Bull. Pharm. Sci., Assiut University, Vol. 33, Part 1, June 2010, pp. 19-31.

PHYTOCHEMICAL AND BIOLOGICAL STUDY OF *ERANTHEMUM NERVOSUM* (VAHL) R. BR., CULTIVATED IN EGYPT

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أدت تجزئة وتنقية خلاصة الكحول الميثيلى للأجزاء الهوائية لنبات البستاشيا الزرقاء التابع للعائلة الأكانتاسية والمنزرع فى مصر إلى فصل أحد عشر مركباً وهم بيتا أميرين (١)، ألفا أميرين (٢)، لوبيول (٣)، بيتا سيتوستيرول (٤)، أبجينين (٥)، كمبيفرول (٦)، بيتا سيتوستيرول-٣-أ-جلوكوبيرانوزيد (٧)، حمض البنزويك (٨)، كامبيفيرول-٣-أ- جلوكوبيرانوزيد (٩)، سيرنجين (١٠) وأبيجنين-٧-أ-نيوهسبريدوزيد (١١). وقد تم التعرف على المركبات المفصولة عن طريق الصفات الطبيعية والكيميائية وأيضا الطرق الطيفية المختلفة وكذلك المقارنة بالعينات القياسية. تم تعيين الجرعات القاتلة لنصف عدد الفئران للخلاصات المختلفة للنبات كما اجريت الدراسة البيولوجية التى شملت تأثير الخلاصات كمضادة للالتهابات وكخافضة للحرارة وحامية لخلايا الكبد وأيضا تأثيرها على الجهاز العصبى المركزى.

*Fractionation and purification of the methanolic extract of the aerial parts of Eranthemum nervosum (Vahl) R. Br. (Acanthaceae) cultivated in Egypt yielded eleven compounds named: β-amyrin (**1**), α-amyrin (**2**), Lupeol (**3**), β-sitosterol (**4**), Apigenin (**5**), Kaempferol (**6**), β-sitosterol 3-O-β-D-glucopyranoside (**7**), Benzoic acid (**8**), Kaempferol-3-O-β-D-glucopyranoside (**9**), Syringin (**10**) and Apigenin 7-O-neohesperidoside (**11**). Identification of these compounds has been established by physical, chemical and spectral data as well as comparison with authentic samples. The LD_{50}; anti-inflammatory; antipyretic; hepatoprotective and the effect on CNS were studied.*

INTRODUCTION

Family Acanthaceae is one of the richest families with medicinal plants, comprising about 250 genera and 2500 species, distributed in tropics, Mediterranean regions, Australia, Central America, Brazil, Africa and Indo-Malaysia[1-3]. The genus *Eranthemum* contains 14 species[4] which are perennial tropical herbs, shrubs or sub-shrubs with blue or rose-coloured flowers, some of them are cultivated for their foliage and others for their flowers. *Eranthemum nervosum (E. pulchellum)*[4], cultivated in Egypt is one of these ornamentals. On reviewing current literature, no biological study for this plant could be traced while chemical investigation led to isolation of one iridoid (eranthemoside)[5] and one alkaloid (3-methoxy vasicinone)[6]. The present study is dealing with the isolation and identification of the chemical constituents in addition to the biological activities of the aerial parts of this plant.

EXPERIMENTAL

General: melting points were measured on Electrothermal 9100 Digital Melting Point Instrument (England Ltd., England). UV/visible spectral data were determined on JASCO Uvidec Model 320 spectrophotometer (Tokyo, Japan) and Ultrospec 1000, UV/visible Spectrometer, Pharmacia Biotech, Cambridge (England). IR spectra were recorded on Shimadzu IR-470 (Japan) spectrometer using KBr pellets. NMR spectra were measured on JEOL TNM-LA FT NMR spectrometer (Japan) using TMS as internal standard. EI-MS were recorded with JEOL JMS 600 Hz (Japan). Column chromatography was performed on Kieselgel 60 (60-230 mesh, Merck) and RP-18 (E-Merck, Germany), precoated silica gel $G_{60}F_{254}$ and RP-18 plates for TLC (E-Merck, Germany) and Whatman sheets No. 1 (Whatman, Ltd., England) for paper chromatography.

Received in 1/4/2010 & Accepted in 20/6/2010

Plant material

The aerial parts of *Eranthemum nervosum* (Vahl) R.Br. were collected in the period from April to July 2004 from the Experimental Station of Faculty of Agriculture, Assiut University; the plant was kindly identified by Prof. Dr. Naeim El-keltawy, Department of Horticulture, Faculty of Agriculture, Assiut University. A voucher sample (No. 20102) was kept in the Herbarium of Faculty of Pharmacy, Assiut University, Assiut, Egypt.

Extraction and isolation

The air-dried powdered aerial parts of *Eranthemum nervosum* (1.5 kg) were exhaustively extracted by maceration in methanol (70%). The methanolic extract was concentrated under reduced pressure to give dark green syrupy residue. The solvent-free residue (150 g) was subjected to fractionation using *n*-hexane, chloroform, ethyl acetate and *n*-butanol respectively till complete exhaustion and dried over anhydrous sodium sulphate yielding (40 g), (5 g), (15 g) and (25 g) for *n*-hexane, chloroform, ethyl acetate and *n*-butanol respectively.

Eight grams of the *n*-hexane fraction was chromatographed on silica gel CC using *n*-hexane-EtOAc gradient as eluting systems (fractions of 300 ml were collected and the similar fractions were combined together) to give five fractions (F-I to F-V). F-II eluate (2 g) from *n*-hexane-EtOAc (9.8:0.2) was re-chromatographed on silica gel CC using *n*-hexane-EtOAc gradient which afforded compounds **1** (20 mg) and **2** (40 mg). F-III (1 g) eluted with *n*-hexane-EtOAc (9.5:0.5) afforded compound **3** (15 mg) after repeated silica gel CC using *n*-hexane-EtOAc gradient. F-IV (2 g) eluted with *n*-hexane-EtOAc (9:1) was also subjected to CC repeatedly using silica gel and *n*-hexane-EtOAc gradient as eluting systems which afforded compound **4** (55 mg).

