Bull. Pharm. Sci., Assiut University, Vol. 34, Part 1, 2011, pp. 9-20.

PHYTOCHEMICAL AND BIOLOGICAL STUDY OF *PETREA VOLUBILIS* L. (VERBENACEAE)

M. F. Abdelwahab[1], A. Abdel-Lateff[2], M. A. Fouad[1*], S. Y. Desoukey[1] and M. S. Kamel[1]

[1]*Department of Pharmacognosy, Faculty of Pharmacy, Minia University, 61519 Minia, Egypt*
[2]*Department of Health Information Technology, Jeddah Community College, King Abdul-Aziz University, 21589 Jeddah Saudi Arabia*

في هذا البحث تم دراسة النواتج الطبيعية وبعض التأثيرات البيولوجية لنبات بيتريا فوليوبيلس (ل) من العائلة الفيربينيه وقد اظهر المسح الكيميائي لخلاصه نبات بيتريا فوليوبيلس وجود مجموعه من المركبات الكيميائية مثل الإستيرولات والتربينات وبعض المركبات الفلافونويدية. ولقد تم دراسه بعض التأثيرات البيولوجية لمختلف الخلاصات لنبات بيتريا فوليوبيلس مثل مضادات الاكسده والالتهاب والتأثير المسكن والخافض للحرارة والتي أظهرت وجود تأثيرات واضحة ومؤثره لمعظم الخلاصات. وقد تم فى هذا البحث الفصل والتعرف على عدد سبعه مركبات لأول مره من نبات البيتريا فوليوبيلس وذلك من خلاصه خلات الإيثيل وهم إبيجينين والكورسيتين و٤ و٦ ثنائى ميثيل سكوتيلارين وحمض ٣ و٤ ثنائى هيدروكسى بنزويك وحمض الترانس كافيك وحمض الفانيلك بالاضافه إلى مركب الاكتيوزيد. وتم ذلك بإستخدام طرق الفصل الكروماتوجرافى المختلفة وتم التعرف على البناء الكيميائي من خلال تطبيق طرق طيفية مختلفة بالإضافة إلى استخدام الرنين النووي المغناطيسي ومطياف الكتلة.

*Petrea volubilis L. was studied for its secondary metabolites and biological activities. The phytochemical screening of dried aerial parts revealed the presence of different constituents such as unsaturated sterols, triterpens, and flavonoids. The biological activities of the total extract and different fractions were evaluated in a series of bioassays (antioxidant, anti-inflammatory, analgesic, antipyretic and antibacterial), the majority of them showed significant activities in the applied test systems. Extensive purification of the ethyl acetate extract led to isolation of apigenin (**1**), quercetin (**2**), 4`, 6-dimethylscutellarien (**3**), hypogallic acid (3,4-dihydroxybenzoic acid) (**4**), trans-caffeic acid (**5**) vanillic acid (**6**) and acteoside (**7**). The structure elucidation of **1**-**7**, was carried out by ^{1}H-NMR, UV and MS analyses.*

INTRODUCTION

Petrea volubilis L. (fam. Verbenaceae) is an ornamental perennial woody climber, sub-shrub or shrub. Traditionally, it is used for treatment of burns, wounds, inflammation and abscess[1]. The main task of the current study is proving the folk use of *Petrea volubilis* as well as isolation of the secondary metabolites, particularly the analgesic, antioxidant, anti-inflammatory, antipyretic and antibacterial activities. This paper describes, for the first time, the biological activities of the total extract and fractions of *Petrea volubilis*, in addition to isolation and identification of seven compounds **1**-**7** **(**Fig. 1) which have been isolated for the first time from this genus.

EXPERIMENTAL

General

^{1}H and ^{13}C-NMR spectra were recorded on a BRUCKER 500 MHz spectrometer. EI-MS data were obtained with a JEOL JMS-600 mass spectrometer. Pre-coated silica gel 60 F_{254} plates (E. Merck) were used for TLC. The compounds were detected by UV absorption at λ_{max} 254 and 366 nm followed by spraying with anisaldehyde/H_2SO_4 reagent and heating at 110°C for 1-2 min.

