Bulletin of Faculty of Pharmacy, Cairo University (2015) **53**, 129–145

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Cairo University

Bulletin of Faculty of Pharmacy, Cairo University

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ORIGINAL ARTICLE

Comparative DNA profiling, botanical identification and biological evaluation of *Gazania longiscapa* DC and *Gazania rigens* L.

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Received 13 August 2014; accepted 25 July 2015

KEYWORDS

Gazania;
Botany;
DNA finger print;
Antinociceptive;
Anti-inflammatory

Abstract *Gazania longiscapa* DC and *Gazania rigens* L. are species of cultivated ornamental plant that grow in Egypt. Genus *Gazania* has a role in folk medicine to prevent toothache; this study presents a comparative investigation of genetic and botanical features of root, rhizome, leaves and flowers of the two *Gazania* species and comparing their biological activity as analgesic and antiinflammatory as related to their folk medicinal use.

The genetic and botanical differences between the two *Gazania* species are reported for the first time in this study. The results contribute toward validation of the traditional use of *Gazania* showing that both species are safe for oral administration and they exhibit significant antinociceptive and anti-inflammatory effects in a dose dependent manner.

1. Introduction

Gazania species belonging to Arctoteae trib of family Asteraceae[1] contain about 25 herbaceous species native to South Africa. Several *Gazania* species are cultivated in Egypt among which is *Gazania longiscapa* also called *Gazania linearis* (Thunb.) Druce, *Gazania stenophylla*, *Gorteria linearis* (Thunb) and known by the common name, treasure flower and *Gazania rigens* also called *Gazania splendens*, *Gorteria rigens* known by the common name, coastal gazania. They are perennial or rare annual herbs. In folk medicine genus *Gazania* has been reported to prevent miscarriage, tooth ache and incorporated in purgative preparations with aloes.[2] Reviewing literature few studies were traced concerning this genus; *Gazania nivea*

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Peer review under responsibility of Faculty of Pharmacy, Cairo University.

http://dx.doi.org/10.1016/j.bfopcu.2015.07.001

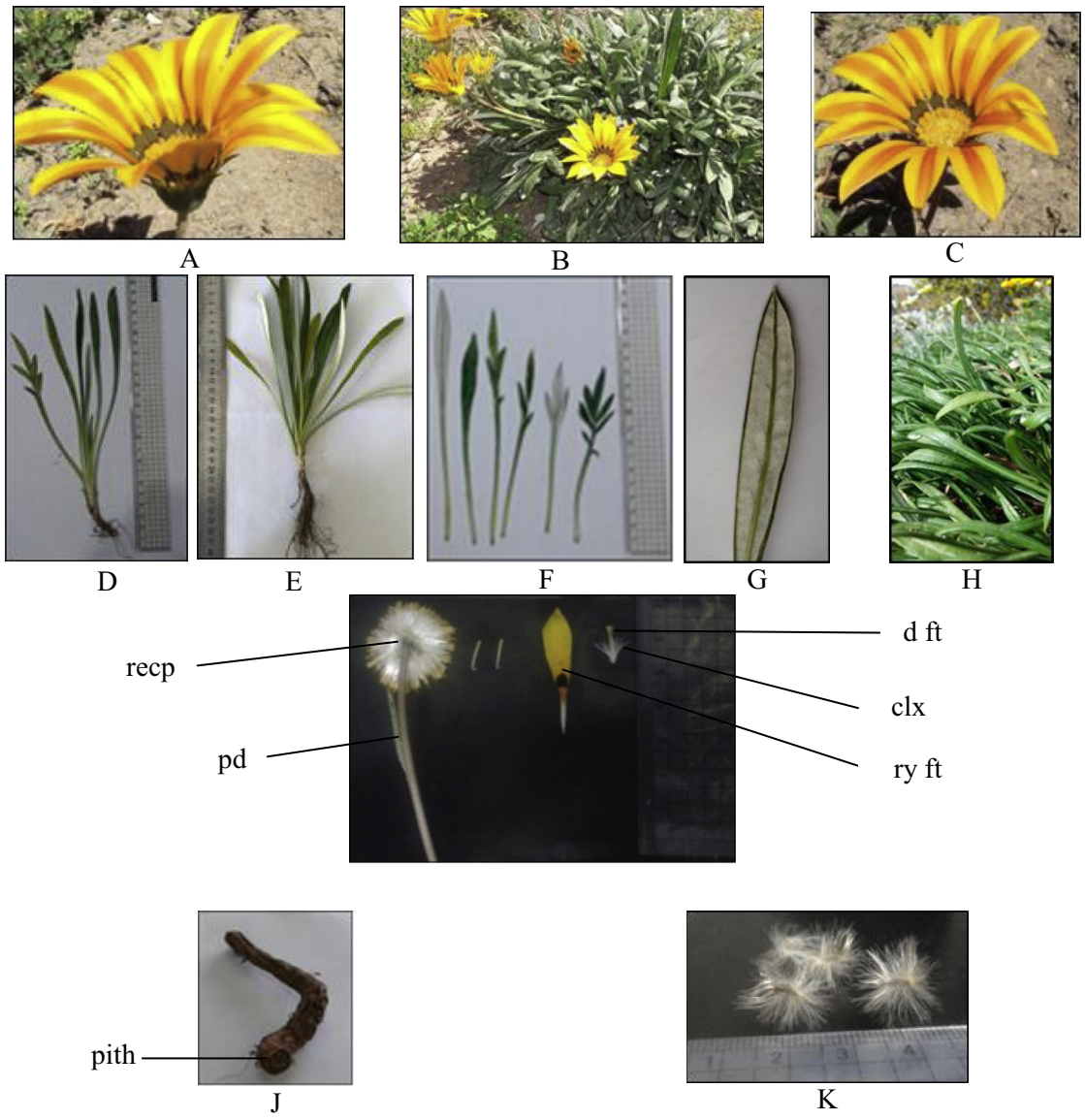


Figure 1 Photograph of *G. longiscapa*. (A) The flower ($X = 0.4$); (B) Whole plant ($X = 0.1$); (C), Top view of the flower ($X = 0.4$); (D and E) Photos show the insertion of leaves ($X = 0.1$); (F) Leaves show variable lamina ($X = 0.2$); (G & H) Leaves show revolute margins ($X = 0.5$) disk and ray floret ($X = 0.5$), (J) The rhizome ($X = 0.2$); (K) The seed ($X = 1.5$); clx, calyx; d ft, disk floret; pd, peduncle; recp, receptacle; ry ft, ray floret.

has antioxidant and hepatoprotective activities,[3] while antimicrobial activity was observed in *G. rigens*.[4]

However not much could be traced in the available literature concerning the botanical characters of the chosen species.[5] It seemed interesting to throw light on the macro and micromorphological features of both *Gazania* species to help in their identification and differentiation in the entire and powdered forms.

Botanical characterization of closely related plant species is nowadays greatly supported via examination of various decisive genetic criteria such as DNA fingerprinting. It is reported as a promising tool for the authentication of medicinal plant species and especially useful in species or varieties that are morphologically and/or phytochemically indistinguishable.[6]

Protein profiling, as well, is frequently used in taxonomy to provide valid evidence for addressing various taxonomic problems and has found wide application in resolving systematic relationships.[7] Since differences between species is based on gene differences, direct comparison of genes is both difficult and time consuming consequently, these differences could be easily measured by comparing the products of gene activity using protein as genotype markers.[8] This technique is considered rapid and inexpensive.[9] Hence, the objective of this work was targeted toward discrimination between *G. longiscapa* and

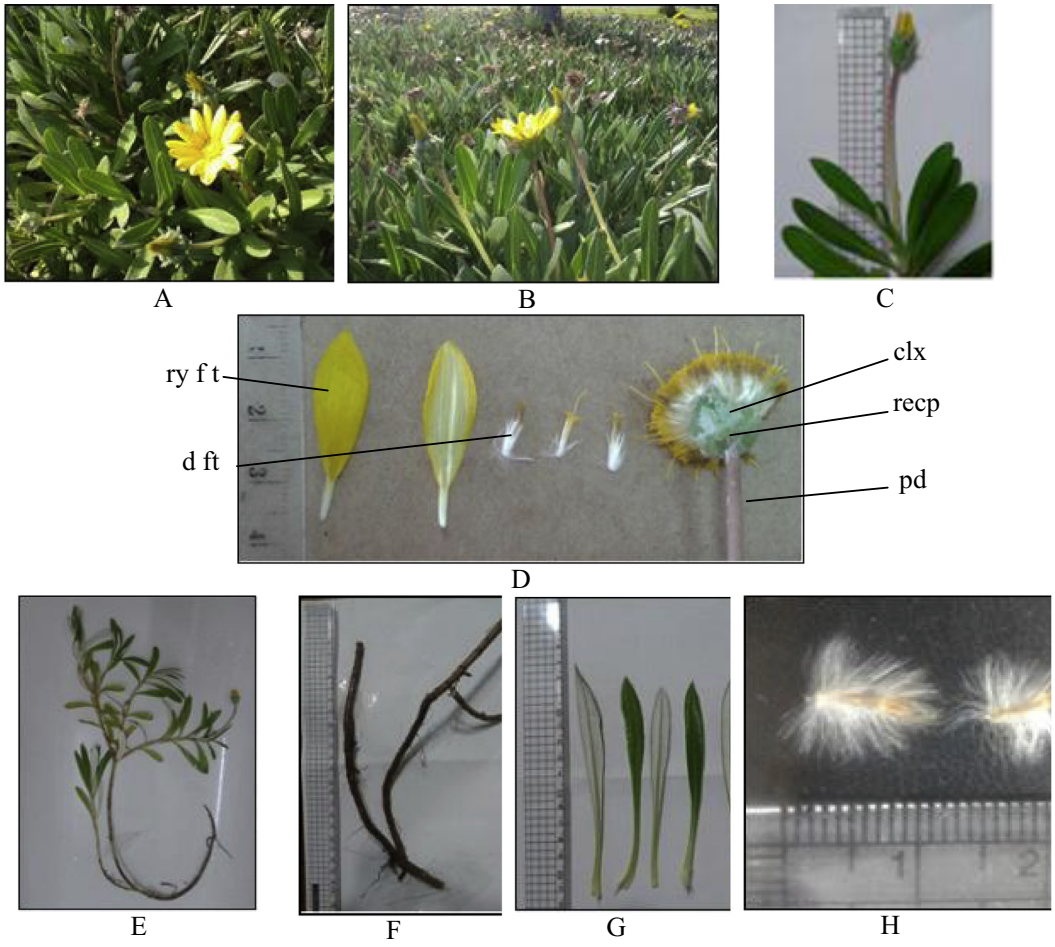


Figure 2 Photograph of *G. rigens*. (A and B) The whole plant ($X = 0.4$). (C) The flower head and peduncle ($X = 0.3$). (D) Disk and ray floret ($X = 1$). (E) Photos show the stem branches ($X = 0.15$). (F) The rhizome and roots ($X = 0.1$). (G) Upper and lower surface of leaves ($X = 0.6$). (H) The seed ($X = 2$). clx = calyx; d ft = disk floret; pd = peduncle; recp = receptacle; ry ft = ray floret.