The chloroform fraction (5 g) was chromatographed on silica gel column and eluted with $CHCl_3$-MeOH (fractions of 100 ml were collected) in gradient elution manner giving four fractions (F-I to F-IV). F-II (0.8 g), eluted with $CHCl_3$-MeOH (9:1) was subjected to CC on silica gel and $CHCl_3$-MeOH system gradient afforded compounds **5** (40 mg) and **6** (45 mg). F-III (0.6 g) and F-IV (0.5 g) eluted with $CHCl_3$-MeOH (8.5:1.5 and 8:2) respectively were separately re-chromatographed on silica gel columns using $CHCl_3$-MeOH with gradient elution manner that afforded compounds **7** (120 mg) from F-III and **8** (50 mg) from F- IV.

The ethyl acetate fraction (15 g) was subjected to CC using silica gel and eluted with $CHCl_3$-MeOH in gradient manner (fractions of 200 ml were collected) giving five fractions (F-I to F-V). F-II (0.3 g) eluted with $CHCl_3$-MeOH (8.5:1.5) afforded compound **9** (45 mg). F-III (0.5 g) eluted with $CHCl_3$-MeOH (8:2) was re-chromatographed using RP-18 CC and 70% MeOH in water as eluent afforded compound **10** (10 mg) while F-V (0.5 g) eluted with $CHCl_3$-MeOH (7:3) afforded compound **11** (20 mg) after re-column on RP-18 using 50% MeOH in water.

Acid hydrolysis

About 10 mg of compounds **9** and **11** were separately dissolved in 5 ml MeOH to which an equal volume of 10% sulphuric acid was added. The mixture was refluxed on a boiling water bath for 3 hrs. The hydrolyzate after cooling was shaken with ether (3x50 ml). The combined ethereal layer was distilled off and the aglycone was subjected to TLC and PC. The acidic mother liquor containing the sugar moiety(s) was neutralized with barium carbonate, concentrated and separately spotted alongside authentic sugars on Whatman No. 1 sheets using *n*-butanol-acetic acid-water (4:1:2 v/v) as a solvent system.

Compound (3): white needle crystals, m.p. 215-216°C. ^{1}H-NMR (500 MHz, $CDCl_3$): δ 3.18 (1H, m, H-3), 2.38 (1H, m, H-19), 0.95 (3H, s, H-23), 1.24 (3H, s, H-24), 0.98 (3H, s, H-25), 1.01 (3H, s, H-26), 0.93 (3H, s, H-27), 0.81 (3H, s, H-28), 4.67 (1H, br.s, H-29a), 4.55 (1H, br.s, H-29b), 1.33 (3H, s, H-30). ^{13}C-NMR (125 MHz, $CDCl_3$): δ 38.7 (t, C-1), 27.5 (t, C-2), 79.0 (d, C-3), 38.9 (s, C-4), 55.1 (d, C-5), 18.4 (t, C-6), 34.3 (t, C-7), 40.8 (s, C-8), 50.4 (d, C-9), 37.2 (s, C-10), 20.9 (t, C-11), 25.1 (t, C-12), 38.1 (d, C-13), 42.9 (s, C-14), 27.4 (t, C-15), 35.6 (t, C-16), 43.0 (s, C-17), 48.3 (d, C-18), 48.0 (d, C-19), 151.0 (s, C-20), 29.9 (t, C-21), 40.0 (t, C-22), 28.0 (q, C-23), 15.5 (q, C-24), 16.2 (q, C-25), 16.0 (q, C-26), 14.5 (q, C-

27), 18.1 (q, C-28), 109.4 (t, C-29), 19.4 (q, C-30).

Compound (5): yellow amorphous powder. The UV/Vis. spectral data (Table 1). ^{1}H-NMR (400 MHz, DMSO-d_6): δ 6.71 (1H, s, H-3), 6.19 (1H, d, *J*= 2.6 Hz, H-6), 6.47 (1H, d, *J*= 2.6 Hz, H-8), 7.81 (2H, d, *J*= 7.1 Hz, H-2`, 6`), 6.87 (2H, d, *J*= 7.1 Hz, H-3`, 5`). ^{13}C-NMR (Table 2).

Compound (6): yellow amorphous powder. UV/Vis. spectral data (Table 1). ^{1}H-NMR (400 MHz, DMSO-d_6): δ 6.21 (1H, br.s, H-6), 6.42 (1H, br.s, H-8), 7.97 (2H, d, *J*= 6.2 Hz, H-2`, 6`), 6.93 (1H, d, *J*= 6.2 Hz, H-3`, 5`), 12.68 (1H, s, 5-OH). ^{13}C-NMR (Table 2).

Compound (8): white needles, m.p.: 122°C. The ^{1}H-NMR (500 MHz, $CDCl_3$): δ 8.1 (2H, br.dd, *J*= 1.6, 8.4 Hz, H-2, 6), 7.6 (3H, m, H-3, 4, 5). EI-MS *m/z*: 122 $[M]^+$, 105 $[M\text{-}OH]^+$ and 77 $[M\text{-}COOH]^+$.

