 0.01 compared to normal control group, ^{c}, p< 0.001 compared to normal control group, *, p< 0.05, **, p< 0.01, ***,p< 0.001 compared to glycerol treated group, $^{\Omega}$, p< 0.001 compared to E50 treated group respectively, using ANOVA followed by Bonferroni as a post-ANOVA test.

Table 3. Effects of treatment with the pure isolates 1, 2, 4 on lipid peroxidation, non-enzymatic, and enzymatic antioxidants in kidney tissues of glycerol treated rats.

Group	TBARS (n mol/g tissue)	GSH (µ mol/g tissue)	SOD (U/mg protein)	CAT (U/mg protein)	GPX (n mol/min/mg protein)	GST (µmol/min/g tissue)
Control	151.31 ± 6.14	4.22 ± 0.78	2.53 ± 0.095	33.45 ± 1.64	40.57 ± 1.25	21.45 ± 3.85
Gly	303.23 ± 41.12^{a}	1.036 ± 0.04^{a}	1.37 ± 0.035^{a}	22.15 ± 2.08^{a}	23.97 ± 0.64^{a}	5.42 ± 1.13^{a}
Gly/1	147.15 ± 2.21***	2.92 ± 0.19^{c}***	2.24 ± 0.11^{b}***	28.73 ± 0.681^{c}**	36.54 ± 2.60***	10.81 ± 1.29^{a}*
Gly/2	149.02 ± 14.44***	2.64 ± 0.30^{b}**	1.81 ± 0. 039^{a}***$^{\#\#\#}$	26.66 ± 0.919^{a}*	30.50 ± 1.58^{a}**$^{\#\#}$	7.28 ± 1.74^{a}
Gly/4	157.29 ± 24.35***	2.16 ± 0.40^{a}*	1.87 ± 0.024^{a}***$^{\#\#\#}$	26.19 ± 0.91^{a}*	32.24 ± 1.58^{a}***$^{\#}$	8.80 ± 2.15^{a}

Data (n = 7) are presented as mean ± S.D. ^{a}, p< 0.001, ^{b}, p< 0.01, ^{c}, p< 0.001, compared to normal control group, *, p< 0.05, **, p< 0.01, ***, p< 0.001 compared to glycerol treated group, $^{\#}$,p< 0.05, $^{\#\#}$, p< 0.01 , $^{\#\#\#}$, p< 0.001 compared to Gly/1 treated group, respectively, using ANOVA followed by Bonferroni as a post-ANOVA test.

Renal antioxidant enzymes

Tables 2 and 3 showed the effects of rhabdomyolysis on the activity of SOD, CAT, GPX and GST in renal tissues. Glycerol injection caused significant decrease in renal antioxidant enzyme activities in all treated groups. This reduction was significantly improved by treatment with either the EE (100 mg/kg) or the major isolates as compared to glycerol treated group ($p < 0.05$).

Between the isolated compounds, the highest improvement in SOD and GXP and GST activity was shown in Gly/1-treated group as compared to Gly/2 or Gly/4 treated groups, indicating the strongest renoprotective effect of 1.

Caspase-3 activity

Caspase-3 is an important enzyme in the execution phase of apoptosis. Compared with the control, glycerol treatment significantly increased caspase-3 activity in kidney homogenate of all treated groups ($p < 0.05$).

Treatment with EE (100 mg/kg) or any of the isolated compounds significantly decreased ($p < 0.05$) the activity of caspase-3 compared to gly-intoxicated group (Figures 3 and 4). However, the most prominent alteration in caspase-3 activity was shown in gly/1-treated group as compared to either control group ($p < 0.01$) or gly-treated rats ($p < 0.001$).gly/1-treated group as compared to either control group ($p < 0.01$) or gly-treated rats ($p <0.001$).