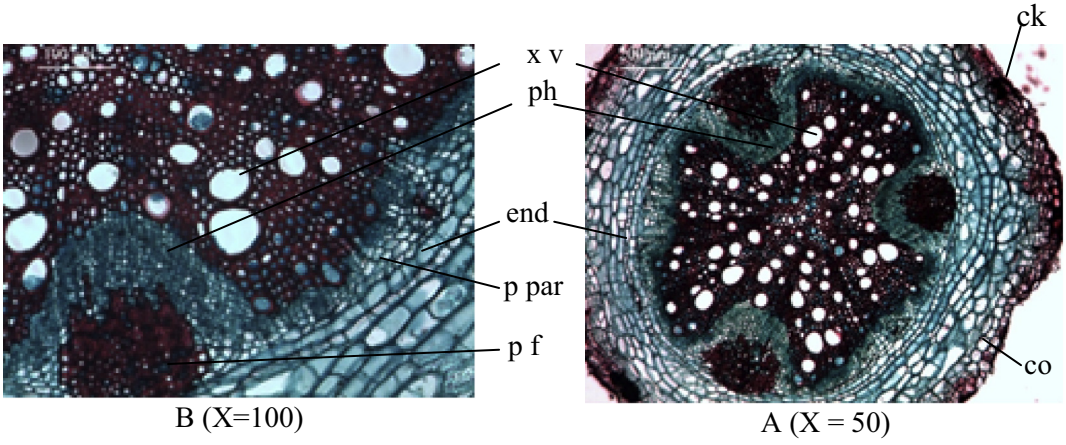


Figure 3 Micromorphology of *G. longiscapa* root; ck = cork, co = cortex, end = endodermis, p f = pericycle fiber, p par = pericycle parenchyma, ph = phloem, x v = xylem vessels.

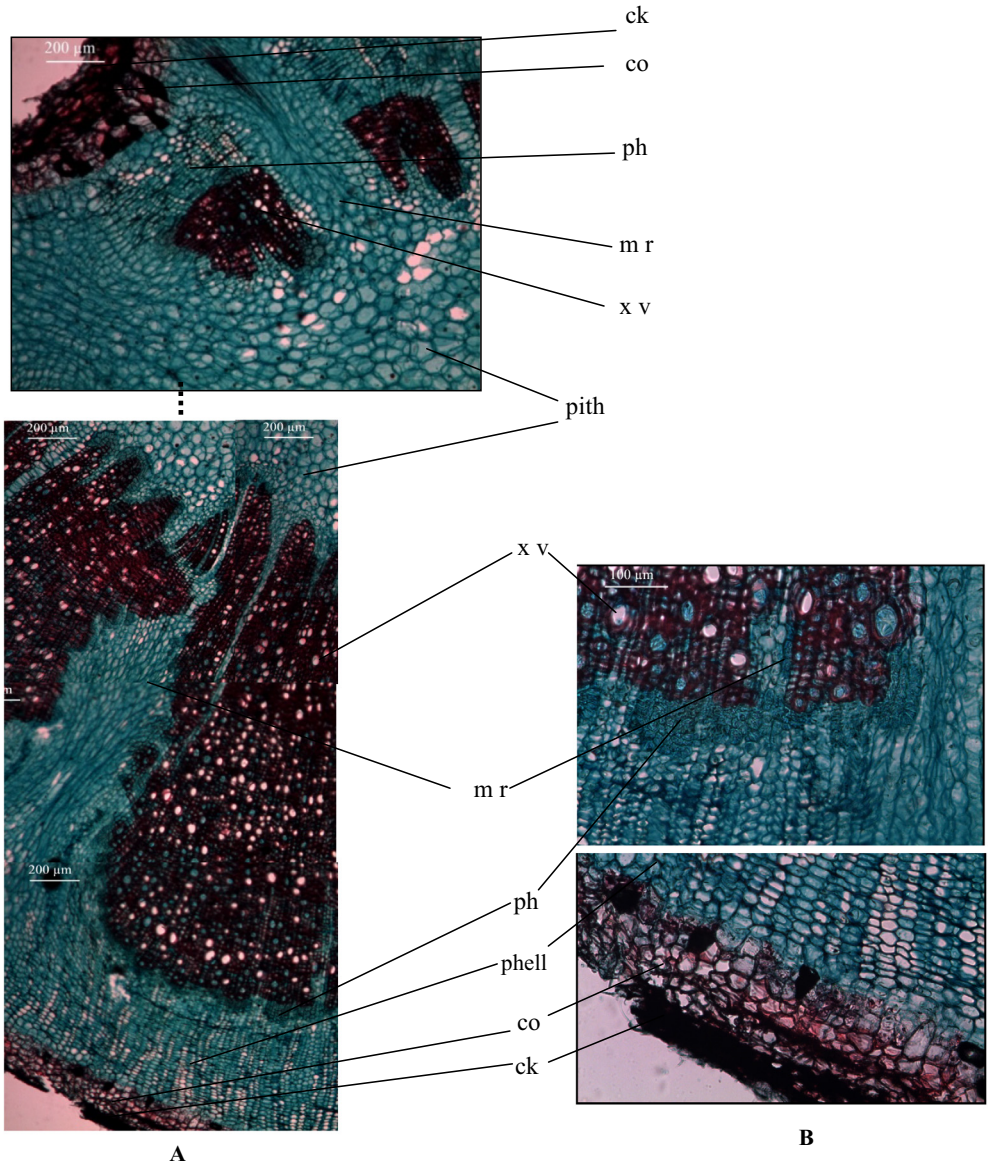


Figure 4 Micromorphology of *G. longiscapa* rhizome [detailed sector in the rhizome A ($X = 50$); B ($X = 100$)]; ck = cork, co = cortex, m r = medullary ray, phell = phelloderm, ph = phloem, x v = xylem vessels.

G. rigens through establishment of different botanical and genetic criteria and also evaluation of their biological activity.

2. Materials and methods

2.1. Botanical profiling

Samples of *G. longiscapa* and *G. rigens* herbs including the underground parts (roots and rhizomes) were collected from the plantation of the Ministry of Agriculture located in the fifth settlement, new Cairo, Egypt. The plant was kindly identified and authenticated by Prof. Dr. Abd Alsalam El Noiehy, Professor of Plant Taxonomy, Botany department, Faculty of Science, Ain Shams University. Fresh plant materials of different organs were preserved in 70% ethyl alcohol containing 5% glycerin soon after collection.

Specimens for morphological studies were dried according to standard herbarium techniques and voucher samples were kept in the Department of Pharmacognosy, Faculty of Pharmacy, Future University in Egypt (FUE). Photographs were taken using a Samsung Digital Camera (DV 150F Dual-view (16.2) Megapixels).

The figures were taken using the Carl Zeiss optical microscope (Switzerland) model DM1000, fitted with camera leica microsystems (Switzerland) Ltd CH-9435 Heerbrugg type: EC3T.

2.2. Genetic profiling

Genetic profiling was done at Biotechnology Research Lab, Horticulture Research Institute, Agriculture Research Center.

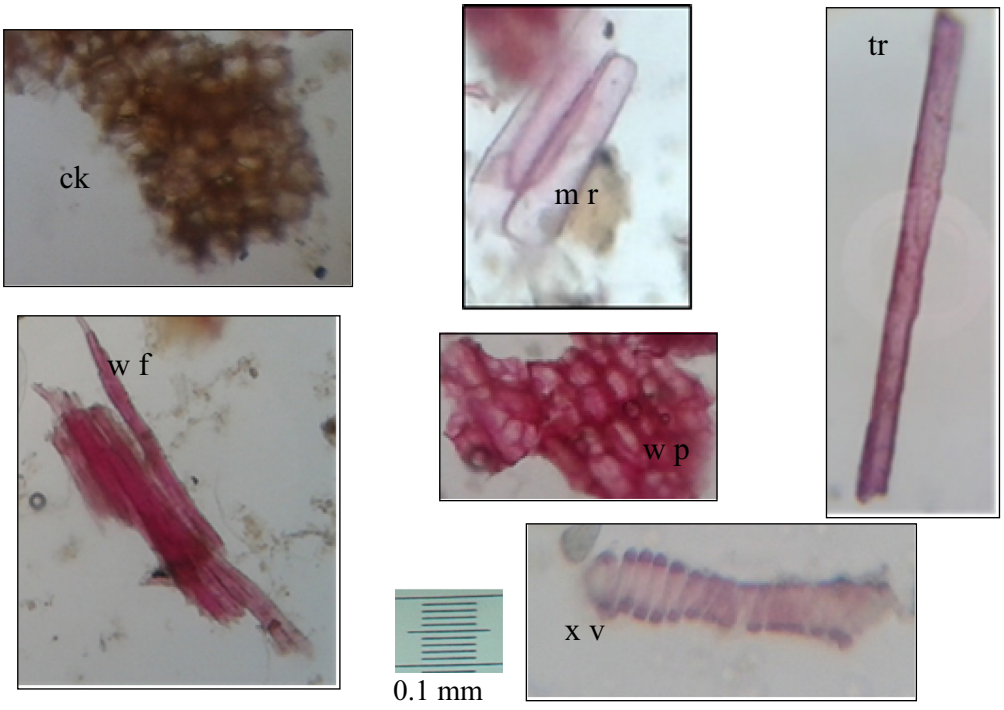


Figure 5 Micromorphology of powdered *G. longiscapa* rhizome and root; ck = cork (*X* = 100), m r = medullary ray (*X* = 150), tr = tracheid (*X* = 400), w f = wood fiber (*X* = 100), w p = wood parenchyma (*X* = 100), x v = xylem vessels (*X* = 100).

