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Phytochemical Investigation and α -Cellulose Content Determination of *Gazania splendens* Moore*

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Abstract: Chemical constituents investigation of *Gazania splendens* Moore (Compositae family) led to isolate two flavonoids via luteolin and luteolin 7-*O*-glucoside, 11 *n*-alkanes (C₁₄-C₃₀), β -amyrin, cholesterol, β -sitosterol, stigmasterol and eight fatty acids. The structures of these compounds were established by Mass Spectrometry (MS), Gas-Liquid Chromatography (GLC) and spectroscopic techniques, including Ultra-violet (UV), Infra-Red (IR). The percentage of α -cellulose content in the leaves and flowers of the plant were 70.50 and 73.11%, respectively, hence the percentage in whole plant was 71.81%.

Key words: α -cellulose content, *Gazania splendens*, hollocellulose content, luteolin, luteolin 7-*O*-glucoside, β -amyrin, stigmasterol, cholesterol, β -sitosterol

INTRODUCTION

Gazania splendens Moore belongs to the family Compositae which is one of the largest plant families, comprising 1,000 genera and 23,000 species. It is the source of relatively few products of economic and medical importance (Hutchinson, 1959). The most common species of genus *Gazania* are represented in Egypt by six species viz., *Gazania hybridus*, *Gazania rigens* R.Br., *Gazania krebsiana* Less, *Gazania lichtenstenii* Less, *Gazania linearis* Druce and *Gazania splendens* (Ahmed, 1981).

Uses in Traditional Medicine

To prevent miscarriage, relieve toothache, relieve earache, in cases of stricture of the urethra (Ahmed, 1981 and Watt *et al.*, 1962).

Previously Isolated Constituents

To the best of our knowledge there are no concrete studies on *Gazania splendens* Moore, other than few studies which report the separation of dry rubber, resin and some pigment compounds from flowers (Zechmeister *et al.*, 1943; Valadon *et al.*, 1967; Bohlmann *et al.*, 1979). In spite of the phytochemical screening of *Gazania* species revealed the isolation of xanthophylls, gazaniaxanthin, lutein, leafxanthophyll, γ -carotene, β -carotene, lycopene, chrysanthemaxanthin, rubixanthin (Zechmeister *et al.*, 1943; Valadon *et al.*, 1967), lupe-13-ene-3-acetate, lupe-13 ene-3-ol, lupeol, lupe-20 (29) ene-3-acetate, two sesquiterpene lactones via gazaniolid, 8 α -isovaleryloxy-gazaniolid (Bohlmann *et al.*, 1979), alkaloids and flavonoids (Rizk *et al.*, 1986).

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The aim of this research deals with the chemical study of the plant as regards their constituents particularly flavonoids, lipids, as well as study of α -cellulose content and proximate analysis.

MATERIALS AND METHODS

Plant Material

Fresh aerial parts of *Gazania splendens* MOORE were collected from East and West Suez Canal region, Ismailia-Port Said road, Ismailia, Egypt in April 2005 during the flowering stage. The identity was established by Prof. Dr. Moustafa Zaghoul, Prof. of Floriculture and Medicinal plants, Department of Horticulture, Faculty of Agriculture, Suez Canal University. A voucher specimen (Number AMYM-1002) has been deposited in the Herbarium of Botany Department, Faculty of Science, Suez Canal University, Ismailia, Egypt.

General Methods

Melting points were determined on Büchi 535 melting point apparatus. IR spectra were recorded on 1430 Ratio Recording, Perkin-Elmer, in the Micro-analytical unit, Faculty of Science, Suez Canal University (SCU). IR-data station Epson FX-86e, in KBr disks. UV/Vis spectra were obtained on lambda 4B UV/Vis spectrophotometer (Perkin-Elmer) in the region of 190-900 nm, in the Micro-analytical unit, SCU. EIMS (ionization voltage 70 eV) was measured on GC-MSQP 1000EX Shimadzu, in the Chemistry Dept., Cairo University (CU), Giza, Egypt. The fractions obtained were subjected to gas-liquid chromatographic analysis (PYE UNICAM Series 304GC), in Faculty of Agriculture, CU, using coiled glass column (2.8 m \times 0.4 mm I.D.), packed with Diatomite C (100-120 mesh) and coated with 1% OV-17, programmed at 10°C/min from 70 to 270°C, then isothermally at 270°C for 25 min, injector temperature and FID detector at 300°C and the nitrogen carrier gas at a flow rate of 30 mL min⁻¹. GLC of the methyl esters of the fatty acids was carried out by PYE UNICAM Series 304 Gas Chromatograph equipment with FID and SGE injector split mode, using capillary column (25 m \times 0.22 mm I.D., 0.2 μ m thickness) packed with vitreous silica coated with free fatty acid phase, programmed at 12°C min⁻¹ from 70 to 190°C, injector temperature at 250°C, FID detector at 270°C and the flow rate of hydrogen is 41.0 cm sec⁻¹.

