Phenolic and biological activities of *Vitex trifolia* aerials parts

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Abstract: *Vitex trifolia* (Family: Verbenaceae) grows as an herbaceous plant in Egypt. Most of the Verbenaceae plants contain phenolic compounds which have important pharmacologically properties. *V. trifolia* aerial parts methanol extract was fractionated by repeated column chromatographic separation to obtain a phenyl ethanoid which isolated for the first time from the genus *Vitex* along with five phenolic metabolites. The identification and structure elucidation of the isolated compounds were based on chemical and spectral data (UV, ESI-MS, ^{1}HNMR, ^{13}CNMR, HMQC and HMBC) and also by direct comparison with respectively published data. Cytotoxic activities of the plant aerial parts extracts (methanol, ethyl acetate and chloroform) have been studied herein for the first time using, brine shrimp bioassay method (LC_{50} values 140 mg ml^{-1}, 165 mg ml^{-1} and 180 mg ml^{-1}, respectively). Total phenolic content of different aerial parts extracts and the antioxidant activity of the major isolates has been studied as well. The radical scavenging activities of compounds 1 - 3 were measured and compound 1 have been identified as the most promising compound.

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1. Introduction

The *Vitex* genus family Verbenaceae is comprised of about 250 species of shrubs and trees; it's widely cultivated in warm temperate and subtropical regions. *Vitex trifolia* L. is a deciduous shrub which commonly known as common chaste tree (1) , the plant has been used as an anti-inflammatory (2), antibacterial (3,4), antipyretic (5), hepatoprotective (6), trypanosidal, and sedative for headache, rheumatism, and the common cold in Asian countries (7), It's also used for the treatment of cough, febrifuge, fever and amenorrhea (8). The plant is known to possess various active constituents viz., essential oil (9), halimane-type diterpenes, vitetrifolins (10,11), flavonoids (12,13), chalcones (14), triterpens (15,16), lignans (17,18), iridoides (19, 20), and ecdysteroids (21). In continuation of our studies on biologically active substances from medicinal plants, we reported herein the isolation and characterization of six phenolic compounds **1-6** from *V. trifolia* aerial parts methanol extract. In addition the cytotoxicity towards brine shrimps larva was determined for the three aerial parts extracts (methanol, chloroform and ethyl acetate). Total phenolic content of different aerial parts extracts and the antioxidant capacity of compounds **1 - 3** have been studied as well.

2. Material and Methods

Plant material

Aerial parts of *Vitex trifolia* were collected from shibin El-kanater Garden (El- kaluobia city, the North - East of Egypt) in January 2008, 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-1) are kept in the herbarium of City for Scientific Research and Technological Application.

Experimental

The NMR spectrum was recorded at 300 (^{1}H) and 75 (^{13}C) MH_Z. on a Varian Mercury 300 NMR spectrometers and δ values were reported as ppm relative to TMS in DMSO-d_6. ESI-MS analyses were measured on a Finnegan LCQ deco LC/MS and double focusing sector field MAT 90 MS spectrometers (Finnegan, Bremen, Germany). For column chromatography (CC), Sephadex LH-20 (Pharmacia, Uppsala, Sweden), microcrystalline cellulose (Merck, Darmstadt, Germany) and polyamide 6S (Riedel de Haën AG, Seelze, Germany) were used. For paper chromatography Whatman No. 1 sheets (England) were used. The spots were detected by spraying anisaldehyde-H_2SO_4 reagent followed by heating. UV spectra of pure samples were recorded, separately, as MeOH solutions and with different diagnostic UV shift reagents on a Shimadzu UV 240 spectrophotometer.