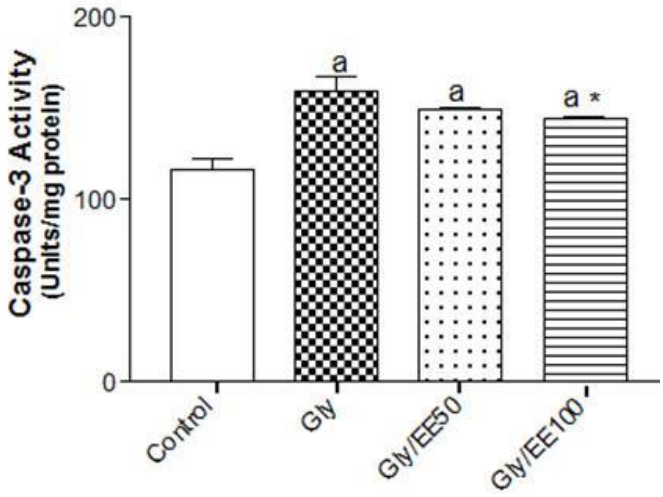


Figure 3. Effect of EE (50 and 100 mg/kg) on caspase-3 activity in kidney tissue of glycerol treated rats. The values are expressed as mean ± S.D. [a], p< 0.001 compared to normal control group, *, p< 0.05 compared to glycerol treated group,respectively, using ANOVA followed by Bonferroni as a post-ANOVA test.

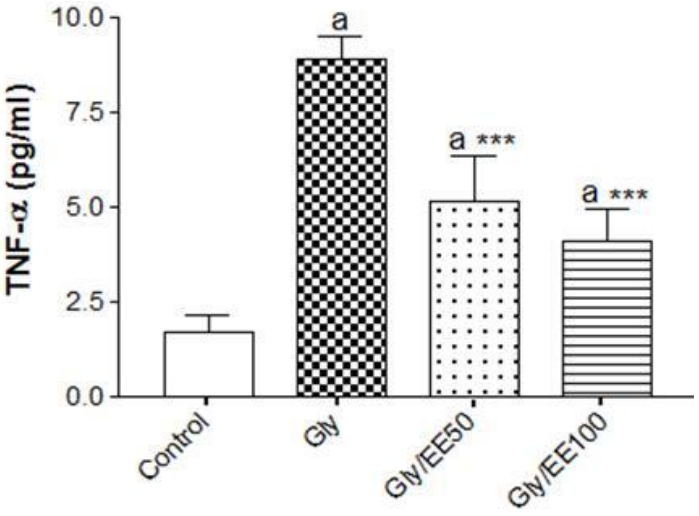


Figure 5. Effect of EE (50 and 100 mg/kg) on serum TNF-α level in glycerol treated rats. The values are expressed as mean ± S.D. [a]P< 0.001 compared to normal control group, ***P < 0.001 compared to glycerol treated group,respectively, using ANOVA followed by Bonferroni as a post-ANOVA test.

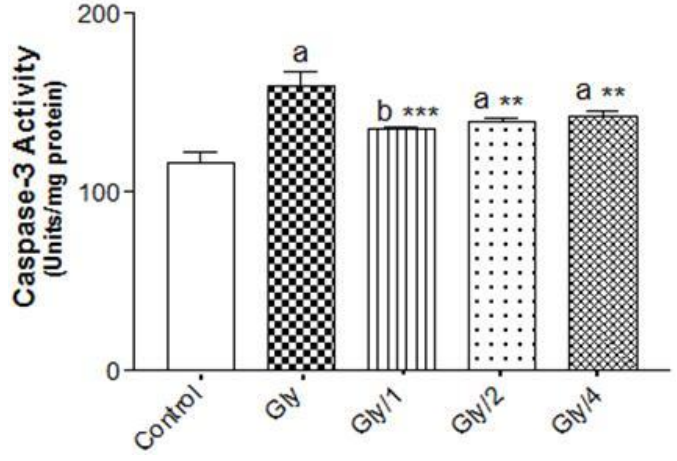


Figure 4. Effect of isolated Compounds 1, 2, and 4 (50 mg/kg) on caspase-3 activity in kidney tissue of glycerol treated rats. The values are expressed as mean ± S.D. [a],p< 0.001, [b], p< 0.01 compared to normal control group, *, p< 0.05; **, p< 0.01, compared to glycerol treated group,respectively, using ANOVA followed by Bonferroni as a post-ANOVA test.

