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CHARACTERIZATION OF BIOACTIVE PHYTOCHEMICAL FROM THE LEAVES OF *Vitex trifolia*

Mona A. Mohamed*, Allia M. Abdou, Manal M.Hamed and Amal M.Saad

Medicinal chemistry department, Theodor Bilharz Research Institute,
Warak El-Hadar, Imbaba, Giza , Egypt
***Corresponding author:** Email: tbi20042003@yahoo.co.uk, Tel: +22-22722766

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ABSTRACT:
Vitex trifolia leaves ethanolic extract was further fractionated with solvent-solvent extraction technique using the following solvents successively, petroleum ether, ethyl acetate and *n*-butanol. Among the last two fractions the *n*-butanol fraction showed the strongest cytotoxic activity against brine shrimp lethality bioassay and Hep-G2 cell line, while the ethyl acetate fraction exhibited the strongest antioxidant activity using 1,1-diphenyl-2-picrylhydrazyl (DPPH) and phosphomolybdenum methods. Phytochemical investigation of the ethyl acetate fraction afforded, stigmasterol **1**, *P*-methoxy benzoic acid **2,** *E*/*Z*-caffeic acid **3**, 3,4'-dimethoxy quercetin 7- *O*-glucopyranoside **4,** 3,6,4'-trimethoxy quercetin 7-*O*-glucopyuranoside **5**, quercetin 7-*O*-neohespridoside **6**, while phytochemical investigation of the *n*-butanol extract yielded a new 23-hydroxy-3- α -*O*- α-L-rhamnopyranosyl-(1'''→4'')-*O*-[β-D-(*E*-6′′′-*O*-caffeoyl)- glucopyranoside] -oxy- olean-12-en-28-oic **12**, along with β-amyrine **7**, oleanolic acid **8**, hedragenin **9**, β- amyrine-3-*O*-glucopyranoside **10**, and 23-hydroxy-3α-(*O*-sulfate-oxy)- olean-12-en-28-oic acid *O*-α-L-rhamnopyranosyl - (1'''→4'') - *O*-β-D- glucopyranosyl (1''→6')- *O*-β-D-glucopyranoside ester **11**. The structures of the isolated compounds were determined by spectroscopic methods (UV, ESI-MS, ^{1}H-, ^{13}C-NMR, ^{1}H-^{1}H COSY, HSQC, and HMBC). Moreover the radical scavenging activity of the ethyl acetate extract major compounds (**3**, **4** and **6**) was measured by (DPPH) and phosphomolybdenum methods. Cytotoxic screening of the *n*-butanol extract major isolates (**8**, **9** and **12**) was carried out on brine shrimps and Hep-G2 cell line as well.

Keywords: *Vitex trifolia*, Antioxidant, Cytotoxic, Brine shrimp, DDPH, Hep-G2.

[I] NTRODUCTION

The *Vitex* genus, family Verbenaceae includes approximately 270 known species of shrubs and trees; in tropical and sub-tropical regions, although species may be found in temperate zones. *Vitex trifolia* L. is a deciduous shrub which commonly known as common chaste tree. *Vitex* species are commonly used in traditional medicine to treat a wide range of ailments, such as depression, venereal diseases, asthma, allergy, wounds, skin diseases, snake bite, body pains[1], diarrhea, gastrointestinal diseases[2-4] . Many plants of the genus *Vitex* are used for their interesting biological activities such as treatment of cough, wound healing, larvicidal, anti HIV, anticancer, trypanocidal, antibacterial and antipyretic [5-12]. *V. trifolia* has been used as an anti-inflammatory [13], antibacterial[14,11], antipyretic[12], hepatoprotective[15], trypanosidal, and sedative for headache, rheumatism, and the common cold in Asian countries[10], It's also used for the treatment of febrifuge, fever and amenorrhea[16]. The plant is

known to possess various active constituents viz., essential oil[17], halimane-type diterpenes, vitetrifolins[18] Flavonoids[19,20], Chalcones[21], Triterpens[22,23], Lignans [24,25], Iridoides[26 ,27] , and ecdysteroids[28]. As part of a our program to assess the chemical and biological diversity of genus *Vitex* found in Egypt[29], we carried out a novel trial to compare the phytochemical constituents of the ethyl acetate and *n*-butanol extracts of *V. trifolia* leaves, and investigates the major constituents of the ethyl acetate extract as antioxidant. Investigation of the *n*-butanol extract major constituents as cytotoxic agents against brine shrimp and HepG2 cell lines was carried out as well.

MATERIALS AND METHODS

2.1. Equipments

NMR spectra for known compounds were recorded at 300 (^{1}H) and 75 MHz (^{13}C) on a Varian Mercury 300, while new compound was recorded at 500 (^{1}H) and 125 MHz (^{13}C) on a JEOL GX-500 NMR spectrometer and δ values are reported in ppm relative to TMS in the convenient solvent. ESI-MS analyses were measured on a Finnigan LCQ deca LC/MS and double focusing sector field MAT 90 MS spectrometers (Finnigan, Bremen, Germany). UV analyses of pure samples were recorded, separately, in MeOH solns. on a Shimadzu UV 240 spectrophotometer.

