Available online on www.ijppr.com
International Journal of Pharmacognosy and Phytochemical Research 2016; 8(7); 1121-1131

ISSN: 0975-4873

Research Article

Hepatoprotection and Antioxidant Activity of *Gazania longiscapa* and *G. rigens* with the Isolation and Quantitative Analysis of Bioactive Metabolites

Samar Y Desoukey[1*], Wafaa M El Kady[2], Abeer A A Salama[3], Eman G Hagag[4], Siham M El-Shenawy[3], M A El-Shanawany[5]

[1]*Pharmacognosy Department, Faculty of Pharmacy, Minia University, Minia, Egypt*
[2]*Pharmacognosy Department, Faculty of Pharmaceutical Sciences & Pharmaceutical Industries, Future University in Egypt, New Cairo, Egypt.*
[3]*Pharmacology Department, National Research Centre, Giza, Egypt.*
[4]*Pharmacognosy Department, Faculty of Pharmacy, Helwan University, Cairo, Egypt.*
[5]*Pharmacognosy Department, Faculty of Pharmacy, Assiut University, Assiut, Egypt.*

Available Online:12th July, 2016

ABSTRACT
Gazania longiscapa and *G. rigens* are two species belonging to family Asteraceae. The present study aimed the isolation of the main active constituents from the methanol extracts using different chromatographic methods and their identification using different spectroscopic techniques, beside the quantitation of some biologically important active constituent as rutin using HPLC technique, together with estimation of total polyphenolic content calculated as gallic acid and estimation of total flavonoid content calculated as rutin using UV technique. Concomitantly the determination of the antioxidant and hepatoprotective activity of the total methanol extracts of the aerial parts of *G. longiscapa* and *G. rigens*. This work resulted in the isolation of 4 flavonoids (Apigenin, Luteolin, Luteolin 7-*O*-β-D-glucopyranosid, Apigenin 7-*O*-β-D-glucopyranosid), 3 phenolic acids (Caffeic acid, Chlorogenic acid and 3,5- di- O-caffeoylquinic acid) from *G. longiscapa* for the first time; these 3 phenolic acids were also isolated from *G. rigens,* together with one flavonoid (rutin), The quantitative determination of the methanol extracts showed that *G. longiscapa* is a richer source of phenolic acids than *G. rigens* and both *Gazania* species are valuable sources of rutin beside having hepatoprotective and antioxidant activity.

Keywords: Gazania, flavonoids, phenolic acids, hepatoprotective, antioxidant.

INTRODUCTION

Genus *Gazania* belongs to family Asteraceae, a rich family with valuable medicinal plants, and revealed a rich harvest of biologically active principles[1]. *Gazania longiscapa* DC. and *G. rigens* L. are native to South Africa, perennial or rarely annual and cultivated for their ornamental value[2]. Few reports were traced on the chemical constituents and the biological activities of *Gazania* speciesdemonstrating the presence of flavonoids, coumarins, polyphenolic acids as well as terpenes and sterols[3-6]. From the biological point of view, it was found that other species as *G. nivea* have hepatoprotective and antioxidant activities[7], while antimicrobial activity was observed in *G. rigens*[8]. This prompted us to investigate the bioactive constitutes of both *G. longiscapa* and *G. rigens*; beside the quantitation of some important biologically active constituent as rutin using HPLC technique and estimation of total phenolic and flavonoid using UV technique; together with the determination of the hepatoprotective and antioxidant activity of the total aqueous methanol extracts of the aerial parts (without the flower heads) of both *Gazania* species.

MATERIALS

Plant material

G. longiscapa and *G. rigens* aerial and underground parts were collected from the plantation of the Ministry of Agriculture located in the Fifth Settlement, New Cairo, Egypt in June 2013 during the flowering stage. Authentication of the plant was performed by Prof. Dr. Abd Alsalam El Noiehy, Prof. of Plant Taxonomy, Botany department, Faculty of Science, Ain Shams University, Cairo, Egypt. Voucher specimens for each species were kept in the department of Pharmacognosy, Faculty of Pharmacy, Future University in Egypt (FUE).

Materials for chromatographic study:

Column chromatography; microcrystalline cellulose (Merck, Darmstadt, Germany), polyamide (Fluka, Switzerland), sephadex LH-20 (Fluka, Switzerland), silica gel for column chromatography (CC, E. Merck, Darmstadt, Germany). For paper chromatography; Whatman no. 1 and Whatman no. 3 sheets (Whatman Ltd, Maidstone, Kent, England). For thin layer chromatography; pre-coated cellulose plates (20 × 20 cm) E. Merck, Darmstadt, Germany. The pure compounds

**Author for Correspondence: drsamaryehia@gmail.com*

were visualized by spraying with Naturstoff reagent[9] [(a)1% diphenyl boryloxyethanolamine in ethanol, (b) 5% polyethylene glycol 400 in ethanol, heating the dry chromatogram at 120°C for 10 min. and visualizing under UV light (365 nm)] and $FeCl_3$ (1% in ethanol). Solvent systems used were S_1 (15% aqueous HOAc), S_2 (n-BuOH: HOAc: H_2O (4:1:5)). The NMR spectra were recorded at 300, 400 and 500 (^{1}H) and 75, 100 and 125 (^{13}C) MHz, on a Varian Mercury 300, a JEOL GX-400 and a Bruker Top Spin 3.0 Software on the Upgraded 500 MHz Spectrometer. The results were reported as δ ppm values relative to TMS in the convenient solvents. ESI-MS analyses were run on LCQ (Finnigan MAT, Bremen, Germany) and LCQ-FT-MS mass spectrometers (Thermo Electron 400, Waltham, USA). UV analyses for pure samples were recorded on MeOH solutions and with different diagnostic UV shift reagents on a (Shimadzu 1800, Germany) spectrophotometer.

Materials for quantitation of rutin using HPLC

High performance (pressure) liquid chromatography [HPLC] apparatus

KNAUER Smart Line High-performance liquid chromatography (HPLC) including**:** Smart line pump 100 V5010, an ultraviolet detectorV2139, Injection switching valves V7452, Smart line degasser V7620, Smart line column oven 4050 V7335, Smart line RI detector V7607 (KNAUER, Germany).

Methanol HPLC grade (Scharlau, Spain).

Acetonitrile HPLC grade (Scharlau, Spain).

Water HPLC grade (Scharlau, Spain).

Materials for estimation of total polyphenolics and flavonoid content

UV- visible spectrophotometer (Shimadzu 1800, Germany).

Reference compound for authenticated standard (gallic acid & rutin) were obtained from E. Merck, Darmstadt, Germany; were used to construct linear standard curves

Aluminum Chloride reagent for assay of flavonoids (Peach and Tracey, 1955); 0.1 M Aluminum Chloride prepared by dissolving 2.4 g/100 ml distilled water.

Folin Ciocalteu reagent [10] obtained from LOBA chemie PVT, Ltd. India.

Materials for pharmacological study

Extract preparation

Aerial parts of the two *Gazania* species (without the flower heads) were separately, air dried, powdered and extracted with 80% methanol till exhaustion. The obtained methanol extracts were evaporated under reduced pressure and the obtained residues were exhaustively extracted with $CHCl_3$. The remaining methanol fractions were used for biological investigations.