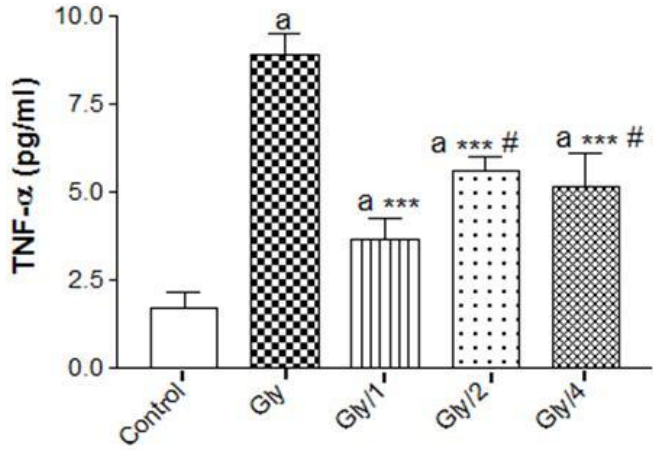


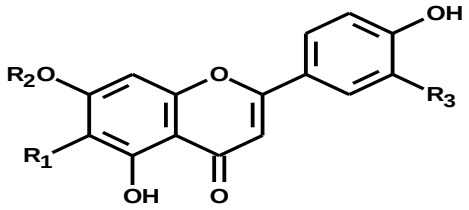
Figure 6. Effect of isolated Compounds 1, 2, and 4 (50 mg/kg) on serum TNF-α level in glycerol treated rats. The values are expressed as mean ± S.D. [a]P< 0.001 compared to normal control group, ***P < 0.001 compared to glycerol treated group, [#]P < 0.01 compared to Gly/1-treated group, respectively, using ANOVA followed by Bonferroni as a post-ANOVA test.

Serum tumor necrosis factor-α

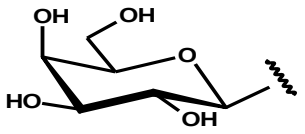
The level of TNF-α was highly increased by glycerol administration about 5 folds compared with normal group. Whereas, treatment with either EE (either 50 or 100mg/kg) or the isolated compounds, decreased serum TNF-α level compared with only-glycerol treated group ($p < 0.05$). Gly/1 treated rats showed the highest significant reduction in serum TNF-α level as compared to either gly/2 or gly/4–treated group ($p < 0.01$) (Figures 5 and 6).

DISCUSSION

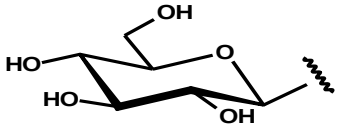
Based on the obtained data, Compound 1 was expected to be luteolin O-glycoside depending on its chromatographic properties (R_f-values, responses towards UV-light and spray reagents). This expectation was also recommended on acid hydrolysis that released luteolin in the organic phase and glucose in the aqueous one (CoTLC with specific spray reagents). Negative and positive ESI-MS showed molecular ion peaks at m/z



1: R_1= H, R_2= β-glucopyranoside, R_3= OH
2: R_1= β-galactopyranoside, R_2= H, R_3= OH
3: R_1= β-galactopyranoside, R_2= R_3= H
4: R_1= R_2=H, , R_3= OH



β-galactopyranoside



β-glucopyranoside

Figure 7. Structures 1 and 2: The characteristic difference between sugar moiety.

