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Caffeoyl Derivatives and Flavonoids from Three Compositae Species

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ABSTRACT
Four flavones; chrysoeriol (**1**), apigenin (**3**), luteolin (**6**), orientin (**11**), and three flavanones; naringenin (**2**), eriodictyol (**4**) and 3',4',5,5',7-pentahydroxy flavanone (**5**), five flavonols; rhamnetin (**13**), hyperoside (**15**), rhamnetin-3-O-ß-D-glucoside (**17**), mearnsitin-3-O-ß-D-glucoside (**19**) and isoquercitrin (**20**), an aurone; 3',4',5',6-tetrahydroxyaurone-4-O-glucoside (bractein] (**7**), six caffeoyl derivatives of quinic acid; 3,4-dicaffeoylquinic acid-methyl ester (**8**), 3,5-dicaffeoylquinic acid-methyl ester (**9**), 3,5-dicaffeoylquinic acid (**10**), neochlorogenic acid (**12**), 1,4,5-tricaffeoylquinic acid (**14**) and 1,5-dicaffeoylquinic acid (**16**) and caffeic acid (**18**) were isolated from the flowers and the remaining aerial parts of *Helichrysum bracteatum* (Vent.) Andrews, *Gazania nivea* DC. and *Dimorphotheca ecklonis* DC. Compounds **8** and **9** were isolated for the first time from genus *Helichrysum*, compounds **10** and **12** from *H. bracteatum*, compounds **12**-**17** from genus *Gazania*, compounds **10, 12, 18** and **20** from genus *Dimorphotheca* and compound **19** from genus *Dimorphothec*a and family Compositae. The anti-inflammatory, analgesic and antipyretic effects of the flowers and the remaining aerial parts of the three studied plants as well as the major isolated compounds of the most potent extract were studied. Bractein (**7**) was the most potent anti-inflammatory and antipyretic, while 3,5-dicaffeoylquinic acid (**10**) was the most potent analgesic. The flowers of the three plants showed a significant hepatoprotective effect.
KEY WORDS: flavonoids, caffeoyl quinic acid derivatives; *Helichrysum bracteatum*, *Gazania nivea*, *Dimorphotheca ecklonis*.; analgesic, antipyretic, anti-inflammatory, hepatoprotective activities.

INTRODUCTION
Helichrysum bracteatum (Vent.) Andrews, *Gazania nivea* DC. and *Dimorphotheca ecklonis* DC. are three plants belonging to family Compositae and cultivated in Egypt for ornamental purposes. Many flavonoids were previously isolated from *H. bracteatum* growing in Germany and Spain (1-5). Phenolic acids such as caffeic, chlorogenic, neochlorogenic, as well as 1,3-, 1,4-, 1,5-, 3,5-, and 4,5 dicaffeoylquinic acids were isolated before from other *Helichrysum* species(6-8). Some reports were found concerning the anti-inflammatory, hepatoprotective, antimicrobial, anticancer and antioxidant effects of different *Helichrysum* species. (9-12) Meanwhile, no literature was traced concerning the phenolic acids content of *H. bracteatum*, the flavonoid content of the plant cultivated in Egypt or on the chemical constituents or the biological activity of *Gazania nivea*, and *D. ecklonis*. Therefore, the phenolic contents as well as some of the biological properties which may be related to these constituents of the three plants cultivated in Egypt have been investigated in this study.

MATERIALS AND METHODS
General
Melting points were determined on electrothermal 9100 (UK). ^{1}H- , ^{1}H-^{1}H COSY and ^{13}C- NMR analyses spectra were recorded on Varian Mercury (^{1}H-NMR, 300 MHZ, ^{13}C, 75 MHZ), Jeol JNM ECA 500 instrument (^{1}H-NMR, 500 MHZ, ^{13}C, 125 MHZ) and Jeol JHA-LAA 400 WB-FT (^{1}H, 400 MHz; ^{13}C, 100 MHz) spectrometer. Thin-layer chromatography (TLC) was performed on silica gel GF_{254} precoated plates (Machery Nagel, Germany) and silica gel G_{60} (E-Merck) for preparative TLC. Silica gel 60 (Machery Nagel 230-400 mesh ASTM) and silica gel H (E-Merck) for VLC were used for column chromatography.

Materials for biological study
Paracetamol (Paramol)®, Misr Co., Egypt; indomethac

-in (Indomethacin)®, Eipico, Egyptian Int. Pharmaceutical Industries Co.; Dipyron-metamizol sodium (Novalgin)®(Aventis, S.A.E., Egypt); Carrageenan (Sigma Co., USA); Brewer's yeast (MEPACO, Cairo, Egypt); Silymarin (Sedico Pharmaceutical Co., 6 October City, Egypt) and Carbon tetrachloride (analar, El-Gomhoreya Co., Cairo, Egypt). Transaminase Kits (Bio-Meriéux Co.): biochemical kits for assessment of serum alanine aminotransferase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase enzymes. Adult male albino rats of Sprauge Dawely Strain weighing 100-150 g and albino mice (20 - 25g). All animals were kept on standard laboratory diet and under hygienic conditions.

Plant material

The flowers and remaining aerial parts (after separation of the flowers) of *Helichrysum bracteatum* (Vent.) Andrews (the orange cultivar), *Gazania nivea* DC. and *Dimorphotheca ecklonis* DC. were supplied from the Experimental Research Station of Faculty of Pharmacy, Cairo University, Giza, Egypt. Identification of the plant materials was carried out by Prof. Dr. Moneir M. Abdel Ghani, Prof. of Plant Taxonomy, Botany Department, Faculty of Science, Cairo University, Egypt. Voucher specimens were deposited at the Museum of the Department of Pharmacognosy, Faculty of Pharmacy, Cairo University.