Animals

Wiser male rats, weighing from 125-150 g were used throughout the experiments. Rats used for the hepatoprotective study were obtained from the animal house colony of the National Research Centre, Dokki, Giza, Egypt. The animals were housed in standard metal cages in an air conditioned room at 22 ± 3°C, 55 ± 5% humidity and provided with standard laboratory diet and water ad libitum. Experiments were performed between 9:00 and 15:00 h. Animal procedures were performed in accordance with the Ethics Committee of the National Research Centre and followed the recommendations of the National Institutes of Health Guide for Care and Use of Laboratory Animals.

Drugs, Chemicals and Diagnostic kits:

Paracetamol (EIPICO, Egypt)

Silymarin (SEDICO, pharmaceutical Co., 6 October City-Egypt).

DPPH [2, 2-Diphenyl-1-picrylhydrazyl radical] (Sigma, USA).

Diagnostic kits ALT (alanine aminotransferase) and AST (aspartate aminotransferase) according to Reitman and Frankel[11] All kits depend on colorimetric method and were obtained from Biodiagnostic Co., Egypt.

METHODS

Methods are done after approval of the research ethics committee; the approval form has serial no. REC-FPSPI-2/7.

Isolation and identification

Extraction of aerial parts of G. longiscapa & G. rigens

The air-dried powdered aerial parts of *G. longiscapa* (1 Kg) and *G. rigens* (0.6 Kg) were separately exhaustively extracted with 80% aqueous methanol. The residues left after evaporation of the solvent were (250 g) & (140 g) respectively, successively pre-purified with $CHCl_3$ under reflux (2 L x 2, 50°C) and solvent evaporated under vacuum, leaving 80 g of $CHCl_3$ extract and 160 g of methanol extract from *G. longiscapa* and 45 g of $CHCl_3$ extract and 90 g of methanol extract from *G. rigens*. The methanol extracts were precipitated from H_2O with excess methanol (1:10) for desalting. The desalted residues obtained from the filtrates (115 g) & (75 g) respectively, were each suspended in water and chromatographed on polyamide S column (650 g, 120 x 5 cm) using a step-gradient of H_2O-MeOH.

Fractionation of G. longiscapa methanol extract

Five major collective fractions (I - V) were obtained from the polyamide S column, monitored by comparative paper chromatography, TLC cellulose and UV-light. Fraction I was found to be polyphenolic-free (NH_3vapour and $FeCl_3$ spray reagent/PC). Fraction II; was chromatographed on a consecutive microcrystalline cellulose column using gradient aqueous MeOH to give compound **Gl (1)** (15 mg). Purification of fraction III was achieved by sephadex LH-20 column using gradient aqueous MeOH to afford **Gl (2)** (25 mg) and **Gl (3)** (18 mg). Fraction IV has 2 major separate spots which were best viewed on pre-coated cellulose plates using S_1 as developer, where preparative Whatmann No. 3 was used with S_1 as developer for separation and isolation of **Gl (4)** (5 mg) and **Gl (5)** (7 mg). Fraction V was chromatographed on silica columns using CH_2Cl_2/MeOH with gradual increase in polarity with methanol, this resulted in 2 major fractions that were purified on sephadex columns using MeOH (50%) for elution in each fraction to give **Gl (6)** (14 mg) and **Gl (7)** (11 mg).

Fractionation of G. rigens methanol extract

Similar to *G. longiscapa*, five major collective fractions (I

Table 1: ^{1}H NMR spectral data of compound Gl (1) & Gr (1).

Chemical shift (ppm)		Assignment
Gl (1)	Gr (1)	
7.40 d (15.9)	7.60 d (15.9)	1H, H-7
7.03 br s	7.30 d (2.5)	1H,H-2
6.95 br d (8.1)	7.10dd (8.1, 2.5)	1H, H-6
6.76 d (8.4)	6.85 d (8)	1H,H-5
6.15 d (15.9)	6.40 d (15.9)	1H, H-8

J-values (Hz) were reported in parenthesis

- V) were obtained from the polyamide S column of *G. rigens*, monitored by comparative paper chromatography, TLC cellulose and UV-light. Fraction I was found to be polyphenolic-free (NH_3vapour and $FeCl_3$ spray reagent/PC). Fraction II; was chromatographed on sephadex LH-20 column using 50% MeOH to afford **Gr (1)** (25 mg). Fraction III was purified on a consecutive microcrystalline cellulose columns using (*n*-Butanol/ Isopropyl alcohol /H_2O; 4: 1: 5 v/v/v -top layer-) BIW for elution to give compound **Gr (2)**15 mg. Fraction IV has a major spot, best viewed on pre-coated cellulose plates using S_1 as developer, and was isolated using preparative Whatmann No. 3 with S_1as developer giving **Gr (3)** (18 mg). Fraction V has a major spot that can be isolated using preparative Whatmann No. 3 with S_1as developer to afford **Gr (4)** (20 mg).

HPLC quantitation of rutin in the aerial and underground parts of G. longiscapa and G. rigens

Plant material and sample preparation

One gram of the aerial and underground parts of each of the two species was air dried, powdered and exhaustively extracted using MeOH/HPLC grade, four times using 25 ml for 48 hours, the solvent in each extract was evaporated under reduced pressure, filtered through 0.045 millipore filter, completed to 10 ml by MeOH/HPLC grade in a volumetric flask then subjected to HPLC quantitation of rutin under specific condition.

Preparation of standard solution

Ten mg of authentic rutin was dissolved in MeOH /HPLC grade in a 25 ml volumetric flask to give 400 µg/ml (stock solution), serial dilutions were made and a calibration curve was constructed over a range of (40- 400 µg).

Assay

Based on the method adopted by Shanmugam[12]. with some changes; the chromatographic separation was performed on C18 Agilent column (150 × 4.6 mm, 5 µm) as stationary phase with a mobile phase comprised of phosphate buffer: acetonitrile (70:30) v/v. at a flow rate 1.2 ml/min. and UV detection at 280 nm with a run time of eight min. and injection volume of 20 µl. N.B. Phosphate buffer was prepared by dissolving 4 gm KH_2PO_4 in 1 L water HPLC grade and adjusted with phosphoric acid to 3.2 pH.

Colourmetric UV investigation of total polyphenol content in aerial parts and underground parts of G. longiscapa and G. rigens

Plant material and sample preparation

Ten grams of each of the powdered plants under investigation were separately exhaustively extracted with 80% ethanol; the filtrates were evaporated to give crude polyphenol extracts of (2, 1.75, 1.9, 1.8 g) for *G. longiscapa* aerial & underground parts and *G. rigens* aerial & underground parts, respectively. A stock solution was prepared in distilled water with a final concentration of 1 mg/ml.

Preparation of gallic acid standard solution

The standard solution was prepared by dissolving 5 mg of gallic acid in 100 ml water that the concentration is 50 µg /ml. A calibration curve was constructed over a range of 0.5-2.5 µg/ml.

Assay

Total polyphenol content was determined according to the procedure adopted by using Folin Ciocalteu colourimetric method[10] and expressed as mg of gallic acid equivalent to plant dry weight.

UV investigation of total flavonoid content in aerial and underground parts of G. longiscapa and G. rigens calculated as rutin.