Compound (9): yellow amorphous powder. UV/Vis. spectral data (Table 1). ^{1}H-NMR (400 MHz, DMSO-d_6): δ 6.19 (1H, d, *J*= 1.7 Hz, H-6), 6.42 (1H, d, *J*= 1.7 Hz, H-8), 8.02 (2H, d, *J*= 8.7 Hz, H-2`, 6`), 6.88 (2H, d, *J*= 8.7 Hz, H-3`, 5`), 5.42 (1H, d, *J*= 7.3 Hz, H-1``), 3.10-3.80 (6H, m, H-2`` to H-6``). ^{13}C-NMR (Table 2).

Compound (10): colourless needles, m.p.: 195-197°C. ^{1}H-NMR (400 MHz, DMSO-d_6): δ 6.72 (2H, s, H-2, 6), 6.56 (1H, d, *J*= 15.7 Hz, H-7), 6.35 (1H, dd, *J*= 15.7, 5.8 Hz, H-8), 4.21 (2H, br.d, *J*= 5.8 Hz, H-9), 4.85 (1H, d, *J*= 7.7 Hz, H-1`), 3.50-3.85 (6H, m, H-2` to H-6`), 3.77 (6H, s, 2-OMe at C-3, 5). $^{13}$C-NMR (100 MHz, DMSO-$d_6$): δ 133.8 (s, C-1), 104.4 (d, C-2, 6), 152.6 (s, C-3, 5), 132.5 (s, C-4), 130.1 (d, C-7), 128.3 (d, C-8), 61.0 (t, C-9), 103.0 (d, C-1`), 74.1 (d, C-2`), 77.5 (d, C-3`), 70.1 (d, C-4`), 77.0 (d, C-5`), 61.4 (t, C-6`), 56.5 (q, two OMe).

Compound (11): yellow amorphous powder. UV/Vis. spectral data (Table 1). ^{1}H-NMR (300 MHz, DMSO-d_6): δ 6.81 (1H, s, H-3), 6.36 (1H, d, *J*= 1.9 Hz, H-6), 6.78 (1H, d, *J*= 1.9 Hz, H-8), 7.91 (2H, d, *J*= 8.8 Hz, H-2`, 6`), 6.87 (2H, d, *J*= 8.8 Hz, H-3`, 5`), 5.24 (1H, d, *J*= 7.3 Hz, H-1``), 5.19 (1H, br.s, H-1```), 0.97 (3H, d, *J*= 6.2 Hz, H-6```), 3.20-3.95 (10H, m, sugar protons).^{13}C-NMR (Table 2).

Preparation of fatty acids[7]

Five grams of the *n*-Hexane fraction was saponified by refluxing with 0.5N ethanolic KOH for 3 hrs. The alcohol was distilled off and the aqueous liquid was diluted with water then extracted with ether till exhaustion. The combined ethereal extract was washed with water and the ether was distilled off under reduced pressure and dehydrated over anhydrous sodium sulphate. The alkaline aqueous solution that remained after removal of the unsaponifiable matter was acidified with dilute sulphuric acid and the liberated fatty acids were extracted with ether (50 ml x 3). The combined ethereal extract was washed with distilled water to remove any acidity, and dried over anhydrous sodium sulphate. The solvent was distilled off under reduced pressure to give a viscous residue of the free fatty acids which has yellowish brown colour. A part of the residue was subjected to methylation as follow[8&9]:

About one gram of the fatty acids was esterified by mixing with anhydrous K_2CO_3 (2 g) and $(CH_3)_2SO_4$ (5 ml) in dry acetone and refluxed on water bath for 4 hrs. After filtration, the filtrate was concentrated to remove acetone, diluted with water and extracted with ethyl acetate. The ethyl acetate layer was washed with water, dried over anhydrous sodium sulphate and concentrated to yield an oily residue, dissolved in methanol and analyzed by GC.

Gas-liquid chromatography (GLC) of the fatty acid methyl esters

GLC analysis of the fatty acid methyl esters was performed using JEOL GC mate Ionization Mode 0020. Gas chromatograph equipped with flame ionization detector and fitted with 3% OV-17 on Carbowax HP 80/100 (6` x 1/8`` x 0.085``) SS column, programmed at 160°C for 2 min then increase by 15°C/min till 300°C and isothermal for 15 min Injector and detector temperature were 250°C and 320°C respectively.

Biological study

The biological studies were performed on the different fractions: *n*-hexane, chloroform, ethyl acetate, *n*-butanol, total methanolic and aqueous extracts.

Preparation of the extract for of the LD_{50} determination: weighed amounts of *n*-hexane, chloroform, ethyl acetate, *n*-butanol and total methanolic extract were dissolved in distilled water with the aid of 2% Tween 80. Subsequent dilutions were made in normal saline. Control solution was made using 2% of Tween 80 as a negative control.

Preparation of the extracts for pharmacological study: the *n*-hexane, chloroform, ethyl acetate, *n*-butanol and total methanolic extract were separately taken in weighed amounts and solublized in normal saline with the aid of 2% Tween 80. Aqueous extract (100 mg/kg) was also used.

Experimental animals: male albino rats (100-120 g) and mice (20-25 g) were used. They were fed with standard diet and free access to water and housed under standardized environmental conditions in the preclinical animal house, Pharmacology Department, Faculty of Medicine, Assiut University.

Statistical Analysis

All the results were expressed as mean ± standard error of the mean. The significance in results from control group was calculated using the student's t-test[10]. *P< 0.05 or less considered as positive result.

Materials for biological study

Normal saline 0.9% [El-Nasr Pharmaceutical and Chemical Co., Egypt.] (ADWIC). Indomethacin as antipyretic and anti-inflammatory drug (El-Nile Co., Egypt). Carrgeenan as a factor inducing edema. Yeast as a factor inducing pyrexia. Pentylenetetrazol as a factor inducing convulsions and death (Sigma Chemical Co., St. Louis, USA). Carbamazepine as a standard anti-convulsant agent (Novarts Co., Switzerland). Carbon tetrachloride (CCl_4) to induce liver damage.