Extraction, Isolation and Characterization

The upper parts of the plant (leaves, flowers and stems) were air dried and ground altogether as a fine powder. The phytochemical screening and the proximate analysis included ash and moisture contents were performed in accordance with AOAC (1990) and Balbaa, (1976).

Acid-insoluble Lignin

The acid-insoluble Lignin of leaves and flowers were determined using Tappi Standard Methods (1954).

Determination of Cellulose Content

Hollocellulose and α -cellulose contents were calculated according to Wise *et al.*, (1946) and Tappi Standard Methods (1954).

Lipids

Air dried and powder aerial parts (2 kg) of the plant were extracted with petroleum ether (40-60°C) to yield 60 g of a dark green oily residue (lipid fraction). The marc was macerated with ethanol (80%) at room temperature till exhaustion. The resulting alcoholic extract was concentrated in vacuo to obtain a crude residue (950 g). About 35 g of the obtained lipid fraction were purified by

treatment with fuller's earth. The purified residue treated in accordance with (Berry, 2004) to effort 4.5 g of acetone insoluble fraction and 18 g of acetone soluble fraction as oily material. Ten grams of the acetone soluble fraction were saponified to yield 4.4 g of yellowish brown, semi-solid residue of unsaponifiable matter and 3.8 g of semi-solid residue of fatty acids. Three grams of the unsaponifiable matter were subjected on a silica gel column chromatography (80×2 cm) with petroleum ether and increasing the polarity with benzene.

Flavonoids

The flavonoids were isolated by treating the alcoholic extract of the plant with Hyflo super cel, then evaporated till dryness. The obtained homogeneous powder was packed into a glass column (diameter 5×60 cm) eluted with petroleum ether (40-60°C), followed by extraction with chloroform (CHCl₃) then ethyl acetate, to effort 7.5 and 11.5 g, respectively. Paper Chromatography (PC) and Preparative Paper Chromatography (PPC) were carried out in accordance with (Jung *et al.*, 2004). Detection was carried out using UV-light at 366 nm before and after exposure to ammonia vapor and spraying with Naturstoff reagent-A (Neu, 1956). Fractionation of the flavonoids was affected by column chromatography. Five grams of the ethyl acetate extract were applied on a glass column packed with silica gel G. (60 cm × 3 cm I.D.) in CHCl₃. Elution was carried out with CHCl₃, followed by CHCl₃/ MeOH with an increasing amounts of the later till 80%. Moreover, application of sephadex LH-20 column chromatography was applied for the separation and purification of the flavonoids, elution was affected with 90% MeOH. The purity of the isolated components G-I and G-II was checked by two-dimensional TLC on a cellulose plate. UV- absorption spectra of the isolated flavonoidal components were measured in MeOH as well as in MeOH after the addition of shift reagents (Mabry *et al.*, 1970).

Acid Hydrolysis of the Flavonoidal Compound (G-II)

Acid hydrolysis of Compound (G-II) (25 mg) was carried out in accordance with Brown and Rice-Evans, (1998).

Compound (G-I). (55 mg), m.p. 327-329°C, R_f: [0.54, BAW (4:1:5)]. IR bands (KBr) ν_{max} : 3417, 1657, 1616, 1579, 1515, 1397, 1313, 1266, 1190, 1165 and 1120 cm⁻¹. UV λ_{max} nm (MeOH): 245sh, 255, 268.7, 292sh, 350.7. EIMS *m/z* (rel. Int.): 286 (100), 258 (14.6), 153 (30.5), 152 (7.1), 137 (2.8), 134 (12.9), 109 (1.0).