447.2 $[M - H]^-$ and 449.2 $[M + H]^+$ together with an adduct ion at 471.2 $[M + H + Na]^+$ to confirm the tentative identification of 1 as 5,3′,4′-trihydroxyflavone 7-O-glucoside that corresponds to MWt of 448. ^{1}H NMRshowed a singlet at δ 12.97 as an evidence for H-bounded OH-5.An ABX-spin coupling system was recorded at 7.44, 7.42 and 6.90 in the form of dd, (J = 8.1, 1.8), d (J = 1.8), and ortho-doublet (J = 8.1), for H-6′, 2′ and 5′ of 3′, 4′-dihydroxy ring B. An AM-coupling system of two meta-doublets (J = 2.1) was interpreted downfield (~Δ + 0.25 ppm) at 6.78 (H-8) and 6.44 (H-6) to confirm the glycosidation of OH-7 in 5, 7-dihydroxy ring A. The H-3 resonance was assigned at 6.74 (s) as an evidence for ring C in flavone. In the aliphatic region, a β-anomeric proton doublet was located at 5.08 (J= 7.2) and also one of the two diastereomeric CH_2-6″ was assigned at 3.72 (9.9 Hz, H-6″a), proving β–4C-stereostructure for glucoside moiety. The ^{13}C NMR spectrum showed 15 signals typical for luteolin 7-O-substituted aglycone including five key signals at δ = 181.8 (C-4), 162.9 (C-7), 149.9 (C-4′), 145.70 (C-3′) and 103.1 (C-3) (Agrawal and Bansal, 1989). Furthermore, the β-4C-stereostructure of glucoside moiety was confirmed due to its six ^{13}C resonances particularly those of C-1″, C-5″ and C-3″ at 99.9, 77.1 and 76.3, respectively. The full assignment of all ^{1}H and ^{13}C-resonances were confirmed by comparing with previously published data (Agrawal and Bansal, 1989; Williams and Harborne, 1994). Therefore, 1 was established as luteolin 7-O-β- D-glucopyranoside.

As for 1, Compound 2 was expected to be luteolin C-glycoside according to its chromatographic behavior (Mabry et al., 1970). Normal acid hydrolysis failed to release the aglycone and sugar, referring the C-glycosidation. It showed molecular ion peaks at m/z 447.2 $[M - H]^-$ and 449.1 $[M + H]^+$ in negative and positive modes with an adduct at 471.1 $[M + H + Na]^+$ corresponding to a MWt. of 448 for luteolinhexoside. As well as, it showed the same splitting pattern of luteolinaglycone in 1 (ABX- and AM-coupling systems) and showed H-1″ signal at 4.59 (d, J = 9.6) and δ and J-values characteristic for C-β-glycoside. The glycoside moiety was assigned as β-galactpyranoside based on the splitting pattern of all sugar proton signals. Final confirmation of the structure was obtained from ^{13}C NMR that showed more or less the same pattern of 15 signals for luteolinaglycone like in 1. The confirmative ^{13}C-evidence for C-glycosidation at C-6 was followed from the intrinsic downfield (~ + 10 ppm) of C-6 at 108.6. The characteristic difference between sugar moiety in both structures of 1 and 2(Figure 7) could be clearly explained by the appearance of C-5″ at 81.3 and sharp upfield location of C-1″ at 72.9 in ^{13}C NMR of 2. All other resonances were assigned by comparison with previous published data. Thus, 2 was identified as luteolin 6-C-β-D-galactopyranoside.

Depending on the chromatographic behavior of 3 specially R_f-values and fluorescence with $AlCl_3$, it was suggested to be 5,7,4′-trihydroxyflavone C-glycoside (Mabry et al., 1970). Similarly, it did not cleave on normal acid hydrolysis. ESI-MS analysis in negative mode gave a molecular ion at m/z 431.2 $[M - H]^-$ and in positive mode it was detected at 433.1 $[M + H]^+$ alongside an adduct with sodium at 455.2 $[M + H + Na]^+$ to support the structure as apigenin C-hexoside of 432 Da (MWt). Confirmation of the glycosidation position and type of sugar and aglycone was followed from the ^{1}H NMR depending on splitting pattern (J-values/multiblicity) and δ-values. The 1,4-disubstituted ring B was deduced from the A_2X_2-coupling system of two ortho-resonances for H-2′/6′ and H-3′/5′. Moreover, the connectivity of sugar was proved to be at C-6 due to the absence of H-6 signal and appearance of H-8 as singlet instead of meta-doublet. Finally, the structure of sugar was determined as β-galactopyranoside according to the splitting pattern of all resonances. The upfield location of anomeric proton at 4.7 and the characteristic δ-value of H-2″ were confirmative for C-glycosidic linkage. Thus, 3 was identified as apigenin 6-C-β-D-galactopyranoside Chromatographic properties led us to expect the structure of 4 as luteolinaglycone. This evidence was then reinforced

byESI-MS that showed a molecular ion peak at 285.1 $[M - H]^-$ and 287.1 $[M + H]^+$ in both negative and positive ionization modes with an adduct at 309.0 $[M + H + Na]^+$. Like in case of 1, ^{1}H NMR confirmed the structure of 4 as luteolin due to an ABX- and AM-spin coupling systems describable for 3′,4′-dihydroxy ring B and 5,7-dihydroxy ring A together with a singlet at 6.57 for H-3 in ring C. Therefore, 4 was established as luteolin.