(a) Determination of the LD_{50}

Mice were divided into several groups (6 mice, each). The tested fractions were i.p. injected and continuously observed for 2 hrs to detect any changes in the autonomic or behavioral responses and then monitored for any mortality. A group of mice was treated with the vehicle (2% Tween 80) as control group. The LD_{50} was determined according to the reported method[11].

(b) Preliminary pharmacological study

The different fractions, total methanolic and aqueous extracts of *Eranthemum nervosum* were screened for their effects on inflammation, hyperpyrexia, motor co-ordination, convulsion and hepatoprotective activity.

1- Anti-inflammatory activity

The method described by Winter *et al*.[12] was used. Seven groups each of five male albino rats were used. The pedal inflammation was induced in rat paws by injection of 0.1 ml, 1% carrageenan suspension in 0.9% NaCl solution into the subplantar tissue of the right hind paw. At the beginning of the experiment, the paws thickness was measured in mm using Varnier Caliper. The first group was kept as negative control injected i.p. by 2% Tween 80 in normal saline; the second group was injected by indomethacin (reference group, 8 mg/kg). The other groups were separately injected i.p. with the different fractions (200 mg/kg). After 30 minutes, the inflammation was induced by injection of the carrageenan suspension in the right hind paw while the left one was injected by an equal volume of saline solution. The increase in linear paw circumference was taken as a measure of edema[13]. The anti-inflammatory efficacy of the tested fractions was estimated by comparing the magnitude of paw swelling in the pretreated animals with those induced in control animals receiving saline. The measurement was carried out at 1, 2, 3 and 4 hrs after injection of the inflammatory agent. The percentage of inhibition were calculated as follows: (V_0-V_t) x100/ V_0, where: V_0: the average paw thickness of control group and V_t: the average paw thickness of the treated group.

2- Antipyretic activity

Seven groups (5 rats, each) were used. Hyperthermia was induced by subcutaneous injection of 20% (w/v) aqueous suspension of yeast in a volume of 10 ml/kg. The first group was kept as negative control injected i.p. by 2% Tween 80 in normal saline, while the second group injected by indomethacin (8 mg/kg). The other groups were separately injected i.p with the different fractions (200 mg/kg). Rectal temperatures were taken at 0, 1, 2 and 3 hrs after administration of tested fractions[14] using a thermometer.

3- Activity on the central nervous system

The activity on the central nervous system were evaluated by performing assays of their effects on:

a- Motor co-ordination (Rota-rod test)
b- Pentylenetetrazol induced convulsion

a- Assay of the effects on motor co-ordination (Rota-rod test)

Boissier's method[15&16] was used where mice were placed on a Rota-rod and those staying on the rod for more than three minutes were selected for the experiment. Six groups (5 mice, each) were placed on the rod 30 and 60 minutes after i.p. injection of the control or test fractions with a dose (200 mg/kg). The time they stayed on the rod was recorded.

b- Effect on pentylenetetrazol (PTZ) induced convulsion

Seven groups (5 mice, each) were used. The control group was injected i.p with PTZ (100 mg/kg) alone. The second group was i.p. injected with PTZ after 30 minutes from i.p. injection of 5 mg/kg carbamazepine as anticonvulsant drug. Other groups were i.p. injected with the tested fractions (200 mg/kg) 30 minutes before the i.p. injection of PTZ. Latencies to the onset of clonic, tonic and the mortality were evaluated during 30 min after PTZ injection[17].

4- Hepatoprotective activity
CCl_4-induced hepatotoxicity

Carbon tetrachloride (CCl_4) is a widely used chemical in experimental studies to induce liver damage which is reflected by an increase in the levels of hepatospecific enzymes; these are cytoplasmic and are released into circulation after cellular damage[18]. The increase in the total bilirubin content, GOT (glutamate oxaloacetate transaminase) and GPT (glutamate pyruvate transaminase) in the CCl_4 treated group could be taken as an index of liver damage and by centrilobular necrosis[19-21]. For inducing hepatotoxicity, four groups (5 rats, each) were injected i.p. with CCl_4 in olive oil (1:1 v/v) at a dose of 0.7 ml/kg of body weight for 7 days. The aqueous extract of the plant was forcefully fed (by feeding needle) at a dose of 100 mg/kg of body weight during CCl_4 intoxication and continued for 15 days. After completing the treatment, blood was collected from the retro-orbital plexus of respective animals. The blood samples after coagulation were centrifuged and the sera isolated were used for estimation of the biochemical markers of liver damage viz. GOT, GPT, total lipid and total bilirubin levels.

RESULTS AND DISCUSSION

All compounds were isolated from the methanolic extract of the air-dried aerial parts of *Eranthemum nervosum.* Compounds **1**-**4** and **7** gave positive colour reactions for sterols and/ or triterpenes[22&23], while compounds **5**, **6**, **9** and **11** gave positive tests for flavonoids[24].