Plant material and sample preparation

One gram of the aerial and underground parts of both *Gazania* species under study was, each, separately exhaustively extracted with ethanol 80%. The different ethanol extracts obtained were dried, and then the residues were each dissolved in ethanol, transferred to a volumetric flask (100 ml capacity) and completed to volume with ethanol. After mixing well, 1 ml of each prepared solution was transferred to a test tube and evaporated to dryness. 10 ml of 0.1M $AlCl_3$ solution was added to each and shaken. Then the intensity of the developed yellow colour was measured at 415 nm against blank.

Preparation of standard solution

Aliquots (2-8 ml) of 0.002% solution of rutin in ethanol equivalent to (40-160 µg) were separately transferred to test tubes and evaporated to dryness on a water bath at 60°C. The residue in each case was treated and shaken with 10 ml of 0.1 M $AlCl_3$ solution (2.4 g in 100 ml H_2O).

Assay

The adopted method was based on measuring the intensity of the colour developed when flavonoid was complexed with aluminum chloride, the intensity of the developed yellow colour was measured at 415 nm against a blank and the percentage was calculated as rutin with reference to a pre-established standard calibration curve[13].

Biological study

Acute toxicity study

Both extracts of *Gazania* species showed low toxicity profile with high safety margin up to 5 g/ kg[14].

Hepatoprotective activity study

Hepatic damage was induced in rats by a single oral dose of paracetamol 1000 mg/kg[15]. fifty-four rats were divided into nine groups of six animals each, for each extract as following:

Group 1: Normal control group, rats were given a daily oral dose of 1ml distilled water.

Group 2: Paracetamol group, rats were given a single oral dose of paracetamol (1000mg/kg)

Group 3: Silymarin group (reference heptoprotective drug), rats were given a daily oral dose of silymarin (25 mg/kg)[16] alone for successive 14 days before paracetamol administration.

Table 2: ^{1}H, ^{13}C-NMR spectral data of compound Gl (2)&Gr (3) (500, 125 MHz, DMSO-d_6).

C	δ_H		δ_C
No.	Gl (2)	Gr (3)	Gl (2)
1			79.11
2	1.79 *m*	1.98*dd*(3, 15)	38.67
3	4.20 *m*	4.10 *m*	70.50
4	3.70 *dd* (8.8, 2.9)	3.95*m*	73.13
5	5.37 *m*	5.15 *m*	71.90
6	2.03 *m*	2*br d* (13)	37.54
7			173.00
2`	7.05 *d* (2)	7.08 *d* (2)	114.85
3`			144.68
4`			148.11
5`	6.75 *d* (6.8)	6.70 *d* (8)	116.22
6`	6.80*dd* (8, 2)	6.93*dd* (8, 2)	121.67
7`	7.54 *d* (15.9)	7.43 *d* (16)	145.45
8`	6.25 *d* (15.9)	6.20 *d* (16)	115.25
9`			163.42

J-values (Hz) were reported in parenthesis

Table 3: ^{1}H NMR spectral data (500 MHz, DMSO– d_6) of compound Gl (3)

Chemical shift(ppm)		Assignment
Gl (3)	Gr (2)	
7.47 d (15.9)	7.58 d (15.9)	1H, H-7
7.39 d (15.9)	7.52 d (15.9)	1H, H-7`
7.01 s	7.06 s	1H,H-2
6.99 s	7.05 s	1H,H-2`
6.88 dd (8.2, 2)	6.96 dd (8.1, 2)	1H, H-6
6.83 dd (8.2, 2)	6.91 dd (8.1, 2)	1H, H-6`
6.71 d (7.8)	6.76 d (8.1)	1H, H-5
6.68 d (7.8)	6.73 d (8.1)	1H, H-5`
6.20 d (15.9)	6.35 d (15.9)	1H, H-8
6.13 d (15.9)	6.27 d (15.9)	1H, H-8`
5.22 m	5.30 m	1H, H-5``
5.19 m	5.15 m	1H, H-3``
2.32 m	2.11 m	2H, H-2``
2.09 m	1.81 m	2H, H-6``

J-values (Hz) were reported in parenthesis

Group 4-9: Treated groups, rats were given a daily oral dose of methanol extracts of the aerial parts of either *G. longiscapa* or *G. rigens* (250, 500 and 1000 mg/kg) alone for successive 14 days before paracetamol administration, and at the end of the experimental period (24 h after oral paracetamol administration), the blood was obtained from all groups of rats after being lightly anaesthetized with ether by puncturing rato-orbital plexus[17], the blood was allowed to flow into a clean dry centrifuge tube and left to stand 30 minutes before centrifugation to avoid hemolysis. Then blood samples were centrifuged for 15 minutes at 2500 rpm, the clear supernatant serum was separated and collected by Pasteur pipette into a dry clean tube for the determination of serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST).

In-vitro Antioxidant activities by (DPPH radical scavenging activity)

The DPPH assay [2,2-Diphenyl-1-picrylhydrazyl radical (DPPH)] was performed according to the method adapted from[18,19]. A solution of DPPH in methanol (0.004% solution) was prepared and stored in the dark until use. Different concentrations of the extracts mentioned above for biological study were prepared for both *G. longiscapa* and *G. rigens* aerial parts. Twenty µl of each of these extracts were added to 180 µl DPPH solution in 96 well plates. The resultant reaction mixtures were vortex-mixed and incubated at room temperature for 30 min. The absorbance of the reaction mixtures was measured at 520 nm. Methanol was used as blank and DPPH solution without addition of extract was used as control. Ascorbic acid was used as a reference standard. The scavenging activity was calculated by the following formula:

Scavenging activity (%) = $(A_1\text{-}A_0/A_1) \times 100$

Where A_1 is the absorbance of the control and A_0 is the absorbance of the extract or reference standard.

RESULTS AND DISCUSSION

Identification of isolated compounds

Compounds Gl (1) & Gr (1)

Both compounds from *G. longiscapa* **Gl (1)** and *G. rigens* **Gr (1)** gave the same physical, chemical and spectroscopic characters, both are off-white amorphous powder m.p. 194-195°C; R_f values 0.42 (S_1), 0.72 (S_2) for ***Gl (1)*** and 0.43 (S_1), 0.73 (S_2) for **Gr (1)** on pre-coated cellulose plate. They gave sky blue fluorescence under UV-light, changing to greenish blue fluorescence with Naturstoff (NA/PE) and blue color with $FeCl_3$ spray reagent. UV max nm: (MeOH) 283, 313 (+NaOMe) 298, 350 for **Gl (1)** and UV max nm: (MeOH) 218, 297 sh, 327 nm for **Gr (1)**. ^{1}H NMR (300 MHz, DMSO-*d6*) data are listed in Table 1.

From the above data and comparison with previous reports[7,8,20] compound **Gl (1) & Gr (1)** were identified as Caffeic acid (Figure 1).

*Compounds **Gl (2) & Gr (3)***

Both compounds have similar physical properties, isolated as pale yellow needle from methanol fraction. They are soluble in methanol, m.p. 208-210ºC. They gave blue fluorescence, the intensity of this blue colour increased upon exposure to NH_3, using pre-coated cellulose plates. They showed R_f value of 0.67 (S_1). ^{1}H, ^{13}C-NMR (500, 125 MHz, DMSO-d_6) data are listed in Table 2.