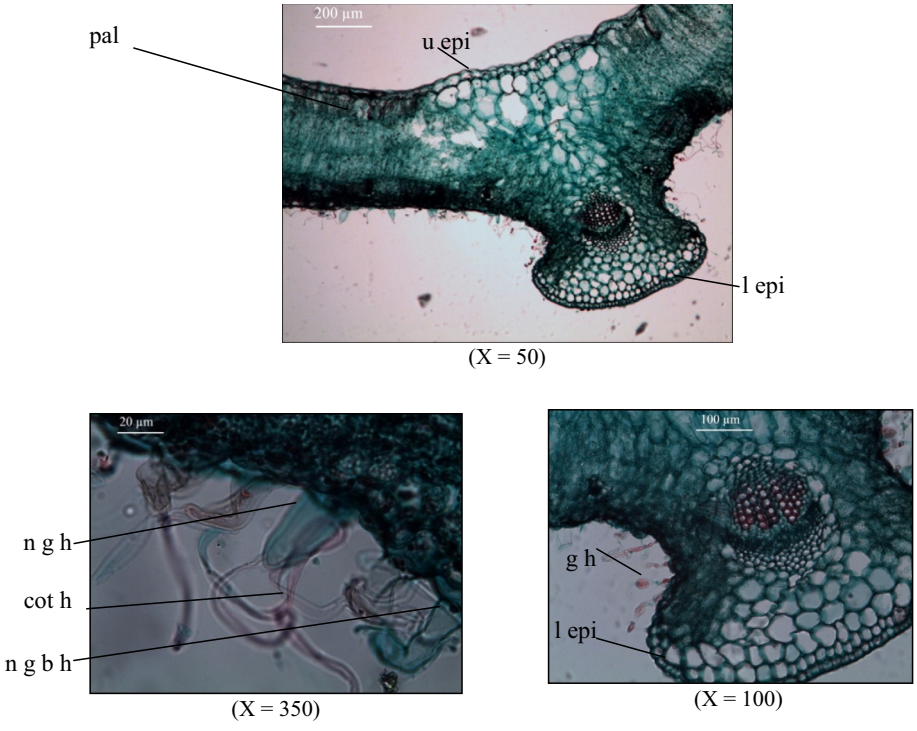


Figure 6 Micromorphology of *G. longiscapa* leaf; cot h = cottony hair, g h = glandular hair, l epi = lower epidermis, l v b = lateral vascular bundle, n g b h = nonglandular bent hair, n g h = non-glandular hair, pal = palisade cells, u epi = upper epidermis.

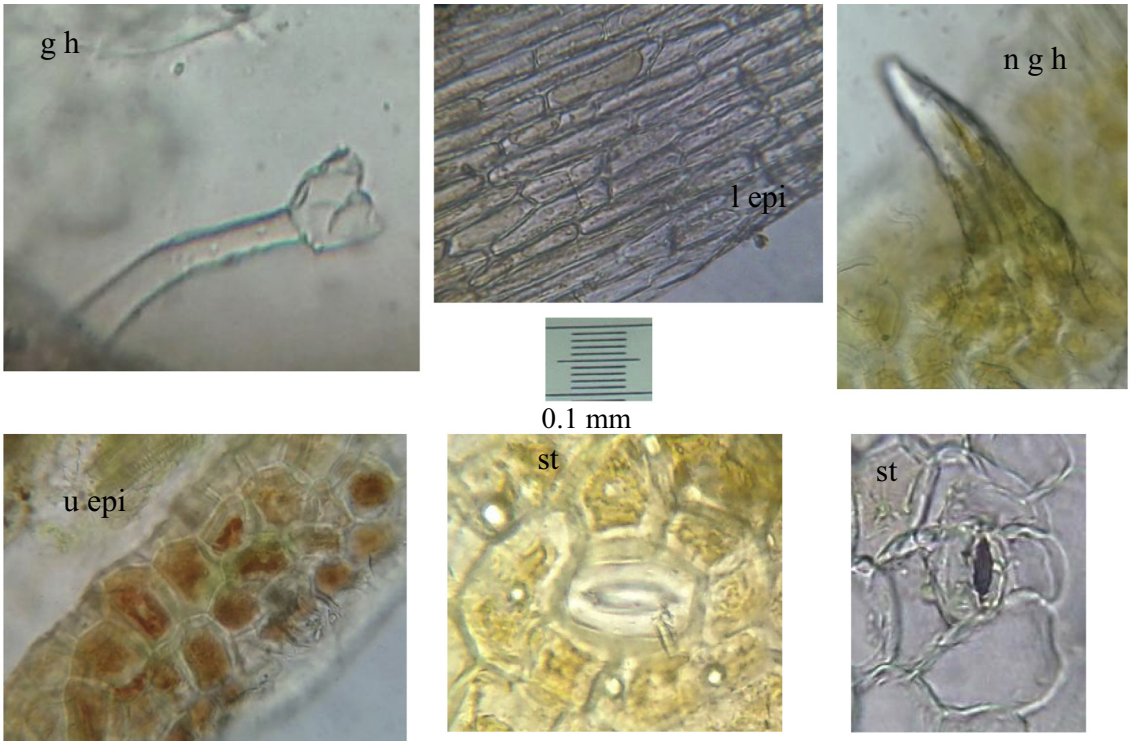


Figure 7 Micromorphology of powdered *G. longiscapa* leaves; g h = glandular hair (X = 300), l epi = lower epidermis (X = 150), n g h = non-glandular hair (X = 300), u epi = upper epidermis (X = 400), st = stomata (X = 400).

2.2.1. DNA fingerprinting

Entire fresh leaves of both plants under investigation were separately freeze-dried and ground to fine powder under liquid nitrogen prior to DNA isolation.

2.2.1.1. DNA extraction. DNA was extracted from 0.2 g of leaf tissues in 1.5 ml microfuge tubes using the DNA extraction method described in Williams et al.[10]

Table 1 Dimensions of different elements of the organs under investigation in microns of *G. longiscapa*.

Parameter measured	Size (min–max) μm		
	L	W or D	H
Root and rhizome			
Cork	40,60	30,70	20, 30
Medullary ray	166, 130	40, 46	
Tracheid	175, 250	10, 15	
Wood fiber	350, 200	20, 30	
Xylem vessels		50, 90	
Leaf			
Lower epidermis	133, 100	20, 26	2
Upper epidermis	22.5, 25	22.5, 30	5
Non glandular hair	100, 110	50, 65	
Stomata	25, 30	25, 50	
Glandular hair			
Stalk	106, 110	16, 20	
Head		40,45	
Xylem vessels		10, 5	

2.2.1.2. Oligonucleotide primers. Two techniques were performed for good statistical analysis.

- RAPD-PCR analysis (randomly amplified polymorphic DNA):

PCR amplification was performed using six random deca-mer arbitrary primers synthesized by (Operon biotechnologies, Inc. Germany, with the following sequences OP-A09: 5′ GGG TAA CGC C 3′; OP-B09: 5′ TGG GGG ACT C 3′; OP-B11: 5′ GTA GAC CCG T 3′; OP-C04: 5′ GAT GAC CGC C 3′; OP-C19: 5′ GTT GCC AGC C 3′; OP-M01: 5′ ACG GCG TAT G 3′.

- ISSR-PCR analysis (inter simple sequence repeat):

PCR amplification was performed using six ISSR primers synthesized by (Operon biotechnologies, Inc. Germany) with the following sequences HB-08: 5′ GAG AGA GAG AGA GG 3′; HB-10: 5′ GAG AGA GAG AGA CC 3′; HB-11: 5′ GTG TGT GTG TGT TGT CC 3′; HB-12: 5′ CAC CAC CAC GC 3′; HB-15: 5′ GTG GTG GTG GC 3′.

2.2.1.3. Polymerase chain reaction (PCR). The PCR amplification in both RAPD and ISSR analysis was done according to the method described by Williams et al.[10]

2.2.2. Protein profiling

2.2.2.1. Sample preparation. Protein extraction was conducted by separately mixing 0.2 g of powdered plant tissue of each species with 1 ml sample buffer [Tris borate solution (pH

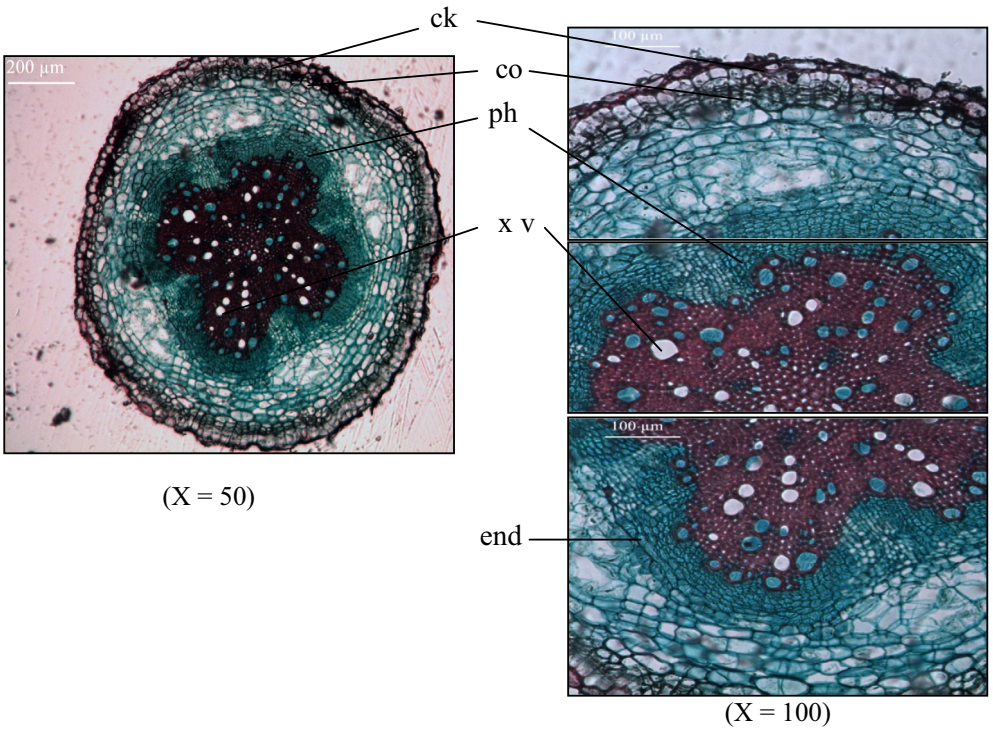


Figure 8 Micromorphology of *G. rigens* root; ck = cork, co = cortex, end = endodermis, ph = phloem, x v = xylem vessels.