The identification of compounds **1**, **2**, **4** and **7** as β-amyrin, α-amyrin, β-sitosterol and β-sitosterol glucoside respectively were established through different physical, chemical and spectral data together with co-chromatography with authentic samples and to the best of our knowledge these compounds were isolated here for the first time from the genus *Eranthemum.*

Compound 3: The ^{13}C-NMR showed 30 signals corresponding to 30 carbon atoms including 7 methyl, 11 methylene, 6 methine groups including one oxymethine and 6 quaternary atoms. The chemical shifts suggest the presence of triterpenoid skeleton[25]. The carbon resonances at δ_C 151.0 (s, 1C) and δ_C 109.4 (t, 1C) with δ_H 4.67, 4.55 (each 1H, br.s) indicated the presence of terminal methylene moiety[26]. The chemical shifts of methine groups including the oxymethine group at δ_C 79 with δ_H 3.18 (1H, m) in addition to other signals for methyls at δ_C 28.0 with δ_H 0.95; δ_C 15.5 with δ_H 1.24; δ_C 16.2 with δ_H 0.98; δ_C 16.0

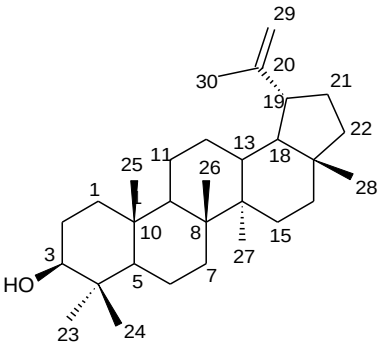
with δ_H 1.01; δ_C 14.5 with δ_H 0.93; δ_C 18.1 with δ_H 0.81 and δ_C 19.4 with δ_H 1.33 and methylene cluster in the ^{1}H-NMR confirmed the triterpenoid nature of the compound. Comparing the spectral data with that reported for triterpenoids indicated the presence of lupeol[25&26]. The β-configuration of OH group at C-3 was deduced from the ^{13}C-NMR and comparing the data with that reported for related compounds[25]. Lupeol is isolated here for the first time from the genus *Eranthemum*.

Compound 5: Different colour reactions[24&27] and the ^{13}C-NMR spectral data (Table 2) of the compound showed 15 carbon atoms indicating a flavonoid skeleton[28&29]. The UV/Vis. spectrum confirmed the flavonoid skeleton from the two characteristic bands (band I at 338 and II at 268 nm) most probably of flavone nucleus[27]. The UV/Vis. data with different ionizing and complexing reagents (Table 1) showed the presence of free OH group at C-4` from the bathochromic shift (+52) in band I after addition of NaOMe comparing with MeOH; presence of free OH group at C-7 from the bathochromic shift (+8) of band II after addition of NaOAc; presence of free OH at C-5 and absence of ortho-dihydroxy group from the bathochromic shift in band I (+46) after addition of $AlCl_3$ and the stability of the formed complex after addition of HCl and confirmed from the absence of bathchromic shift in band I after addition of NaOAc/H_3BO_3. The ^{1}H-NMR spectral data showed a characteristic pattern for a flavone[27], represented by two doublets at δ_H 6.47 and 6.19 (each 1H, *J*= 2.6 Hz) for *meta*-coupling protons assigned for H-8 and H-6 respectively. In addition, a typical A_2B_2 pattern represented by two sets of *ortho*-coupled aromatic protons at δ_H 7.81 and 6.87 (each 2H, d, *J*= 7.1 Hz) assigned to H-2`, 6` and H-3`, 5` respectively. Furthermore, a singlet proton at δ_H 6.71 was assigned for H-3. The above data were found to be identical with those reported for apigenin[27-30], and reported here for the first time from the genus *Eranthemum*.

Compound 6: The UV data of the compound (Table 1) showed a typical flavonol structure[24&27] with free hydroxyl groups at C-4`, C-7 and C-5 and lacking of *ortho*-dihydroxy groups in both rings from the bathochromic shifts after addition of NaOMe for C-4`, NaOAc for C-7, $AlCl_3$/HCl for C-5 and NaOAc/H_3BO_3 for *ortho*-dihydroxy groups. The ^{1}H-NMR showed typical flavonol structure, noted from the absence of a singlet proton signal characteristic for H-3 of flavone nucleus[27]. The spectral data as in compound **5** showed an A_2B_2 pattern from the two doublet signals at δ_H 7.97 and δ_H 6.93 (each 2H, *J*= 6.2 Hz) assigned for H-2`, 6` and H-3`, 5` respectively, indicating *a para*-disubstituted ring; two broad singlet signals at δ_H 6.21 and 6.42 (each 1H) assigned to H-6 and H-8 respectively. In addition; a downfield signal at δ_H 12.68 42 (1H, br.s) was assigned for 5-OH. The ^{13}C-NMR data (Table 2) confirmed the flavonol structure from the presence of 13 signals representing 15 carbon atoms nearly similar to those of **5** with the exception of a carbon doublet at δ_C 102.6 for compound **5** and a singlet at δ_C 134.7 for compound **6** which affect the chemical shifts of C-2 and C-4[22&28]. These chemical shifts were found to be in good agreement with those reported for kaempferol[28] that reported here for the first time in the genus *Eranthemum*.

Compound 8: The ^{1}H-NMR data of compound **8** showed aromatic signals at δ 8.13 (2H, br.dd, *J*= 1.6, 8.4 Hz, H-2, 6), 7.62 (3H, m, H-3, 4, 5) attributed for monosubstituted benzene ring. The EI-MS which gave M^+ at m/z 122 and fragments at 105 [M-OH] and at 77 [M-COOH] are characteristic for benzoic acid[31]. These data and co-chromatography with authentic sample indicated that compound **8** is benzoic acid and this is the first report about its isolation from the genus *Eranthemum*.