From the above data and comparison with previous reports[7,8,20]. compound **Gl (2) & Gr (3)** were identified as Chlorogenic acid (Figure 1).

*Compounds **Gl (3) & Gr (2)***

Both compounds were isolated as off-white amorphous powder, the R_f value was 0.61 (S_1), m.p. 223-225ºC., they gave violet fluorescence, the intensity of the fluorescence increases upon exposure to NH_3vapour. **UV**-spectrum of **Gl (3)** in **MeOH** gave two intrinsic absorbance at λ_{max} 299 nm and 324 nm and **UV**-spectrum of **Gr (2)** in **MeOH** gave 220,244 and 328 nm characterized for cinnamoyl chromophore The **^{1}H NMR** spectral data of compound **Gl (3)** are recorded in (Table 3). From the above mentioned physical, chemical, chromatographic and spectroscopic studies and comparing with previous published data[21], compound **Gl (3)** and **Gr (2)** were identified as (3,5- di- O-caffeoyl quinic acid) (Figure 1).

*Compound **Gl (4)***

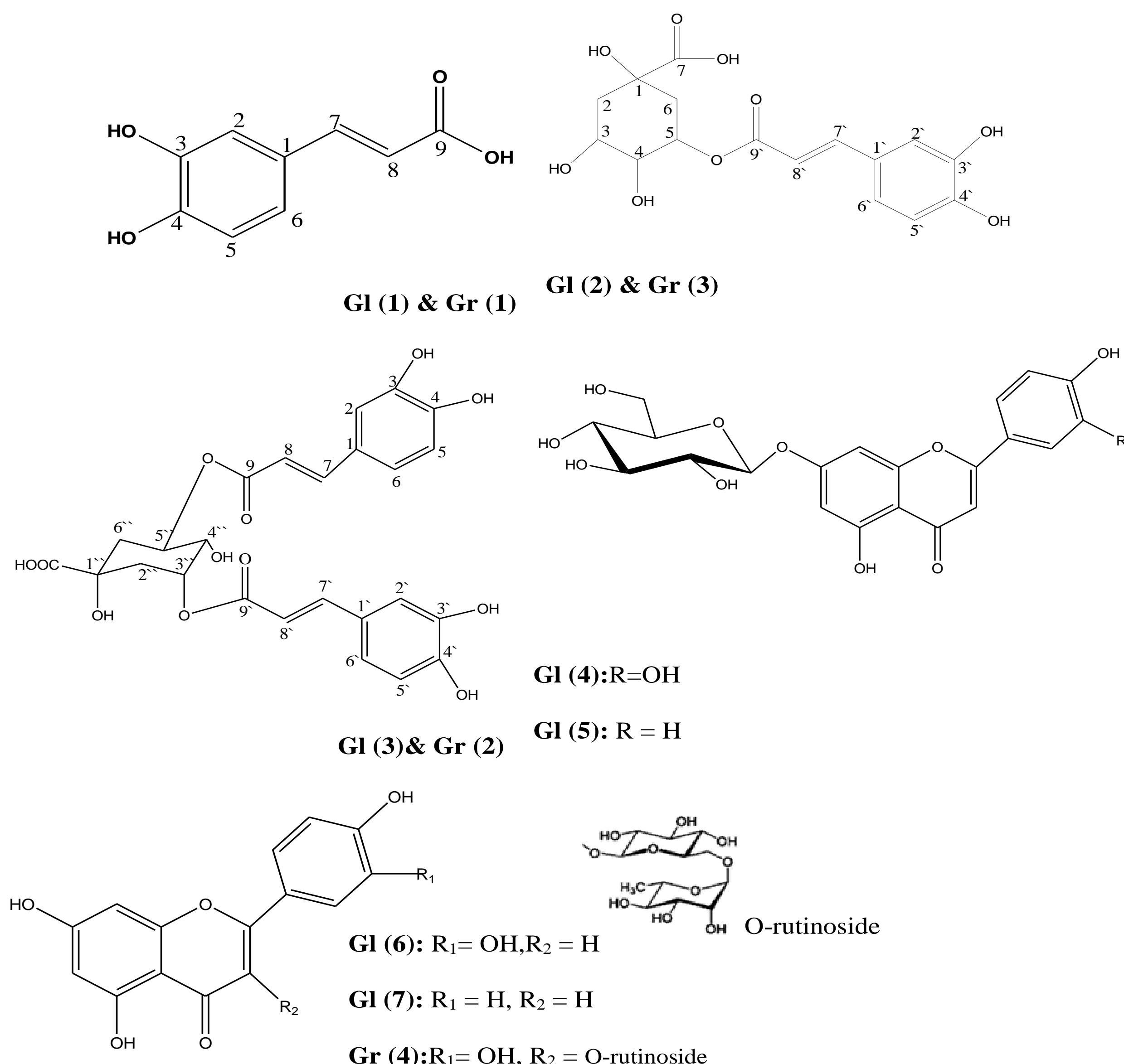


Figure 1: Structures of the identified compounds from *G. longiscapa* and *G. rigens*.

Isolated as reddish brown amorphous powder, m.p. 266-268°C., Negative ESI/MS of **Gl (4)** showed a molecular ion peak at *m/z*447.09 $[M-H]^-$for luteolin monohexoside with a fragment ion peak of the luteolin aglycone at 284.04

*Compound **Gl (5)***

Isolated as brown amorphous powder, m.p. 230-237°C., Negative ESI/MS of **Gl (5)** revealed a molecular ion peak at *m/z* 431.2 $[M-H]^-$ corresponding to M.wt of 432 of apigenin monohexoside with a fragment ion at *m/z* 269.17 for apigenin aglycone.

*Compound **Gl (6)***

Isolated as yellow amorphous powder, m.p. 328-330°C. Negative ESI/MS of **Gl (6)** revealed a molecular ion peak at *m/z* 284.04 $[M-H]^-$.

*Compound **Gl (7)***

Isolated as yellow amorphous powder, m.p. 345-350°C. Negative ESI/MS of **Gl (7)** revealed a molecular ion peak at *m/z* 269.17 $[M-H]^-$.

*Compound **Gr (4)***

Isolated as yellowish amorphous powder, m.p 242 °C. Negative ESI/MS of **Gr (4)** revealed a molecular ion peak at *m/z* 611.03 $[M-H]^-$corresponding to M.wt of quercetin dihexoside with fragment ions at *m/z* 465 $[M-H]^-$ and 303 $[M-H]^-$, consistent with the sequential loss of two hexose units from the molecular ion. The chromatographic properties, UV data and ^1H NMR data of **Gl (4)**, **Gl (5), Gl (6), Gl (7)** are listed in Tables 4 and 5. The structures of **Gl (4)**, **Gl (5), Gl (6), Gl (7)** (Figure 1) were fully elucidated on the basis of their physicochemical and spectral data (in particular UV, ^1H and ESI-MS) and by comparison with published data[22,23] and were identified as following

***Gl** (4) Luteolin **7-O-β-D-**glucopyranoside*

***Gl** (5) Apigenin **7-O-β-D-**glucopyranoside*

***Gl** (6) Luteolin*

***Gl** (7) Apigenin*

***Gr** (4) Rutin*

HPLC quantitation of rutin in the aerial and underground parts of G. longiscapa and G. rigens