Extraction and isolation

The air-dried powdered aerial parts of *V. trifolia* (800 g) were exhaustively extracted under reflux with

hot 80% MeOH (3 x 5 L). After evaporation of the solvent, the dry residue obtained, was defatted with petroleum ether (60-80 °C) (3 x 1 L) to give residue which was extracted by suspended in water and extracted with $CHCl_3$ (3 x 500 mL) and ethyle acetate separately (3 X 500). The water soluble portions from $CHCl_3$ and EtOAc were collected and desalted by precipitation with excess MeOH. After evaporation of the MeOH, the extract (100 g) was suspended in H_2O and fractionated on a polyamide column (110 × 6 cm, 300 g) using a stepwise gradient from H_2O, H_2O/MeOH mixtures up to pure MeOH for elution. Based on chromatographic properties (Co-PC) with the use of UV light, 1% $FeCl_3$ and Naturstoff spray reagents for detection, the individual 65 fractions (150 ml) each were collected in 6 fractions (A – F). Fractions A and B (H_2O, 15 g) were exhibited free sugar characters. 2D – PC of fraction C (10% methanol, 20 g) showed a major blue spot, it was purified by a Sephadex LH – 20 column using 20% aqueous MeOH to afforded a pure sample of compound **1**. Five dark purple spots were detected on 2D – PC of fraction D (20-30%- MeOH, 20 g), which gave yellowish green fluorescence under UV after spraying with Na / PE. Separation of the individual major compounds was carried out on a cellulose column using H_2O / MeOH (7:3), this fractionation led to the isolation of chromatographically pure samples of compounds **2** and **3**. Fraction E (40-70% MeOH, 15 g) showed three dark purple minor spots on the 2D – Pc of this fraction, they were changed to orange and greenish yellow upon spraying with Naturstoff reagent. Application of fraction E on cellulose column using *n*-butanol saturated with water resulted in three subfractions. Final purification of this subfractions were carried out by successive fractionation on Sephadex LH-20 CC using H_2O / MeOH (60%), resulted in chromatographically minor samples of compounds. Two dark purple spots and a blue one were detected on 2D-PC of fraction F (80-100% MeOH, 20 g). It was rechromatographed on a Sephadex LH-20 column using 95% EtOH as an eluent to afford pure samples of compounds **4**, **5** and **6**. All separation processes were followed up by Comp. PC using Whatman No. 1 paper with (S_1) *n*-BuOH – HOAc – H_2O (4:1:5, upper layer) and (S_2) 15% aqueous HOAc as a solvent systems.

Determination of total phenolic content

Total phenolic content determined with the Folin-Ciocaltea (FC) (22). 100 µl of the aerial parts extract dissolved in methanol (equivalents to 1mg of extract) were mixed with 750 µl of (FC) reagent (previously diluted 10-fold with distilled water) and allowed to stand at 22°C for 5 min; 750 µl of Na_2CO_3 (60 g /l) solution were added to the mixture. After 90 min, the absorbance was measured at 750 nm. All determinations were carried out in triplicate. The standard curve of gallic acid was carried out and the equation Y = 2.4412 X was obtained. This equation was used to obtain the gallic acid equivalents (mg of gallic acid per mg dry weight extract). The results were represented in table 3

Antioxidant activity

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.

a- **DPPH radical scavenging activity**

The ability of *V. trifolia* pure compounds **1-3** to scavenge DPPH radicals was evaluated according to the procedure described by **Molyneux and Songklanakarin** (23). 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 4).

B-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 (24).

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).

Brine shrimp lethality bioassay

The cytotoxicity was measured by brine shrimp lethality bioassay (25), where eggs of brine shrimp *Artemia salina* (Atremia Inc., California) were allowed to hatch into their larvae under convenient conditions. Assays were performed in test tubes with ten larvae in each, and methanol, ethyl acetate, and chloroform extracts were separately dissolved in distilled water to give six assay concentrations (1000, 800, 600, 400, 200, 100 and, 10 mg /ml) solubility was aided by DMSO; the final volumes were adjusted to 5 ml sea salt solution immediately after adding the shrimp. After 24 h, the number of surviving shrimp at each dose was recorded. Each dose was examined in triplicate. The same steps carry out for potassium dichromate as positive control and DMSO as negative control. The values and the statistical analysis of the results were calculated and carried according to **Reed-Muench** method (26, 27)

The statistical analysis

Values were expressed as mean ± SEM. Statistical difference between groups were computed by one-way analysis of variance (ANOVA). Tukey-Kramer multiple comparison tests were used to compare between treated and control groups. The level of significance was accepted at $p < 0.05$. Statistical analysis was performed by the aid of Instat version 2 computer program (Graph pad software, Inc., San Diego, USA).