Compound (G-II). (70 mg), R_f: [0.34, BAW (4:1:5)]. IR bands (KBr) ν_{max} : 3303-3362, 1663, 1618, 1571, 1520, 1515, 1388, 1260 and 1181 cm⁻¹. UV λ_{max} nm (MeOH): 255.6, 268.7sh, 349.7, 400sh. EIMS *m/z* (rel. Int.) (after hydrolysis): 286 (100), 258, 153, 152, 137, 134, 109.

RESULTS AND DISCUSSION

The results obtained from the preliminary phytochemical screening of *Gazania splendens* Moore revealed the presence of flavonoids conmarins, carbohydrates and/or glycosides, sterols and/or triterpenes and tannins. The average percentages of the constants of the plant were calculated as shown in Table 1.

The percentage of α -cellulose content in the leaves and flowers of the plant were 70.5 and 73.11% respectively, as shown in Table 2. The percentage in whole plant was 71.81%. This percentage represented approximately twice the percentages present in each of *Bagasse* (40%) and *Rice straw* (38%) (Anonymous, 2000; Chan, 1999), The amount of dried leaves of the plant from feddan, in one season was 15.864 ton. While the amount of dried flowers of the plant from feddan, in one season was 3.890 ton, this shows the economic importance of the *Gazania splendens* plant as a source for paper manufacturing industries.

Table 1: Constants of *Gazania splendens* Moore

Plant material	Moisture ^a (%)	Ash ⁱⁱⁱ (%)	Acid-insoluble lignin ^{iv} (%)	Extractives ^v (%)					
				Petroleum ether (60-80°C)	Benzene (%)	CHCl ₃ (%)	Ethyl acetat (%)	MeOH (%)	Hot H ₂ O (%)
Leaves	14.0	18.2	13.40	3.4	1.6	1.4	1.1	5.8	13.4
Flowers	11.0	14.7	10.07	3.8	0.7	1.4	1.6	4.6	8.0

^aCalculated on the air-dried plant material, ⁱⁱⁱAverage Percentages (means of duplicate analysis), ^vCalculated on the oven-dried material

Table 2: α -cellulose content of *Gazania splendens* Moore

Plant material	α -cellulose content (%)	Amount of dried plant per Feddan (ton)	Accepted amount of α -cellulose in the plant from Feddan, in one season (ton)
Leaves and Stalks	70.50	15,864	11.18
Flowers	73.11	3,890	2.84
Total	71.81	19,754	14.02

Table 3: Gas liquid chromatographic analysis of the hydrocarbon fraction

<i>n</i> -alkane	Retention time (min)	Relative percentage (%)
<i>n</i> -C ₁₄	7.37	10.01
<i>n</i> -C ₁₆	9.96	3.53
<i>n</i> -C ₁₈	12.49	4.06
<i>n</i> -C ₂₀	14.18	7.59
<i>n</i> -C ₂₂	16.45	21.04
<i>n</i> -C ₂₃	17.22	4.57
<i>n</i> -C ₂₄	18.12	5.67
<i>n</i> -C ₂₅	19.05	4.77
<i>n</i> -C ₂₆	20.39	6.50
<i>n</i> -C ₂₈	21.25	12.69
<i>n</i> -C ₃₀	25.38	19.57

ⁱ*n*: normal

The results obtained from the GLC chromatogram of hydrocarbon fraction, eluted with petroleum ether-benzene (90:10), revealed the presence of a series of 11 *n*-alkanes (*n*-C₁₄ to *n*-C₃₀) with C₂₂H₄₆ and C₃₀H₆₂ as the major constituents of the mixture 21.04 and 19.57 % respectively and the percent of the other constituents ranging from 3.53-12.69% as shown in Table 3 and Fig. 1.

The residue obtained from fractions eluted with petroleum ether-benzene (75:25) white crystalline needles in MeOH. It melted at 197-199°C both alone and upon admixture with authentic β -amyrin. It gives positive Liebermann-Burchardt reaction, indicating that it is a triterpenoid in nature. TLC on Silica gel using different solvents showed to be a single spot possessing the same retention time as authentic β -amyrin. The IR-spectrum of the isolated substance showed the same absorption bands characteristic for authentic β -amyrin.