The retention time of standard rutin was detected (Figure

Table 4: Chromatographic properties and UV data of *Gl (4)*, *Gl (5), Gl (6), Gl (7)*

	R_f		Fluorescence in long UV	Spray reagent response		UV data λ max (nm)					
	S_2	S_1		$FeCl_3$	NA/PE UV	MeOH	+ NaOMe	+ NaOAc	+NaOAc +H_3BO_3	+ $AlCl_3$	+ $AlCl_3$ + HCl
Gl (4)	0.53	0.18	d.pr	gr	or.	254, 268 sh 348	264 , 300 sh, 388	256 ,267 sh, 354, 405 sh	259, 376	274, 299 sh, 331, 431sh	275, 291sh,346,390
Gl (5)	0.51	0.12	d.pr	gr	gr. y.	267, 336	271, 296 sh, 382	268, 269 sh, 347, 382	268, 332	274, 300, 339, 384	275, 300 sh, 345, 385
Gl (6)	0.80	0.20	d.pr	gr	or.	242sh, 252, 266, 348	266, 329 sh, 400	266, 301 sh, 383	260, 295, 371	273, 300 sh, 327, 425	265 sh, 275, 293 sh, 354, 386
Gl (7)	0.73	0.07	d.pr	gr	gr. y.	267, 296 sh, 336	275, 324, 392	274, 301, 376	268, 302 sh, 338	276, 301, 348, 384	276, 299, 340, 381
Gr (4)	0.37	0.26	d.pr	gr	or.	257, 359	272, 411	273, 387	260, 381	275, 430	271, 293sh, 400

d.pr=deep purple; gr = green; gr. Y= greenish yellow; or=orange; sh=shoulder; y= yellow.

Table 5: ^{1}H NMR data of Gl (4), Gl (5), Gl (6), Gl (7)

Gl (4)	Gl (5)	Gl (6)	Gl (7)	Gr (4)	Assignment
6.70 s	6.83 s	6.53 s	6.59 s		H-3
6.40 d (2.4)	6.42 d (2.0)	6.20 d (2.1)	6.20 d (2.1)	6.20 d (2.0)	H-6
6.73 d (2.4)	6.76 d (2.4)	6.43 d (2.1)	6.45 d (2.1)	6.40 d (2.0)	H-8
6.91 d (8.4)	-	6.89 d (8.7)	-	6.80 d (8.0)	H-5\`
7.44 d (2.4)	-	-	-		H-2\`
7.40dd (2.4, 8.4)	-	-	-		H-6\`
-	6.94 d (8.4)	-	6.92 d (9.0)		H-3\`/5\`
-	7.97 d (8.4)	7.37 m	7.84 d (9.0)	7.66 m	H-2\`/6\`
5.06 d (7.6)	5.05 d (7.6)	-	-	5.30d (8.0)	H-1\`\`
				4.40 d (2.0)	H-1\`\`\`
3.80 – 3.00 m	3.80 – 3.00 m	-	-	3.80–3.00m	Remaining sugar protons

J-values (Hz) were reported in parenthesis

Table 6: Concentration of rutin in *G. longiscapa* and *G. rigens* in both aerial and underground parts

Plant	AUP	Conc. µg/ml	Conc. (mg/g)
G.l ar	3640	218.33	2.1
G.l ug	3519	211.66	2.1
G.r ar	3739	223.79	2.2
G.r ug	4740	278.99	2.7

3.a.) and a calibration curve was constructed (Figure 3.b.) from which the determination of rutin concentration in the aerial and underground parts of both *Gazania* species were calculated by substitution of area under the peak (Figure 4) in each equation as presented in (Table 6) and the results were calculated in g % dry weight of each plant organ.

Colourmetric UV investigation of total polyphenol content in aerial parts and underground parts of G. longiscapa and G. rigens.

From the linear regression analysis obtained from the calibration curve (Figure 5), the polyphenol content of each extract was calculated as in (Table 7).

UV investigation of total flavonoid content in aerial and underground parts of both G. longiscapa and G. rigens calculated as rutin

The total flavonoid content in both *Gazania* species can be calculated by substitution in the regression equation of the calibration curve (Figure 6). Results are listed in (Table 8).

BIOLOGICAL RESULTS

Hepatoprotective result

Table 7: Results of estimation of the total polyphenolic content in 80% ethanol extract of aerial and underground parts in *G. longiscapa* and *G. rigens*.

Plant extract	Absorbance	Conc. µg/ml	CPP (g /10 g dry wt)	TP in mg (GAE) /g dry wt
G.l ar	0.8485	38.74	2	3.09
G.l ug	0.7683	35.03	1.75	2.45
G.r ar	0.8122	37.06	1.9	2.81
G.r ug	0.7346	33.46	1.8	2.40

CPP = crude polyphenol extracts, TP = Total polyphenol, GAE = gallic acid equivalent, *G.l* ar = *G. longiscapa* aerial parts, *G.l*ug = *G. longiscapa* underground parts, *G.r*ar = *G. rigens* aerial parts, *G.r*ug = *G. rigens* underground parts.

Table 8: Result of estimated flavanoidal content in 80% ethanol extract of aerial and underground parts of *G. longiscapa* and *G. rigens*

Plant extract	Abs.	Corresponding concentration in µg rutin / ml	Total flavonoid(mg /g) calculated as rutin
G.l ar	0.069	23.16	2.3
G.l ug	0.041	19.62	1.9
G.r ar	0.082	24.8	2.5
G.r ug	0.053	21.13	2.1

G.l ar= *G. longiscapa* aerial parts, *G.l* ug = *G. longiscapa* underground parts, *G.r* ar = *G. rigens* aerial parts, *G.r* ug = *G. rigens* underground

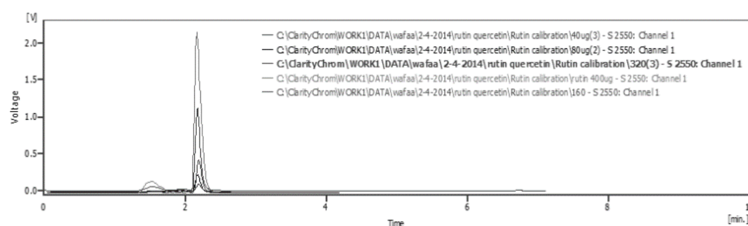


Figure 3.a: HPLC chromatogram of different concentration of rutin

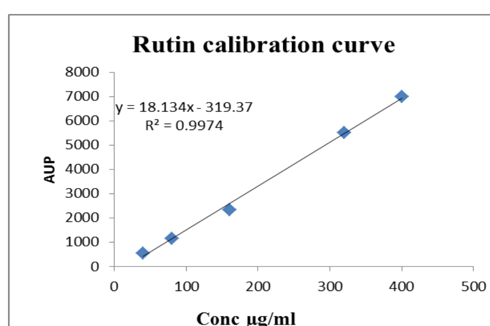


Figure 3.b: Calibration curve for rutin

Rats were given a single oral dose of paracetamol (1000 mg/kg) showed significant elevation in their serum enzyme levels of ALT and AST by 55.42 and 20.38 % after 24 hours as compared with control group. The extract of *G. longiscapa* aerial parts given at dose levels of (250, 500 and 1000 mg/kg) showed reduction in elevated serum ALT and AST levels by 20.25 and 12.17 % at the dose of 250 mg/kg and significant reduction by 32.97 and 22.07 % at the dose of 500 mg/kg and by 26.94 and 20.33% at the dose of 1000 mg/kg respectively, as compared with paracetamol treated group. Regarding silymarin (25 mg/kg) exhibited significant reduction in serum ALT and AST levels by 14.86 and 17.83 % respectively, as compared with paracetamol treated group (Figure 7). While oral administration of *G. rigens* aerial parts extract given at dose of (250,500 and 1000 mg/kg) showed reduction in elevated serum ALT and AST levels by16.61 and 17.79% at the dose level of 250 mg/kg, by 28.87 and 21.96 % at dose 500 mg/kg and by 20.17 and 12.99 % at the dose level of 1000 mg/kg respectively, compared with paracetamol treated group (Figure 8).