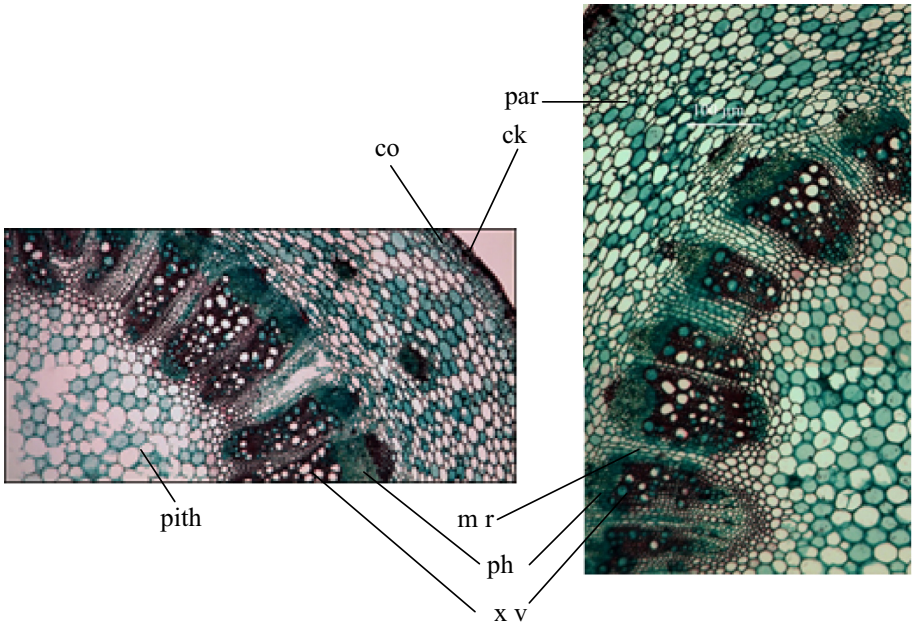


Figure 9 Micromorphology of G. *rigens* rhizome (X = 100); ck = cork, co = cortex, end = endodermis, m r = medullary ray, ph = phloem, w p = wood parenchyma, x v = xylem vessels.

8.2) (1:3 v/v)]. The slurry was centrifuged at 6000 rpm for 10 min. The supernatant containing the protein extract was used immediately for electrophoresis. The proteins of *G. longiscapa* and *G. rigens* were analyzed using continuous polyacrylamide gel electrophoresis according to the method described by Stegmann.[11]

2.2.2.2. Scoring of protein data. The protein binding profile in the gel was photographed. The binding profile was scanned using SynGene System, version 4.00A (Gene tools electrophoresis analysis system). The densitometer determined the position and percentage of each protein fraction in the seed protein profile of both samples. The molecular weight of each

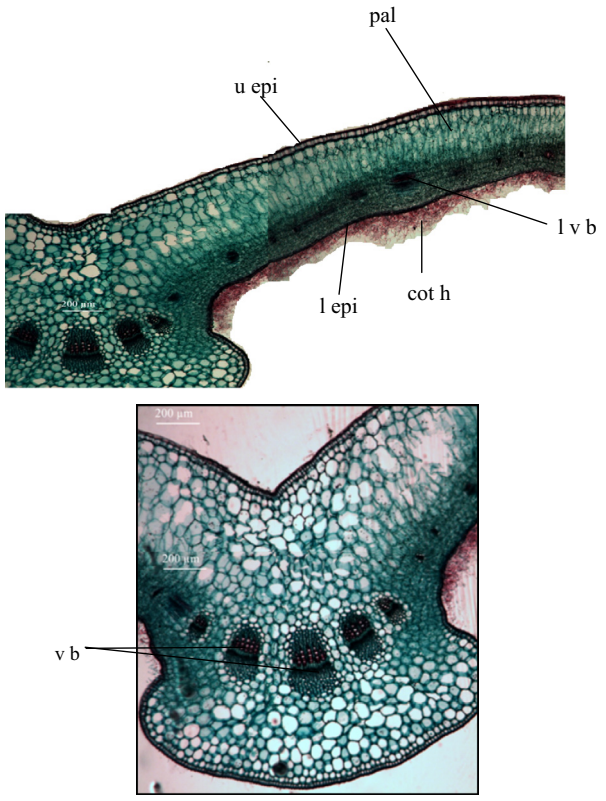


Figure 10 Micromorphology of *G. rigens* leaf ($X = 50$); cot h = cottony hair, l epi = lower epidermis, l v b = lateral vascular bundle, pal = palisade cells, u epi = upper epidermis, v b = vascular bundle.

band was calculated by comparison to a molecular weight marker.

2.3. Biological activity

2.3.1. Extract preparation

Aerial parts of both *Gazania* species were separately collected and air dried then extracted with 80% methanol till exhaustion, the obtained aqueous methanol extracts were separately evaporated under reduced pressure and the obtained residues were separately exhausted with $CHCl_3$. The remaining methanol residues were used for biological testing.

2.3.2. Animals

Wiser male rats, weighing from 125 to 150 g and Swiss mice of 20–30 g body weight were used throughout the experiments. Rats were used for the acute toxicity study, anti-inflammatory, central analgesic activity, while mice were used for peripheral analgesic activity. The rats and mice 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.

2.3.3. Drugs, chemicals and diagnostic kits

- Indomethacin was obtained from Epico, Egypt Int. pharmaceutical Industries Co., ARE under license of MERCK Co. Inc-Rahaway, NJ, USA and used at dose of 25 mg/kg according to Suleyman et al.[12]
- Tramadol was obtained from October Pharma S.A.E., 6-October City – Giza – EGYPT and used at dose of 20 mg/kg according to Sacerdote et al.[13]
- Acetic acid – Carrageenan were obtained from Sigma, USA.
- Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were analyzed according to Reitman and Frankel.[14]
- Creatinine and Blood urea Nitrogen (BUN) according to Teger-Nilsson[15] and Tabaco et al.[16]
- All were obtained from Biodiagnostic Co., Egypt.

2.3.4. Acute toxicity study

The extracts of both *Gazania* were dissolved in distilled water then given orally to rats in graded doses up to 5 g/kg. The control group received the same volumes of distilled water. The percentage mortality for extracts was recorded 24 h later.

Then rats were divided into 2 groups of 12 rats each (6 males and 6 females) as follows: – 1st control group: rats were given distilled water. 2nd group: rats were given a single oral dose of 5000 mg/kg (maximum dose that didn't cause mortality).

Rats were observed for 14 days, for any changes in the skin and fur, respiratory, circulatory, autonomic, central nervous systems, somatomotor activity and behavioral pattern. Particular observation for tremors, convulsions, salivation, diarrhea, lethargy, sleep, and coma was done. Blood was collected from retro-orbital venous plexus of anesthetized rats after 14 days, serum was separated and used for hematological analysis (red blood cells (RBCs), mean corpuscular volume (MCV), hemoglobin, platelets and white blood cells (WBCs)) and for assessment of liver function tests (serum ALT, AST) and kidney function tests (creatinine and BUN).

2.3.5. Anti-inflammatory activity (carrageenan induced paw edema)

Paw swelling was elicited by a sub-plantar injection of 100 µl of 1% sterile lambda carrageenan suspension in saline into the right hind paw.[17]

Contralateral paw received an equal volume of saline. The edema component of inflammation was quantified by measuring hind footpad immediately before carrageenan injection and 1–4 h after carrageenan injection with a micrometer caliber.[18] Edema was expressed as a percentage of change from control (pre-drug) values. Rats were divided into eight groups each of six. 1st group: rats received orally saline (0.2 ml/rat) as control, 2nd group: rats were given indomethacin (25 mg/kg). 3rd–5th groups: rats received *G. rigens* aerial parts extract (250, 500, 1000 mg/kg, respectively. 6th–8th group: rats received *G. longiscapa* aerial parts extract (250, 500, 1000 mg/kg), respectively. Indomethacin and the extracts were given

OP-A09

Band No.	M.W Bp	Species	
		1	2
1	620	1	1
2	580	0	1
3	540	1	1
4	500	1	1
5	470	1	1
6	450	1	0
7	325	1	1
8	310	1	1
9	250	1	1
10	175	0	1
Total		8	9

OP-B09

Band No.	M.W Bp	Species	
		1	2
1	600	0	1
2	500	1	1
3	420	1	1
4	380	0	1
5	290	1	1
6	240	1	0
Total		4	6

OP-B11

Band No.	M.W Bp	Species	
		1	2
1	900	1	0
2	650	0	1
3	620	1	1
4	590	1	0
5	400	1	0
6	370	1	1
7	350	0	1
8	240	0	1
9	200	1	0
10	160	1	0
Total		7	5

OP-C04

Band No.	M.W Bp	Species	
		1	2
1	500	1	1
2	330	1	1
3	290	1	1
4	270	1	1
5	180	1	1
6	160	1	1
Total		6	6

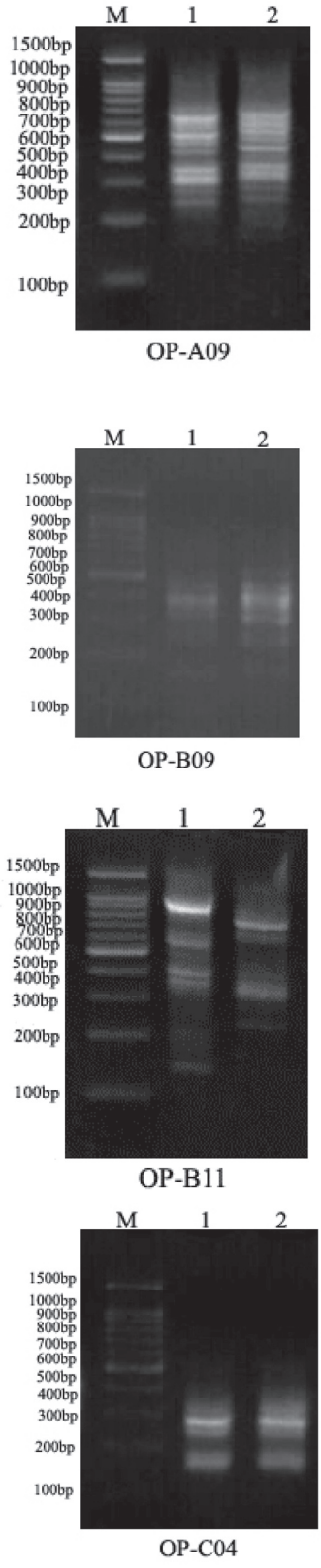


Figure 11 The RAPD electrophoretic profiles of *G. longiscapa* (Species 1) and *G. rigens* (Species 2).