Extraction and Isolation:

The ethanolic extracts of each of the flowers (1 kg) and the remaining aerial parts (1.5 kg) of *Helichrysum bracteatum*, *Gazania nivea* and *Dimorphotheca ecklonis* were prepared by cold maceration with 95 % ethanol (7 X 10 L). Each extract was fractionated successively with petroleum ether (10 x 200 ml), chloroform (8 x 200 ml), ethyl acetate (10 x 200 ml) and *n*-butanol saturated with water (12 x 200 ml).) to yield 23.22, 17.3, 12 and 10 g. of the ethyl acetate fractions of *H. bracteatum* flowers, *H. bracteatum* remaining aerial parts, *G. nivea* flowers and *D. ecklonis* flowers and 53 g of the *n*-butanol fraction of *G. nivea* flowers. The fractions rich in phenolic compounds, based on their TLC investigation, were used for the isolation of these compounds.

Each of the investigated fractions was chromatographed over a vacuum liquid chromatography column (VLC, Si gel H, 70 g, 20 x 5 cm). Gradient elution was carried out using ethyl acetate-chloroform mixtures and ethyl acetate-methanol mixtures with increasing polarity. Fractions 200 ml each were collected and monitored by TLC. Similar fractions were pooled together. Each of the pooled fractions was purified on several sephadex LH-20 columns (40 × 2 cm) using methanol and /or methanol-water (1:1 v/v) as an eluent.

The ethyl acetate fraction (20 g) of the flowers of *H. bracteatum* yielded six main fractions (A_{HF}-F_{HF}). Rechromatography of fraction $\mathbf{A}_{HF}$ (10-25% ethyl acetate / chloroform, 3 g) on sephadex LH-20 yielded two main subfractions. Fraction $\mathbf{A}_{HF}$-1 was purified using preparative TLC technique developed with ($CHCl_3$- MeOH 9.5: 0.5), double run to yield compounds **1** (yellow powder, 15 mg) and **2** (orange yellow powder, 12 mg). Fraction $\mathbf{A}_{HF}$-2 was evaporated to give yellow powder of compound **3** (20 mg). Fraction $\mathbf{B}_{HF}$ (30-45% ethyl acetate / chloroform, 2.9 g) upon purification on sephadex LH-20 gave two main subfractions. Fraction $\mathbf{B}_{HF}$-1 was rechromatographed on a Si gel column (30g, 25 × 1.5 cm) eluted with *n*-hexane-ethyl acetate (8:2 v/v) to give orange yellow powder of compound **4** (110 mg) and orange yellow powder of compound **5** (40 mg). Fraction $\mathbf{B}_{HF}$-2 was evaporated to yield yellow powder of compound **6** (170 mg). Fraction $\mathbf{F}_{HF}$ (25-30% methanol / ethyl acetate, 3.9 g) afforded compound **7** (orange powder, 200 mg). Fractions $\mathbf{C}_{HF}$,-$\mathbf{E}_{HF}$ (50 % ethyl acetate / chloroform - 20% methanol / ethyl acetate) contained major compounds **8**, **9**, **10** and **11** which were found in lager proportions and isolated from the ethyl acetate extract of the remaining aerial parts of the plant .

The ethyl acetate fraction (15 g) of the remaining aerial parts of *H. bracteatum* yielded five major fractions (A_{HA}-E_{HA}). Fraction $\mathbf{A}_{HA}$ (30-45% ethyl acetate / chloroform, 2.5 g) afforded 54 mg yellow powder of compound **6**. Rechromatography of fraction $\mathbf{B}_{HA}$ (50-90% ethyl acetate / chloroform, 2.8 g) on a Si gel column (30g, 25 × 1.5 cm) using chloroform-methanol (9.2:0.8 v/v) as eluting solvent yielded, 23 mg brownish yellow powder of compound **8** and 30 mg brownish yellow powder of compound **9**. Fraction $\mathbf{C}_{HA}$ (95 % ethyl acetate /chloroform-5% methanol /ethyl acetate, 1.2 g) afforded brownish yellow powder of compound **10** (20 mg). Fraction $\mathbf{D}_{HA}$ (10-20 % methanol / ethyl acetate, 3 g) gave 50 mg of compound **10** and 108 mg of dark yellow powder of compound **11** and finally compound **12** was obtained from fraction $\mathbf{E}_{HA}$ (25-50 % methanol / ethyl acetate, 1.8 g) as yellowish white powder (105 mg).

The ethyl acetate fraction (10 g) of the flowers of *G. nivea* yielded three main fractions (A_{GF}-C_{GF}). Compound **13** was obtained as yellow powder (20 mg) from fraction $\mathbf{A}_{GF}$ (55-65% ethyl acetate / chloroform,

1.8 g), compound **14** as brownish yellow powder (115 mg) from fraction $\mathbf{B}_{GF}$ (85% ethyl acetate / chloroform-5% methanol /chloroform, 2.5 g) and compound **15** as yellow powder (30 mg) from fraction $\mathbf{C}_{GF}$ (10-20% methanol / ethyl acetate, 1.2 g).

n-Butanol fraction (10 g) of the flowers of *G. nivea* yielded three main fractions (D_{GF}-F_{GF}). Fraction $\mathbf{D}_{GF}$ (5-10% methanol / ethyl acetate, 1.5 g) gave brownish yellow powder of compound **16** (110 mg). Fraction $\mathbf{E}_{GF}$ (15-20% methanol / ethyl acetate, 1.9 g) was further purified on reversed phase C_{-18} column eluted with methanol-water (30:70 v/v) to give compounds **17** (yellow powder, 29 mg) and **15** (yellow powder, 10 mg). Compound **12** (120 mg) was obtained from fraction $\mathbf{F}_{GF}$ (25-50% methanol / ethyl acetate, 2 g) as a yellowish white powder.