In vitro study of antioxidant activities

The antioxidant activity of methanol extracts of both *G. longiscapa* and *G. rigens* aerial parts were studied in ***vitro*** using the DPPH method; the results of the kinetics of DPPH scavenging reaction of tested extracts and L-ascorbic acid were demonstrated in (Figure 9) and revealed that *G. longiscapa* aerial parts methanol extract in different concentrations (10, 20, 40, 60, 80 and 100 mg/ml) showed a remarkable significant scavenging activity, the maximum reactive reaction rate after 45 sec. was (82.3, 82.9, 84.0, 89.0, 96.0 and 98.8 %), respectively (Figure

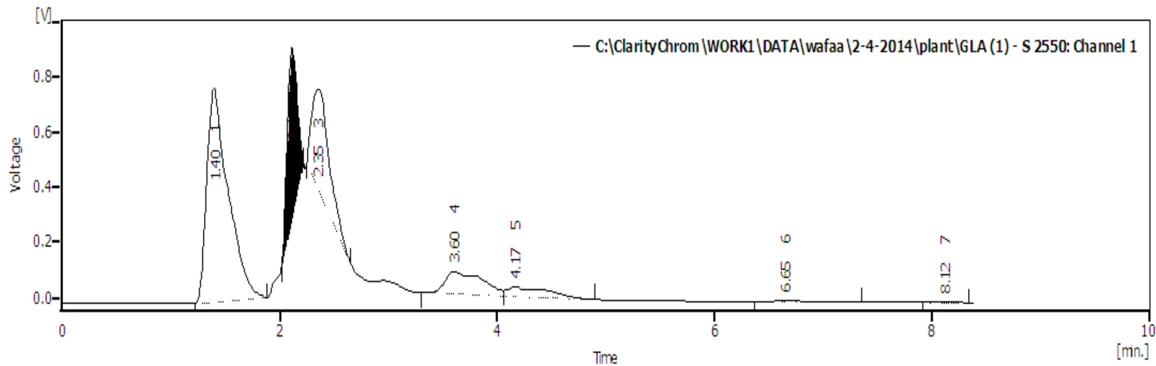


Figure 4.a: HPLC chromatogram of methanol extract of *G. longiscapa* aerial parts at 280 nm.

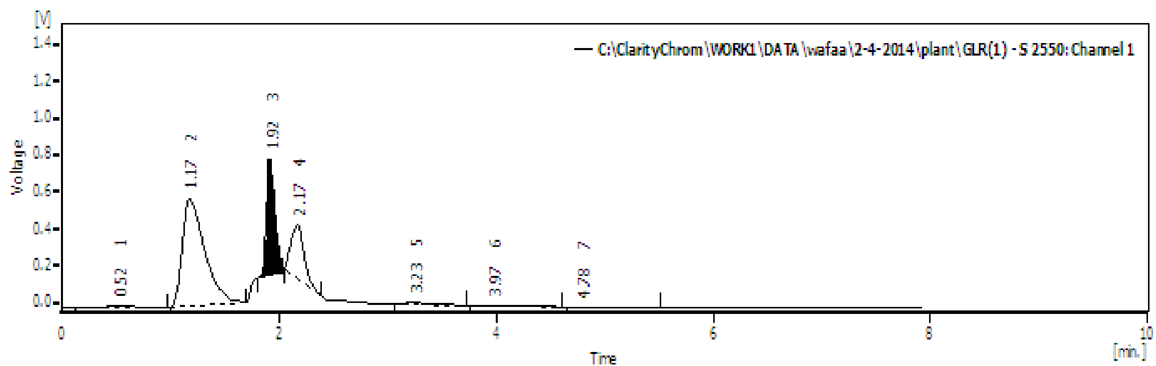


Figure 4.b: HPLC chromatogram of methanol extract of *G. longiscapa* underground parts at 280 nm.

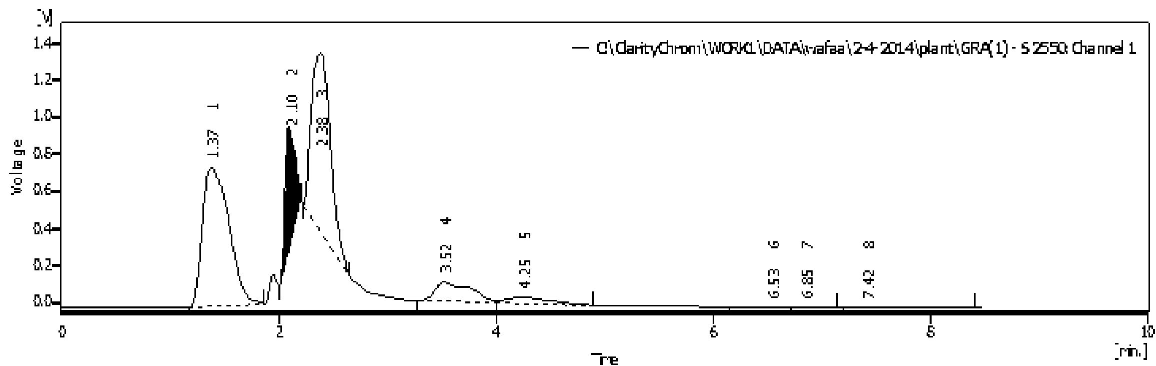


Figure 4.c: HPLC chromatogram of methanol extract of *G. rigens* aerial parts at 280 nm.

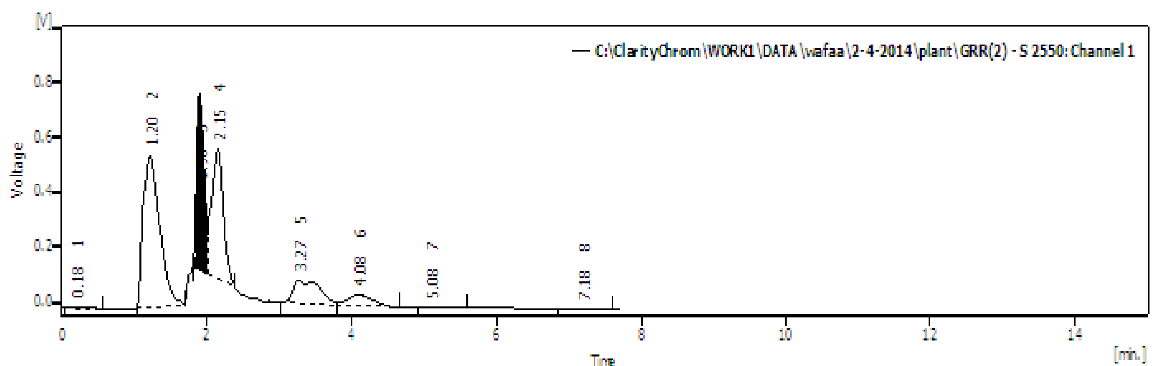


Figure 4.d: HPLC chromatogram of methanol extract of *G. rigens* underground parts at 280nm.