Received in 5/12/2010 & Accepted in 2/4/2011

*Corresponding author E-mail: m_fouad2000@yahoo.com

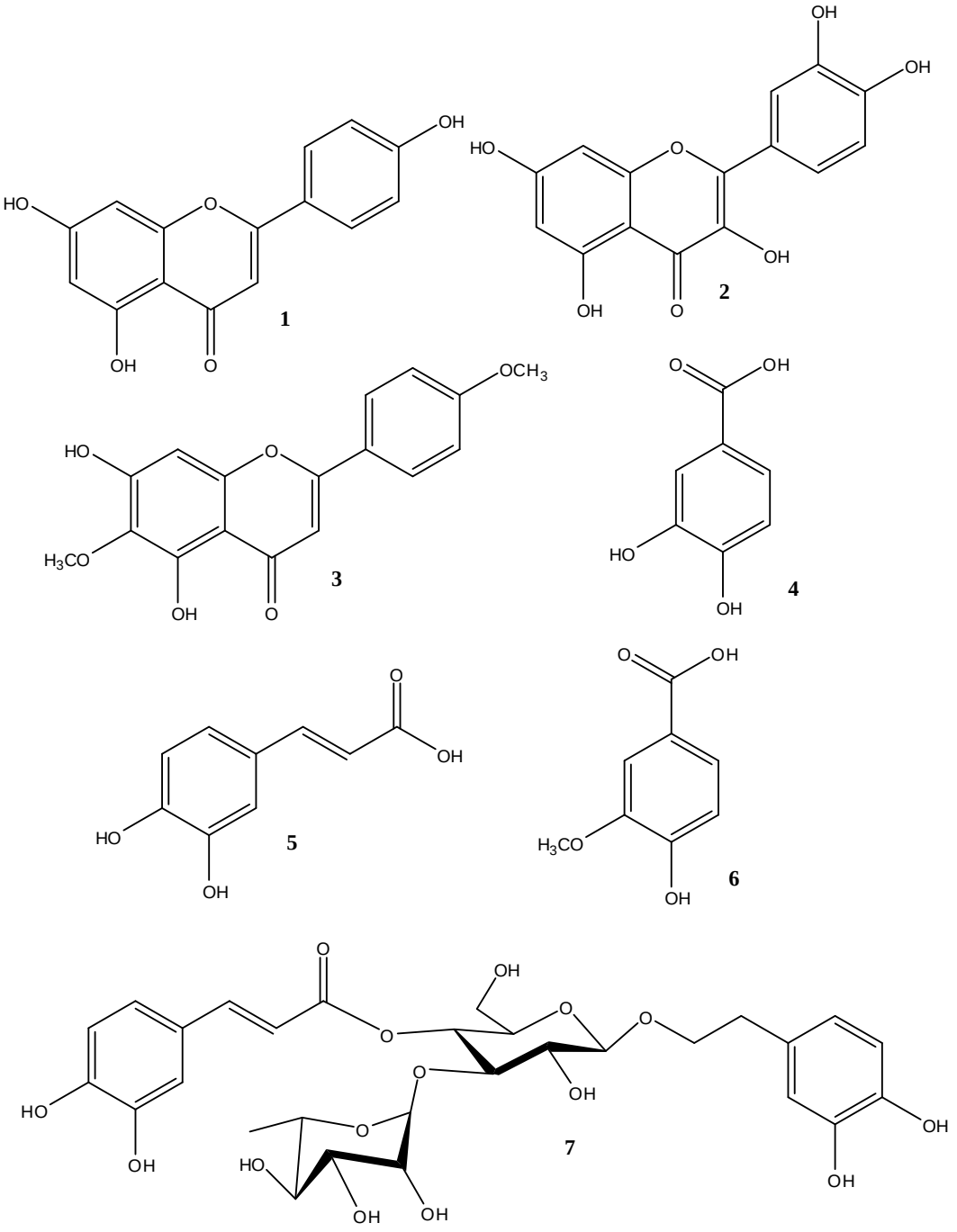

Fig. 1: Structure of the isolated compounds (**1-7**).

Plant materials

Petrea volubilis L. is cultivated in El-Zohria garden, Cairo, Egypt. The plant material (leaves and stems) was collected in January 2005 before the flowering stage of the plant. The plant was identified by Dr. Mamdoh Shokry, director of El-Zohria garden, and Prof. Dr. Abdel-Aziz Fayed, Professor of plant taxonomy, Botany Dept, Faculty of Science, Assuit University.

Extraction and isolation

The dried leaves and stems (1.8 kg) was extracted with methanol and concentrated to yield viscous brown extract (130 g). Successive fractionation of the extract using n-hexane, EtOAc and n-butanol, yielded 30, 33, 58 g of the different fractions, respectively. After extensive TLC study of different fractions using different visualizing reagents, indicated that the ethyl acetate and n-butanol fractions

are promising for further fractionation. Thus ethyl acetate fraction (33 g) was fractionated by C.C. on silica gel, employing gradient elution from $CHCl_3$ to MeOH, yielded six fractions (Fraction I – Fraction VI). According to the TLC, Fraction IV was subjected to column chromatography (CC) on silica gel using chloroform-methanol gradient with increasing polarities as mobile phase to afford two fractions: Fraction IV-1 (75:25) and Fraction IV-2 (55:45). Fraction IV-1 was further purified by CC on silica gel using ethyl acetate-methanol gradient as mobile phase to give: Fraction IV-1-1 (98:2) and Fraction IV-1-2 (92:8). Fraction IV-1-1 was subjected to CC on polyamide using methanol, isocratic elution, as mobile phase to afford compound **4** (4 mg) and compound **5** (2 mg). Fraction IV-2 was subjected to CC on polyamide employing methanol, isocratic elution, as mobile phase to afford compound **6** (4 mg). Purification of fraction IV-1-2 with CC. on polyamide using methanol as mobile phase, yielded two fractions: Fraction IV-1-2-1 and Fraction IV-1-2-2. Fraction IV-1-2-1 was subjected to CC on silica gel using ethyl acetate-methanol (99:1) as mobile phase to afford compound **3** (2 mg). While Fraction IV-1-2-2 was subjected to CC. on silica gel using ethyl acetate-methanol, gradient elution with increasing polarities of the mobile phase to yield **7** (4 mg), **1** (2 mg) and **2** (2 mg).

Biological activities

The acute toxicity of the total extract was determined by measuring the lethal dose for 50% of the laboratory animals 'LD_{50} method[2]. Different dose levels (0.5, 1, 2, 4, 8 and 16 g/kg, p.o.) of the total methanolic extract were administered to different groups of mice (20-25 g). Both the test and control groups were observed for 48 hrs under normal environmental conditions, with free access to food and water.

The anti-inflammatory activity was evaluated by the carrageenan-induced paw edema test in the mouse[3]. The extracts were diluted and administered orally to mice (20-25 g) at doses of 0.5 and 1 g/kg. After 1 hr, mice were received the injection of 0.1 ml carrageenan suspension (1% w/v in normal saline solution) into the subplantar area of the right hind paw[4]. The contralateral paw was injected with 0.1 ml normal saline solution and used as control. The first group was kept non-treated (negative control) and given 10 ml/kg of the total extract vehicle, orally. While the second group was given indomethacin (positive control) at a dose of 8 mg/kg, p.o. Edema measurements were determined in mm with a plethysmometer[5] prior and 1, 2, 3 and 4 hrs after carrageenan injection. The difference between the thicknesses of the right and left paws was taken as a measure of edema.

The antioxidant activity was performed by measuring the gastric mucosal content of lipid peroxides using the thiobarbituric acid method[6] which measures the thiobarbituric acid reactive substances (TBARS) concentration, sometimes referred to as malondialdehyde (MDA) concentration, that are the breakdown products of lipid peroxides. The mice (20-25 g) were given the total methanolic extract or the different fractions of *Petrea volubilis* L., orally at doses of 0.5 and 1 g/kg. After one hour, they were given a large dose (40 mg/kg) of indomethacin to induce gastric ulceration and eventually increasing the level of gastric mucosal lipid peroxides. The first group was kept as negative control (non-ulcer, non-treated). Eight hours later, mice were killed by overdose of ether. Their stomachs were removed, opened along the greater curvature and washed with ice-cold saline. 1,1,3,3-Tetramethoxypropane (TMP) was used as an external standard to prepare standard concentrations of malondialdehyde (1, 2, 4, 6, 8 and 10 nmol/ml) and the procedure was repeated to prepare a standard curve using TMP instead of gastric mucosa. From this curve, the peroxide concentration in the unknown sample was deduced from the corresponding absorbance using the regression line from the standard curve.