OP-C19

Band No.	M.W Bp	Species 1	2
1	630	1	1
2	600	1	1
3	500	1	1
4	380	1	1
5	300	1	1
6	270	1	1
7	230	1	1
Total		7	7

OP-M01

Band No.	M.W Bp	Species 1	2
1	625	1	1
2	400	1	1
3	340	1	1
4	200	1	1
Total		4	4

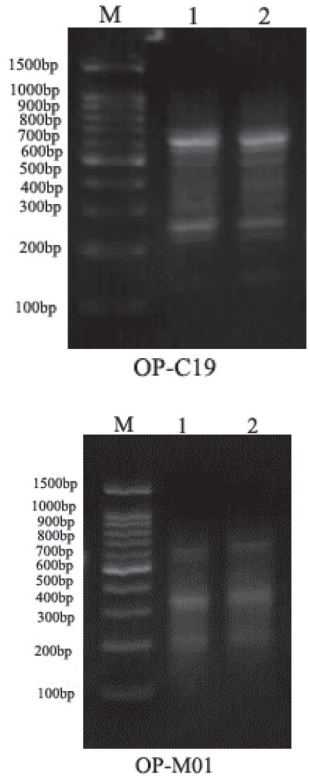


Figure 11 (*continued*)

orally 60 min before the injection of the carrageenan suspension.

2.3.6. Antinociceptive activity

This activity was determined by measuring the responses of animals to the chemical and thermal stimuli.

2.3.6.1. Chemical test. Acetic acid induced writhing in mice.[19–21] Mice were divided into six groups and received different concentrations of both extracts orally as under anti-inflammatory activity. After 30 min interval, the mice received 0.6% acetic acid i.p (0.2 ml/mice). The number of writhing in 30 min period was counted and compared.

2.3.6.2. Thermal test. Hot-plate test was conducted according to Eddy and Leimback[22] using an electronically controlled hot-plate (Ugo Basile, Italy) adjusted at 52 ± 0.1 °C and the cut-off time was 60 s. The rats were divided into eight groups as mentioned before under antiinflammatory activity, the time elapsed until either paw licking or jumping occurs is recorded before and 1 and 2 h after oral administration of saline, tramadol and both extracts.

3. Results and discussion

3.1. Botanical profiling

3.1.1. Macromorphology

*3.1.1.1. Macromorphology of **G. longiscapa***. *G. longiscapa* is an ornamental plant, flowering from March to June. The herb is about 50 cm long. It shows a mat-forming or clumping perennial herb growing from rhizomes.

3.1.1.1.1. The root (Fig. 1E). The root is adventitious almost cylindrical, 10–15 cm long and about 0.3–0.5 cm in diameter at the middle part. It bears numerous spreading short tapering lateral roots which bear in turn numerous rootlets. Externally the root is earthly brown in color with a rough surface.

3.1.1.1.2. The rhizome (Fig. 1J). *G. longiscapa* is stemless or nearly so[23]; it has an underground stem (rhizome); it is nearly cylindrical, 10–30 cm long and about 0.5–1 cm in diameter. Externally it is brown in color and has a rough surface.

3.1.1.1.3. The leaf (Fig. 1F). They are simple leaf crowded in a basal tuft, as it is stemless, the lamina is variable in shape, lanceolate and entire or pinnatifid, it has acute to acuminate apex, white-tomentose from the lower side, glabrous or nearly so from the upper side, margins somewhat revolute and ciliate (Fig. 1G and H), they are about 18–20 cm. long, and 2–4 cm wide. They are odorless and tasteless.

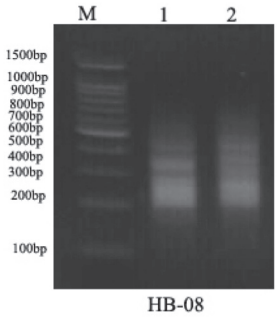
3.1.1.1.4. The flower (Fig. 1A). The inflorescence is a terminal capitulum attached to a reddish green peduncle. The capitulum has shades of bright yellow and orange color. The ray florets have dark spots near the bases, curled upward along their edges, and close at night. The flower has no characteristic odor or taste.

3.1.1.1.4.1. The peduncle (Fig. 1I). It is erect, cylindrical, slightly curved, smooth, reddish green in color and measures from 10 to 20 cm length and 3 to 5 mm in diameter.

3.1.1.1.4.2. The receptacle (Fig. 1G). It is disk like, flat to nearly curved, about 1–2 mm in height and 1–2 cm in diameter, surrounded by an involucre of 2–4 sealed rows of bracts, cap like at the base, toothed at the apex.

HB-08

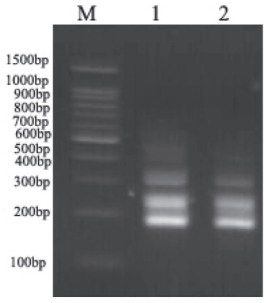
Band No.	M.W Bp	Species 1	2
1	500	0	1
2	460	1	1
3	370	1	1
4	350	1	0
5	290	1	0
6	270	1	1
7	220	1	1
Total		6	5



HB-08

HB-10

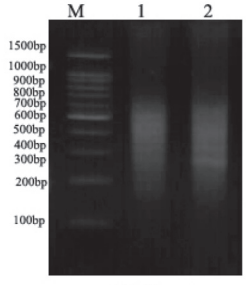
Band No.	M.W Bp	Species 1	2
1	470	1	0
2	400	1	1
3	300	1	1
4	270	1	1
5	250	1	0
6	180	1	1
Total		6	5



HB-10

HB-11

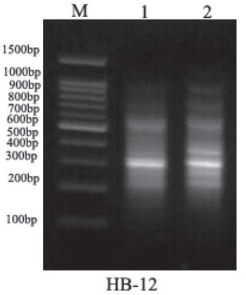
Band No.	M.W Bp	Species 1	2
1	600	1	1
2	500	1	1
3	460	1	1
4	430	1	1
5	280	1	1
6	180	1	0
Total		6	5



HB-11

HB-12

Band No.	M.W Bp	Species 1	2
1	940	1	1
2	700	1	1
3	540	1	1
4	360	0	1
5	300	1	1
6	270	0	1
7	220	1	1
Total		6	7



HB-12

HB-15

Band No.	M.W Bp	Species 1	2
1	540	1	1
2	300	0	1
3	270	1	1
4	200	1	1
5	160	1	1
Total		4	5

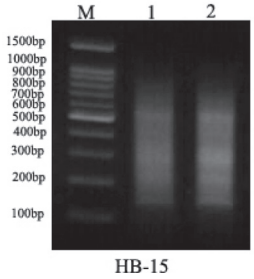


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Figure 12 The ISSR electrophoretic profiles of *G. longiscapa* (species 1) and *G. rigens* (species 2).

Table 2 The percent of monomorphic and polymorphic bands.

PCR technique	Total band	Unique bands	Monomorphic bands	Polymorphic bands	Polymorphic (%)
RAPD	43	14	29	14	32.5
ISSR	31	9	22	9	29
Total	74	23	51	23	31

3.1.1.1.4.3. The ray florets (Fig. 1I). They are arranged in one whorl, of 15–20 florets, they are zygomorphic, pistillate, sessile measure from 6 to 7 cm in length and 0.5 to 0.8 cm in breadth at the middle part of the corolla.

3.1.1.1.4.3.1. The calyx (Fig. 1I). It is shown by a pappus of few bristles.

3.1.1.1.4.3.2. The corolla (Fig. 1I). Each floret has a strap like corolla, oblong lanceolate in shape orange in color in the upper two thirds and has dark orange brown spot at the lower third. The surface is smooth; it has an entire slightly curved margin.

3.1.1.1.4.3.3. The androecium. Absent.

3.1.1.1.4.3.4. The gynoecium. An indistinct ovary; where the style and stigma are absent.

3.1.1.1.4.4. The disk florets (Fig. 1I). They are many florets arranged in many whorls. The disk florets are actinomorphic, hermaphrodite and sessile.

3.1.1.1.4.4.1. The calyx. It is represented by a pappus of numerous bristles hairy like calyx, each measuring about 0.5–0.7 cm in length.

3.1.1.1.4.4.2. The corolla. It consists of 5 yellow tubular petals, they are free at the top, but fused from the lower side forming tubular corolla it measures about 1 cm in length and about 1 mm in diameter.

3.1.1.1.4.4.3. The androecium. It consists of 5 epipetalous syngenesious stamens, inserted on the upper part of the corolla with short free filaments and orange united anthers. The stamens are 0.2–0.3 cm in long, fertile arranged in several whorls and are of the same length. The anthers are about 0.1 cm in length; they are cylindrical, orange, glabrous and bilobed.

3.1.1.1.4.4.4. The gynoecium. Hardly visible ovary by naked eye; the style is filiform, orange in color about 0.4–0.6 cm and with bifid stigma.

3.1.1.1.5. The seed (Fig. 1K). It is a tiny achene covered with very long hairs several times the length of the seeds body.