9a). The *G. rigens* aerial parts methanol extract in different concentrations (10, 20, 40, 60, 80 and 100 mg/ml) showed a marked significant scavenging activity, the maximum reactive reaction rate after 45 sec. was (90.6, 92.0, 92.2, 94.7, 95.1 and 99.6 %), respectively (Figure 9b). So *G. longiscapa* and *G. rigens* have more antioxidant activity than L-ascorbic acid especially at high dose level as the reactive reaction rate (inhibition %) of L-ascorbic acid was 96.7%.

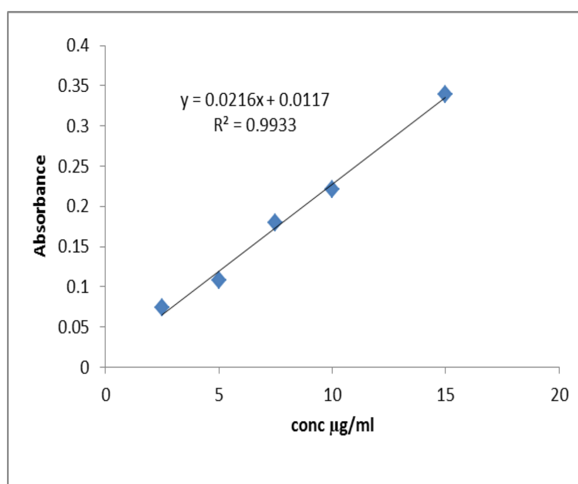


Figure 5: Calibration curve for Gallic acid

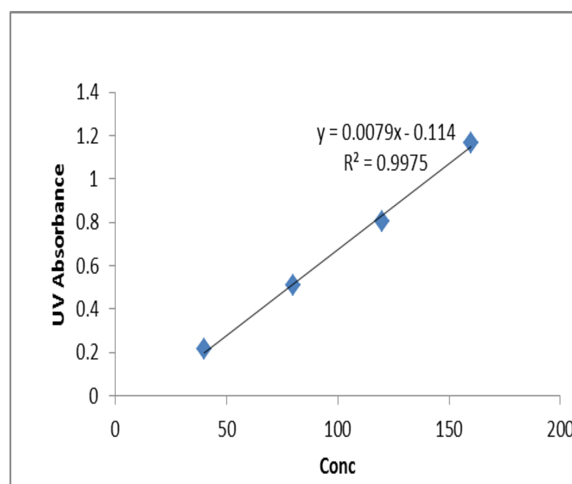
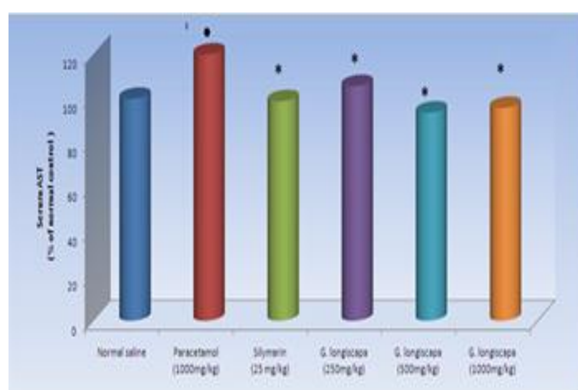
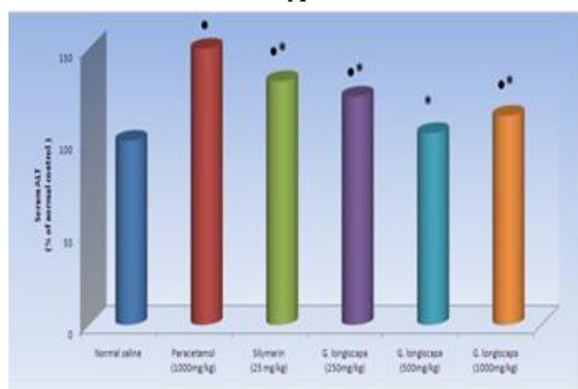


Figure 6: Calibration curve for rutin

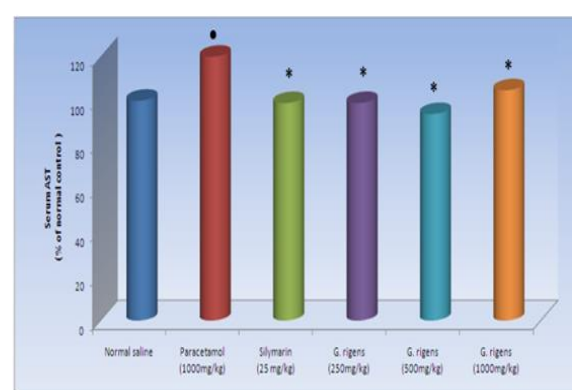


A

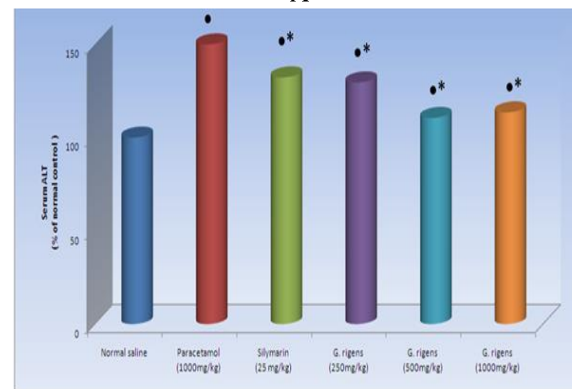


B

Figure 7: Effect of oral administration of methanol extract of *G. longiscapa* aerial parts (250,500: and 1000mg/kg) on a) AST and b) ALT serum activity in paracetamol induced hepatotoxicity in rats, (n=6). Values represent the mean ± S.E. of six animals for each group using one way ANOVA followed by LSD. ● = P < 0.05: Statistically significant from saline control group. *=P <0.05: Statistically significant from paracetamol group.



A



B

Figure 8: Effect of oral administration of methanol extract of *G. rigens* aerial parts (250, 500 and 1000 mg/kg) on a) AST and b) ALT serum activity in paracetamol induced hepatotoxicity in rats, (n=6). Values represent the mean ± S.E. of six animals for each group using one way ANOVA followed by LSD. ● = P < 0.05: Statistically significant from saline control group. *=P <0.05: Statistically significant from paracetamol group.

CONCLUSION

The present study revealed that both *G. longiscapa* and *G. rigens* could be considered as a rich source for phenolic and flavonoid compounds and has valuable hepatoprotective and antioxidant activities in a dose dependent manner which was quite comparable with reference standards silymarin and ascorbic acid, respectively. This biological activity could be due to the presence of phenolic and flavonoid compounds which were isolated from both species.