The combined fractions eluted with petroleum ether-benzene (25:75) gave white crystalline needles in CHCl₃/MeOH, m.p. 133-135°C. The substance showed a single spot on TLC, Silica gel G, using toluene- acetone system (90:10), possessing R_f 0.47. It was identical with authentic β -sitosterol. However, several authors (Bennet and Heftman 1966; Marsill and Moreill 1968; Hornero-Mendez and Minguez-Mosquera, 2000; Singh *et al.*, 1970) reported that the sterols isolated from plants, showed single substances on TLC and their physical properties confirmed this. Yet on subjecting to GLC they were proved to be mixtures. So the sterol fraction was subjected to GLC. The obtained chromatogram revealed that, the isolated sterol fraction is a mixture of three compounds possessing the same retention times (18.69, 19.85 and 20.04%, respectively) as authentic Cholesterol, β -sitosterol and Stigmasterol. The percentages of them were 17.3, 39.2 and 43.5% respectively from the total sterol fraction. GLC chromatogram of the methyl ester of fatty acids revealed the presence of myristic, pentadecanoic,

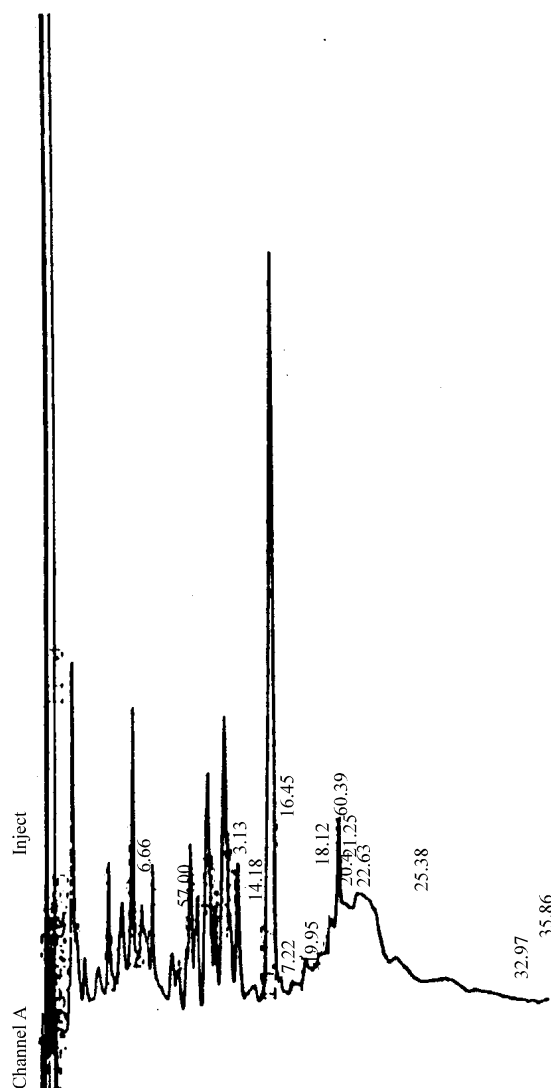


Fig. 1: GLC chromatogram of hydrocarbon fraction

palmitic, heptadecanoic, stearic, oleic, linoleic and linolenic acids according to their retention times in comparison with authentic (3.06, 0.38, 67.89, 0.37, 1.52, 2.87, 19.61 and 4.30%, respectively). The saturated fatty acids represent 73.22% while the unsaturated fatty acids represented by 26.78% of the total fatty acids. The major fatty acids were palmitic acid 67.89% and linoleic acid 19.61% as shown in Table 4 and Fig. 2.

TLC and PC of the ethyl acetate extract were performed better separation and revealed the presence of four flavonoid constituents, two major flavonoid R_f (0.54, 0.34; BAW 4:1:5) and two minor flavonoid constituents R_f (0.86, 0.77; BAW 4:1:5) beside fluorescent constituent R_f (0.61; BAW 4:1:5). On the other hand, the chloroform extract was found to contain two flavonoid spots R_f (0.54, 0.34, BAW 4:1:5) as minor constituents. Compound (G-I). UV-spectrum of (G-I) in MeOH (Table 5 and Fig. 3), exhibited λ_{max} at 350.7 nm (band-I). This indicates that it is a flavone type. The

Table 4: Gas liquid chromatographic analysis of the methyl esters of fatty acids fraction