3.1.1.2. Macromorphology of G. rigens. *G. rigens* could be differentiated from *G. longiscapa* by the following:

1. *G. rigens* has short creeping stems above the ground of 30–50 cm supporting a number of flower heads.
2. *G. rigens* has a stem (Fig. 2E) which is cylindrical, solid, herbaceous when fresh, cartilaginous and brittle when dry. It has monopodial branching where the leaves are alternate in arrangement and usually terminates with a capitulum. The stem reach up to 30–50 cm long and up to 0.5 cm in diameter. The surface is nearly smooth, pale green in color, with a short fibrous fracture. It is tasteless and odorless.
3. The leaves (Fig. 2G) are simple, long and narrow (i.e. narrowly-oblanceolate) 10–15 cm long and 0.7–1 cm width with green or greyish upper surfaces and whitish hairy undersides. Leaves are alternately arranged or densely clustered along the stem. They have entire margins.

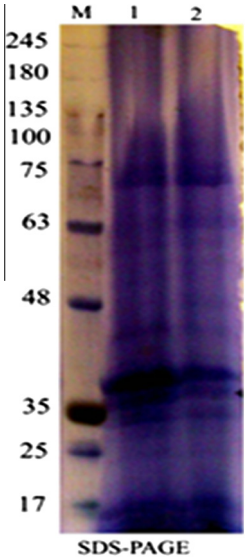

Figure 13 The protein profile of *G. longiscapa* (species 1) and *G. rigens* (species 2).

Table 3 Data matrix illustrating the presence and absence of bands in the protein electrophoretic banding pattern for *Gazania* species.

Band No.	M.W (kDa)	Species	
		1	2
1	115	1	0
2	102	1	1
3	94	1	1
4	77	1	1
5	68	1	1
6	61	1	1
7	56	0	1
8	50	0	1
9	44	1	1
10	37	1	1
11	33	1	1
12	30	1	1
13	27	1	1
14	24	1	1
15	22	1	1
16	21	0	1
17	17	1	1
18	15	1	1
Total		15	17

Table 4 Effect of single oral administration of the methanolic extracts of *G. longiscapa* and *G. rigens* aerial parts (5 g/kg) on some hematological parameters (RBCs, MCV, Hemoglobin, platelets, WBCs and Lymphocytes) in rats of both sex.

Parameters	RBC (10^3/μl)	MCV (fL)	Hemoglobin (g/dL)	Platelets (10^3/μl)	WBCs (10^3/μl)	Lymphocytes (10^3/μl)
Male normal control	6.87 ± 0.28	58.25 ± 1.80	14.45 ± 0.15	466.50 ± 11.70	14.95 ± 0.47	63.15 ± 1.48
G. longiscapa	6.41 ± 0.21	59.75 ± 0.93	13.65 ± 0.45	461.50 ± 34.50	15.60 ± 1.41	63.03 ± 0.50
G. rigens	6.87 ± 0.11	55.26 ± 1.68	13.70 ± 0.22	465.40 ± 13.22	14.60 ± 1.81	67.70 ± 4.99
Female normal control	6.44 ± 0.26	55.47 ± 0.13	13.23 ± 0.60	459.33 ± 39.49	9.97 ± 0.84	63.77 ± 0.76
G. longiscapa	6.58 ± 0.16	54.63 ± 0.80	13.23 ± 0.17	450.33 ± 42.37	10.30 ± 1.36	63.77 ± 1.67
G. rigens	6.73 ± 0.14	53.98 ± 0.89	13.48 ± 0.24	451.75 ± 19.89	10.60 ± 1.61	65.23 ± 2.56

Table 5 Effect of single oral administration of the methanolic extracts of *G. longiscapa* and *G. rigens* aerial parts (5 g/kg) on some serum biochemical parameters (ALT, AST, Creatinine and Urea) in rats of both sex.

Parameters	ALT (U/ml)	AST (U/ml)	Creatinine (mg/dl)	Urea (g/dl)
Male normal control	13.08 ± 0.56	12.51 ± 0.24	16.14 ± 0.39	5.09 ± 0.04
G. longiscapa	13.18 ± 0.26	12.74 ± 0.19	15.94 ± 0.09	5.19 ± 0.09
G. rigens	13.17 ± 0.44	12.84 ± 0.21	16.30 ± 0.45	5.20 ± 0.07
Female normal control	13.08 ± 0.49	12.53 ± 0.33	16.30 ± 0.44	5.06 ± 0.04
G. longiscapa	12.91 ± 0.49	12.44 ± 0.16	16.01 ± 0.41	5.16 ± 0.07
G. rigens	13.38 ± 0.17	13.09 ± 0.12	16.54 ± 0.29	5.06 ± 0.12

Data in both tables were expressed as mean ± SE (n = 6). Statistical analysis was carried out by one-way (ANOVA) followed by Least Significant Difference test.
RBC = red blood cells, MCV = mean corpuscular volume, WBCs = white blood cells, ALT = Alanine aminotransferase, AST = aspartate aminotransferase.

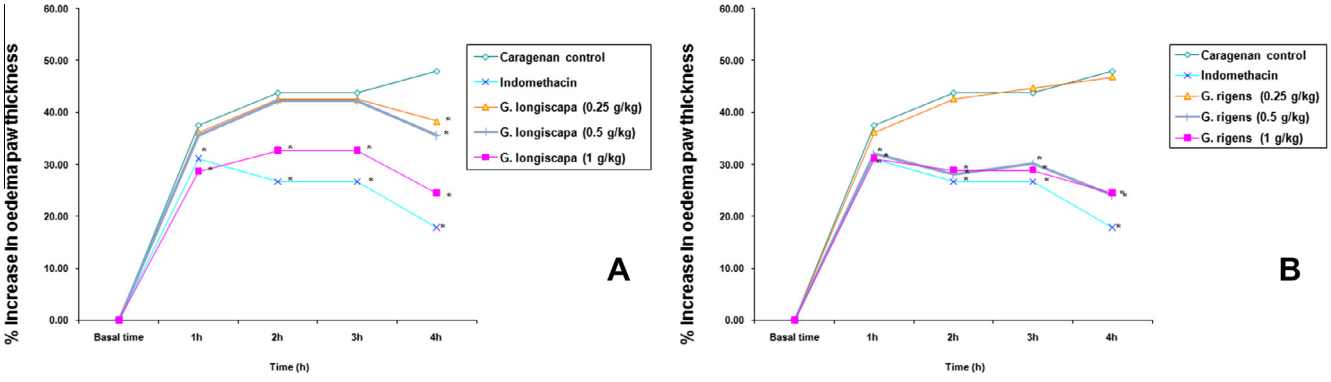

Figure 14 Time course of the effect of oral administration of indomethacin, extracts of *G. longiscapa* and *G. rigens* aerial part (250 mg, 500 mg and 1000 mg/kg) on rat increase in edema paw thickness induced by sub-plantar injection of 1% carrageenan.

4. The flower-heads (capitula) (Fig. 3A–C) are borne singly on peduncles that originate at the leaf axis. The peduncle is erect and 8–15 cm long. The capitulum (3–4 cm across) has numerous ray florets (1–2 cm long) which are narrowly oval to elongated in shape.

The macromorphological characters of *G. rigens* were similar to those previously reported.[4]

3.1.2. Micromorphology

3.1.2.1. Micromorphology of G. longiscapa

3.1.2.1.1. The root. A transverse section in the root (Fig. 3A and B) is nearly circular in outline. It is formed of a narrow cork followed by a considerably wide cortex, and a pericycle surrounding a complete ring of a wide stele which occupy three fourth the diameter of the root. The stele consists of wide inner xylem vessels and the phloem at the outer side.

The cork (Fig. 3A and B). It is formed of 3–4 rows of brownish cells radially arranged; they are polygonal in shape with non-lignified thin walls (Fig. 5).

The cortex (Fig. 3A and B). It is formed of 6–7 rows of thin-walled tangentially elongated cells free of starch, calcium oxalate and sclerenchyma.

The endodermis (Fig. 3A and B). It is indistinct composed of tangentially elongated with thin walls.

The pericycle (Fig. 3A and B). It is formed of 3–4 rows of thin walled parenchyma cells interrupted with about 3 groups

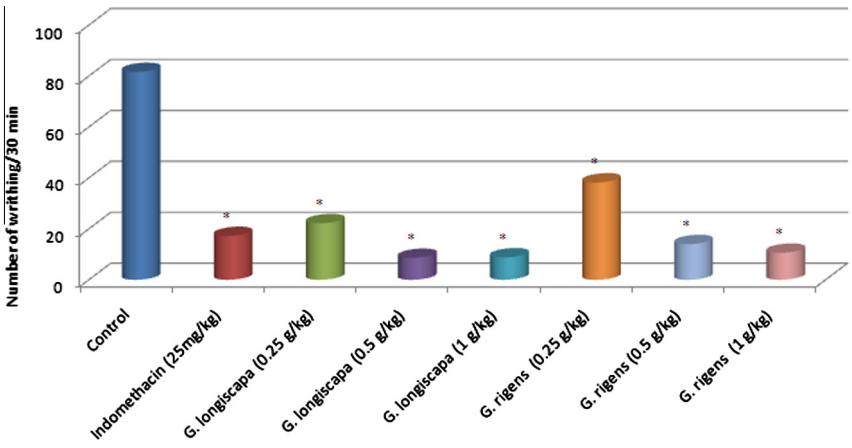

Figure 15 Analgesic effect of oral administration of indomethacin (25 mg/kg), *G. rigens* and *G. longiscapa* aerial part extract (250 mg, 500 mg and 1000 mg/kg), on visceral pain in mice using writhing test.

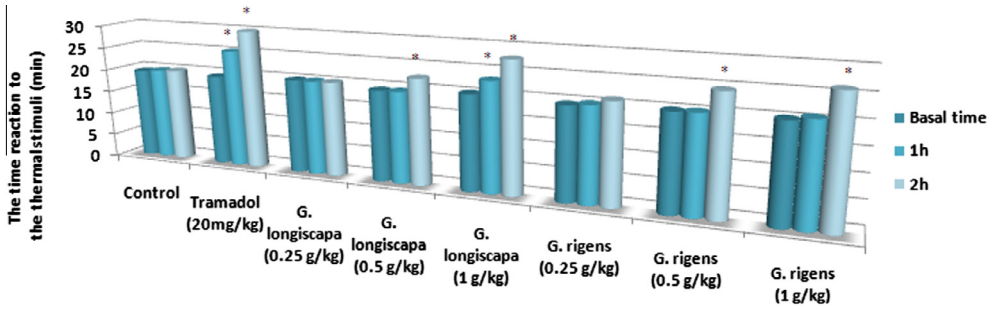

Figure 16 Analgesic effect of oral administration of tramadol (20 mg/kg), *G. longiscapa* and *G. rigens* aerial parts extracts (250 mg, 500 mg and 1000 mg/kg), on thermal pain using the hot plate test.

of pericyclic fiber which is composed of thick walled lignified fiber with narrow lumen.