The ethyl acetate fraction (10 g) of the flowers of *D. ecklonis* yielded four main fractions (A_{DF}-D_{DF}). Fraction $\mathbf{A}_{DF}$ (40-50% ethyl acetate / chloroform, 0.9 g) afforded white powder of compound **18** (15 mg). Further purification of fraction $\mathbf{B}_{DF}$ (55-100 % ethyl acetate / chloroform, 2 g) on a Si gel column (25 × 1.5, 30 g) using a mixture of chloroform-methanol (9.6:0.4 v/v) as an eluent gave brownish yellow powder of compound **10** (40 mg). Fraction $\mathbf{C}_{DF}$ (5-10% methanol in ethyl acetate, 1.4 g) yielded compounds **19** (yellow powder, 16 mg) and **20** (yellow powder, 18 mg). Finally, compound **12** was obtained as brownish yellow powder (109 mg) from fraction $\mathbf{D}_{DF}$ (25-50% methanol / ethyl acetate, 2.3 g).

The yield of the isolated compounds in mg/g extract from the plants under investigation is given in Table (1).

Compound 7

^{1}H-NMR: δ ppm [300 MHz, DMSO] 5.08 (1H,d,*J*=7.2, H-1``), 6.29 (1H, s, H-5), 6.33 (1H, s, H-7), 6.38 (1H,s, benzylic proton =CH-), 6.9 (2H, s, H-2`,6`).^{13}C-NMR: δ ppm [75 MHz, DMSO] 92.9 (**C-5**), 98.04 (**C-7**), 103.71(**C-3a**), 111.19 (**C-2`,6`**), 112.16 (**=CH**), 122.6 (**C-1`**), 146.22 (**C-2**), 146.52 (**C-3`,4`,5`**), 157.15 (**C-4**), 167.89 (**C-6**), 168 (**C-7a**) and 179.52(**C-3**). The sugar moiety: 60.80 (**C-6``**), 69.82 (**C-4``**), 73.26 (**C-2``**), 76.55 (**C-3``**), 77.33 (**C-5``**) and 99.95 (**C-1``**).

Analgesic, antipyretic and anti-inflammatory effects

The aqueous and ethanolic extracts (100 mg/kg body weight) of *H. bracteatum*, *G. nivea* and *D. ecklonis* flowers and the remaining aerial parts , the petroleum ether and ethyl acetate fractions of the ethanolic extract of *H. bracteatum* flowers as well as bractein (**7**), 3,4-dicaffeoyl quinic acid methyl ester (**8**), 3,5-dicaffeoyl quinic acid methyl ester (**9**) and 3,5-dicaffeoyl quinic acid (**10**) at a dose of 50 mg/kg. body weight were tested for their analgesic, antipyretic and anti-inflammatory activity. In addition, eriodictyol (**4**), luteolin (**6**) and orientin (**11**) were tested for their analgesic and antipyretic properties at a dose of 50 mg/kg body weight.

Anti-inflammatory activity

The tested extracts as well as the isolated compounds were tested for the anti-inflammatory activity and compared with that of indomethacin (20 mg/kg body weight) as a standard using carrageenan-induced rat hind paw oedema test on male rats of albino Sprague Dawely strain (130-140 g) according to the method of Winter *et al*. [13] Results are shown in Table (2). One hour after the administration of a single dose of the tested extracts, inflammation was induced by sub planter injection of 0.1 ml of 1% carrageenan solution in saline in the right hind paw while 0.1 ml saline was injected in the left hind paw. Four hours after oral administration of the different extracts, both hind paws were separately weighed to calculate the weight of oedema. The percentage of oedema (inflammation) was calculated according to the following equation:

$$\frac{Weight\ of\ right\ paw - weight\ of\ left\ paw}{weight\ of\ left\ paw} \times 100$$

Analgesic activity

The tested extracts as well as the isolated compounds were evaluated on male rats of albino Sprague Dawely strain (130-140 g) and compared with that of novalgin (50 mg/ kg. body weight) as standard, using electric current as anxious stimulus as described by Charlier *et al*. [14]

Animals were received the tested extracts orally in a dose of 100-mg/kg b. wt., the isolated compounds in a dose of 50 mg/kg b. wt. or 50 mg/kg novalgin to serve as a positive control. Electrical stimulation was applied to the rat tail by means of 515 Master shocker using alternative current of 50 cycle / second for 0.2 second. The minimum voltage required for the animal to emit a cry was recorded before treatment at zero time v_o, as well as treated groups v_t. The results (Table 3) were measured after 1 and 2 hours from zero time. The percentage of change was calculated according the following equation:

$$Percentage\ of\ change = (v_t - v_o)\ x\ 100 / v_o$$

Antipyretic activity

The activity of the tested extracts and the isolated compounds were evaluated on male rats of albino Sprague Dawely strain (130-140 g) and compared with that of paracetamol (100 mg/kg. body weight) as standard, using yeast–induced hyperthermia method as

described by Tomazetti *et al* [15] Pyrexia was induced to each animal by injecting a dose of 1ml/100 g b. wt. of yeast suspension (44%) intramuscularly. After 18 hours, the rectal temperature of each animal was recorded and the obtained temperature represented the base line of elevated body temperature to which the antipyretic activity will be compared. The animals were given the plant extracts orally in a dose of 100 mg/kg b. wt., the isolated compounds in a dose of 50 mg/kg.b.wt. and paracetamol (100 mg/kg b. wt.) to serve as a positive control. The induced raise in temperature was recorded before treatment at zero time T_o and in treated groups T_t. Results were measured after one and two hours. Percentage of change was calculated according the following equation:

$$Percentage\ of\ change = (T_o - T_t)\ x\ 100 / T_o$$

Results are recorded in Table (4).