Compound 9: The positive test for carbohydrate indicated its glycosidic nature while the ^{13}C-NMR indicated its flavonoid skeleton. UV/Vis. data (Table 1) confirmed the flavonoid structure[27] and showed the presence of free OH groups at C-4`, C-5 and C-7 as in compound **5**. The ^{1}H-NMR data were similar to that of compound **7** in addition to signals for the sugar moiety at δ_H 5.42 (1H, d, *J*= 7.3 Hz) for the anomeric proton H-1`` and 3.10-3.80 for the remaining protons. The acid hydrolysis was carried out giving the sugar which was identified as glucose and the aglycone as



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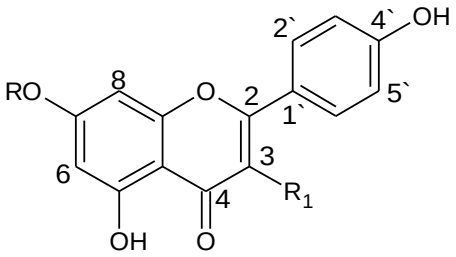
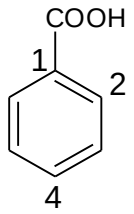
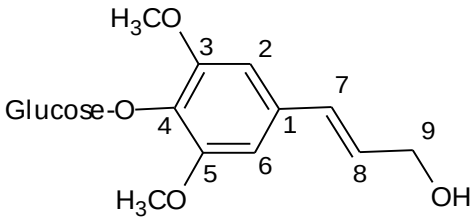


	5	**6**	**9**	**11**
R	**H**	**H**	**H**	**neohesperidoside**
R_1	**H**	**OH**	**O-glucose**	**H**



8



10

The structures of the isolated compounds

Table 1: UV spectral data of compounds **5, 6, 9** and **11** with different ionizing and complexing reagents.

Cpd. No.	Bands	λ_{max} and $\Delta\lambda_{max}$ nm										
		MeOH	NaOMe		NaOAc		$NaOAc/H_3BO_3$		$AlCl_3$		$AlCl_3$/HCl	
		λ_{max}	λ_{max}	$\Delta\lambda$	λ_{max}	$\Delta\lambda$	λ_{max}	$\Delta\lambda$	λ_{max}	$\Delta\lambda$	λ_{max}	$\Delta\lambda$
5	I	338	390	+52	375	+37	340	+2	384	+46	384	+46
	II	268	274	+6	276	+8	269	+1	278	+10	278	+10
6	I	374	442	+68	378	+4	377	+5	434	+60	434	+60
	II	270	288	+18	276	+6	270	-	276	+6	274	+4
9	I	356	410	+54	373	+20	356	-	402	+46	402	+46
	II	272	280	+8	280	+8	272	-	278	+6	279	+7
11	I	339	386	+47	386	+47	341	+2	388	+49	386	+47
	II	269	274	+5	269	-	269	-	275	+6	277	+8

Table 2: ^{13}C-NMR spectral data of compounds **5, 6, 9** and **11** in DMSO-d_6.

C	**5**	**6**	**9**	**11**
2	163.6 (s)	145.9 (s)	156.7 (s)	164.1 (s)
3	102.6 (d)	134.7 (s)	133.5 (s)	103.5 (d)
4	181.3 (s)	175.0 (s)	177.7 (s)	182.0 (s)
5	161.2 (s)	160.2 (s)	161.5 (s)	161.0 (s)
6	99.6 (d)	97.3 (d)	99.0 (d)	98.0 (d)
7	163.6 (s)	162.8 (s)	164.4 (s)	162.5 (s)
8	94.2 (d)	92.4 (d)	94.0 (d)	94.6 (d)
9	157.3 (s)	154.8 (s)	156.7 (s)	157.3 (s)
10	103.5 (s)	102.3 (s)	104.3 (s)	105.4 (s)
1`	121.3 (s)	121.1 (s)	121.2 (s)	121.3 (s)
2`	128.4 (d)	128.6 (d)	131.2 (d)	128.5 (d)
3`	116.2 (d)	114.6 (d)	115.4 (d)	116.0 (d)
4`	161.0 (s)	158.1 (s)	160.2 (s)	156.9 (s)
5`	116.2 (d)	114.6 (d)	115.4 (d)	116.0 (d)
6`	128.4 (s)	128.6 (d)	131.2 (d)	128.5 (d)
1``			101.2 (d)	99.3 (d)
2``			74.5 (d)	77.1 (d)
3``			76.6[a] (d)	76.3 (d)
4``			70.1 (d)	69.7 (d)
5``			77.6[a] (d)	77.2 (d)
6``			61.0 (t)	60.5 (t)
1```				100.4 (d)
2```				70.3[a] (d)
3```				70.4[a] (d)
4```				71.8 (d)
5```				68.3 (d)
6```				18.0 (q)

[a] data in each column may be interchangeable.

kaempferol by paper chromatography and TLC alongside authentic samples. The β-configuration of glucose was determined from the large coupling constant of the anomeric proton (7.3 Hz) and it's attachement to C-3 by comparing the UV colour of the spots on the TLC before and after hydrolysis; the upfield shift of C-3 (-1.2 ppm), downfield shift of C-4 (+2.7 ppm) and pronounced downfield shift of C-2 (+10.8 ppm) comparing with kaempferol[28&29]. From the above-mentioned data, compound **9** was identified as kaempferol-3-O-β-D-glucopyranoside which is isolated here for the first time from the genus *Eranthemum*.

Compound 10: The ^{1}H-NMR data of compound **10** showed the presence of a singlet aromatic signal at δ_H 6.72 (2H) which in addition to six signals at δ_C 132.5 (s, 1C), 152.6 (s, 2C), 104.4 (d, 2C), 133.8 (s, 1C), in the ^{13}C-NMR data suggested the presence of tetrasubstituted benzene ring. These data with the signals at δ_H 6.56 (1H, d, *J*= 15.7 Hz) with δ_C 130.1 (d, 1C); δ_H 6.35 (1H, dd, *J*= 15.7, 5.8 Hz) with δ_C 128.3 (d, 1C) and δ_H 4.21 (2H, br.d, *J*= 5.8 Hz) with δ_C 61.0 (t, 1C), indicated the presence of tetrasubstituted *trans* cinnamoyl alcohol moiety. A signal at δ_H 3.77 (6H, s) with δ_C 56.5 (q, 2C) indicated the presence of two aromatic methoxy groups. Other NMR data showed signals at δ_C 103.0 (d, 1C) with δ_H 4.85 (1H, d, *J*= 7.7 Hz) and five carbon signals at δ_C 74.1, 77.5, 70.1, 77.0 (each d) and 61.4 (t) with δ_H 3.50-3.85 (6H, m) which indicated the presence of glucose as a sugar

moiety in β-configuration. The attachment of the two methoxy groups to the aromatic ring was deduced from their chemical shift values and by comparison with related compounds (syringinoside, solargin I, II, III and IV)[32&33], while the attachment of glucose moiety to C-4 not to C-9 was deduced from the chemical shift of C-9 (δ_C 61.0) and also from the reported data[32-36]. The above-mentioned data proved that compound **10** is Syringin[36] which is isolated here for the first time from the genus *Eranthemum.*