The vascular tissue (Fig. 3A and B). It consists of a complete ring of radial vascular bundles (tri-arched), separated by batches of pericycle and traversed by uni or biseriate medullary rays. *The phloem* is formed of sieve tubes, companion cells and phloem parenchyma. *The xylem* is formed of radially arranged elements. The vessels show lignified annular thickening. Wood fibers are fusiform with wide lumen and blunt apex. Wood parenchyma consists of medullary rays which are formed of elongated rectangular cells (Fig. 5).

3.1.2.1.2. The rhizome. A transverse section in the rhizome (Fig. 4) shows a rounded and slightly wavy outline, a narrow cork followed by a wide cortex and several collateral vascular bundles about 14–18 surrounding a central wide pith. The bundles are large on one side and much smaller on the other side and are separated from each other by medullary rays.

The cork: Consists of 4–6 rows of tangentially elongated, radially arranged cells which appear polygonal in surface view with straight suberized anticlinal walls measuring 0.4–0.5 μm.

The phelloderm: Two rows of tangentially arranged parenchyma cells below the cork layer and *cortex* which consists of several rows (23–25) of tangentially elongated parenchymatous cells with thin cellulosic walls free from starch and calcium oxalate crystals.

The vascular tissue: Several vascular bundles are arranged radially surrounding the parenchymatous pith. There are 7–8 large vascular bundles from one side and 7–10 smaller ones from the other side; they are separated by multiseriate medullary rays.

The phloem: Consists of several rows of phloem elements and phloem parenchyma.

The xylem: Consists of lignified wood fibers, tracheids and wood parenchyma.

The vessels: They have a wide lumen with lignified annular, reticulated walls, measuring 0.1 μm in diameter.

The tracheids (Fig. 5): They are narrow and elongated, with slightly blunt apexes and pitted lignified walls.

The wood fibers (Fig. 5): They are lignified straight or irregular with moderately thickened walls, each having a wide lumen with a blunt apex.

The wood parenchyma (Fig. 5): They are polyhedral to rectangular in shape, more or less elongated and having moderately thickened lignified walls.

The medullary rays (Fig. 5): Consist of 2–5 rows traversing both phloem and xylem and having elongated polygonal cells with non-lignified cellulosic walls in the xylem region and thin cellulosic walls in the phloem area.

Pith: Consists of large thin walled cellulosic parenchyma cells, slightly rounded to irregular in shape and free from starch and calcium oxalate crystals.

- *Powdered rhizome and roots* are brownish yellow in color, odorless and the taste is slightly salty. Microscopically (Fig. 5), it is characterized by the following (Table 1):
  a. Fragments of brown, polygonal and suberized cork cells.
  b. Fragments of lignified wood fibers with straight walls having wide lumina and blunt apices.
  c. Fragments of polyhedral to rectangular wood parenchyma having lignified polyhedral to rectangular cells.
  d. Fragments of tracheids which have long pitted lignified walls and wide lumens.
  e. Fragments of xylem vessels showing annular thickening.

3.1.2.1.3. The leaf. Leaf lamina: a transverse section through the leaf blade (Fig. 6) shows a prominent midrib on the lower surface, while the upper surface is nearly flat. It reveals a dorsiventral structure with the upper palisade layer consisting of 2–3 rows of columnar cells with no intercellular space, interrupted in the midrib region by subepidermal spongy parenchyma from both upper & lower sides. The vascular system of the midrib is formed of a collateral vascular bundle forming a ring and consisting of a radiating xylem, phloem and surrounded by a pericycle. The upper epidermis is nearly free of hairs, while the lower epidermis has different types of hairs both glandular & non glandular. The upper epidermal cells stain yellow with KOH reagent indicating the probable presence of flavonoids.

Epidermis consisting of:-

(i) *The upper epidermis*: is formed of one row of subrectangular to square cells in side view while in surface view the cells are polygonal, usually isodiametric to slightly elongated with straight anticlinal walls. Stomata are present showing anomocytic type (Fig. 7).
(ii) *The lower epidermis*: is formed of one row of subrectangular to square cells in side view and it appears to be smaller than the upper epidermis while in surface view the cells appeared polygonal, elongated with straight anticlinal walls and covered with a smooth cuticle. The majority of the lower epidermal cells are carrying different type of trichomes except the region below midrib. The glandular hairs appear to have a unicellular head with a unicellular stalk. The non- glandular hairs are unicellular or bicellular uniseriate straight and bent, also cottony hairs are numerously present (Figs. 6 and 7).
(iii) *The neural epidermis*: the upper neural epidermal cells consists of one row of polygonal isodiametric or slightly elongated with straight anticlinal walls showing few anomocytic stomata; while the lower neural epidermal cells appeared more elongated than that of the upper one with straight anticlinal walls, covered with few glandular and non-glandular trichomes and devoid of stomata (Fig. 7).

The mesophyll: is homogenous showing an upper palisade layer consisting of 2–3 rows of columnar, cylindrical, thin walled cells. The spongy tissue consists of 3–4 rows of more or less rounded to irregular parenchyma cells with wide intercellular spaces.

The midrib region: consisting of:

(i) *The cortical tissue*: which is formed of parenchyma surrounding the main vascular bundle of the midrib; 7–8 rows of parenchyma cells above the bundle, but only 3–4 rows below followed by 1–2 layers of collenchyma cells. The endodermis is indistinct composed of one row of tangentially elongated cells.
(ii) *The pericycle:* consists of 3–6 rows of thin walled parenchyma cells.
(iii) *The vascular tissue*: is represented by the main vascular bundle of the midrib and lateral bundles of the veins. Each vascular bundle is formed of a xylem, a phloem and surrounded by a pericycle. *The phloem*: is represented by a narrow zone of soft elements between xylem and pericycle. It consists of sieve tubes, companion cells and phloem parenchyma. *The cambium*: is formed of about 3 rows of cambiform cellulosic cells. *The xylem:* is formed of lignified vessels which are present with spiral and sometimes annular thickening with thin walled cellulosic non-lignified wood parenchyma. The medullary rays are about one cell wide, radially elongated thin-walled cellulosic cells.

Powdered leaves are pale green in color, odorless and the taste is slightly salty. Microscopically (Fig. 7) (Table 1), it is characterized by the following:

(a) Fragments of upper epidermis which appear polygonal isodiametric to slightly elongated with straight anticlinal walls, it stains yellow to orange yellow with KOH due to the presence of flavonoids.
(b) Fragments of lower epidermis that appeared more elongated than the upper one.
(c) Fragments of stomata showing anomocytic type.
(d) Fragment of glandular hair with a bicellular head and a bicellular uniseriate stalk.
(e) Fragment of non-glandular unicellular hair.

3.1.2.2. Micromorphology of G. rigens. The micromorphological structure of different organs of *G. rigens* is more or less similar to their respective ones in *G. longiscapa* and also to that reported previously[4], but could be differentiated and characterized as follows

- The root (Fig. 8)
  a. Absence of pericyclic fiber patches.
  b. The phloem forms a continuous ring around the xylem.

- The stem (Fig. 9)
  a. Vascular bundles in *G. rigens* stem are scattered radially and all are more or less of the same size.

- The leaf (Fig. 10)
  a. In the mid rib region it has about 5 separated small collateral vascular bundles.
  b. Only cottony hairs could be observed.

3.2. Genetic profiling

3.2.1. DNA fingerprinting

In this study the extracted DNA of each of the two *Gazania* species were amplified using 2 different techniques (RAPD & ISSR) to detect genetic variability between them, the obtained band profiles produced by the primers used in the RAPD & ISSR analysis are represented in (Figs. 11 and 12).

The RAPD and ISSR techniques produced multiple band profiles with the number of amplified DNA fragments ranging from 22 in ISSR analysis to 29 in RAPD analysis in both species. On the other hand, the total number of fragments was 74 bands, 23 were polymorphic representing a level of polymorphism of 31%. The highest degree of similarity (100%) was observed using primers OPC-04, OPC-19 and OPM-01, while the least degree of similarity (10%) was observed using primers OPB-09 and OPB-11, the percent of monomorphic and polymorphic bands resulting from ISSR and RAPD techniques can be observed in (Table 2).

3.2.2. Protein profiling (Fig. 13)

The protein profiles of both *Gazania* species were studied and the observed protein banding profile is shown in Fig. 13. The molecular weight of the revealed bands and their distribution among the studied samples are given in (Table 3). Protein band patterns were coded 0 or 1 depending on their absence or presence in each species. The highest number of bands (17) is recorded for *G. rigens*. The molecular weight observed within the studied samples ranges between 115 and 15 kDa. The total number of bands recorded in both samples is 18, among these fourteen are common to both species, 16, 8, and 7 kDa were absent from proteins of *G. longiscapa* and 115 kDa was absent from proteins of *G. rigens*. The similarity coefficient between both samples was 77.7%.

3.3. Biological activity

3.3.1. Acute toxicity study

The results showed no mortality after 24 h of oral administration of both extracts (*G. longiscapa* and *G. rigens*) at graded doses up to a 5 g/kg and according to Semler[24] who reported that if just one dose level at 5 g/kg is not lethal, regulatory agencies no longer require the determination of an LD50 value. Hence, the chosen experimental doses were 1/20, 1/10 and 1/5 of 5 g/kg of each *Gazania* aerial parts extract (250, 500 and 1000 mg/kg). After 15 days of single oral administration of both species extracts, the results revealed that no significant change was determined in hematological parameters [RBC, MCV, hemoglobin, platelets and WBCs] (Table 4) as well as in liver functions (serum ALT and AST) and kidney functions [serum creatinine and BUN] (Table 5).

3.3.2. Carrageenan induced paw edema

The subplanter injection of 100 μL of 1% sterile carrageenan into the rat hind paw elicited an inflammation (swelling and erythema) and a time-dependent increase in paw edema by 37.5%, 43.75% and 43.75% at 1st, 2nd and 3rd hours, respectively, and the paw thickness was maximal by 47.91% at 4 h post-carrageenan injection as compared with pre-carrageenan control values.