The antipyretic activity was performed using the yeast-induced fever[7]. The test was performed on mice (20-25 g) by subcutaneous injection (in the back, below the nape of the neck) of 20% aqueous suspension of yeast in a dose of 10 ml/kg to induce pyrexia[8]. Rectal temperature of each animal was recorded, using digital thermometer, inserted 2 cm into the rectum, before and 18 hrs after the yeast injection, when the temperature was at peak[9].

The first group of animals was kept as control, while the second group was given acetylsalicylic acid (100 mg/kg, p.o.). The other groups were separately, orally, administered the different extracts at doses of 0.5 and 1 g/kg. The rectal temperature of each animal was again recorded at 30 min interval for 4 hrs following drug treatment.

The analgesic activity was determined by the "Hot-plate" test method[10&11]. The mice (20-25 g) were treated orally with two doses (0.5 and 1 g/kg) of the total methanolic extract and the different fractions. While the reference group animals were treated orally with 100 mg/kg acetylsalicylic acid[12]. The temperature of the hot-plate was regulated to 55±0.5°C. Each mouse was placed on the hot plate in order to obtain the animal's response to heat-induced pain. Jumping was taken as an indicator of the animal's response to heat-induced pain. The time taken for each mouse to jump out (i.e. reaction time) was noted and recorded in seconds. Each mouse served as its own control. Thus, the initial reaction time was determined prior to drug administration. The reaction time was measured in a 180-min period at 30 min intervals after drug treatment.

The antibacterial activity was tested against Gram-positive organisms (*Staphylococcus aureus* and *Bacillus subtilis*) and Gram-negative organisms (*Escherichia coli*, *Klebsiella pneumonia* and *Pseudomonas aeruginosa*). The well technique[13] was used to detect the minimal inhibitory concentration (MIC) of the tested extracts. The bacterial strains used in this study were obtained from Microbiology Department, Faculty of Pharmacy, Minia University. Certain weights of the total extract and its different fractions were separately taken and dissolved in 20% methanol (this solvent showed no inhibitory effects on the tested organisms) to obtain certain concentrations 2.5, 5, 10, 20, and 40 mg/ml. The test organisms were grown in nutrient agar for 24 hrs at 37°C then added separately into the same medium in a concentration of 1×10^6 CFU/ml at 50°C. Equal portions (about 5 ml) of the seeded agar were transferred, under laminar flow, into sterile 90 mm glass Petri-dish to give a depth of 2 mm in each plate. After solidification of the inoculated agar, circular wells of 10 mm diameter were carefully punched using a sterile cork porer. For each concentration a 50 µl were applied to each well. Each concentration was applied triplicate. The plates were incubated overnight at 37°C. Any zone of inhibition was recorded as positive result.

RESULTS AND DISCUSSION

Compound 1 was isolated as a yellow amorphous powder with m.p. 195-198°C and R_f value of 0.31 (EtOAc: MeOH: HCOOH; 90: 10: 0.5). The UV spectral data, in methanol and with different ionizing and complexing reagents (Table 1), revealed that compound **1** is a flavone having free hydroxyl groups at C-4', C-5 and C-7 and confirming the absence of ortho-dihydroxyl groups. ^{1}H-NMR spectral data (Table 2), showed a typical flavone structure, confirmed by the presence of a singlet proton signal at δ_H 6.58 characteristic for H-3. The spectrum showed two superimposed (overlapping) signals as a doublet signal appeared at δ_H 7.9 (J= 8.8 Hz) which was assigned to H-2' and H-6' due to ortho-coupling of H-2' with H-3' and H-6' with H-5' and two superimposed (overlapping) signals as a doublet signal appeared at δ_H 6.92 (J= 8.8 Hz) which was assigned to H-3' and H-5' due to ortho-coupling of H-3' with H-2' and H-5' with H-6'. The ^{1}H-NMR spectrum revealed also two aromatic proton broad singlets at δ_H 6.18 and δ_H 6.82 assigned to H-6 and H-8 respectively. These data were similar to those reported in literature[14]. Consequently, compound **1** was concluded to be Apigenin.

Compound 2 was isolated as a yellow amorphous powder with m.p. 310-312°C and R_f value of 0.29 (EtOAc : MeOH : HCOOH; 90:10:0.5). The nucleus was identified as flavone based on the UV spectral data obtained from different ionizing and complexing reagents (Table 1), has free hydroxyl groups at C-3', C-4', C-5 and C-7, and confirmed the presence of ortho-dihydroxy groups in ring B. ^{1}H-NMR spectral data of **2** (Table 2), showed a typical flavonol structure, noted from the absence of a singlet proton signal characteristic for H-3. The spectrum showed a doublet signal at δ_H 7.72 (J= 2.0 Hz) for H-2' due to meta-coupling with H-6', a doublet doublet signal at

Table 1: UV spectral data of compounds **1**-**3** with different shift reagents.

Compound No.	Bands	MeOH	NaOMe		$AlCl_3$		$AlCl_3$/HCl		NaOAc		NaOAc/H_3BO_3	
		λ_{max}	λ_{max}	$\Delta\lambda_{max}$	λ_{max}	$\Delta\lambda_{max}$	λ_{max}	$\Delta\lambda_{max}$	λ_{max}	$\Delta\lambda_{max}$	λ_{max}	$\Delta\lambda_{max}$
1	II	288	310	+22	296	+08	306	+18	303	+15	290	+02
	I	330	380	+50	382	+52	382	+52	356	+26	327	-03
2	II	297	306	+09	303	+06	290	-07	308	+11	300	+03
	I	358	398	+40	395	+37	380	+22	378	+20	378	+20
3	II	282	286	+04	308	+26	306	+24	300	+18	284	+02
	I	335	340	+05	384	+49	384	+49	338	+03	336	+01

Table 2: ^{1}H (MeOH-d_4, 500 MHz) NMR Spectral Data for Compounds **1**-**6**.