2.2. Plant material

Leaves of *Vitex trifolia* were collected from El-Orman Garden; the plant was authenticated by Mohamed El-Kassas, Professors of Taxonomy, Department of Botany, Faculty of Science, Cairo University, Giza, Egypt. Voucher specimens (Reg. No.: V-XI) are kept in the herbarium of Medicinal Chemistry Department, Theodor Bilharz Research Institute.

2.3. Antioxidant activity

2.3.1. DPPH radical scavenging activity

The ability of *V. trifolia* EtOAc and *n*-BuOH extracts as well as the isolated pure compounds (**3**,**4**,**6**) to scavenge DPPH radicals was evaluated according to the procedure described by Molyneuxand (2004)[30] . To 1 ml of each sample at a concentration of 100µg/ml was mixed with 1ml of 0.1m M DPPH in methanol. The mixture was then shaken and left for 20 min. at room temperature in the dark. The absorbance was measured at 517 nm using a spectrophotometer. Ascorbic acid was used as a reference standard. All experimental were carried out in triplicate. The activity of each sample was expressed as percentage DPPH radical scavenging relative to the control using the following equation: *DPPH radical scavenging % = [(control absorbance - sample absorbance)/control absorbance] × 100.* The scavenging effect (antioxidant activity) of each sample was expressed as SC_{50} which is the concentration of the extract required for 50% scavenging of DPPH radicals compared with that of the standard ascorbic acid (table II).

2.3.2. Evaluation of total antioxidant capacity

An aliquot of 0.1 ml of sample solution was combined in an Eppendorf tube with 1 ml of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate). The tubes were capped and incubated in a thermal block at 95°C for 90 min. After the samples had cooled to room temperature, the absorbance of the aqueous solution of each was measured at 695 nm against a blank. A typical blank solution contained 1 ml of reagent solution and the appropriate volume of the same solvent used for the sample, and it was incubated under the same conditions as the rest of the sample [31].

All chemicals and solvents used in the two previous antioxidant methods were of analytical grade. DPPH (2, 2-diphenyl 1-picrylhydrazyl) were purchased from Sigma Co. (USA), while the other reagents sodium phosphate, ammonium molebdate, ascorbic acid and sulfuric acid were purchased from Merk Chemical Co. (Germany).

2.4. Cytotoxicity assay

2.4.1. Brine shrimp lethality bioassay

Eggs of *Artemia salina* were allowed to hatch into their larvae [32]. The dried ethyl acetate, *n*-butanol

extracts and compounds **8**, **9** and **12** were separately dissolved in distilled water to give four assay concentrations (1000, 500, 100 and 10 mg mL-1). Solubility was aided by Tween 80 and each dose was examined in triplicate. Potassium dichromate was used as a reference drug and dissolved in sea water, to obtain concentrations of 1000, 100 and 10 μg mL-1. Assays were performed in test tubes with ten larvae each and the final volumes were adjusted to 5 mL sea salt soln. immediately after adding the shrimps. After 24 h, the number of surviving shrimps at each dose was recorded. The LC50 values were calculated by the use of the Instate computer program.

2.4.2. Measurement of potential cytotoxicity by SRB assay

Potential cytotoxicity of the *V. trifolia* leaves ethyl acetate, *n*-butanol extracts and the isolated compounds **8**, **9** and **12** were tested at the National Cancer Institute, Egypt using the method of [33]. Cells were plated in a 96-well plate (104 cells/well) for 24 h before treatment to allow the attachment of cells to the wall of the plate. Different concentrations of the fractions under investigation (0, 1, 2.5, 5 and 10 μg/mL) were added to the cell monolayer. Triplicate wells were prepared for each individual dose and they were incubated for 48 h at 37 ºC in 5% CO_2. After 48 h cells were fixed, washed and stained with sulforhodamine B stain. Excess stain was washed with acetic acid and attached stain was recovered with Tris-EDTA buffer and the color intensity was measured in an ELISA reader. The survival curve of the tumor cell line was plotted for each tested fraction [33].