Oral administration of *G. longiscapa* extract (250 and 500 mg/kg) showed non-significant inhibition of edema formation at 1st, 2nd and 3rd hours, respectively, while induced a significant edema inhibition by 20.07% and 25.79% after the 4th hour. Meanwhile *G. longiscapa* (1000 mg/kg) induced a significant edema inhibition by 23.81%, 37%, 37% and 48.90% at 1st, 2nd, 3rd and 4th hours, respectively, as compared with carrageenan control group at the same time post carrageenan injection.

Oral administration of *G. rigens* extract (250 mg/kg) showed non-significant inhibition of edema formation while (500 mg/kg) induced significant edema inhibition by 25.33%, 36%, 17.71% and 49.90% at 1st, 2nd, 3rd and 4th hours, respectively. Also, *G. rigens* extract (1000 mg/kg) showed a significant edema inhibition by 17.04%, 33.99%, 33.99% and 48.99% at 1st, 2nd, 3rd and 4th hours, respectively, as compared with carrageenan control group at the same time post carrageenan injection.

Indomethacin (25 mg/kg) showed significant inhibition of edema formation by 17.04%, 39.06%, 39.06% and 62.90% at 1st, 2nd, 3rd and 4th hours, respectively, as compared with carrageenan control group at the same time post carrageenan injection.

Comparing the results of both extracts with indomethacin, results revealed that the extract of *G. rigens* 500 mg/kg has anti-inflammatory activity more than *G. longiscapa* 1000 mg/kg .So this means that that *G. rigens* has more potent anti-inflammatory activity than *G. longiscapa* (Fig. 14).

Data represent the mean value ± S.E. of six rats and % increase in edema paw thickness Data were analyzed using a one way ANOVA and LSD comparison test $^{*}P < 0.05$ vs carrageenan control value at respective time point.

3.3.3. Antinociceptive activity

This activity was determined by measuring the responses of animals to the thermal and chemical stimulus.

3.3.3.1. Chemical test. In the chemical test (visceral pain test), *G. longiscapa* extract showed a significant decrease in the number of writhing in mice after acetic acid injection by 72.79%, 89.46% and 62.5% at dose of 250, 500 and 1000 mg/kg, respectively. Also *G. rigens* extract administration showed a significant decrease in the number of writhing by 53.19%, 82.84%, 87.01% at dose of 250, 500, 1000 mg/kg, respectively as compared with the control group. While indomethacin treated group showed a significant decrease in the number of writhing by 78.92% as compared with the control group.

These results showed that *G. longiscapa* at doses 250 and 500 mg/kg. is more potent than *G. rigens* in peripheral antinociceptive activity as compared with the indomethacin group (Fig. 15).

Data represent the mean value ± S.E. of six mice per group and the number of writhing/30 min. Statistical comparison of the difference between the control group and treated groups (indomethacin, *G. longiscapa* and *G. rigens*) was done using a one way ANOVA and LSD comparison test $^{*}P < 0.05$.

3.3.3.2. Thermal test. G. longiscapa extract at dose 500 mg/kg induced significant prolongation in the reaction time to the thermal stimulant by 16.11% at 2nd hour only but 1000 mg/kg by 14.9% and 37.12% at 1st and 2nd hours, respectively.

While *G. rigens* extract (500 and 1000 mg/kg) showed a significant prolongation in the reaction time to the thermal stimulant by 22.47% and 29.81% at 2nd hours only, as compared with pre-drug values (basal time).

The group of rats treated with tramadol orally showed a significant prolongation in the reaction time to the thermal stimulant by and 30.19% and 54.55% at 1st and 2nd hours, respectively, as compared with pre-drug values (basal time).

G. longiscapa is also more potent than *G. rigens* in the central antinociceptive activity compared with the tramadol group. (Fig. 16).

Data represent the mean value ± S.E. of six rats per group, shown at the basal (zero time), 1 h and 2 h values for each group (saline, tramadol, *G. longiscapa* and *G. rigens*). Statistical comparisons between basal (pre-drug values) and post-drug values were done using a one way ANOVA and LSD comparison test $^{*}P < 0.05$.

4. Conclusion

Our results highlight the differences between the macro and micro-morphological criteria of both plants that can be used for distinction between the two species, on macromorphological basis we can find that *G. longiscapa* has no clearly observed aerial stem and has large flower compared with that of *G. rigens*, while on micromorphological point of view it showed a great similarity and most reliable criteria which can be used for distinction between the two species that are prominent in fresh samples and not evident in the powdered ones. On the other hand, the genetic characterization of both *Gazania* species using random amplified polymorphic DNA (RAPD) and Inter Simple Sequence Repeat (ISSR) showed that primers OPC-04, OPC-19 and OPM-01, could be used as indicators for obtaining genetic markers. In addition, the primers OPB-09 and OPB-11 were found to be the most effective in generating polymorphic bands on application of the RAPD technique to both plants, and therefore can act as markers for species authentication beside the morphological characteristics.

Protein fingerprint revealed that the distance between the two studied *Gazania* species did not exceed 22.2%, which indicates little variation among their SDS–PAGE profiles and might further explain any resemblance on the chemotaxonomical level.

The acute toxicity study of both *Gazania* aerial part extracts revealed that the plant is safe for oral administration. *G. rigens* is more potent as anti-inflammatory than *G. longiscapa*. On the other hand *G. longiscapa* exhibit more potency in peripheral and central antinociceptive activity. This biological activity could be due to the presence of phenolic and flavonoidal compounds in the Genus as was previously reported.

5. Conflict of interest

None declared.

Acknowledgements

This work was financially supported by Future University in Egypt.

References

1. Bruce AB, Tod FS. *Flavanoid of the sunflower Family (Asteracea)*. New York: S. Wien; 2001.
2. Watt JM, Breyer-brandwijk MG. *The medicinal and poisonous plants of southern and eastern Africa*. London: Livingstone; 1962.
3. Taha SE, Mona HH, Shahira ME, Amany AS. Pharmacognostical and biological studies on the rhizomes of *Gazania nivea* DC cultivated in Egypt. *Bull Fac Pharm., Cairo Univ*. 2008;**46**(2):253–65.
4. Hala MH. Biologically-guided phytochemical investigation of *Gazania rigens* L.. *Alex J Pharm Sci* 2009;**23**(2):96–100.
5. Ahmed SI. Pharmacognostical study of *Gazania rigens* R.B. family Compositae cultivated in Egypt [M.sc. thesis]. Faculty of Pharmacy, Assuit University; 1981.
6. Herbal Drug Standardization, Posted July 1999. Retrieved at: <www.swatijaininst.com/etechno/arunjain.doc>.
7. Karihaloo JL, Kaur M, Singh S. Seed protein diversity in *Solanum melongena L.* and its wild and weedy relatives. *Genet Res Crop Evol* 2002;**49**(6):533–9.
8. Miege MN. Protein types and distribution. In: Boulter D, Parthier B, editors. Nucleic acids and proteins in plants, Encyclopedia of plant physiology. 1982;14:291–345.
9. Ladizinsky G, Hymowitz T. Seed protein electrophoresis in taxonomic and evolutionary studies. *Theoretical Appl Genet* 1979;**54**:145–51.
10. Williams JK, Kubelisk AR, Livak KJ, Rafalski JA, Tingey SV. DNA polymorphism amplified by arbitrary primers are useful as genetic markers. *Nucl Acids Res* 1990;**18**:6531–5.
11. Stegmann H. Characterization of proteins from potatoes, and the index of European varieties. In: Hawkes JG, Lester RN, Skelding AD, editors. *Biology and taxonomy of the Solanaceae*. Academic Press; 1979. p. 229–84.
12. Suleyman H, Demircan B, Karagoz Y, Oztasan N, Suleyman B. Anti-inflammatory effects of selective COX-2 inhibitors. *Pol J Pharmacol* 2004;**56**:775–80.
13. Sacerdote P, Bianchi M, Manfredi B, Panerai AE. Effect of tramadol on immune responses and nociceptive thresholds in mice. *Pain* 1997;**72**:325–30.
14. Reitman S, Frankel S. A colorimetric method for the determination of glutamic oxaloacetic and glutamic pyruvic transaminases. *Am J Clin Pathol* 1957;**28**:56–63.
15. Teger-Nilsson AC. Serum creatinine determination using an ion exchange resin. *Scand J Clin Lab Invest* 1961;**13**:326–31.
16. Tabaco A, Meiattini F, Moda E, Tarli P. Simplified enzymic/colorimetric serum urea nitrogen determination. *Clin Chem* 1979;**25**:336–7.
17. Winter CA, Risley EA, Nuss GW. Carrageenan-induced oedema in hind paws of the rat as an assay for anti-inflammatory drugs. *Proc Soc Exp Biol Med* 1962;**111**:544–52.
18. Obukowicz MG, Welsch DJ, Salsgiver WJ, Martin-Berger CL, Chinn KS, Duffin KL. Noval, selective 6 or 5 fatty acid desaturase inhibitors as antiinflammatory agents in mice. *J Pharmacol Exp Ther* 1998;**287**:157–66.
19. Koster R, Anderson M, De Beer EJ. Acetic acid for analgesic screening. *Fed Proc* 1959;**18**:412.
20. Collier HDJ, Dinnin LC, Johnson CA, Schneider C. The abdominal response and its suppression by analgesic drugs in mouse. *Br J Pharmacol* 1968;**32**(4):295–310.
21. Egesie UG, Chima KE, Galam NZ. Anti-inflammatory and analgesic effects of aqueous extract of Aloe Vera (*Aloe barbadensis*) in rats. *Afr J Biomed* 2011;**4**:201–9.
22. Eddy NB, Leimback D. Synthetic analgesic. . Dithienyl-butenyl and dithienylbutilamines. *J Pharmacol Exp Ther* 1953;**107**(4):385–93.
23. Bailey LH. *Manual of cultivated plants*. New York: Cornell University, The Macmillan Company; 1958, p. 1012.
24. Semler DE. *The rat toxicology in animal models in toxicology*. NewYork, Basel, Hong Kong: Marcel Dekke; 1992, p. 39.