Hepatoprotective activity

The aqueous and ethanolic extracts (100 mg/kg body weight) of *H. bracteatum*, *G. nivea* and *D. ecklonis* flowers as well as the petroleum ether and ethyl acetate fractions of the ethanolic extract of *H. bracteatum* were tested for their hepatoprotective activity. The tested extracts were administered at a daily dose of 100 mg/kg body weight for one month before induction of liver damage by intraperitoneal injection of 5ml / kg of 25% carbon tetrachloride (CCl_4) in liquid paraffin according to the method of Klassan and Plaa (1969)(16) using silymarin 25 mg / kg body weight as a reference drug. The extracts as well as the reference drug was continued to be administered to the rats for another month after liver damage. The levels of aspartate aminotransferase (AST)(17), alanine aminotransferase (ALT)(17) and alkaline phosphatase (ALP)(18) enzymes were measured in the blood of each group at zero time, after one month of receiving the tested drug, 72 hours after induction of liver damage and after one month of treatment with the tested samples. Results are shown in Table (5).

The LD_{50}

The LD_{50} of the ethanolic and aqueous extracts of the flowers and the remaining aerial parts of *H. bracteatum G. nivea* and *D. ecklonis* and the most active extracts were estimated following Karber, G. (1931) procedure (19).

RESULTS AND DISCUSSION

Twelve compounds were isolated from *H. bracteatum;* compounds **1-7** from the flowers and compounds **6 & 8-12** from the remaining aerial parts. On the other hand, six compounds (**12-17**) were isolated from *G. nivea* flowers and five compounds (**10, 12** and **18-20**) from *D. ecklonis* flowers.

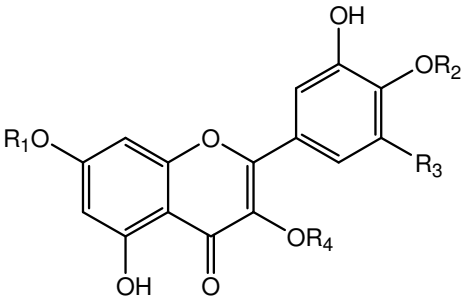
Compounds **1**, **3**, **6** and **11** were identified as **chrysoeriol**, (20, 21) **apigenin** (22), **luteolin** (22-24) and **orientin** (23) flavones. Compounds **1** and **3** were isolated before from the ivory white flowers of *H. bracteatum* growing in Spain, (4) while compounds **6** and **11** were isolated before from the highly coloured involucral bracts and the leaves respectively of *H. bracteatum* growing in Germany. (1)

The UV spectral data of **2, 4** and **5** showed their flavanone nature. (22,25) Compounds **2, 4** and **5** were identified as **naringenin,** (20, 21) **eriodictyol** (26) and **3',4',5,5',7-pentahydroxy flavanone** (3) which were isolated before from the bracts of *H. bracteatum* growing in Germany. (3)

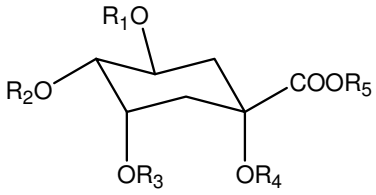
The UV spectral data of **7** in methanol with λ_{max} 405 nm indicated that it is an aurone and it was comparable to that of bractein.(20) No change in band II on the addition of $AlCl_3$, which suggested that compound **7** is a 4-O-glycoside.(27) ^{1}H-NMR spectrum of **7** showed the presence of four aromatic protons suggested a pentasubstituted aurone.(22,25,27) A singlet at δ 6.90 ppm integrated as two protons assigned to H-2` and H-6`, two singlets at δ 6.33 and 6.29 ppm, each integrated as one proton assigned to H-7 and H-5, respectively and a singlet at δ 6.38 ppm integrated as one proton and assigned to the benzylic proton (=CH-) were displayed in ^{1}H-NMR of the compound. In addition, it showed a doublet at δ 5.08 ppm with a coupling constant 7.2 Hz, assigned to the anomeric proton and indicated a ß-linked sugar. ^{13}C-NMR data of the compound are in accordance with the data published for a pentasubstituted aurone with glucose as a sugar moiety (24). From the above data compound **7** was identified as **3`,4`,5`,6-tetrahydroxyaurone-4-O-glucoside [bractein]**. This compound was isolated before from the leaves and flowers of six variously coloured cultivars of *H. bracteatum* growing in Germany. (3) We report here the NMR data of the compound due to lack of literature.

All the previous compounds are reported for the first time from the plant cultivated in Egypt.

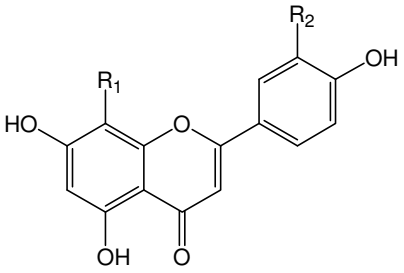
Compound **13, 15, 17** and **20** were identified as **rhamnetin,** (22) **hyperoside** (28)**, rhamnetin-3-O-ß-D-glucoside** (29) and **isoquercitrin** (28)**.** This is the first report for the isolation of these compounds from genus *Gazania*.