Positions	Compounds					
	1	**2**	**3**	**4**	**5**	**6**
2				7.35 (d, *J*= 2.2, 1H)	7.15 (br.s, 1H)	7.52 (d, *J*= 1.7, 1H)
3	6.58 (s, 1H)		6.72 (s, 1H)			
5				6.89 (d, *J*= 8.2, 1H)	6.77 (d, *J*=7.3, 1H)	6.83 (d, *J*= 8.2, 1H)
6	6.18 (br.s, 1H)	6.17 (d, *J* = 2.1, 1H)		7.28 (dd, *J*= 8.2, 2.2, 1H)	6.93 (br.d, *J*= 9.8,1H)	7.54 (dd, *J*= 8.2, 1.7, 1H)
7					7.54 (d, *J*= 16.0, 1H)	
8	6.82 (br.s, 1H)	6.38 (d, *J*= 2.1, 1H)	6.98 (s, 1H)		6.25 (d, *J*=16.0, 1H)	
2'	7.90 (d, *J* = 8.8, 1H)	7.72 (d, *J*= 2.0, 1H)	8.00 (d, *J*= 8.5, 1H)			
3'	6.92 (d, *J* = 8.8, 1H)		7.10 (d, *J*= 8.5, 1H)			
5'	6.92 (d, *J* = 8.8, 1H)	6.87 (d, *J* = 8.5, 1H)	7.10 (d, *J*= 8.5, 1H)			
6'	7.90 (d, *J* = 8.8, 1H)	7.62 (dd, *J* = 2.0,8.5, 1H)	8.00 (d, *J*= 8.5, 1H)			
6, 4` OCH_3			4.50 (s, 6H)			
5 OCH_3						3.90 (s, 3H**)**

δ_H 7.62 (*J*= 2.0 Hz and *J*= 8.5 Hz) for H-6' due to ortho-coupling with H-5' and meta-coupling with H-2', a doublet signal at δ_H 6.87 (*J*= 8.5 Hz) for H-5' due to ortho-coupling with H-6'. The ^{1}H-NMR spectrum showed, also, two doublet signals at δ_H 6.38 (*J*= 2.1 Hz) for H-8 and at δ_H 6.17 (*J*= 2.1 Hz) for H-6 indicating meta coupling). These data were similar to those reported in literature[15]. Therefore, compound **2** was concluded to be Quercetin.

Compound 3 was isolated as a yellow amorphous powder with m.p. 215-218°C and R_f value of 0.4 (EtOAc : MeOH : HCOOH; 85:15:0.5). The nucleus was identified as flavone based on the UV spectral data obtained from different ionizing and complexing reagents, indicated that compound **3** has free hydroxyl groups at C-7and C-5, and confirmed the absence of a free hydroxyl group at C-4' as well as the absence of ortho-dihydroxyl groups at rings A and B[16] as shown in table 1, ^{1}H-NMR spectral data (Table 2), showed a typical flavone structure, confirmed by the presence of a singlet proton signal at δ_H 6.72 characteristic for H-3[17]. It revealed also one aromatic proton singlet at δ_H 6.98 assigned to H-8. The spectrum showed two superimposed (overlapping) signals as doublets appeared at δ_H 8.00 with (*J*= 8.5 Hz) which was assigned to H-2' and H-6' due to ortho-couplings of H-2' with H-3' and H-6' with H-5', and at δ_H 7.1 with (*J*= 8.5 Hz) which was assigned to H-3' and H-5' due to ortho-couplings of H-3' with H-2' and H-5' with H-6'(representing [AA'BB'] spin-system). The spectrum also revealed the presence of two aromatic OCH_3 groups appeared as a singlet signal at δ_H 4.5. The molecular formula of **3** was established as $C_{17}H_{13}O_6$ based on the ESI-MS (negative mode) spectrum. It showed a quassi-molecular ion peak at *m/z* 313 $[M-H]^+$ corresponding to a molecular formula of $C_{17}H_{13}O_6$ in addition to a characteristic fragment peak at *m/z* 283 for $[M-H-2(CH_3)]^+$,which proved the presence of two OCH_3 groups[18]. Thus, compound **3** was concluded to be 6,4'-dimethyl-scutellarein.

Compound 4 was isolated as white powder with m.p. 197-200°C and R_f value of 0.36 (n-Hexane – EtOAc, 30:70). ^{1}H-NMR spectral data (Table 2), showed three signals assigned to three aromatic protons, a doublet signal at δ_H 7.35 (*J*= 2.2 Hz) for H-2 due to meta-coupling with H-6[19], a doublet doublet signal at δ_H 7.28 (*J*= 2.2 Hz and *J*= 8.2 Hz) for H-6 due to ortho-coupling with H-5 and meta-coupling with H-2, and a doublet signal at δ_H 6.89 (*J*= 8.2 Hz) for H-5 due to ortho-coupling with H-6. This confirmed the presence of a tri-substituted benzene ring.^{13}C-NMR and DEPT experiments revealed the presence of 7 carbon atoms of which 3 methine and 4 quaternary carbons, one of the latter was downfield shifted at δ_C 168 assignable to a carboxylic group[20]. In addition to 2 quaternary oxygenated aromatic carbons appearing at δ_C 145 and 151 (for C-3 and C-4, respectively). The other signals were assigned for aromatic carbons. EI-MS spectral data of compound **4** revealed that the molecular ion peak $[M]^+$ appearing at *m/z* 154 is the most intense peak (the base peak) which is characteristic for aromatic acids[18], coincident with the molecular formula $C_7H_6O_4$. The other significant peaks were attributed to loss of OH $[M-17]^+$ at m/z 137 and loss of CO_2H $[M-45]^+$ at *m/z* 109. The spectrum showed no peak due to loss of H_2O $[M-18]^+$ proving the absence of a hydrogen-bearing ortho group[18]. Therefore, the OH groups are in meta and para-positions, confirming the identity of the structure. Therefore, compound **4** was identified as 3,4-dihydroxybenzoic acid (hypogallic).

Compound 5 was isolated as yellow needles (methanol) with m.p. 211-213°C. It was obtained as a single spot with R_f value of 0.46 (n-Hexane - EtOAc, 30:70). ^{1}H-NMR spectral data (Table 2), revealed the presence of two doublets at δ_H 7.54 and 6.25 characteristic for two olefinic protons (H-7 and H-8 respectively) with *J* constants of 16.0 Hz, indicating a *trans*-orientation[21]. Other signals appeared at δ_H 7.15 (1H, br.s), δ_H 6.77 (1H, d, *J*= 7.3) and δ_H 6.93 (1H, br.d, *J*= 9.8) were assigned to H-2, H-5 and H-6 respectively, revealing a trisubstituted benzene ring[22]. The identity was confirmed by positive FAB-MS analysis, which showed a quassi-molecular ion peak at *m/z* 181 $[M+H]^+$ corresponding to a molecular formula $C_9H_9O_4$, in addition to characteristic fragment peaks at *m/z* 164 for $[M+H-(OH)]^+$ and m/z 136 for $[M+H-(CO_2H)]^+$. Therefore, compound **5** was concluded to be *trans*-caffeic acid.