3. Result and Discussion

The defatted total aqueous methanol extract of *V. trifolia* aerial parts was fractionated by repeated column chromatographic separation to obtain compounds **1-6**. Based on chemical and physicochemical analyses, they were identified as 2-(3,4-dihydroxyphenyl)ethyl-2-*O*-[6-deoxy- *α*-L-mannopyranosyl-4′-(3,4-dihydroxyphenyl)- 2-propenoate]-β-D-glucopyranoside **1**, vitexin **2**, isovitexin **3**, luteolin-7-*O*-β- glucuronopyranoside **4**, quercitrin **5** and methyl caffeiate **6**.

Compound **1:** was expected to be a phenylethanoid on the basis of its chromatographic properties, UV spectral data and acid hydrolysis products. Its negative ESI-MS spectrum gave a dimeric adduct and molecular ion peaks at *m/z* = 1247.0 and 623.1 assignable to $[2M-H]^-$ and $[M-H]^-$, respectively, together with three fragment ions at 461.1, 179.1 and 161.1 for $[M-H\text{-caffeate}]^-$, $[\text{caffeate}]^-$ and $[\text{caffeate-}H_2O]^-$, respectively to prove its identity as 3,4- dihydroxyphenethyl-alcohol-caffeoyl-rhamnosylglucoside. In the aromatic region of its ^{1}H NMR spectrum (Table 1), an A^2X^2-spin coupling system of H-7''' and H-8''' at 7.59 and 6.29 ppm, respectively (each *d*, *J* = 15.9 Hz) together with an ABM one of H-2''', H-6''' and H-5''' at 7.17, 7.05 and 6.87 ppm, respectively were indicative an *E*-caffeoyl moiety. The characteristic signals of the aglycone moiety (3, 4-dihydroxyphenethyl alcohol) were assigned in the form of an ABM spin coupling system at 6.77, 6.73 and 6.57 ppm for H-2, H-5 and H-6, respectively in the aromatic region and its characteristic AX-system of two triplets for H-7 and H-8 at 2.78 and 3.84, respectively in the aliphatic region. The ^{1}H-NMR also showed two anomeric proton signals at 4.43 ppm (H-1', *d*, *J* = 7.8 Hz) and at 5.29 (H-1", brs) together with the signal at 1.11 (3H, *d*, *J* = 6 Hz- CH_3-6"). The glucoside moiety adopts an *O*-β-$^4C_1$-pyranose, while rhamnosyl adopts an *O*-α-$^1C_4$-pyranose depending on all of their *δ*- and *J*-values in ^{1}H and ^{13}C-spectra (Table 1). The connectivity of the caffeoyl moiety on OH-4 of glucose was followed from the downfield location of H-4' as t-like at 4.90, whereas, the connectivity of *O*-rhamnosyl on C-2-glucoside was concluded from the characteristic downfield shift of its ^{13}C NMR signal to 79.66 ppm. Depending on the assignment of all other signals of ^{1}H and ^{13}C NMR with the aid of the cross peaks in 2D-NMR spectra (^{1}H^{1}H-COSY, HMQC and HMBC) and comparison with previously reported data. (28, 29), compound **1** was identified as 2-(3,4-dihydroxyphenyl)ethyl-2-*O*-[6-deoxy-α-L-mannopyranosyl-4-(3,4-dihydroxyphenyl)- 2-propenoate]-β-D-glucopyranoside (28, 29).