Thus, this study provides convincing evidence for the oxidative stress-related renal dysfunction in this rat model of myoglobinuric ARF. A marked reduction in renal oxidative stress coupled with a significant improvement in renal function by the major isolates 1, 2, 4, indicates that their protective effect is based on free radical scavenging activity (Eldahshan et al., 2009; Tsai et al., 2011). Moreover, our results point to the potential role of cynaroside (1) in attenuating glycerol-induced ARF in rats. These results are in accordance with data reported on the antioxidant and anti-inflammatory effects of 1 and 4 (Lewis, 1989; Wolniak et al., 2007). Cynarosidealso showed the highest cytotoxic activity against the three tumor cell lines tested. These results are in accordance with data reported on the anticarcinogenic potential of Compound 1 (Baskar et al., 2011). As far as the available literature is concerned, this is the first report on either the bioactivity or chemical composition of the plant. In addition, this is the first report for the isolation of these compounds from the genus Horwoodia.

Conclusion

Three flavonoid glycosides and one aglycone have been isolated from EE of *H. dicksoniae* (Brassicaceae), for the first time, and their structures were established from negative ESI-MS, ^{1}H-, ^{13}C-NMR and DEPT as luteolin 7-O-β-D-glucopyranoside (1), luteolin 6-C-β-D-galactopyranoside (2), apigenin 6-C-β-D-galactopyranoside (3) and luteolin (4). The SRB cytotoxicity assay was used to investigate the antitumor activity of the EE, Compounds 1, 2 and 4. Compound1 showed the highest cytotoxic activity against the three human cell lines viz. HEP-G2, HCT-116 and MCF-7 cell lines (IC_{50} = 10.7, 9.3 and 9.9 µg/ml, respectively), compared with the reference drug. Compound 4 showed selective antitumor activity against the colon cell line (IC_{50} = 9.5 µg/ml). The present investigation also demonstrates the protective effect of Compounds 1, 2 and 4 with strong antioxidant potential, in glycerol–induced myoglobinuric acute renal failure in rats. Moreover, all tested compounds separately attenuated renal dysfunction, and restored the oxidant balance by decreasing renal MDA levels, increasing the activity of the depleted renal antioxidant enzymes, and the non enzymatic antioxidant GSH. Theyalsodecreasedtheelevatedserum inflammatory marker (TNF-α), and ameliorated apoptotic kidney damage by reduction in caspase-3 activity. Taken together, 1 was found to be the most biologically active compound.