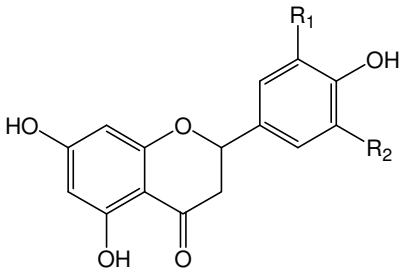
	R_1	R_2	R_3	R_4
13	CH_3	H	H	H
15	H	H	H	Galactose
17	CH_3	H	H	Glucose
19	H	CH_3	OH	Glucose
20	H	H	H	Glucose



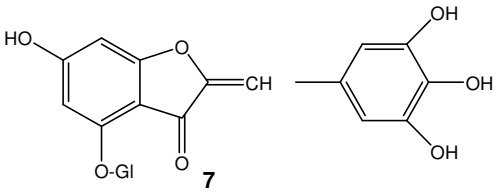
	R_1	R_2	R_3	R_4	R_5
8	H	Caf	Caf	H	CH_3
9	Caf	H	Caf	H	CH_3
10	Caf	H	Caf	H	H
12	H	H	Caf	H	H
14	Caf	Caf	H	Caf	H
16	Caf	H	H	Caf	H



	R_1	R_2
1	H	OCH_3
3	H	H
6	H	OH
11	Glucose	OH



	R_1	R_2
2	H	H
4	OH	H
5	OH	OH



7

The UV spectral data and ^{1}H-NMR spectrum of **19** displayed the characteristic protons of mearnsetin aglycone (30). PC analysis of the acid hydrolysate of the compound showed the presence of D-glucose. Therefore, compound **19** was identified as **mearnsetin-3-O-ß-D-glucoside.** This is a rare flavonoid isolated only before from *Licania heteromorpha* (31). Therefore, it is the first report for the isolation of this compound from genus *Dimorphotheca* and from family Compositae.

The UV spectral data of compounds **8, 9, 10, 12, 14, 16** and **18** showed the characteristic features of hydroxycinnamic acids in methanol (32).

^{1}H-NMR spectrum of compound **12** showed the presence of one caffeic and one quinic acid moieties. By comparing with the published data, (33) compound **12** was identified as **3-caffeoylquinic acid [neochorogenic acid]**. This is the first report for the isolation of this compound from *H. bracteatum*, genus *Gazania* and genus *Dimorphotheca*.

^{1}H-NMR spectrum of **8, 9, 10** and **16** showed the characteristic signals for a quinic acid and two caffeic acid moieties (32-34). The determination of the sites

of acylation in quinic acid moiety was based on their ^{1}H and ^{13}C-NMR data. Compounds **8, 9, 10** and **16** were identified as **3,4-dicaffeoylquinic acid-methyl ester,** (35) **3,5-dicaffeoylquinic acid-methyl ester**, (32) **3,5-dicaffeoylquinic acid** (32) and **1,5-dicaffeoylquinic acid** (36). This is the first report for the isolation of compounds **8** and **9** from genus *Helichrysum*, compound **10** from *H. bracteatum* and from genus *Dimorphotheca* and compound **16** from genus *Gazania*.

^{1}H-NMR spectrum of **14** showed three caffeic acid and a quinic acid moieties. The downfield shift of H-4 and H-5 showed that the acylation of the quinic acid by two caffeic acid molecules was on the hydroxyl groups at C-4 and C-5, and the third caffeic acid molecule acylated the OH on C-1. So, compound **14** was identified as **1,4,5-tricaffeoylquinic acid.** This is the first report for the isolation of this compound from genus *Gazania*. This is a rare phenolic acid which was isolated only from the flowers of *Arnica Montana* L. and *A. chamissonis* Less. (37)

The assignment of the protons of the quinic acid moiety in compounds **8, 9, 10, 12, 14** and **16** was determined using ^{1}H-^{1}H COSY.

Compound **18** was identified as **caffeic acid** (32)**.** This is the first report for the isolation of this compound from genus *Dimorphotheca*

The absence of flavonols and the presence of the flavones, aurones and flavanones in *H. bracteatum*, in addition to C-glycosyl flavones, which appear to be rare in the tribe Inulae but only reported in *H. bracteatum* was in agree with the reported chemotaxonomy of the tribe Inulae. (38)

Too little is known yet regarding the distribution of flavonoids in tribes Calenduleae and Arctotideae to make use of them as taxonomic markers. Meanwhile, we could notice that only flavonols could be detected in *G. nivea* (tribe Arctotideae) and *D.* ecklonis (tribe Calenduleae) but other classes of flavonoids were absent, which may be of chemotaxonomic interest. The presence of the rare flavonol mearnsetin-3-O-ß-D-glucoside is in contrary to that previously reported which was the absence of flavonols with trihydroxylated B-ring pattern in the family Compositae. (38) Concerning the phenolic acids, tricaffeoyl quinic acid was isolated only from *G. nivea* which belongs to tribe Arctotideae, while the mono and dicaffeoyl quinic acids were isolated from the three plants under investigation.

The analgesic, antipyretic and anti-inflammatory effects: The observed data revealed that all the tested extracts showed significant analgesic, antipyretic and anti-inflammatory effects when compared to control group as shown in Tables (2-4). The most potent extract was the ethanolic extract of the flowers of *H. bracteatum*. So, the petroleum ether and ethyl acetate fractions of this extract were evaluated for these activities. The ethyl acetate fraction showed a higher activity than the petroleum ether fraction. While many flavonoids isolated from the ethyl acetate fraction were reported to exhibit anti-inflammatory activity such as naringenin (**2**), apigenin (**3**), eriodictyol (**4**), luteolin (**6**) and orientin (**11**) (39), no studies were found concerning the anti-inflammatory activity of the aurone; bractein (**7**) and the phenolic acids; 3,4-dicaffeoyl quinic acid methyl ester (**8**), 3,5-dicaffeoyl quinic acid methyl ester (**9**) and 3,5-dicaffeoyl quinic acid (**10**) which were isolated from the ethyl acetate fraction of the flowers of *H. bracteatum* in considerable amounts. Thus, these compounds were tested for the anti-inflammatory activity and it was found that all had significant anti-inflammatory effect at a dose of 50 mg/kg, when compared to the control group as shown in Table (2). Bractein (**7**) was the most potent (85.14% of that of indomethacin).