2.5. Extraction and isolation

The air-dried powdered leaves of *V. trifolia* (800 g) were extracted with ethanol (5 x3L) at room temperature. The solvent was removed under reduced pressure. The obtained dry ethanol extract (60 g) was suspended in H_2O (400 ml), extracted with petroleum ether (250 ml x 3), ethyl acetate (250 ml x 3) followed by *n*-butanol (150 ml x3), successively. 2 D-PC and TLC analysis proved that the petroleum ether extract is free from polyphenols and flavonoids are nearly taken by ethyl acetate, while saponins were concentrated in the *n*- butanol extract. Both EtOAc and *n* -BuOH extracts were washed with 100 mL distilled water (x3) and then concentrated to dryness by removing the solvent in a rotary evaporator. Concentrated ethyl acetate soluble phase (20 g) was subjected to column chromatography (3 x 110 cm, 200 g) on silica gel 60 (28-200 mesh) and eluted with petroleum ether (60-80 ºC), petroleum ether/$CHCl_3$ and then $CHCl_3$/MeOH mixtures for gradual increase of the polarity up to pure MeOH. On the basis of comparative TLC and PC with the use of UV light, 10% H_2SO_4 in ethanol and Naturstoff spray reagents, the individual 25 fractions (250 ml each) were collected into five collective fractions (I-V). Fraction 1 and II gave oily dark brown material of no phenolic character. Fraction III (20% MeOH in $CHCl_3$, 220 mg), which exhibited a major component on TLC, was crystallized in heat from CH_3Cl to give needle crystals of **1** (50 mg). Two major spots were detected in fraction IV (30-50 % MeOH in $CHCl_3$, 5.6 g), which was subjected to repeated CC on Sephadex LH- 20 with 20-60% aqueous EtOH as an eluent. This fractionation resulted in pure **2** (40 mg) and **3** (65 mg). Fraction V (50-100 % MeOH in $CHCl_3$, 8.5 g) was fractionated on a Sephadex column 30 - 60% aqueous EtOH to give three main subfractions. Each was then separately chromatographed on a Sephadex column with MeOH, whereby pure **4** (68 mg), **5** (29 mg) and **6** (85 mg) were isolated from the 1^{st}-3^{rd} subfractions, respectively. The *n*-butanol extract (17 g) was subjected to CC over silica gel with $CHCl_3$ - MeOH [1:0 (1L), 1:1 (3L) and 0:1(2L)]. Four fractions (A - D) were obtained according to the differences in composition indicated by TLC analyses. Fr.A ($CHCl_3$- MeOH, 8:2, 3 g) was purified by CC over silica gel, eluting with MeOH-EtOAc, (2:8), to give two main subfractions. The first subfraction was then separately chromatographed on Sephadex with

MeOH as eluant, whereby a pure sample of **7 (**25 mg) was isolated, which was the major component of this subfraction. Crude **8** was crystallized from Fr.B ($CHCl_3$-MeOH, 7:3, 5 g) and purified by repeated crystallization from MeOH to yield a pure sample of **8** (80 mg). Fr.C ($CHCl_3$-MeOH, 1:1, 3 g) was rechromatographed on Sephadex LH-20 with MeOH to afford a pure sample of **9** (90 mg). Fr.D ($CHCl_3$-MeOH, 3:7, 3 g), (a mixture of **10, 11** and **12**) was rechromatographed on a silica gel column eluting with $CHCl_3$-MeOH-H_2O (3:7:0.1) to give pure samples of **10** (20 mg), **11** (30 mg) and **12** (95 mg)**.** All separation processes were followed up by Co-TLC with solvent systems: S3 (MeOH/$CHCl_3$, 2 : 8), S4 (EtOAc/$CHCl_3$ 7 : 3), S5 (MeOH/EtOAc/ $CHCl_3$/H_2O 35:32:28:7) and S6 (*n*-BuOH/MeOH/ H_2O 4:1:1) or by 2D-PC and Comp-PC using Whatman No. 1 paper with S1 [*n*-BuOH/HOAc/H2O (4 :1: 5, top layer)] and S2(15% aqueous HOAc) solvents.

2.6. 23-hydroxy-3- α -O- α-L-rhamnopyranosyl-(1‴→4″)-O-[β-D- (E-6‴-O-caffeoyl) - glucopyranoside] -oxy- olean-12-en-28-oic (12)

Off Wight amorphous powder, Rf = o.55 (S5) and 0.59 (S6). – Negative ESI-MS: *m/z* 941. 44 $[M-H]^-$, *m/z* 769.46 $[M-H\text{-deoxycaffeoyl}]^-$, *m/z*, 633.41 [M-H- deoxycaffeoyl-146]-, *m/z* 471.428 [M-H-deoxycaffeoyl-146-162]$^-$ = $[aglycone-H]^-$.^{1}H and ^{13}C: (Table 1).

RESULTS

3.1. Identification of the isolated compounds

V. trifolia leaves ethanol extract was successively extracted with petroleum ether, Ethyl acetate and *n*-butanol. Both extracts (ethyl acetate and *n*-BuOH) underwent, initially, column chromatography over silica gel. The substances were purified over Sephadex LH-20 and identified through ^{1}H and ^{13}C NMR (1D and 2D) spectra. This identification was supported by comparison with authentic samples on TLC, by comparison of spectral data with those reported in the literature and with those obtained from the authentic samples. The structures of isolated compounds are presented in (figure1) and were identified as: stigmasterol **1**[34], *P*-methoxy benzoic acid **2**[35] **,** *E*/*Z*-caffeic acid **3**[36], 3,4'-dimethoxy quercetin 7-*O*-glucopyranoside **4** 3,6,4'-trimethoxy quercetin 7-*O*-glucopyuranoside **5**, quercetin 7-*O*-neohesperidoside **6**[37-39], β-amyrine **7** [40], oleanolic acid **8**, hedragenin **9**[41], β- amyrine-3-*O*-glucopyranoside **10**[40], and 23-hydroxy-3-(*O*-sulfate-oxy)- olean-12-en-28-oic acid *O*-α-L-rhamnopyranosyl - (1‴→4″) - *O*-β-D-glucopyranosyl (1″→6′)- *O*-β-D-glucopyranoside ester **11**[42].