Compound 6 was isolated as white amorphous powder with m.p. 208-211°C. It was obtained as a single spot with R_f value of 0.36 (EtOAc : MeOH : HCOOH; 90:10:0.5). ^{1}H-NMR spectral data (Table 2) showed 3 signals corresponding to three aromatic protons, and one signal corresponding to protons of an aromatic OCH_3 group as follow: a doublet at δ_H 7.52 (*J*= 1.7 Hz) for H-2 due to meta-coupling with H-6, a doublet at δ_H 6.83 with (*J*= 8.2 Hz) for H-5 due to ortho-coupling with H-6, and a doublet doublet at δ_H 7.54 with (*J*= 8.2 Hz and *J*= 1.7 Hz) for H-6 indicating its ortho-coupling with H-5 and meta-coupling with H-2. These 3 signals confirmed the presence of a trisubstituted benzene ring[22]. The singlet signal appearing at δ_H 3.9 was assigned to the 3 protons of the aromatic OCH_3 group[17]. The HMBC (hetero-nuclear multiple bond coherence) study proved the position of the OCH_3 group. It showed a three-bond H→C correlation of OCH_3 protons (δ_H 3.9) with C-3 (δ_C 148), as well as a three-bond correlation of H-6 (δ_H 7.54) with C-4 (δ_C 152), a correlation of H-6 with C-7 (δ_C 169) and a correlation of H-2 (δ_H 7.52) with C-7, this indicated that OCH_3 group is attached to C-3 and OH group is attached to C-4. Moreover, the EI-MS spectral data of compound 6 revealed that the molecular ion peak $[M]^+$ is the most intense peak (the base peak) which is characteristic for aromatic acids[18], at *m/z* 168, it is coincident with the molecular formula $C_8H_8O_4$. The other prominent peaks are formed by loss of CH_3 $[M-15]^+$ at *m/z* 153, loss of OH $[M-17]^+$ at *m/z* 151 and loss of CO_2H $[M-45]^+$ at *m/z* 123. The mass spectrum also proved the absence of OH group in ortho-position to CO_2H group due to absence of a peak corresponding to loss of H_2O. By comparing the chemical shifts of the ^{1}H-NMR data of compound **6** with those reported in literature, it was found that the shifts of the aromatic protons are more in agreement with values calculated for vanillic acid than in the case of its isomer, isovanillic acid[23]. Therefore, compound **6** was concluded to be vanillic acid.

Compound 7 was isolated as pale yellow amorphous powder. It was obtained as a single spot with R_f value of 0.55 ($CHCl_3$: MeOH : H_2O, 75:25:2). ^{1}H-NMR spectral data (Table 3) suggested the presence of four fragments, including a phenyl ethanoid moiety (aglycone), a *trans*-caffeoyl moiety, a glucosyl and a rhamnosyl units. These data were very similar to those of acteoside reported in literature[24]. The spectrum showed the presence of two doublets at δ_H 7.55 and δ_H 6.23 corresponding to the olefinic protons of caffeoyl H-7''' and H-8''' respectively, with *J*= 16.0 Hz of trans-coupling. The spectrum also indicated the presence of two trisubstituted benzene rings one belonging to caffeoyl moiety at δ_H (7.02, br.s.; 6.74, d, *J*= 8 Hz; 6.9, br.d, *J*= 8Hz) assigned for H-2```, H-5```and H-6``` respectively, the other signals reputed to a 3,4-dihydroxy phenyl ethanoid moiety at δ_H (6.70, d, *J*= 2.0 Hz; 6.65, d, *J*= 8.0 Hz; 6.52, dd, *J*= 2.0, 8.0 Hz) assigned for H-2, H-5 and H-6 respectively. Moreover, a multiplet spletted signal integrated for two protons appeared at δ_H 2.73 assigned to H_2-7. Further investigation of ^{1}H-NMR spectrum, indicated to presence of two multiplet signals at δ_H 4.05 and 3.73 assigned to oxygenated methylen protons H-8_a, H-8_b which confirmed the ethanoid side chain of the phenyl aglycone moiety. Furthermore, the appearance of a doublet signal at δ_H 1.05 (3H, d, *J*= 6.4 Hz, Rha H-6) confirmed the presence of rhamnose moiety. In addition to two anomeric sugar protons, one deduced for β-glucose at δ_H 4.34 (1H, d, 8.0 Hz) and the other deduced for α-rhamnose at δ_H 5.16 (1H, br.s).

Table 3: ^{1}H (MeOH-d_4, 400 MHz) NMR Spectral Data for Compound **7**.

Positions	δ^1H (ppm, *J* in Hz.)
2	6.70 (d, *J*= 2.0, 1H)
5	6.65 (d, *J*= 8.0, 1H)
6	6.52 (dd, *J*=2.0 , 8.0, 1H)
7	2.73 (m, 2H)
8a 8b	4.05 (m, 1H) 3.73 (m, 1H)
H-1' glucose	4.34 (d, *J*= 8.0, 1H)
Sugar protons	3.40 – 3.85 (m, 11H)
H-1" rhamnose	5.16 (br.s, 1H)
CH_3-rhamnose	1.05 (d, *J* = 6.4, CH_3-rhamnose)
2'''	7.02 (br.s, 1H)
5'''	6.74 (d, *J*= 8.0, 1H) '
6'''	6.90 (br.d, *J*= 8.0, 1H)
7'''	7.55 (d, *J*= 16.0, 1H)
8'''	6.23(d, *J*= 16.0, 1H)

From the above mentioned data and by co-chromatography with an authentic sample of acteoside using the system Chloroform – methanol - water (7.5:2.5:0.2), compound **7** was identified as acteoside.

The acute toxicity: the LD_{50} value was 8 g/kg indicating a large margin of safety for the total extract of *Petrea volubilis* L.