On the other hand, the major flavonoids and phenolic acids isolated from the ethyl acetate of the flowers of *H. bracteatum*; luteolin (**6**), eriodictyol (**4**), bractein (**7**), orientin (**11**), 3,4-dicaffeoyl quinic acid methyl ester (**8**), 3,5-dicaffeoyl quinic acid methyl ester (**9**) and 3,5-dicaffeoyl quinic acid (**10**) were not tested before for the analgesic and the antipyretic activity.

They exhibited significant increase in the voltage needed to emit a cry after 1 and 2 hours. 3,5-dicaffeoyl quinic acid (**10**) was the most active compound whose potency was 69.71 % of that of novalgin. Also, all the tested compounds showed significant decrease in the rectal temperature of the yeast-induced hyperthermic rats, but the aurone bractein (**7**) was the most potent and its potency was even higher than that of paracetamol.

The hepatoprotective activity

Early studies of flavonoids investigated their significant hepatoprotective effect.(40) In addition, phenolic acids especially caffeoyl quinic acids were reported to exhibit significant hepatoprotective activity.(35)The chemical analysis revealed that the flowers of the three plants were more rich (TLC investigation) in phenolic compounds than the

Table 1. *The yield of the isolated compounds from H. bracteatum, G. nivea and D. ecklonis.*

Isolated compounds	Amount isolated in mg/g extract.				
	H. bracteatum flowers	*H. bracteatum* remaining aerial parts	*G. nivea* flowers ethyl acetate	*G. nivea* flowers butanol	*D. ecklonis* flowers
Chrysoeriol (1)	0.75	-	-	-	-
Naringenin (**2**)	0.6	-	-	-	-
Apigenin (**3**)	1	-	-	-	-
Eriodictyol (**4**)	5.5	-	-	-	-
3',4',5,5',7-Pentahydroxy flavanone (**5**)	2	-	-	-	-
Luteolin (**6**)	8.5	3.6	-	-	-
Bractein (**7**)	10	-	-	-	-
3,4-Dicaffeoylquinic acid-methyl ester (**8**)	-	1.53	-	-	-
3,5-Dicaffeoylquinic acid-methyl ester (**9**)	-	2	-	-	-
3,5-Dicaffeoylquinic acid (**10**)	-	4.7	-	-	4
Orientin (**11**)	-	7.2	-	-	-
Neochlorogenic acid (**12**)	-	7	-	12	10.9
Rhamnetin (**13**)	-	-	2	-	-
1,4,5-tricaffeoylquinic acid (**14**)	-	-	1.1	-	-
hyperoside (**15**)	-	-	3	1	-
1,5-Dicaffeoylquinic acid (**16**)	-	-	-	11	-
Rhamnetin-3-O-β-D-glucoside (**17**)	-	-	-	2.9	-
Caffeic acid (**18**)	-	-	-	-	1.5
Mearnsitin-3-O-β-D-glucoside (**19**)	-	-	-	-	1.6
Isoquercitrin (**20**)	-	-	-	-	1.8

Table 2. *Acute anti-inflammatory activity of H. bracteatum, G. nivea and D. ecklonis.*

Group	Type of extract.	% Oedema	
		Mean ±S.E.	% of change
Control	-	61.3±2.1	-
H. bracteatum flowers	Aqueous	26.3±0.5*	57.1
	Ethanolic	25.5±0.4*	58.4
	Pet. Ether fr.	37.3±1.1*	39.2
	Ethyl acetate fr.	25.7±0.8*	58.1
	Compound (**7**)	26.8±0.5*	56.3
	Compound (**8**)	27.4±0.8*	55.3
	Compound (**9**)	28±0.4*	54.3
	Compound (**10**)	30.1±.9*	50.9
H. bracteatum remaining aerial parts	Aqueous	29.7±0.6*	51.6
	Ethanolic	28.5±0.7*	53.5
G. nivea flowers	Aqueous	32.2±0.8*	47.5
	Ethanolic	31.3±0.4*	48.9
G. nivea remaining aerial parts	Aqueous	45.6±.9*	25.6
	Ethanolic	41.2±0.6*	32.8
D. ecklonis flowers	Aqueous	36.4±0.9*	40.6
	Ethanolic	35.6±0.7*	41.9
D. ecklonis remaining aerial parts	Aqueous	39.7±1.1*	35.2
	Ethanolic	38.1±1.2*	37.9
Indomethacin	-	21.4±0.5*	66.1

The extracts were given at a dose of 100 mg/kg body weight. Indomethacin was give at a dose of 20 mg/kg body weight. fr., fraction. ; Significantly different from control group at p<0.01