Compound **12**, was isolated as an off-white amorphous powder. Upon complete acid hydrolysis of **12**, glucose and rhamnose were detected in the aqueous phase, while hedragenin was identified along with caffeic acid in the organic phase (Co-TLC and PC with the authentic samples). Its negative ESI-MS showed a molecular ion peak at *m/z* 941.44 $[M-H]^-$. In addition, it gave diagnostic fragment ion peaks at *m/z* 779.46 $[M-H\text{-deoxycaffeoyl}]^-$, 761.3 [M-H – deoxycaffeoyl-$H_2O]^-$, m/z 633.41 $[M-H\text{-deoxycaffeoyl-146}]^-$ (loss of a rhamnosyl) and 471.4280 $[aglycone-H]^-$, corresponding to the loss of a glucoside from the last fragment. These data, together with the acid hydrolysis products, were compatible with a structure of hedragenin caffeoyl rhamnoglucoside. The resonances due to six sp^3 methyl carbons at δ 13.6, 15.9, 17.5, 25.2, 23.3, and 32.9, a primary carbinol at δ 67.7 and two sp2 carbons at δ 121.8 and 143.5 in the ^{13}C NMR spectrum of **12**, coupled with the corresponding information from the ^{1}H NMR spectrum [6 methyl proton singlets, two doublets, one proton each, at δ 4.26 and 3.73 (CH_2-23), and a broad singlet at δ 5.49 of H-12] confirmed the aglycone moiety as 3,23-dihydroxyolean-12-en-28-oic acid. The relative upfield location of C-5 at δ 46.6 ($\Delta \sim +5$ ppm) was also an evidence for the γ-effect of the carbinol-OH-23. The resonances of C-3 at δ 80.7, and C-28 at δ 179.8 were characteristic of a monodesmoside aglycone with 3α-hydroxyl, where H-3 was observed as a broad singlet at δ

4.29. All assigned ^{1}H and ^{13}C-resonances of the aglycone moiety were confirmed by HMQC and HMBC correlation spectroscopy (Table 1) [43]. An *E*-caffeoyl moiety was deduced in the structure of **12** through an AX spin coupling system of two *E*-olefinic doublets at 7.41 and 6.18 (each, *d*, *J* = 16 Hz) assignable to H-7''' and H-8''', respectively, along with an ABX spin coupling system of its phenyl protons at 7.04 (*d*, *J* = 1.8 Hz), 6.97 (*brd*, *J* = 8.0 Hz) and 6.78 (*d*, *J* = 8.0 Hz) of H-2''', H-6'''and H-5''', respectively in ^{1}HNMR spectrum. Additional evidence for the caffeoyl moiety was deduced from the characteristic carbonyl signal at 167.7 in the ^{13}CNMR spectrum. Two anomeric proton signals were assigned at δ 5.05 (*J* = 7.0 Hz, β -glucosyl), 6.26 (*br s*, α-rhamnosyl) in the ^{1}H NMR spectrum through their direct one bond coupling in the HMQC spectrum with their own anomeric carbon signals at δ 104.1 and 101.3, respectively (Table 1). The sugar moieties were deduced to have α-$^{1}C_4$-and β-$^{4}C_1$-pyranose stereostructure in the case of the rhamnosyl and glucoside moieties, respectively, on the basis of the *J*-values of the anomeric protons and δ-values of their ^{13}C NMR resonances (Table 1). The sugar aglycone linkage was deduced from the downfield location of C-3 at 80.7 and the long range three bond HMBC correlation between H-1' at δ 5.05 (glucopyranosyl) and C-3 aglycone. The inter glycosedic linkage at C-3 established as 3- α- *O*-α-L-rhamnopyranosyl-(1"→4')-*O*- β- D-glucopyranoside from the downfield location of C-4' glucose at 78.5. This evidence was further confirmed from the observation of the three bond correlation peak between H-1" of rhamnose proton signal at δ 6.27 and the C-4 signal of glucose in the HMBC spectrum. In addition, the esterification of the *E*-caffeoyl group to C6' was deduced from the characteristic downfield shift of both the H-6' methylene protons and C-6' and the upfield signal of C-5' relative to those of unsubstituted glucose[44,45], together with the long range correlation between this protons and the carbonyl carbon of the caffeoyl moiety at δ 167.7. All other ^{1}H and ^{13}C-resonances were assigned with the aid of HMQC and HMBC-correlation peaks and comparison with the corresponding data of structurally related compounds [46,47]. Therefore, **12** was finally identified as 23-hydroxy-3-*O*- α-L-rhamnopyranosyl-(1'''→4")-*O*-[β-D- (*E*-6‴-*O*-caffeoyl) - glucopyranoside] -oxy- olean-12-en-28-oic.