The anti-inflammatory activity: The different extracts exhibited an anti-inflammatory activity by inhibition of the edema induced by carrageenan. The most powerful reduction on paw edema was obtained with the n-butanol fraction and the total methanolic extract. The maximum effect being obtained after 4 hrs, where the total extract (in a dose of 1 g/kg), was able to reduce the edema more than indomethacin (Fig. 2). The higher anti-inflammatory activity could be attributed to its higher content of flavonoids[25].

The antioxidant activity: The more pronounced antioxidant effect was observed with the total methanolic extract at the dose of 1 g/kg. While the other fractions showed more or less weak antioxidant activities (Fig. 3).

The antipyretic activity: the total methanol extract, ethyl acetate and n-butanol fractions exhibited antipyretic activity by causing reduction in yeast-induced pyrexia. The most effective fractions were n-butanol and the total methanolic extract. The rectal temperature reaches the normal level after approximately 1 hr when the total methanolic extract were given in a dose of 1 g/kg, after 2 hrs in case of n-butanol fraction, and after more than 3 hrs in case of ethyl acetate fraction. On the other hand, the n-hexane fraction showed weak antipyretic activity, where the rectal temperature decreased, but did not reach the normal level even after 4 hrs following the treatment (Fig. 4).

The analgesic activity: The n-hexane fraction considerably increased the animal's reaction time to the heat stimulus. The increase was significant in all cases beginning from 60 min but the dose of 1 g/kg of n-hexane fraction increased the reaction time, similar to acetylsalicylic acid, beginning from 30 min with its maximum effect at approximately 1 hr after treatment. On the other hand, the total methanolic extract showed moderate analgesic activity while the ethyl acetate and n-butanol fractions showed the least and almost the same analgesic activity (Fig. 5).

The antibacterial activity: The total methanolic extract is the most effective antibacterial agent among the tested fractions. This could be attributed to its high content of phenolic constituents. It is effective against Gram-positive bacteria such as *Staphylococcus aureus* and *Bacillus subtilis* at dose of 5 mg/ml for each one. But, it did not show any antibacterial activity against the Gram-negative bacteria such as *Escherichia coli, Klebsiella pneumonia* and *Pseudomonas aeruginosa* even at the higher used concentration (40 mg/ml). The n-butanol also showed antibacterial activity against, only, the Gram-positive bacteria, but the MIC value was higher than that of the total extract (MIC=10 mg/mL). The n-hexane and Ethyl acetate fractions did not show any antibacterial activities against all the tested organisms at the higher used concentration (40 mg/ml).

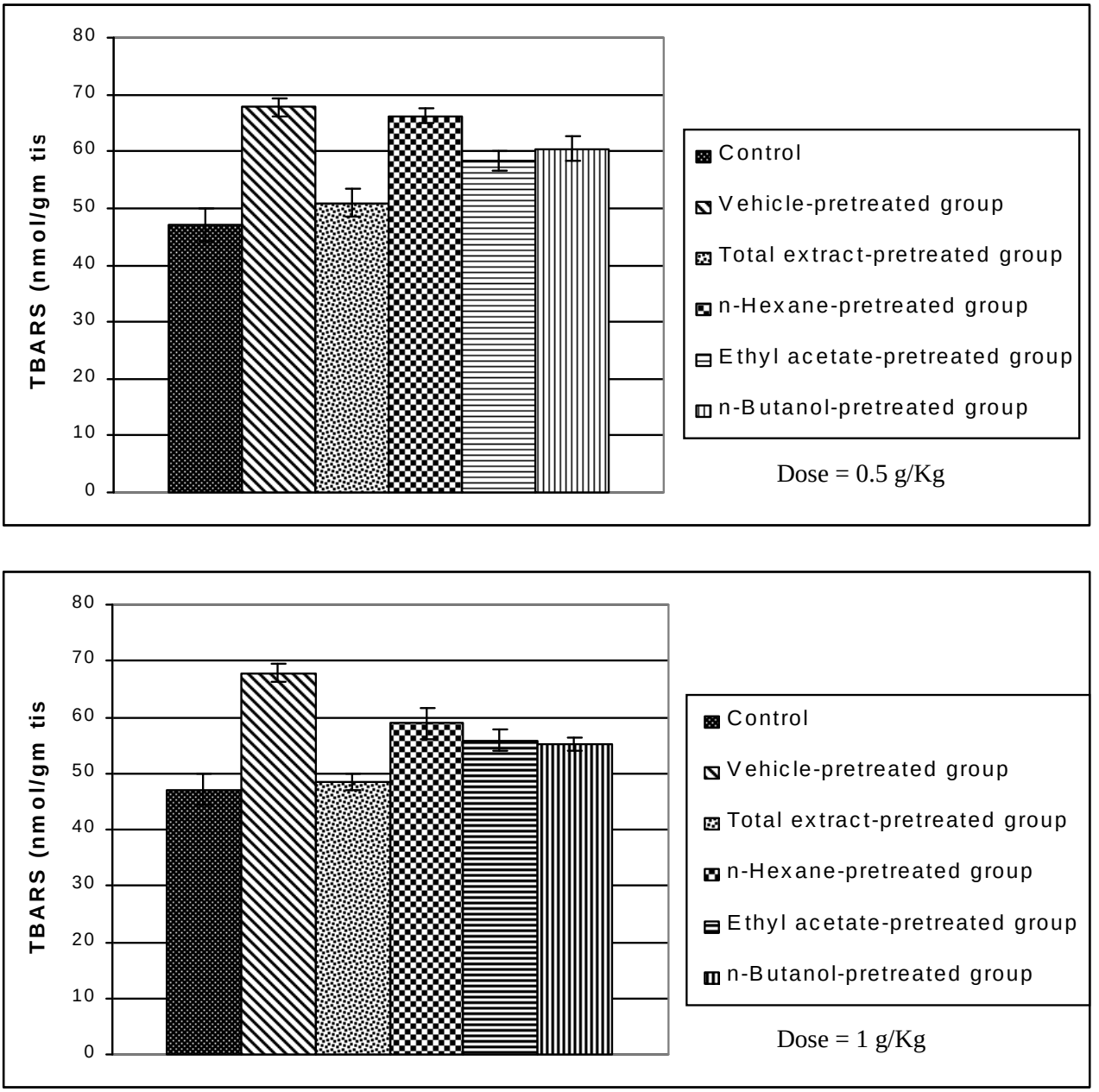

Fig. 3: Effect of extracts on gastric mucosal lipid peroxides which induced by indomethacin.