Methyl esters of fatty acids	Retention time (min)	Relative percentage (%)
Myristic acid	15.13	3.06
Pentadecanoic acid	16.50	0.38
Palmitic acid	18.57	67.89
Heptadecanoic acid	20.05	0.37
Stearic acid	23.10	1.52
oleic acid	24.09	2.87
Linoleic acid	26.36	19.61
Linolenic acid	29.64	4.30

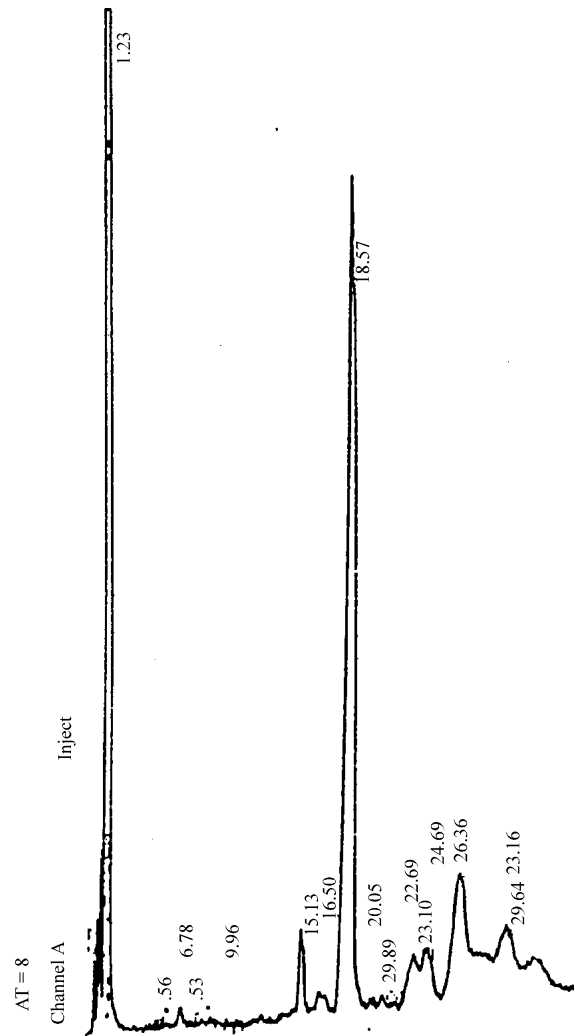


Fig. 2: GLC chromatogram of saponifiable fraction

NaOMe spectrum showed a bathochromic shift of 52.5 nm in band-I, which is indicated to the presence of 4'-OH group. Also, showed a shoulder peak at 328 nm which is an indicative to the presence of 7-hydroxy group. $AlCl_3$ spectrum showed peak > 415 nm (426.2 nm) and $AlCl_3/HCl$ spectrum showed peak < 395 nm (386.8 nm). This indicates that the substance G-I belongs to the