Table I. ^{1}H, ^{13}C NMR and HMBC spectral data of **12** aglycone and sugar moieties (500/125 MHz, Pyredine-d_5)

No	H	C	No	H	C	HMBC
1		38.9	**1'**	5.05 *d* (67)	104.1	C-3, 3'
2		25.18	**2'**	4.58 *brd* (9,6)	72.9	C-4'
3	4.29 *t-like*	80.7	**3'**	4.10 *dd* (8.5,3.5)	74.1	C-1', 5'
4		42.4	**4'**	4.75 *t-like* (10.0)	78.5	C-2', 6', 1"
5		46.6	**5'**	3.69 *m*	74.1	C-3'
6		17.5	**6' a**	4.70 *brd* (12)	65.4	C-4', 9'''
			6' b	4.50*		
7		32.8	**1''**	6.27 *brs*	101.3	C-4', 3"
8		39.1	**2''**	4.25 *brd**	72.0	C-4"
9		47.8	**3''**	4.65 *dd* (9.5,3.5)	72.2	C-1", 5"
10		36.5	**4''**	4.29 *t-like* (10.5)	73.8	C-2", 6"
11		23.9	**5''**	4.40 *m*	69.3	C-3"
12	5.49 *br s*	122.2	**6''**	1.62 *d* (6.5)	18.9	C-4"
13		144.4	**1'''**		125.3	
14		42.4	**2'''**	7.04 *d*, *J* = 2	115.7	C-4''', 6''', 7'''

15		27.6	**3'''**		145.1	
16		23.4	**4'''**		149.0	
17		47.4	**5'''**	6.78 *d*, *J* = 8	122.2	C-3''', 1'''
18	3.27 *dd* (13.5, 3.5)	41.9	**6'''**	6.97 *brd*, *J* = 8	122.8	C-2''', 4''', 7'''
19		46.6	**7'''**	7.41 *d*, *J* = 16	144.5	C-9''', 6''', 2'''
20		31.0	**8'''**	6.18 *d*, *J* = 16	113.4	C-1'''
21		33.5	**9'''**		167.7	
22		32.5				
23	4.26 *d* (10.5) 3.73 *d* (10.5)	63.5				
24	1.02 *s*	13.6				
25	0.99 *s*	15.9				
26	1.09 *s*	17.5				
27	1.21 *s*	25.2				
28		179.8				
29	0.90 *s*	32.9				
30	0.93 *s*	23.3				

*Unresolved proton resonances, δ in ppm and *J* values (Hz), are given in parentheses. All carbon and proton resonances were assigned on the basis of 2D (^{1}H-^{1}H COSY, HSQC and HMBC).

Table II. DPPH radical scavenging activity and total antioxidant capacity of *V. trifolia* EtOAc, *n*-BuOH leaves extracts and compounds **3**, **4**, and **6**.

Sample	**DPPH SC_{50} [µg/ml]**	**Total antioxidant capacity (mg AAE /g ext.)**
Compound 3	3.72 ± 0.12	905.21 ± 0.37
Compound 4	13.19 ± 0.20	620.35 ± 2.31
Compound 6	29.48 ± 0.47	355.71 ± 1.36
MeOH	34.69 ± 0.23	285.80 ± 3.56
***n*-BuOH**	271.28 ± 5.22	72.87 ± 0.45
Ascorbic acid	7.90 ± 0.20	----------

DISCUSSION

Antioxidative and radical scavenging properties of extracts and isolated phenolic were evaluated using 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical and phosphomolybdenum method (Table II). The DPPH is a purple stable organic radical with an absorption band in the range of 515-528 nm; when the radical accept an electron or a free radical species, the result is a visually noticeable discoloration from purple to yellow. Because the DPPH radical can accommodate many samples in a short period of time and is sensitive enough to detect active molecules at low concentrations, it has been extensively used to screen antiradical activities of *Vitex trifolia* extracts and pure isolates. The antioxidant results showed that the ethyl acetate extract is a strong scavenger of the artificial radical DPPH as indicated by the low SC50 value (Table II). Additionally, all the tested ethyl acetate compounds possessed a strong antioxidant capacity in a consequence order of **3** > **4** > **7**. The ethyl acetate extract contains a rich mixture of phenolics, and six major phenolic compounds belonging to three classes. The strong anti-oxidant activity of ethyl acetate extract may be attributed to the corresponding activities of the extract constituents. Presence of 2 sugar moieties in the flavonoid structure for compounds **6** resulted in a diminution of the antioxidant activity.

The high antioxidant activity of **3** may be attributed to the *E*/*Z* caffeic acid moieties and the multi hydroxyl groups [48].. A synergistic effect due to the other phenolic substances present in ethyl acetate extract can explain this result. According to Hsu *et al.* (2005) [49], the combination of different compounds at a certain effective dosage can strengthen or weaken their scavenging free radicals. Such synergistic effects were detected in different antioxidants including phenolic acids, flavonoids and herbal medicines.