Compound 11: different colour reactions and spectral analysis of compound **11** indicated it's flavonoid glycoside nature[24,27-29]. The ^{13}C-NMR showed 25 signals equivalent to 27 carbon atoms, 13 of them are equivalent to 15 carbon atoms with their ^{1}H-NMR data indicated the presence of flavone nucleus while the other 12 signals for a sugar moiety. The UV/Vis. data (Table 1) showed typical flavone structure with free OH groups at C-4` and C-5 and absence of orthodihydroxy groups as in compound **5**. The absence of free OH group or blocked C-7 position was established from the absence of bathochromic shift in band II after addition of NaOAc. ^{1}H and ^{13}C-NMR spectra showed two protons at δ_H 5.24 (1H, d, *J*= 7.3 Hz) with δ_C 99.3 and δ_H 5.19 (1H, br.s) with δ_C 100.4, in addition to a doublet at δ_H 0.97 (3H, d, *J*= 6.2 Hz) with δ_C 18.0 indicating the presence of two sugars most probably glucose and rhamnose in β- and α- configuration respectively. C-1`` of rhamnose was found to be attached to C-2` of glucose from the downfield shift of C-2`` (δ_C 77.1)[37].

Acid hydrolysis yielded glucose and rhamnose (confirmed by PC) as a sugar moiety and apigenin aglycone (co-chromatography with authentic sample and similarity of its UV/Vis. data with that of **5**). The above data were found to be identical with those reported for Apigenin 7-O-neohesperidoside[27,29&38] and this represents the first isolation of **11** from the genus *Eranthemum.*

Biological study

- The LD_{50} results (Table 3) revealed that the different extracts of the aerial parts of *E. nervosum* are safe enough to be used and the ethyl acetate is the safest fraction.
- Different fractions as well as total methanolic extract showed anti-inflammatory activity which reduced the carrageenan-induced edema with maximum effects being obtained after 3 hrs. (Table 4) and well-marked antipyretic activity (Table 5). They reduce yeast-induced fever compared with indomethacin (8 mg/kg) with maximum activity after 2 hrs. The best fractions were the *n*-hexane, ethyl acetate and *n*-butanol.
- All fractions attained a central nervous system depressant activity (Table 6). The total methanolic extract has potent anticonvulsant activity against PTZ induced convulsion comparable to that of carbamazepine while the *n*-hexane fraction (200 mg/kg) has a mild effect. Other fractions have no effect (Table 7).
- Pretreatment of the rats with aqueous extract of the plant inhibited the increase in levels of GPT, GOT, total lipids and total bilirubin (Table 8). This suggests the maintenance of structural integrity of hepatocytic cell membrane or regeneration of damaged liver cells by the extract. The decrease in serum bilirubin after treatment with the extract in liver damage indicates the effectiveness of the extract in normal functional status of the liver. Thus, it is evident that the total aqueous extract has remarkable hepatoprotective effect in CCl_4-induced liver damage.

Fatty acids analysis

The fatty acids analysis showed the presence of saturated acids (lauric, myristic, palmetic, stearic, behenic and cerotic acid); unsaturated acids (palmetoleic, oleic, linoleic, linolenic and erucic acids). The major acids were palmetic, linoleic and oleic (Table 9).

Table 3: The LD_{50} of different fractions and the total methanolic extract of the aerial parts.

Extract	total methanolic extract	*n*-hexane fraction	chloroform fraction	EtOAc fraction	*n*-butanol fraction
LD_{50} (g/Kg)	0.9	3	3	3.5	2.5

Table 4: Results of the anti-inflammatory activity and inhibitory effects of the different fractions on Carrageenan induced edema in rats.

Group	Dose mg/Kg	Thickness of the right paw (mm) after injection (Mean±S.E), n= 5 and Percentage of inhibition			
		1 hr	2 hrs	3 hrs	4 hrs
Control	-----	8.77±0.15 (-)	8.91±0.14 (-)	9.16±0.21 (-)	8.95±0.27 (-)
Indomethacin	8	7.22±0.19** (17.64)	7.41±0.17** (16.79)	7.43±0.18** (18.89)	7.36±0.94** (17.85)
Total extract	200	7.71±0.18 (12.09)	7.31±0.27* (17.97)	7.44±0.16*** (18.86)	7.85±0.19 (12.32)
n-Hexane fraction	200	7.30±0.24 (16.77)	7.05±0.31** (20.79)	7.47±0.18** (18.45)	7.51±0.18** (16.14)
Chloroform fraction	200	6.98±0.15* (20.40)	6.76±0.20*** (24.10)	7.04±0.18*** (23.21)	7.51±0.21** (16.14)
Ethyl acetate fraction	200	7.84±0.55 (10.63)	7.43±0.63* (16.56)	7.37±0.32*** (19.54)	7.67±0.34* (14.38)
n-Butanol fraction	200	7.07±0.261* (19.35)	6.63±0.17*** (25.52)	6.70±0.22*** (26.87)	7.53±0.14* (15.89)

Values are mean of 5 experiment ± standard error (S.E.).
Data between parenthesis indicate the % of inhibition.
Differences with respect to the control group using student's t-test ($^{*}P<0.05$ $^{**}P<0.01$ $^{***}P<0.001$).