Table 3. *Analgesic activity of H. bracteatum, G. nivea and D. ecklonis*

Group	Type of extract	Volts needed before treatment (zero time)	Volts needed after single oral dose			
			One hour		Two hours	
			Mean ±S.E.	% of change	Mean ±S.E.	% of change
Control	-	73.6±1.7	74.1±1.2	0.7	74.5±1.4*	1.2
H. bracteatum flowers	Aqueous	77.5±1.5	131.2±6.1*	69.3	151.6±7.3*	95.6
	Ethanolic	78.4±1.7	138.1±5.9*	76.2	141.5±5.7*	80.5
	Pet. Ether fr.	79.4±1.6	104.9±4.7*	32.1	118.2±6.2*	48.9
	Ethyl acetate fr.	77.8±1.3	121±5.2*	55.5	158.4±6.8*	72.5
	Compound (**4**)	76.4±6.8	107.2±4.9*	40.3	129.8±4.9*	69.9
	Compound (**6**)	78.9±1.4	113.4±5.1*	43.7	131.4±4.2*	66.5
	Compound (**7**)	79.1±1.6	126.8±5.4*	60.3	139.2±5.6*	75.9
	Compound (**8**)	72.9±1.5	121.3±5.2*	66.4	145.3±5.8*	99.31
	Compound (**9**)	73.1±1.4	121.5±3.9*	66.2	144.3±4.8*	97.4
	Compound (**10**)	75.1±1.7	124.6±4.9*	65.9	150.2±6.2*	100
	Compound (**11**)	77.6±2.1	115.2±6.1*	48.4	141.5±6.1*	82.3
H. bracteatum remaining	Ethanolic	79.1±1.8	143.7±6.4*	81.7	165.4±5.8*	109.1
aerial parts .	Aqueous	77.2±2.1	135.4±5.7*	75.4	132.3±4.9*	71.3
G. nivea flowers	Aqueous	761±1.3	117.3±4.6*	54.1	123.1±5.1*	61.8
	Ethanolic	74.3±1.2	128.6±4.3*	73.1	136.2±5.2*	83.3
G. nivea remaining aerial	Aqueous	75.2±1.8	104.2±3.8*	38.6	112.5±5.7*	49.6
parts	Ethanolic	77.6±1.7	121.5±3.9*	43.7	120.2±6.3*	54.9
D. ecklonis flowers	Aqueous	76.4±2.1	102.7±3.5*	34.4	115.1±4.3*	50.7
	Ethanolic	74.8±1.9	108.9±4.1*	45.6	119.2±5.2*	59.4
D. ecklonis remaining	Aqueous	75.9±1.6	112.4±5.2*	48.1	113.9±5.9*	50.1
aerial parts	Ethanolic	76.8±1.4	116.5±6.1*	51.7	118.6±4.9*	54.4
Novalgin	-	74.9±1.6	158.4±5.7*	111.5	182.3±6.4*	143.4

*The extracts were given at a dose of 100 mg/kg body weight. Novalgin was give at a dose of 50 mg/kg body weight. fr., fraction. * Significantly different from zero time at p<0.01. % of change calculated as regard zero time.*

Table 4. *Antipyretic activity of H. bracteatum, G. nivea and D. ecklonis*

Group	Type of extract	Body Temperature change				
		Induced rise in temperature	One hour		Two hours	
			Mean ±S.E.	% of change	Mean ±S.E.	% of change
Control	-	38.6±0.2	38.9±0.3	0.8	39.1±0.4	1.3
H. bracteatum flowers	Aqueous	39.8±0.2	38.1±0.2*	4.3	36.9±0.3*	6.0
	Ethanolic	39.6±0.3	37.6±0.1*	5.8	37.5±0.1*	6.8
	Pet. ether	39.0±0.4	38.9±0.3*	0.3	38.6±0.2*	1.02
	Ethyl acetate	39.3±0.2	38.4±0.3*	2.3	37.1±0.1*	5.6
	Compound (**4**)	38.9±0.2	38.2±0.2*	1.8	37.1±0.1*	4.6
	Compound (**6**)	39.1±0.3	38.1±0.2*	2.6	37.2±0.1*	4.9
	Compound (**7**)	40.2±0.4	38.6±0.4*	4	36.9±0.1*	8.2
	Compound (**8**)	38.7±0.2	38.1±0.2*	1.6	37.6±0.1*	3.3
	Compound (**9**)	38.9±0.1	38.1±0.2*	2.1	37.5±0.1*	3.6
	Compound (**10**)	39.6±0.4	38.2±0.3*	3.5	36.9±0.04*	6.8
	Compound (**11**)	39.4±0.4	38.6±0.2*	2	37.3±0.1*	5.3
H. bracteatum remaining	Aqueous	39.1±0.4	37.9±0.1*	3.1	37.3±0.1*	4.6
aerial parts .	Ethanolic	39.5±0.2	38.4±0.2*	2.8	37.8±0.1*	4.8
G. nivea flowers	Aqueous	38.7±0.3	38.1±0.1*	1.6	37.9±0.2*	2.1
	Ethanolic	39.4±0.3	38.4±0.3*	2.5	38.1±0.3*	3.3
G. nivea remaining aerial	Aqueous	39.3±0.4	38.6±0.1*	1.8	38.1±0.2*	3.1
parts	Ethanolic	38.8±.03	38.5±0.2*	0.8	37.9±0.1*	2.3
D. ecklonis flowers.	Aqueous	38.9±0.1	38.1±0.2*	2.1	37.5±0.1*	3.6
	Ethanolic	39.1±0.2	38.2±0.1*	2.3	37.6±0.1*	3.8
D. ecklonis remaining	Aqueous	39.5±0.4	38.1±0.3*	3.5	37.8±0.2*	4.3
aerial parts	Ethanolic	39.2±0.4	38.2±0.1*	2.6	37.2±0.2*	3.3
Paracetamol	-	39.4±0.3	37.2±0.1*	5.6	36.5±0.03*	7.3

*The extracts were given at a dose of 100 mg/kg body weight. Paracetamol was give at a dose of 100 mg/kg body weight. fr., fraction. * Significantly different from zero time at p<0.01.; % of change calculated as regard zero time.*

Table 5. *Effect of extracts of the flowers of H. bracteatum , G. nivea and D. ecklonis on the serum AST, TLT and ALP level*