Among the tested extracts, the *n*-butanol extract of *V. trifolia* displayed a strong cytotoxic activity against brine shrimp larva of *Artemia salina* and Hep-G2 with LC50 and IC50 values of 75, 14.3, respectively. This prompted us to perform chemical investigation to identify the bioactive compounds responsible for the cytotoxicity of the extract which led to the isolation of six triterpenoids. We found that, compounds **8**, **9** and **12** possessed a strong cytotoxic activity in brine shrimp lithely bioassay with LC50 = 66.4, 50, and 41 mg/L, respectively (fig. II). As well as they have cytotoxic activity on Hep-G2 cell line with IC50 = 9.6, 9.0, and 7.5, respectively (Fig III). We found that oleane type triterpenoids accumulated in the *n*-butanol extract with **8**, **9** and **12** being may be responsible for cytotoxicity. Upon a closer examination to relate the bioactivity with that of the compounds' chemical structures revealed all tested compounds possessed a free hydroxyl moiety. Such a feature is also apparent among the biologically active oleane type triterpenoids such as oleanolic acid and hedragenin. Oleanolic acid is a pentacyclic triterpene existing widely in the plant kingdom and was reported to have anti-cancer and other interesting bioactivity [50]. Hence, we strongly believe this could be an important pharmacophore to be considered for cytotoxicity in this class of compounds. Comparison of the structures of the compounds **8** and **9** bear close resemblance to each other. The only notable difference is the additional hydroxyl group in **9**, which might have been derived biosynthetically from the hydroxylation of **8**. In this investigation, **12** have been identified as the most promising compound due to the additional *E*-caffeoyl group which may enhance its cytotoxic properties to brine brine shrimp lithely bioassay and HepG2 cells as well. Compounds **7**, **10** and **11** were not tested here due the little amount isolated [50] .

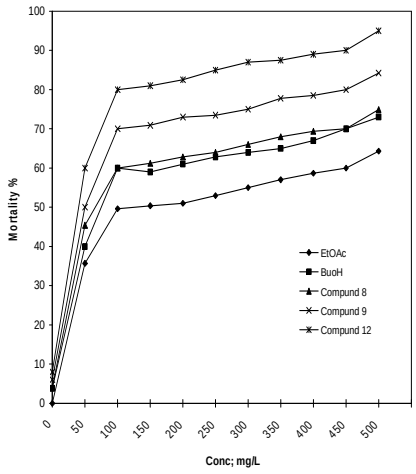

Fig. II- The cytotoxic activity of *V. trifolia* BuOH, EtOAc extracts and compounds **8**, **9** and **12** against brine shrimp (*A. salina*)

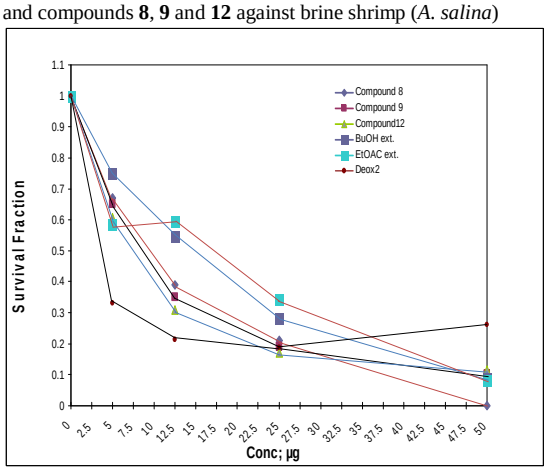

Fig.III The cytotoxic activity of *V. trifolia* EtOAc and BuOH extracts and compounds **8**, **9** and **12** against HepG2 cell line.

CONCLUSION

The high potency of *V. trifolia* ethyl acetate extract in free radical scavenging is shared by few other plants and several studies have shown that hepatoprotective effects are associated with antioxidant rich extracts [51]. Hsu *et al.*, (2005) [49] have described the activity of flavonoids, such as

quercetin, rutin and phenolic acids, to prevent inflammation. Thus, the potency of the *V. trifolia* ethyl acetate extract in free radical scavenging activity, which may be associated with the isolated phenolics, could provide a scientific basis for the health benefits claimed for ethyl acetate extract of this plant in folk medicine and warrant further studies to assess the potential of this plant as effective natural remedies. Furthermore, we are continuing to find new tripertenes from other herbs. It is possible that we can get promising triterpenes for cancer therapy either by isolating them from herbs or by structure-modification from natural triterpenes. Understanding of the cytotoxic mechanism of triterpenes will be helpful to the study and the use of possible promising tripertenes.

REFERENCES

[1] Neuwinger HD. [2000] African Traditional Medicine: A Dictionary of plant use and Applications. *Medpharm Scientific Publishers, Stuttgart*.

[2] Argueta A, Cano LM, and Rodarte ME. [1994] Atlas de las Plantas de la Medicina Tradicional I y III.Instituto Nacional Indigenista, *Mexico*, pp. 537–538 Also pp. 1200, 1397

[3] Ahmad FB, and Holdsworth DK. [1995] Traditional medicinal plants of Sabah State Malaysia. Part III. Int. *J. Pharmacognosy 33, 262–264*.

[4] Bajpai A, Ojha JK, and Sant HR. [1995] Medicobotany of the Varanisi District Uttar Pradesh, India. Int. *J. Pharmacognosy 33, 172–176.*

[5-] Aranda E, Hernandez MM, Heraso C, Villarreal ML, and Arispuro IV. [1999] Biological activities of crude plant extracts from *Vitex trifolia* L. *j. Ethnopharmacol. 67, 37-44*.

[6] Kannathasan K, Senthilkumar A, Chandrasekaran M, and Venkatesalu V. [2007] Differential larvicidal efficacy of four species of *Vitex* against Culexquinquefasciatus larvae. Parasitol. Res. *101, 1721-1723*.