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REFERENCES

Aebi H (1974).Catalase. In: Bergmeyer HU, ed. Methods of Enzymatic Analysis. Chemical Academic Press Inc. Verlag,pp. 673-685.
Agrawal PK, Bansal MC (1989). ^{13}C-NMR of Flavonoids. In: Agrawal PK, ed. Studies in Organic Chemistry 39, New York: Elsevier Science, 6:283-364.
Al Kahtani JSM, Al Yahya MA, Al Meshal IA (2000). Medicinal Plants of Saudi Arabia vol II. Riyadh, Saudi Arabia: King Fahad National Library, 36: 483.
Al Yahya MA, Al Meshal IA, Mossa JS, Al Badr AA, Tariq M (1990). Saudi Plants, A Phytochemical and Biological Approach. Riyadh, Saudi Arabia: King Saud University Press,pp. 225-227.
Baskar AA, Ignacimuthu S, Michael GP, Al Numair KS (2011). Cancer chemopreventive potential of Luteolin 7-O-glucoside isolated from Ophiorrhizamungos Linn. Nutr. Cancer, 63: 130-138.
Chaudhary SA (2001). Flora of the Kingdom of Saudi Arabia Illustrated vol I. Riyadh, Saudi Arabia: Ministry of Agriculture and Water, National Agriculture and Water Research Center, Cruciferae (Brassicaceae), pp. 464-533.
Chaudhary SA, Al Jowaid AA (1999). Vegetation of the Kingdom of Saudi Arabia. Riyadh, Saudi Arabia: Ministry of Agriculture and Water, National Agriculture and Water Research Center, p. 344.
Daduang J, Vichitphan S, Daduang S, Hongsprabhas P, Boonsiri P (2011). High phenolics and antioxidants of some tropical vegetables related to antibacterial and anticancer activities. Afr. J. Pharm. Pharm., 5:608-615.
Deplanque D, Gelé P, Pétrault O, Six I, Furman C, Bouly M, Nion S, Dupuis B, Leys D, Fruchart JC, Cecchelli R, Staels B, Duriez P, Bordet R (2003). Peroxisome proliferator-activated receptor-alpha activation as a mechanism of preventive neuroprotection induced by chronic fenofibrate treatment. J. Neurosci.,23:6264-6271.
Eldahshan OA, Ayoub NA, Singab AB, Al-Azizi MM (2009). Potential antioxidant phenolic metabolites from doum palm leaves. Afr. J. Pharm. Pharm., 3: 158-164.
Ellman GL (1959). Tissue sulfhydryl groups. Arch. Biochem. Biophys., 74: 214-226.
Habig WH, Pabst MJ, Jakoby WB (1974). Glutathion-S-transferase.The first enzymatic step in mercapturic acid formation. J. Biol. Chem.,249: 71130-71139.
Lewis DA (1989). Anti-inflammatory drugs from plant and marine source. In: Flavonoids. Berlin, Germany: AAS, Birkhauser Verlag, pp. 137-167.
Lowery OH, Roesborough MJ, Farr AL, Randall RJ (1951). Protein measurement with 606 Folin-Phenol reagent. J. Biol. Chem., 193: 265-275.
Mabry TJ, Markham KR, Thomas MB (1970).The Systemic Identification of Flavonoids. New York: Springer-Verlag: Berlin-Heidelberg, 5:pp. 41-164.
Marklund SL (1985). Pyrogallolantioxidation. In: Greenwald, R.A. (Ed.), Handbook of Methods for Oxygen Radical Research. Boca Raton, Florida, USA: CRC Press, pp. 243-247.
Mossa JS, Al-Meshal IA, Mekkawi AG, Hifnawy MS, AL Yahya MA (1985).Aromatic Plants of Saudi Arabia Part 10.Essential oil of H. dicksoniae Turril. Proc 8thSymp. Saudi Biol. Soc. Al Hassa, 8: 163-168.
Neshat M, Azarmi Y, Mehdi ZS, Doustar Y, Mousavi G (2011). Effects

of Hypericum perforatum (St. John's Wort) extract on renal function after unilateral ureteral obstruction in rat. Afr. J. Pharm. Pharm., 5: 457-461.
Paglia DE, Valentine WN (1967). Studies on the quantitative characterization of erythrocyte glutathione peroxidase. J. Lab. Clin. Med., 70: 158–169.
Skehan P, Storeng R, Scudeero D, Monks A, McMahon J, Vistica D, Warren JT, Bokesch H, Kenney S, Boyd MR (1990). New colorimetric cytotoxicity assay for anticancer-drug screening. JNCI, 82: 1107-1112.
Tsai JC, Huang GJ, Chiu TH, Huang SS (2011). Antioxidant activities of phenolic components from various plants of Desmodium species. Afr. J. Pharm. Pharm., 5: 468-476.
Uchiyama M, Mihara M (1978). Determination of malondialdehyde precursor in tissues by thiobarbituric acid test. Anal. Biochem., 86: 271-278.
Williams CA, Harborne JB (1994). 1D and 2D Nuclear Magnetic Resonances of Flavonoids. In: Harborne JB, eds. The Flavonoids: advances in research since 1986. Cambridge, London: Chapman & Hall, University Press, p. 441-497.
Wolniak M, Tomczykowa M, Gudej J, Waw er I (2007). Antioxidant activity ofextracts and flavonoids from Bidenstripartita.Acta. Pol. Pharm., 64: 441-447.
Zager RA (1989). Studies of mechanisms and protective manoeuvers in myoglobinuric acute renal injury. Lab. Invest., 60:619–629.