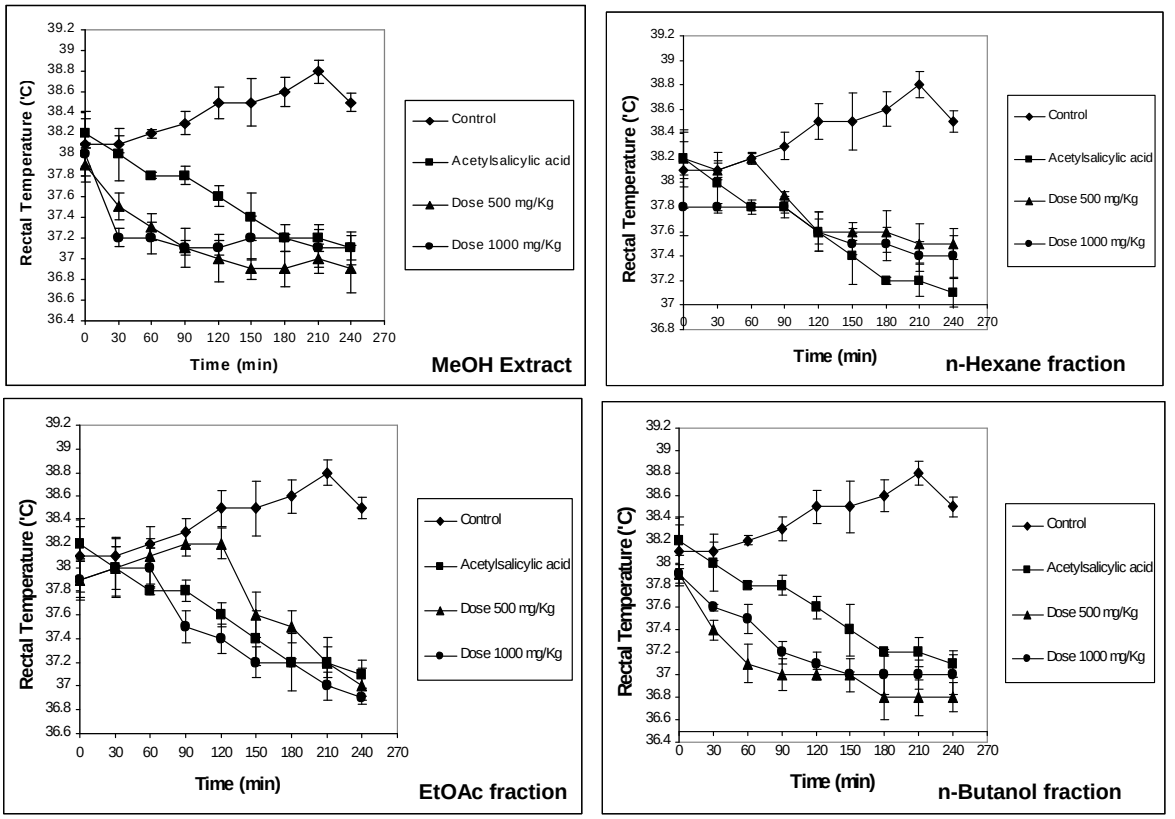

Fig. 4: Antipyretic effect of the total extract and fractions of *Petrea volubilis* L. on yeast-induced pyrexia.

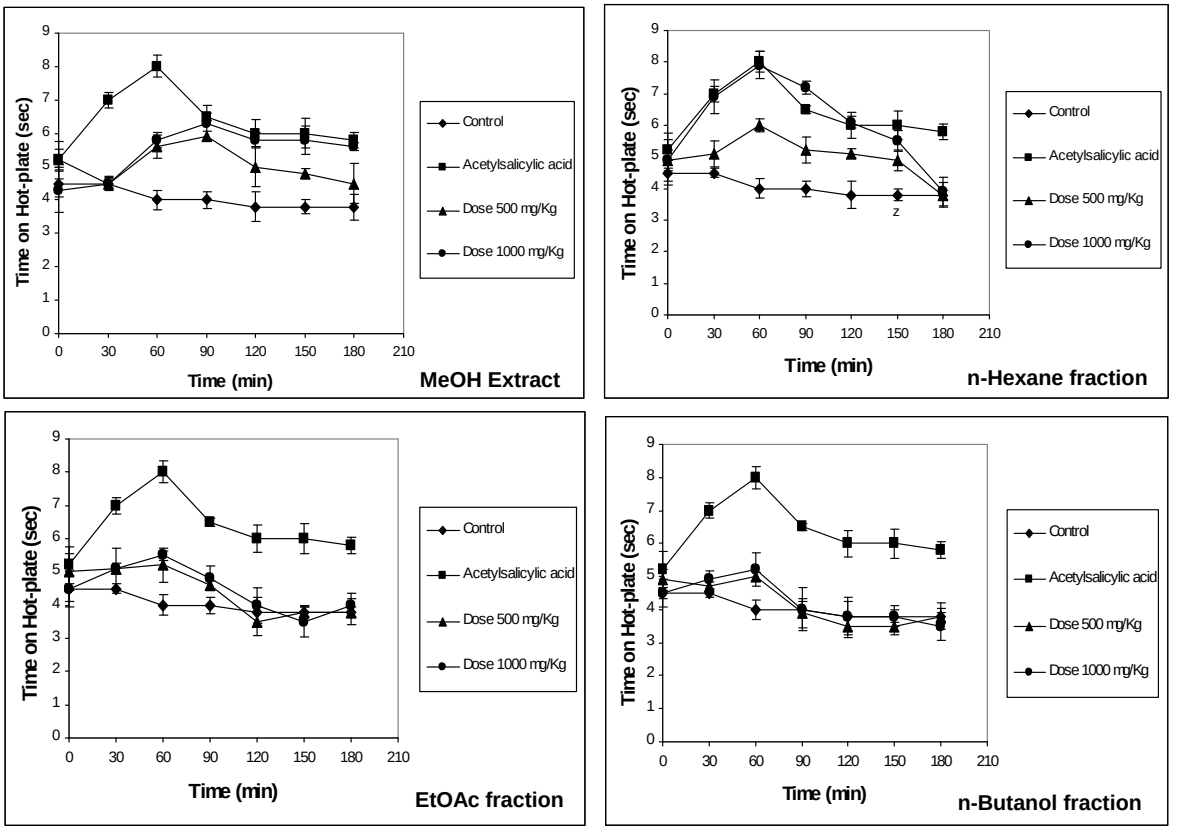

Fig. 5: Analgesic effect of the total extract and fractions of *Petrea volubilis* L. by hot-plate induced pain test.