[7] Manjunatha B K, Vidya SM, Krishna V, Mankani KL, Singh SD, and Manohara YN. [2007] Comparative evaluation of wound healing potency of *Vitex trifolia* L. and *Vitex altissima* L. *Phytother. Res. 21, 457-461*.

[8] Li WX, Cui CB, Cai B, Wang HY, and Yao XS. [2005] Flavonoids from *Vitex trifolia* L. inhibit cell cycle progression at G2/M phase and induce apoptosis in mammalian cancer cells. *J. Asian Nat. Prod. Res. 7, 615- 626.*

[9] Woradulayapinij W, Soonthornchareonnon N, and Wiwat C. [2005] *In vitro* HIV type 1 reverse transcriptase inhibitory activities of Thai medicinal plants and *Canna indica* L. rhizomes. *J. Ethnopharmacol. 101*: (*1-3*) *84- 89*.

[10] Kiuchi F, Matsuo K, Ito M, Qui TK, and Honda G. [2004] New norditerpenoids with trypanocidal activity from *Vitex trifolia. Chem. Pharm. Bill., 52, 1492-1494.*

[11] Hossain MM, Paul N, Sohrab MH, Rahman E, and Rashid MA. [2001] Antibacterial activity of *Vitex trifolia. Fitoterapia 72, 695- 697*.

[12] Ikram M, Khattak SG, and Gilani SN, [1987] Antipyretic studies on some indigenous Pakistani medicinal plants: II. *J. Ethnopharmacol. 19, 185-192*.

[13] Kimura T. [1996] International Collation of Traditional and Folk Medicine. *World Scientific, Singapore 1, 66 - 67*.

[14] Hernandez MM, Heraso C, Villarreal ML, Vargas-Arispuro I, and Aranda E. [1999] Biological activity of crude plant extracts from *Vitex trifolia* L. (Verbenaceae). *J. Ethnopharmacol. 67, 37 -44*.

[15] Anandan R, Jayakar B, Karar B, Babuji S, Manavalan R, Kumar R S. [2009] Effect of ethanol extract of flowers of *vitex trifolia* linn. on CCL(4) induced hepatic injury in rats**.** Vinayaka Mission's College of Pharmacy, Salem-636 008. Tamilnadu, India.

[16] Chopra RN, Nayar SL, and Chopra IC. [1956] Glossary of Indian medicinal plants. *New Delhi, CSIR.*

[17] Mallavarapu GR, Ramesh S, Kaul RN, Bhattacharya AK, and Rajeswara Rao BR. [1994] Composition of the essential oil of the leaves of *Vitex negundo*. *Planta Med. 60, 583-584.*

[18] Ono, Ito Y, and Nohara T. [2001] Four new halimane-type diterpenes, vitetrifolins D-G, from the fruit of *Vitex trifolia*. *Chem. Pharm. Bull. 49, 1220-1221*.

[19] Sathiamoorthy B, Gupta P, Kumar M, Ashok K, Chaturvedi, Shukla PK, and Maurya R. [2007]. New antifungal flavonoid glycoside from *Vitex negundo*. Bioorg. Med. *Chem. Lett. 17, 239-242*.

[20] Bhargava SK. [1989] Antiandrogenic effects of flavonoid-rich fraction of *Vitex negundo* seeds: a histological and biochemical study in dogs. *J. Ethnopharmacol. 27, 327-339.*

[21] Thuy T.T., Porzel A., Ripperger H., Sung T.V. and Adam G. (1998) Chalcones and ecdysteroids from *Vitex leptobotrys*. Phytochemistry 49, 2603-2605.

[22] Chandramu C, Manohar RD, Krupadanam DG, and Dashavantha RV. [2003] Isolation,

characterization and biological activity of betulinic acid and ursolic acid from *Vitex negundo* L. *Phytother. Res. 17, 129-134*.

[23] Chawla AS, Sharma AK, Handa SS, and Dhar KL. [1992] Chemical investigation and anti-inflammatory activity of *Vitex negundo* seeds. *J. Nat. Prod. 55, 163-167*.

[24] Azhar-Ul-Haq, Malik A, Khan MTH, Khan SB, Anwar-Ul-Haq, Ahmad A and Choudhary MI. [2006] Tyrosinase inhibitory lignans from the methanol extract of the roots of *Vitex negundo* Linn. and their structure–activity relationship *Phytomedicine **13**, 255-260.*

[25] Kawazoe K, Yutani A, and Takaishi Y. [1999] Aryl naphthalenes norlignanes from *Vitex rotundifolia. Phytochemistry 52, 1657-1659.*

[26] Kuruüzüm-Uz A_, Ströch_K_, Demirezer LÖ_, and Zeeck **A.** [2003] Glucosides from *Vitex agnus-castus. Phytochemistry 63, 959-964*

.[27] Sridhar C, Subbaraju GV, Venkateswarlu Y, and Venugopal RT. [2004] New acylated iridoide glucoside from *Vitex altissima. J. Nat. prod. 67, 2012-2016.*

[29] EL-Sayed MM., EL-Hashash M., Mohamed MA., and Korany TM. [2011] Cytotoxic activity of *Vitex trifolia* purpurea different extracts. *J. Egypt. Soc. Parasitol, 41, 409-416.*