Compounds **2, 3** both occur as yellow amorphous powder they gave dark purple spots under UV light (365 nm), and this color changes with NH_3, $FeCl_3$ and Naturstoff reagent, (yellow, green and greenish yellow, respectively). In compound **2** R_f: 0.59 (S_1), 0.55 (S_2). -UV spectral data λ_{max} (nm), (MeOH) =: 344.5, 269.5, 229.5, 288.5 and 248 where in compound 3 R_f: 0.51 (S_1), 0.54 (S_2). -UV spectral data λ_{max} (nm), (MeOH) =: 345, 270.5, 228, 289.5 and 247. On complete acid hydrolyses they remained without any change and this indicated that compounds **2, 3** are C-glycosides. -Negative ESI-MS spectrum of both compounds showed a molecular ion peak at *m/z* = 431 $[M-H]^-$, corresponding to a molecular weight of 432, which was in complete accordance with apigenin C-hexoside. The fragment ion peak at *m/z* = 269 $[M\text{ -H- hexose}]^-$ in both compounds corresponding to apigenin. $^{-1}$H-NMR spectrum of compound **2** and **3** (Table 2) showed two doublets at δ (7.95 – 7.84), and δ (6.92-6.9) both integrating for two protons and are assigned to (H-2' / 6') and (H-3' / 5') of the 1', 4' disubstituted B-ring, respectively. Signals at δ 6.55 and 6.50 ppm are due to protons attached at C-3 in the apigenin skeleton of the compounds **2** and 3, respectively. Absence of the

characteristic signal of H-8 led us to identify compound **2** as 8-subistetuted apigenin, while the absence of characteristic signal of H-6 in compound **3** led us to identify it as 6-subistetuted apigenin. Presence of C-β- glycoside moiety in the structure of **2** and **3** were concluded depending on the anomeric protons coupling at δ 4.7 and 4.98 ppm, respectively with characteristic high *J*-values ($9H_Z$) in both compounds. In the ^{13}C NMR spectrum of compound **2** and **3** (Table 2), the signals at (δ128.9 and 128.36 ppm) and (δ 115.9 and 116.11 ppm) revealing to C (2' / 6') and (3' / 5'), respectively. The methane carbon signal at δ 102.59 and 102.6 ppm were characteristic for C-3 apigenin aglycone in **2** and **3**, respectively. The characteristic downfield shifts of C-8 to δ = 104.6 ppm (Δ ≈ 10 ppm) and upfield of both C-7 and C-9 to δ 161.2 and 156 ppm, respectively was diagnostic for 8-*C*-glycosedation in compound **2**, while the characteristic downfield shifts of C-6 to δ 109.1 (Δ ≈ 10 ppm) and upfield of both C-7 and C-5 to δ 161.43 and 160.7 ppm, respectively was diagnostic for 6-*C*-glycosedation in compound **3**. C-β- glycoside moiety in the two structures can be obtained from the signal at around 74 ppm, indicating that the anomeric carbon is attached to C- atom and not to the usual oxygen in the both aglycone. Six C-resonances of the β-C- glucopyranoside moiety were assigned in the range of 82-60 ppm in the both compounds. The remaining carbon resonances of both compounds were completely assigned by comparison with previously corresponding data (30, 31). Therefore, **2** and **3** were finally identified as vitexin and isovitexin, respectively.