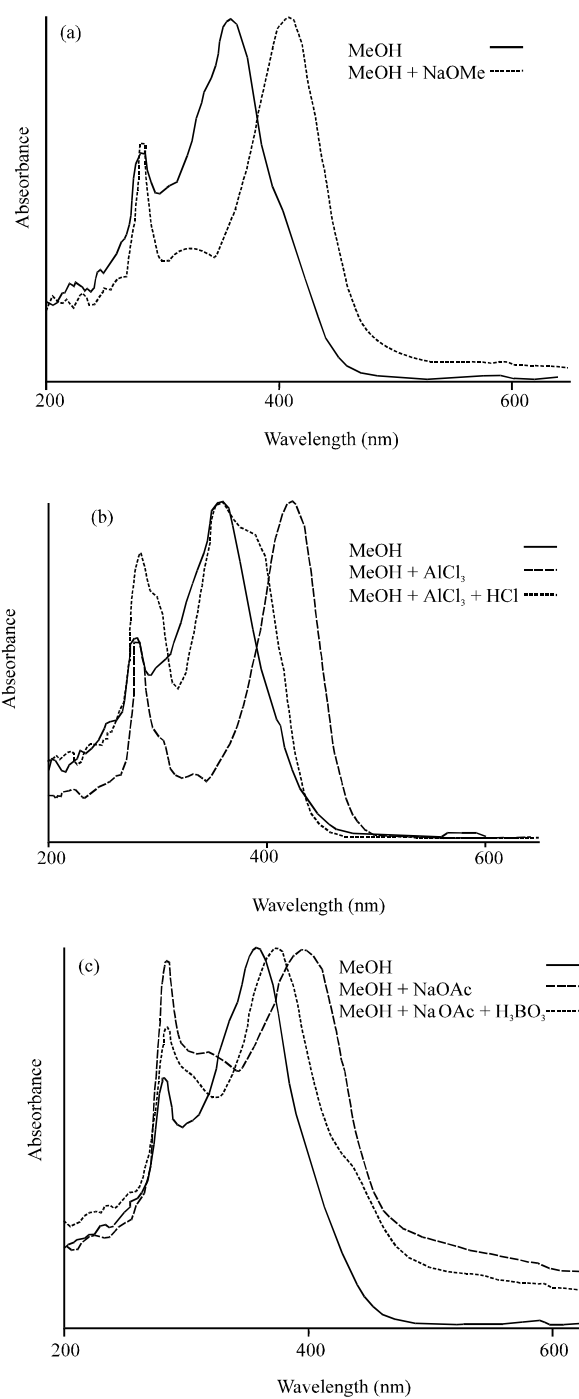


Fig. 3: UV/Vis absorption spectra of compound G-I in (a) MeOH and Me OH/NaOMe, (b) MeOH, MeOH/AlCl₃ and MeOH/AlCl₃/HCl and (c) MeOH, MeOH/NaOAc and MeOH/NaOAc/H₃BO₃

phloroglucinol type A ring (i.e., 5, 7-dihydroxy) (Voirin, 1983). Also, AlCl_3 spectrum showed a relatively large bathochromic shift (75.5 nm) indicating the existence of *ortho*-dihydroxy system and/or 3, 5-di-hydroxy groups. However, AlCl_3/HCl spectrum exhibited hypsochromic shift (39.4 nm) in band-I relative to AlCl_3 spectrum. This performed the presence of 3', 4'- *ortho*-dihydroxy groups. The presence of only a free 5-hydroxyl group is mainly due to the persistence of a bathochromic shift (36.1 nm) in band-I of AlCl_3/HCl spectrum (relative to MeOH spectrum) and not due to 3-hydroxyl group. NaOAc spectrum showed a bathochromic shift (18 nm) in band-II, indicates the presence of free 7-OH group. An *ortho*-dihydroxy system is further proved to exist in ring-B by the bathochromic shift (44.3 nm) in band-I of the NaOAc/ H_3BO_3 spectrum. IR-spectrum of G-I showed a strong band at 3417 cm^{-1} corresponding to -OH group, a strong band at 1657 cm^{-1} corresponding to an α - β -unsaturated ketone (C = O group of (γ -pyrone), absorption bands at 1616, 1579, 1515 cm^{-1} (an aromatic system); in addition to the absorption bands at 1397, 1313, 1266, 1190, 1165 and 1120 cm^{-1} . MS of G-I showed a molecular ion (M^+) at m/z 286 (100) which corresponds to the molecular formula $\text{C}_{15}\text{H}_{10}\text{O}_6$ of four hydroxy substitution pattern. Hence, the fragmentation pathway undergoes the Retero-Diels Alder reaction-giving rise to ring-A fragment at m/z 153 (30.5) and m/z 152 (7.1). However, the hydrogen transfer ion at m/z 153 is much intense than that of the normal fragment ion at m/z 152, indicating that it has 5,7-di-hydroxy grouping. Moreover, the ring-B fragment at m/z 134 (12.9) indicates that the other two-hydroxyl groups must be attached to ring-B. Furthermore, loss of CO directly from the molecular ion (M^+-CO) was also shown, leading to the phenylbenzofuran fragment ion at m/z 258 (14.6) which further fragments giving rise to the benzoyl ion at m/z 137 (2.8). This loses CO directly giving m/z 109 (1.0). The previous data are in agreement with those reported for luteolin (Johnson *et al.*, 2002; Đorđević *et al.*, 2000; Braune *et al.*, 2001; Wouters Rosario-Chirinos, 1992; Geissman, 1955; Mabry *et al.*, 1970; Macek, 1972), (Fig. 4).