[30] Molyneux P. (2004) The use of the stable free radical diphenylpicrylhydrazyl (DPPH) for estimating antioxidant activity. *Songklanakarin J. Sci. Technol. **26**: 211-219.*

[31] Prieto P, Pineda M and Aguilar M. [1999] Spectrophotometric quantization of antioxidant capacity through the formation of a phosphomolybdenum complex: Specific application to the determination of vitamin E1. *Analytical Biochemistry 269**,** 337–341.*

[32] Fatope M , Ibrahim H, and Takeda Y. [1993] Screening of higher plants reputed as pesticides using the brine shrimp lethality assay. Int. *J. Pharmacog. 31, 250-254.*

[33] Skehan P, Storeng R, Scudiero D, Monks A, McMahon J, Vistica D, Warren JT, Bokesch H, Kenney S, and Boyd MR. [1990] New colorimetric cytotoxicity assay for anticancer drug screening. *J. Nat. Cancer Inst., 82, 1107-1112.*

[34] Habib MR, Nikkon F, Rahman M, Haque ME, and Karim MR. [2007] Isolation of stigmasterol and beta-sitosterol from methanolic extract of root bark of *Calotropis gigantea* (Linn). *Pak J. Biol. Sci. 10, 4174-6.*

[35] Gadgoli C, and Mishra SH. [1999] Antihepatotoxic activity of *p*-methoxy benzoic acid from Capparis spinosa. *J. Ethnopharmacol. 66, 187-192.*

[36] Lu Y, and Foo L Y. [2002] Polyphenolics of salvia -a review. *Phytochemistry 59, 114 – 117.*

[37] Agrawal P K, [1989] Studies in organic chemistry 39, ^{13}C NMR of flavonoids. In: Flavonoid Glycosides (Agrawal P K, and Bansal M C, eds.). Elsevier Science, New York, pp. 283 – 310.

[38] Harborne JB. [1984] Phytochemical Methods: A Guide to Modern Techniques of Plant analysis, 2nd ed. Chapman & Hall Ltd, London, pp. 49 – 50.

[39] Harborne JB and Mabry T J. [1982] The flavonoids: Advances in research. In: ^{13}C NMR Spectroscopy of Flavonoids (Markham K. R., and Mohanchari V., eds.). Chapman & Hall Ltd, University Press, Cambridge, London, pp. 119 – 132

.[40] Amico V, Barresi V, Condorelli D, Spatafora C, Tringali C. [2006] Antiproliferative terpenoids from almond hulls (Prunus dulcis): identification and structure-activity relationships. *J. Agric. Food Chem. 54, 810-814.*

[41] Hassan SE, Abdel Rahman EH, and Abd El Monem AZ. [2010] Molluscicidal activity of butanol fraction of *Meryta denhamii* flowers against *Lymnaea natalensis* and *Biomphalaria alexandrina*. *Global Veterinaria 4 , 15-21.*

[42] Elias R., Lanza AMD, Vidalollivier E, Blansard G, Faure R, and Babadjamian A. [1991] Triterpenoid saponins from the leaves of *hedera helix. J. Nat. Prod. 54, 98-103.*

[43] Morota T, Xin C.J, Sasaki H, Qin WZ, Sugama K, Mieo KL, Yoshino T,Xu LH, Marumo M, and Yang BH. [1995] Triterpenes from *Tripterigium wilfordii. Phytochemistry 39, 1153-1157.*

[44] Hirobe C, Qiao ZS, Takeya K, and Itokawa H. [1997] Cytotoxic flavonoids from *Vitex Agnus*-Castus. *Phytochemistry 46, 521-524.*

[45] Kanchanapoom T, Noiarsa P, Tiengtham P, Otsuka H, and Ruchirawat S. [2005] Acetophenone diglycosides from *Erythroxylum cambodianum. Chem. Pharm. Bull. 53, 579-581.*

[46] Crublet M.L., Long C., Sevenet T, Hadi HA and Lavaud C. [2003] Acylated flavonol glycosides from leaves of *Planchoniagrandis. Phytochemistry 64**,** 589-594.*

[47] Crublet ML, Pouny I, Delaude C, and Lavaud C. [2002] Acylated Triterpenoid saponins from the stem bark of *Foetidia africana. J. Nat. Prod. 65**,** 1560-1567.*

[48] Nagao T, Abe F, and Okabe H. [2001] Antiproliferative constituents in the plants 7. Leaves of *Clerodendron bungei* and leaves and bark of *C. trichotomum. Biol. Pharm. Bull. 24, 1338-1341.*

[49] Hsu FH, Houng JY, Chang CL, Wu CC, Chang FR and Wu YC. [2005] Antioxidant activity , cytotoxicity, and DNA information of *glossogyne tenuifolia. J. Agric. Food Chem., 53, 6117- 6125.*

[50] Sultana N, and Ata A. [2008] Oleanolic acid and related derivatives as important compounds. *J. Enzyme. Inhib. Med. Chem.*, ***23***, *739-756*.

[51] Harish R, and Shivanandappa T. [2006] Antioxidant activity and hepatoprotective potential of Phyllanthusniruri. *Food Chem*. *95, 180*.

Fig.1 Structures of isolated compounds