Compound **4**: occurs as yellow amorphous powder, R_f: 0.7 (S1), 0.3 (S2). -UV/V λ_{max} (MeOH) =: 345; (NaOMe): 266 sh, 329sh, 401; (NaOAc): 268, 326sh, 386; (H_3BO_3): 260, 300 sh, 370,428 sh; ($AlCl_3$): 272, 300 sh, 325, 426; (HCl): 272, 290, 355, 383. It was found to be substituted at 7-OH by absence of bathochromic shift upon addition of NaOAc. On complete acid hydrolyses of **4**, letulin was detected in the organic phase while, glucuronic acid was detected in the aqueous phase (Co-PC with authentic samples). ESI–MS of **4** gave a molecular ion peak at *m/z* = 461 corresponding to M.Wt = 462. The fragment ion peak at *m/z* 285 = [aglycone-H] $^-$ which consistence with letulin and was attributed to the loss of glucuronic acid moiety. ^1H NMR (300 MHz DMSO-d_6) δ: = 7.39 (1H, *d*, *J* = 2.1 Hz, H-2'), 7.35 (1H, *d*, *J* = 2.1 Hz, H-6'), 6.89 (1H, *d*, *J* = 8.4 Hz, H-5'), 6.75 (1H, *d*, *J* = 1.8 Hz H-8), 6.66 (1H, *s*, H-3), 6.38 (1H, *d*, *J* = 2.1 Hz, H-6), 5.16 (1H, *d*, *J* = 6.9 Hz, H-1") 3.90 - 3.30 (remaining sugar protons). ^{13}C NMR (75 MHz DMSO-d_6) δ: = 182.53 (C-4), 171.41 (C-6"). 165.24 (C-2), 163.33 (C-7), 161.81 (C-5), 157.62 (C-9), 150.73 (C-4'), 146.53 (C-3'), 121.93 (C-1'), 119.75 (C-6'), 116.89 (C-5'), 114.39 (C-2'),106.1 (C-10), 103.79 (C- 3), 100.16 (C- 1"), 100.05 (C-6), 95.32 (C-8), 76.59 (C-5"), 75.68 (C-3"), 73.54 (C-2"), 72.14 (C-4"). $^{-1}$HNMR spectrum exhibited signals at δ- values 7.39 ppm (*d*, *J* = 2.1 H_Z, H-2'), 7.35 ppm (*dd*, *J* = 8.4 and 2.1 H_Z, H-6'), and 6.89 ppm (*d*, *J* = 8.4 H_Z, H-5'), characteristic for ABX spin coupling system for a 3', 4'- disubstituted B– ring. The glucuronosedation at 7- OH was concluded from downfield shift of both H-6 and H-8 (≈ + Δ 0.2 ppm) and the β – anomeric proton signal at 5.16 (*J* = $7H_Z$) (30). $^{-13}$CNMR spectrum of **4** showed 21 carbon signals fifteen of them were attributed to the aglycone moiety and assigned by comparison with corresponding data (31), while the sex remaining signals for the glucuronic acid moiety. Slight upfield shift of C-7 and downfield shift of both C-6 and C-8 were further confirmation for the glucuronosedation at 7-OH (32). So the structure of **4** was deduced as letulin 7-*O*- glucuronopyranoside. (30, 31)

Compound **5**: occurs as yellow amorphous powder R_f: 0.6 (S1), 0.4 (S2). -UV λ_{max} (MeOH) =: 259, 355, 300 sh.; (NaOMe): 270, 325sh, 399; (NaOAc): 270, 325sh, 460; (NaOAc /H_3O_3): 269, 325,390; ($AlCl_3$): 239, 275, 343, 425. It appeared as dark purple fluorescence under UV light turned to orange with Naturstoff spray reagent. On complete acid hydrolyses quercetin was detected in the organic phase, while rhamnose was detected in the aqueous phase (Co-PC with authentic samples).$^{-1}$H NMR (300 MHz DMSO-d_6) δ: = 7.3 (1H, *d*, *J* = 1.8 Hz, H-2'), 7.26 (1H, *dd*, *J* = 8.4, 1.2 Hz, H-6'), 6.87 (1H, *d*, *J* = 8.4 Hz, H-5'), 6.39 (1H, *d*, *J* = 2.1 Hz, H-8), 6.21(1H, *d*, *J* = 2.1 Hz, H-6), 5.26 (brs, H-1"), 3.98 (brs, H-2"), 3.6 (brd, H-3") 3.80 - 3.30 (remaining sugar protons), 1.2 (1H, *d*, *J* = 5.7 Hz, H-6"). $^{-13}$C NMR (75 MHz DMSO-d_6) *δ*: = 177.33 (C-4), 164.1 (C-7), 161.28 (C-5), 156.31 (C-9), 156.17 (C-2), 148.42 (C-4'), 144.9 (C-3'),132.91 (C-3), 121.73 (C-1'), 121.24 (C-6'), 116.03 (C-5'), 115.18 (C-2'),104.06 (C-10), 102.2 (C-1"), 98.43 (C-6), 93.59 (C-8), 71.94 (C-4"), 70.66 (C-2"), 70.31 (C-3"), 68.34 (C- 5"),17.26 (C-6").