Compound (G-II). UV-spectrum in MeOH, exhibited λ_{max} at 349.7 nm (band-I), which indicates that it is a flavone type. The NaOMe spectrum showed a bathochromic shift of 53.5 nm in band-I with slow degeneration indicated to the presence of 4'-OH group. AlCl_3 spectrum showed peak > 415 nm (427.8 nm) and AlCl_3/HCl spectrum showed also peak I < 395 nm (388.4 nm) (Fig. 5). This indicates

Table 5: UV/Vis absorption maxima of the isolated flavonoidal constituents

Compound	λ_{max} nm	λ_{max} nm	λ_{max} nm	λ_{max} nm	λ_{max} nm	λ_{max} nm
	MeOH	MeOH/ NaOMe	MeOH/ AlCl_3	MeOH/ AlCl_3/HCl	MeOH/ NaOAc	MeOH/ NaOAc/ H_3BO_3
G-I	245sh ⁱⁱ , 255, 268.7, 292sh ⁱⁱ , 350.7	255.3sh ⁱⁱ , 276, 327.8sh ⁱⁱ , 403.2	276.6, 300sh, 327.8sh ⁱⁱ , 426.2	265sh ⁱⁱ , 278.6, 294.9sh ⁱⁱ , 357.3, 386.8	263, 275.3, 311.4sh ⁱⁱ , 321.2sh ⁱⁱ , 395	242.5sh ⁱⁱ , 275.3, 298.6sh ⁱⁱ , 367.1, 439.3sh ⁱⁱ
G-II	255.6, 268.7sh ⁱⁱ , 349.7, 400sh ⁱⁱ	268.7, 300sh ⁱⁱ , 403.2	276.9, 294.9sh ⁱⁱ , 301.5sh ⁱⁱ , 329.4sh ⁱⁱ , 427.8	275.2, 294.9sh ⁱⁱ , 357.3sh ⁱⁱ , 388.4	256.9, 268.7sh ⁱⁱ , 363.9sh ⁱⁱ , 404.8	256.3, 278.6, 360.6