REFERENCES

1- M. Hickey and C. King, "1100 Families of Flowering Plants", Cambridge University press, Great Britain, 2nd Edition. 1988, pp. 385-386.

2- E. E. S. Schapoval, M. R. Winter de Vargas, C. G. Chaves, R. Bridi, J. A. Zuanazzi and A. T. Henriques, "Anti-inflammatory and antinociceptive activities of extracts and isolated compounds from *Stachytarpheta cayennensis*", Journal of Ethnopharmacology, 60, 53-59 (1998).

3- C. A. Winter, E. A. Risley and C. W. Nuss, "Carrageenan induced edema in hind paw of the rat as an assay for anti-inflammatory drugs", Proceeding of the Society for Experimental Biology and Medicine, 111, 544-547 (1962).

4- H. G. Vogel and W. H. Vogel, "Drug Discovery and Evaluation, Pharmacological Assays", Springer-Verlag, Berlin, Heidelberg, New York, 1997, pp. 406-407.

5- S. H. Ferreira, "A new method for measuring variations of rat paw volume", Journal of Pharmacy and Pharmacology, 31, 648 (1979).

6- J. A. Buege and S. D. Aust, Methods of Enzymol., 52, 302-310 (1978).

7- U. M. Teotino, L. P. Friz, A. Ganduni and D. D. Bella, "Thio derivatives of 2,3-dihydro-4H-1,3-benzoazin-4-one synthesis and pharmacological properties", Journal of Medicinal Chemistry, 6, 248-250 (1963).

8- J. Y. Kang, M. N. A. Khan, N. H. Park, J. Y. Cho, M. C. Lee, H. Fujii and Y. K. Hong, "Antipyretic, analgesic and anti-inflammatory activities of the seaweed *Sargassum fulvellum* and *Sargassum. thunbergii* in mice", Journal of Ethnopharmacology, 116, 187-190 (2008).

9- A. Panthong, D. Kanjanapothi, T. Taesotikul, T. Wongcome and V. Reutrakul, "Anti-inflammatory and antipyretic properties of *Clerdendrum petasites* S.Moore", ibid., 85, 151-156 (2003).

10- X. Hu, H. Jin, W. Xu, W. Zhang, X. Liu, S. Yan, M. Chen, J. Li and W. Zhang, "Anti-inflammatory and analgesic effects of *Daphne retusa* Hemsl.", ibid., 120, 118-122 (2008).

11- B. Adzu, S. Amos, S. D. Kapu and K. S. Gamaniel, "Anti-inflammatory and anti-nociceptive effects of *Sphaeranthus senegalensis*", ibid., 84, 169-173 (2003).

12- X. Shu, Z. Gao and X. Yang, "Anti-inflammatory and anti-nociceptive activities of *Smilax china* L. aqueous extract", ibid., 103, 327-332 (2006).

13- J. V. Bennet, J. L. Brodie, J. L. Benner and W. M. M. Kirby, "Simplified accurate method for antibiotic assay of clinical specimens", Applied Microbiology, 14, 2170-2177 (1966).

14- K. R. Markham, "Techniques of Flavonoid Identification", Academic Press, London, New York, Paris, San Diego, San Francisco, Sao Paulo, Sydney, Tokyo, Toronto, 1982, pp. 39-41.

15- S. G. Leitao and F. D. Monache, "2"-*O*-Caffeoylorientin from *Vitex polygama*", Phytochemistry, 49, 2167-2169 (1998).

16- T. J. Mabry, K. R., Markham and M. B. Thomas, "The Systematic Identification of Flavonoids", Springer, Verlarge, New York, Heidelberg, Berlin (1970).

17- C. Hirobe, Z. Qiao, K. Takeya and H. Itokawa, "Cytotoxic flavonoids from *Vitex agnus-castus*", Phytochemistry, 46, 521-524 (1997).

18- R. M. Silverstein and F. X. Webster, "Spectrometric Identification of Organic Compounds", John Wiley and Sons, Inc., New York, 6th Ed., 1998, pp. 26, 27, 229.

19- A. M. Aguinaldo, O. P. M. Ocampo, B. F. Bowden, A. I. Gray and P. G. Waterman, "Tectograndone, an anthraquinone-naphthoquinone pigment from the leaves of *Tectona grandis*", Phytochemistry, 33, 933-935 (1993).

20- E. Helfrich and H. Rimpler, "Iridoid glycosides from *Gmelina philippensis*", ibid., 54, 191-199 (2000).

21- H. Otsuka, T. Yamanaka, Y. Takeda, Y. Sasaki, K. Yamasaki, Y. Takeda and T. Seki, "Fragments of acylated 6-O-α-L-rhamnopyranosyl catalpol from leaves of *Premna japonica*", ibid., 30, 4045-4047 (1991).

22- I. Kouno, M. Inoue, Y. Onizuka, T. Fujisaki and N. Kawano, "Iridoid and phenolic glucoside from *Vitex rotundifolia*.", ibid., 27, 611-612 (1988).
23- B. Ouattara, L. Angenot, P. Guissou, P. Fondu, J. Dubois, M. Frederich, O. Jansen, J. V. Heugen, J. Wauters and M. Tits, "LC/MS/NMR analysis of isomeric divanilloylquinic acids from the root bark of *Fagara zanthoxyloides* Lam.", ibid., 65, 1145-1151 (2004).
24- A. Karioti, H. Skaltsa, J. Heilmann and O. Sticher, "Acylated flavonoid and phenylethanoid glycosides from *Marrubium velutinum*", ibid., 64, 655-660 (2003).
25- G. N. Silva, F. R. Martins, M. E. Matheus, S. G. Leitao and P. D. Fernandes, "Investigation of anti-inflammatory and antinociceptive activities of *Lantana trifolia*", Journal of Ethnopharmacology, 100, 254-259 (2005).