Conflict of interest

None declared.

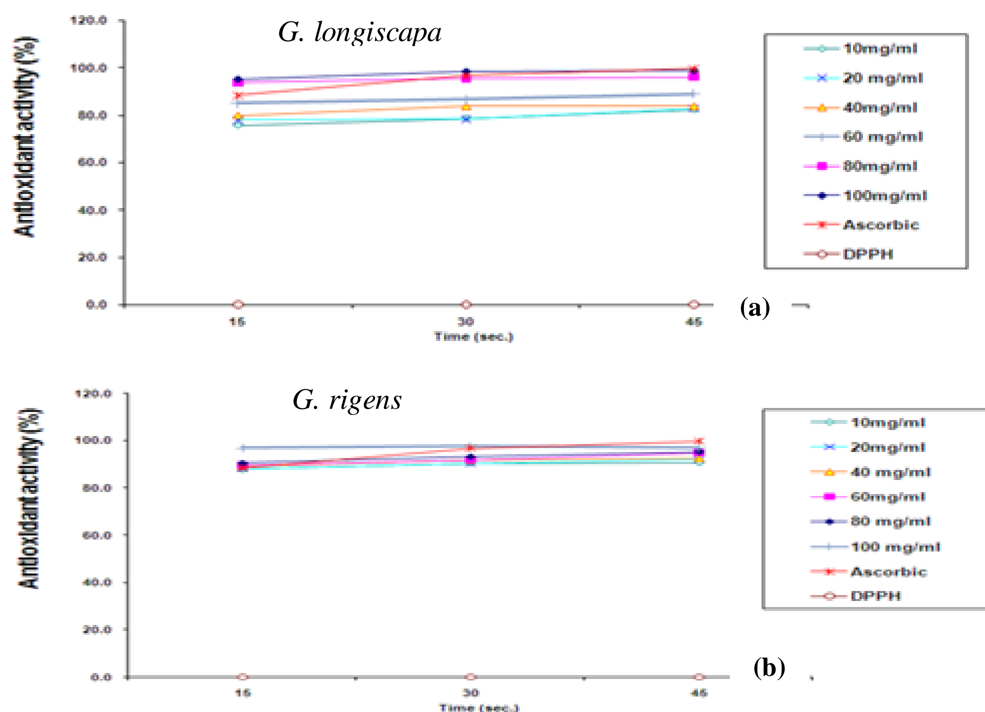


Figure 9: Antioxidant activity of aerial parts methanol extract of a) *G. longiscapa,* b) *G. rigens* (10, 20, 40, 60, 80 and 100 mg/ml) and ascorbic acid (0.1 M concentration) in vitro, using DPPH radical scavenging activity method.

ACKNOWLEDGEMENTS
This work was financially supported by Future University in Egypt.

REFERENCES

1. A Tomb: The biology and chemistry of the Compositae”. Academic Press, London, New York, 1977.
2. Bailey LH.: Manual of cultivated plants. The Macmillan Company, New York, 1958.
3. Ahmed SI., Pharmacognostical study of *Gazania rigens* R.B. family Compositae cultivated in Egypt. Faculty of pharmacy, Assuit University1981.
4. Amal M. Youssef Moustafa, Ahmed Ismail Khodair. Fiaza Mohamed Hammouda. Phytochemical Investigation and α-Cellulose Content Determination of *Gazania splendens* Moore. Research journal of Phytochemistry 2010; 4:182-193.
5. Bohlmann F.Zdero C. New eudesmanolides from *Gazania Krebsiana*. Phytochemistry 1979; 18:332-3.
6. Newman A A.: Chemistry of Terpenes and Terpenoids London and New York, 1972.
7. Taha SE., Mona HH., Shahira ME., *et al.* Pharmacognostical and biological studies on the rhizomes of *Gazania nivea* DC cultivated in Egypt. Bull. of the Faculty of Pharm. Cairo University; 2008; 46:253-265.
8. Hala MH. Biologically-guided phytochemical investigation of *Gazania rigens* L. Alex. J. Pharm. Sci 2009; 23:96-100.
9. Hiermann A. Die untersuchung potetieller Wirkstoffe in *Epilopium*. Scientica Pharmaceutica 1983; 51:158-167.
10. Kumazawa S., Tanguchi M., Suzuki Y., *et al.* Antioxidant activity of polyphenols in carob pods. J. Agric. Food. Chem 2002; 50:373-377.
11. Reitman S.Frankel S. A colorimetric method for the determination of glutamic oxaloacetic and glutamic pyruvic transaminases. American Journal of Clinical Pathology 1957; 28:56-63.
12. Shanmugam R., Gowthamarajan K., Priyanka DL., *et al.* Development and Validation of an RP- UFLC Method for Simultaneous Estimation of Quercetin and Rutin. Hygeia: Journal for Drugs and Medicines 2013; 5:113-120.
13. Kameo J.Furuyak. Quantitative estimation of anthraquinone derivatives and flavonoid content of *Rhamnus lucioides* L. fruit, *Asphodeles micocarpus* Salzm and Viv herb and flavonoid content of *Capparis cartilaginea* Dence herb. Journal of the Pharmaceutical Society of Japan 1952; 72:
14. Wafaa M. El Kady, Abeer A.A. Salama, Samar Y. Desoukey, *et al.* Comparative DNA profiling, botanical identification and biological evaluation of *Gazania longiscapa* DC and *Gazania rigens* L. Bulletin of Faculty of Pharmacy, Cairo University 2015; 53:129-145.
15. VM Silva, Thibodeau MS, Chen C, *et al.* Transport deficient (TR-) hyperbilirubinemic rats are resistant to acetaminophen hepatotoxicity. Biochem Pharmacol 2005; 70:1832-9.
16. Bhandari U., Shamsher A. Pillai K. Antihepatotoxic activity of Ginger Ethanol Extract in Rats. Pharmaceutical Biology 2003; 41:66-71.

17. DA SorgBuckner B. A simple method of obtaining venous blood from small animals. Proc. Sec. Exp. Med 1964; 115:1131-32.
18. Amic. D., Davidovic-Amic. D., Beslo. D., *et al.* Structure-radical scavenging activity relationships of flavonoids. CroaticaChemicaActa 2003; 76:55-61.
19. Phang CW., AbdMalek SN., Ibrahim H., *et al.* Antioxidant properties of crude and fractionated extracts of Alpiniamutica rhizomes and their total phenolic content. African Journal of Pharmacy and Pharmacology 2011; 5:842 - 852.
20. Nakatani N, Kayano S, Kikuzaki H, *et al.* Identification, quantitative determination, and antioxidative activities of chlorogenic acid isomers in prune (*Prunus domestica* L.). J Agric Food Chem 2000; 48:5512-6.
21. Sook Kyung Hyun., Hyun Ah Jung., Byung-Sun Min., *et al.* Isolation of Phenolics, Nucleosides, Saccharides and an Alkaloid from the root of *Aralia cordata*. Natural Product Sciences 2010; 16 20-25.
22. Mabry TJ, Markham KRThomas MB.: The systematic identification of flavonoid. Springer Verlag New York, 1970
23. Harborne JB.: The Flavonoids, Advances in Research. since 1986. Chapman and Hall, London, 1994.