ⁱⁱsh: shoulder

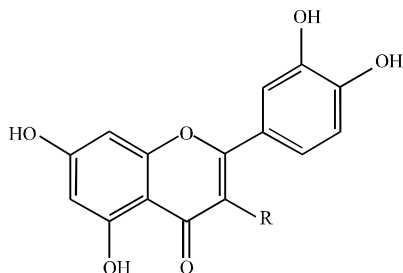


Fig. 4: Luteolin; R = OH, Luteolin 7-O-glucoside; R = O-glu

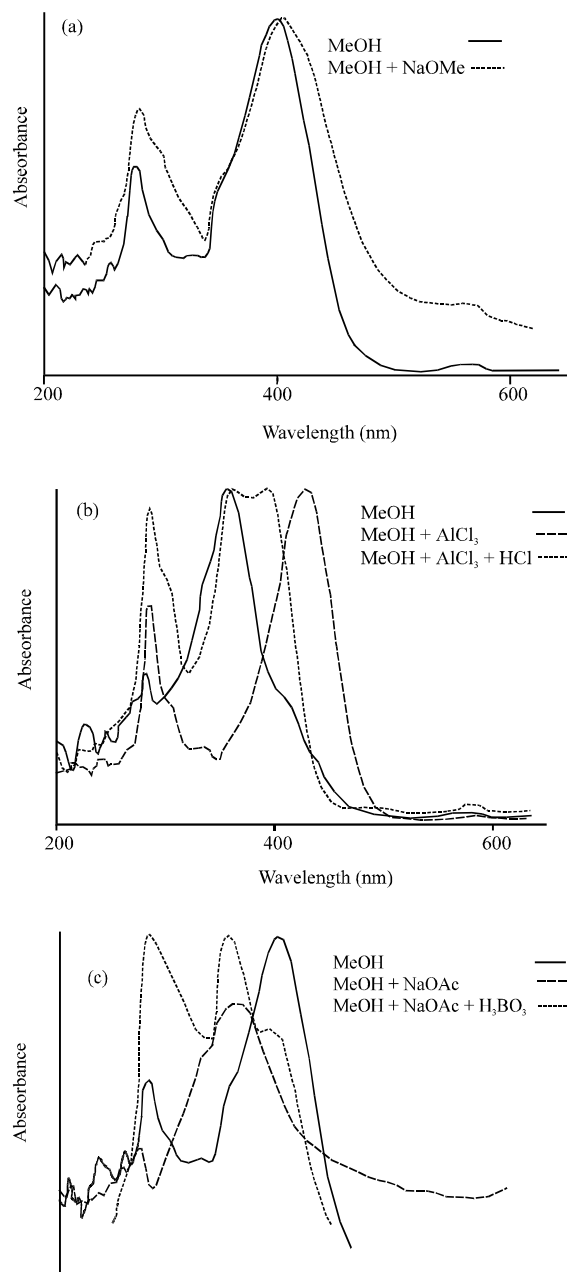


Fig. 5: UV/Vis absorption spectra of compound G-II in (a) MeOH and MeOH/NaOMe, (b) MeOH, MeOH/AlCl₃ and MeOH/AlCl₃/HCl and (c) MeOH, MeOH/NaOAc and MeOH/NaOAc/H₃BO₃

that the substance G-II belongs to the phloroglucinol type A ring (i.e., 5, 7-dihydroxy) (Voinin, 1983). Also, AlCl₃ spectrum showed a relatively large bathochromic shift (78.1 nm) indicating the presence

of *ortho*-dihydroxy system and/or 3 and/or 5-hydroxy groups at ring B. AlCl₃/HCl spectrum exhibited hypsochromic shift (39.4 nm) in band-I relative to AlCl₃ spectrum, which performed the presence of 3', 4'- *ortho*-dihydroxy groups. The presence of only a free 5-hydroxyl group is mainly deduced from the persistence of a bathochromic shift (38.7 nm) in band-I of AlCl₃/HCl spectrum (relative to MeOH spectrum) and not due to 3-hydroxyl group. No bathochromic shift was observed in band-II in the NaOAc spectrum, indicates the absence of free 7-OH group and it may be occupied by the sugar moiety. Furthermore, the presence of free 7-hydroxyl group in its aglycone after acid hydrolysis was confirmed by the bathochromic shift (13.1 nm) in band-II of NaOAc spectrum. IR-spectrum of G-II showed a strong band at 3303-3362 cm⁻¹ corresponding to -OH group, a strong band at 1663 cm⁻¹ corresponding to a α , β -unsaturated ketone (C = O group of γ -pyrone), absorption bands at 1618, 1571, 1520 and 1515 cm⁻¹ (an aromatic system); in addition to the absorption bands at 1388, 1260 and 1181 cm⁻¹. MS of G-II after hydrolysis, showed a molecular ion (M⁺) at m/z 286 (100), which corresponds to the molecular formula C₁₅H₁₀O₆ of parent flavone 222 plus four hydroxy substitution patterns 64. The mass spectral data are in agreement with those reported for luteolin. The previous data are in agreement with those reported for luteolin 7-*O*-glucoside as shown in Fig. 4.

The R_f-values as well as the color under UV-light showed that, it is probably a flavonoidal glycoside in nature. PC analysis of G-II residue after hydrolysis revealed the presence of glucose as a sugar moiety while the aglycone was identified as luteolin by comparison with authentic reference. The previous data are in agreement with those reported for luteolin 7-*O*-glucoside (Godjevac *et al.*, 2004; Nissler *et al.*, 2004; Kader, 2006; Geissman, 1955; Mabry *et al.*, 1970; Macek, 1972).

CONCLUSION

The high α -cellulose content approximately twice that of *Bagasse* (40%) and *Rice straw* (38%), shows the economic importance of the *Gazania splendens* plant as a source for paper manufacturing industries. Investigation of *Gazania splendens* resulted in the isolation and identification of flavonoids and lipid constituents with percentages 2.4 and 3.0% relative to the total powder plant. The flavonoid constituents include luteolin and luteolin 7-*O*-glucoside as the major constituents. The isolation and identification of lipid fraction resulted in 11 *n*-alkanes (C₁₄-C₃₀), β -amyrin, cholesterol, β -sitosterol, stigmaterol and eight fatty acids. The saturated fatty acids represent 73.22 % while the unsaturated fatty acids represented by 26.78% of the total fatty acids. The major fatty acids were palmitic acid 67.89% and linoleic acid 19.61%.

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