Enzyme	Time	Control	Silymarin	*H. bracteatum* Vent.			*G. nivea* DC.				*D. ecklonis* DC.		
				Aqueous	Ethanolic	Ethyl acetate fr.	Aqueous	Ethanolic	Ethyl acetate fr.	*n*-Butanol fr.	Aqueous	Ethanolic	Ethyl acetate fr.
AST (U/L)	Zero	28.3±1.2	28.9±0.8	27.8±0.3	29.3±0.7	26.9±0.4	29.4±0.4	30.1±1.1	27.6±0.8	29.8±0.6	29.1±1.1	28.6±0.8	28.9±0.7
	30 day	27.4±0.6	26.7±1.2	25.3±0.6	26.2±0.9	27.1± 0.6	29.2±0.7	29.4±0.6	27.1±0.9	29.4±0..5	27.5±0.8	27.8±0.9	28.1±0.6
	72 h. after liver damage	106.5±2.9	38.5±1.4*	49.8±0.7*	45.6±1.4*	47.3±2.8*	65.6±2.1*	58.9±1.4*	47.2±1.4*	63.4±1.2*	74.5±2.3*	69.3±2.4*	56.2±1.3*
	30 day after liver damage	138.1±4.5	25.3±0.5*	37.6±0.9*	32.4±1.1*	38.7±1.2*	48.3±1.4*	41.2±1.3*	32.6±1.1*	39.1±1.4*	46.2±1.5*	53.8±1.2*	40.2±1.5*
ALT (U/L)	Zero	29.2±0.8	27.6±0.6	32.4±1.2	31.6±1.1	29.4±0.3	31.2±1.1	29.8±0.5	32.9±1.1	30.4±1.3	26.9±0.6	28.8±0.7	30.9±0.8
	30 day	28.6±0.7	27.3±0.6	29.9±0.8	30.9±1.3	29.6±0.7	30.4±0.6	28.3±0.9	33.5±1.2	29.6±0.5	27.1±0.2	27.9±1.5	31.1±1.1
	72 h. after liver damage	112.8±5.1	39.1±0.8*	46.8±1.3*	52.1±1.6*	51.2±1.8*	67.2±2.3*	63.4±2.1*	49.8±1.6*	51.2±1.7*	76.1±2.3*	74.5±2.6*	62.3±2.8*
	30 day after liver damage	124.7±4.3	23.4±0.3*	38.2±1.2*	35.4±0.9*	32.9±1.2*	38.9±1.2*	43.2±1.7*	32.1±0.9*	35.1±1.1*	48.2±1.4*	45.1±2.3	39.4±1.6
ALP (U/L)	Zero	7.5±0.3	7.3±0.1	7.9±0.1	8.1±0.2	7.2±0.2	7.7±1.2	8.4±0.1	7.4±0.1	6.9±0.1	7.2±0.4	7.5±0.9	7.1±0.1
	30 day	7.6±0.2	7.1±0.2	7.6±0.1	7.8±0.2	7.1±0.3	7.5±0.3	8.2±0.2	7.5±0.2	7.1±0.2	7.1±0.6	7.5±0.1	7.2±0.1
	72 h. after liver damage	30.2±0.6	10.2±0.4*	14.1±0.3*	16.2±0.9*	15.6±0.6*	18.9±0.8*	18.6±0.2*	17.2±0.8*	16.8±0.9*	19.5±0.4*	22.3±1.2*	19.7±1.2*
	30 day after liver damage	32.1±0.9	6.4±0.2*	8.2±0.2*	9.3±0.4*	9.2±0.5*	15.2±0.9*	14.6±1.2*	12.8±0.9*	11.9±0.8*	16.1±1.2*	18.8±1.1*	13.5±1.1*

**Statistically significant different from control at p<0.01*

the remaining aerial parts. So, the aqueous and ethanolic extracts of the flowers of the investigated plants as well as the ethyl acetate and *n*-butanol fractions which were found to be rich in these phenolic compounds were tested for the hepatoprotective activity. Liver damage by 25% CCl_4 (5 ml / kg) led to a significant rise in AST, ALT and ALP. A daily dose of all the tested extracts (100 mg/kg b. wt.) showed no significant change in AST, ALT and ALP levels after one month administration. Administration of these extracts for another month after induction of liver damage leads to a significant decrease in the liver enzymes levels from the control group. It is obvious (Table 5) that the ethanolic extract of the flowers of *H. bracteatum* was the most active. The ethyl acetate fractions of the ethanolic extracts of the flowers of *G. nivea* and *D. ecklonis* as well as *n*-butanol fraction of the ethanolic extract of the flowers of *G. nivea* were more active than the aqueous and ethanolic extracts of the same plants. Rhamnetin(39) and hyperoside(41) were reported to possess hepatoprotective activity, therefore, they may participate in the potent hepatoprotective activity of the ethyl acetate and *n*-butanol fractions of the ethanolic extracts of the flowers of *G. nivea* DC. On the other hand, the activity of the ethyl acetate fraction of the ethanolic extract of *D. ecklonis* DC. may be due to the presence of caffeic acid (42) and 3,5-dicaffeoyl quinic acid (35). Meanwhile, 3,5-dicaffeoyl quinic acid (35) may be responsible also with naringenin and luteolin [(39)], for the hepatoprotective activity of the ethanolic extract of *H. bracteatum*.

Determination of the LD_{50}

The LD_{50} was found to be 8.9, 7.8, 8.1 and 8.7 g/kg for the alcoholic and aqueous extracts of the flowers and remaining aerial parts of *H. bracteatum*, respectively, 6.8, 6.5, 7.6 and 8.2 g/kg for the alcoholic and aqueous extracts of the flowers and remaining aerial parts of *G. nivea*, respectively, 6.3, 6.7, 6.5 and 6.8 g/kg, for the alcoholic and aqueous extracts of the flowers and remaining aerial parts of *D. ecklonis*, respectively, 8.5, 6.4 and 6.1 g/kg for the ethyl acetate fraction of the ethanolic extract of the flowers of *H. bracteatum, G. nivea* and *D. ecklonis*, respectively. Therefore, the tested extracts were found to be of high safety margin at the tested dose level.

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