Table 5: Results of antipyretic activity of the different fractions on yeast-induced pyrexia in rats.

Group	Dose mg/Kg	Average rectal temperature (°C) ± S.E., n= 5			
		T_o hr	1 hr	2 hrs	3 hrs
Control	-----	37.9±0.21	37.8±0.24	37.9±0.20	37.8±0.20
Indomethacin	8	38.0±0.12	36.6±0.13**	35.7±0.15**	36.3±0.09**
Total extract	200	38.1±0.15	36.6±0.24*	36.6±0.20***	37.2±0.20*
n-Hexane fraction	200	38.0±0.17	36.9±0.17	36.3±0.15***	37.0±0.17**
$CHCl_3$ fraction	200	38.1±0.18	36.8±0.33	36.5±0.19***	36.8±0.15***
EtOAc fraction	200	38.1±0.14	36.4±0.29**	36.4±0.25***	36.9±0.12***
n-Butanol fraction	200	38.0±0.18	36.5±0.17**	36.1±0.09***	36.5±0.07***

T_o = average rectal temperature just before plant fractions or solvent injection.
Values are mean of 5 experiment ± standard error (S.E.).
Differences with respect to the control group using student's t-test ($^{*}P<0.05$ $^{**}P<0.01$ $^{***}P<0.001$).

Table 6: Results of the effect of different fractions on motor Co-ordination.

Group	Dose mg/kg	Time on the rod (sec.)(mean ± SE), n= 5	
		0.5 hr	1 hr
Control	-------	150±1.67	150.1±2.1
Total extract	200	27.0±0.84***	24.6±3.27***
n-Hexane fraction	200	111.0±4.12***	85.4±7.53***
Chloroform fraction	200	120±5.23***	93.4±4.32***
Ethyl acetate fraction	200	67.4±3.12***	28.8±2.82***
n-Butanol fraction	200	34.4±2.06***	19.2±2.52***

Values are mean of 5 experiment ± standard error (S.E.).
Differences with respect to the control group using student's t-test ($^{***}P<0.001$).

Table 7: Results of the effect of different fractions on pentylenetetrazole induced convulsion and death.

Group	Dose mg /kg	Time of clonic convulsion (min) (mean±S.E.), n= 5	Time of tonic convulsion (min) (mean±S.E.), n= 5	Time until death (min) (mean±S.E.), n= 5
Control	-----	1	1.92± 0.21	2.31± 0.12
Standard	5	3.50± 0.18**	10.32± 0.31**	14.21± 0.21**
Total extract	200	3.51± 0.32**	12.11± 0.43**	17.51±0.21**
n-Hexane fraction	200	1.52± 0.53*	2.13±0.21**	2.63±0.56
Chloroform fraction	200	1.32± 0.23	1.91± 0.12	2.32± 0.32
EtOAc fraction	200	1.12± 0.45	1.81± 0.53	2.01± 0.23
n-Butanol fraction	200	1.31± 0.21	2.11± 0.36	2.82±0.32

Values are mean of 5 experiment ± standard error (S.E.).
Differences with respect to the control group using student's t-test ($^{*}P<0.05$ $^{**}P<0.01$).

Table 8: Effect of total aqueous extract on CCl_4 treated rats.

Animal group	GOT	GPT	Total lipid	Total bilirubin
Control (Normal)	23.2± 0.860	32.6±1.077	84.8±2.835	0.56±0.017
CCl_4 treated	86.0 ±4.868	48.8±1.772	163.6±7.985	1.11±0.083
Aq. Ext. alone	27.4±1.6	34.0±1.414	91.2±1.855	0.59±0.020
Aq. Ext. and CCl_4	52.8±6.888**	40.4±1.435**	108.6±3.027**	0.77±0.024**

Values are mean of 5 experiment ± standard error (S.E.).
Differences with respect to the control group using student's t-test ($^{**}P<0.01$).

Table 9: Results of GLC analysis of fatty acids methyl esters.

Peak No.	Fatty acids	No. of carbons	No. of unsaturation	R_t/min	Relative area %
1	Lauric acid	12	0	12.78	1.61
2	Myristic acid	14	0	14.53	1.53
3	Unknown	----	----	16.59	0.57
4	Palmetic acid	16	0	18.50	28.49
5	Palmetoleic acid	16	1	18.85	1.11
6	Unknown	----	----	19.88	0.84
7	Unknown	----	----	20.45	0.53
8	Stearic acid	18	0	21.27	1.59
9	Oleic acid	18	1	21.90	10.33
10	Unknown	----	----	22.43	7.21
11	Linoleic acid	18	2	23.30	12.96
12	Unknown	----	----	24.51	18.66
13	Linolenic acid	18	3	25.24	0.54
14	Unknown	----	----	25.95	0.53
15	Behenic acid	22	0	27.40	1.02
16	Erucic acid	22	1	28.30	1.04
17	Unknown	----	----	30.06	0.90
18	Cerotic acid	26	0	32.65	1.04
19	Unknown	----	----	33.20	0.84
20	Unknown	---	----	33.82	1.10
21	Unknown	----	----	35.69	0.69
22	Unknown	----	----	36.42	0.53
23	Unknown	----	----	37.08	0.60
24	Unknown	----	----	39.86	5.62

Acknowledgement

The authors thanks Prof. Dr. Ahmed Othman, Professor of Pharmacology, Faculty of Medicine, Assiut University for his great help and assistance through the pharmacological studies.

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