Negative ESI-Ms spectrum of compound **5:** showed a molecular ion peak at *m/z* = 447.1 $[M-H]^-$, together with a fragment ion peak at 301.2 [M-H - 146]$^-$, corresponding to the loss of rhamnose moiety which confirmed the presence of quercetin as aglycone**.** The UV methanol spectrum showed characteristic absorption bands at λ_{max} 259 and 355 nm for band II and I, respectively for quercetin moiety. The bathochromic shift observed upon addition of NaOMe (+42 nm in band I) with increase in the intensity proved the presence of free 4-OH. Compound **5:** showed in the aromatic region of its ^{1}HNMR spectrum two characteristic spin coupling systems, the first one occur as an ABX of three types

of protons at δ = 7.3 (1H, *d*, *J* = 1.8 Hz), 7.26 (1H, *dd*, *J* = 8.4, 1.2 Hz) and 6.87 (1H, *d*, *J* = 8.4 Hz) assignable to H-2', 6' and 5', respectively of 3', 4'-dihydroxy B-ring. The second coupling system occurs as an AM of two meta coupled protons at δ = 6.39 (1H, *d*, *J* = 2.1 Hz), 6.21 (1H, *d*, *J* = 2.1Hz) assignable for H-8 and H-6, respectively of 5, 7-dihydroxy A-ring. The presence of 3-*O*-rhamnopyranosyl in **5** was concluded from its aliphatic proton signal (brs) at δ = 5.26 ppm. Stereo structure of sugar moiety was established as α-$^{1}C_{4}$-pyranose based on the typical *J*- and δ-values in both of its $^{-1}$H- and $^{-13}$C-NMR signals. The remaining carbon resonances of **5** were completely assigned by comparison with previously corresponding data (31). Therefore, compound **5**: was finally identified as quercetin 3-*O*- α -L-$^{1}C_{4}$-rhamnopyranoside (querecterin) (32).

Compound **6**: occurs as pale yellow amorphous powder. -UV-spectral data λ_{max} (nm), (MeOH): 235 sh., 325, 340; (+NaOMe): 250, 300, and 350. It gave a blue fluorescent spot under -UV- light which turned into greenish blue with NA/PE and gave blue color with $FeCl_3$ spray reagents. $^{-1}$H-NMR spectral data revealed the presence of caffeoyl moiety from the ABM spin coupling system at *δ* = 6.90 ppm (1H, *d*, *J* = 1.8 H_Z), *δ* = 6.82 ppm (1H, *dd*, *J* = 1.8, 8.5 H_Z) and *δ* = 6.68 ppm (1H, *d*, *J* = 8.5 H_Z) for the three aromatic protons H-2, H-6 and H-5, respectively. In addition the AX spin coupling system at *δ* = 7.46 ppm and 6.16 ppm (each 1H, *d*, *J* = 16 H_Z) for the *E*-olefinic protons of H-7 and H-8, respectively. Furthermore the singlet signal at 3.51(3H, *s*, OCH_3) in the aliphatic region was assigned to methoxy group. Compound **6** was completely assigned by comparison with authentic samples and previously corresponding data (33). From the previous data compound **6** was identified as *E*- methyl caffeate. The results in (Table 3) showed that methanol extract had the highest amount of total phenolic content (3.5 ± 0.054 mg gallic equivalent per mg dry extract) followed by ethyl acetate extract (1.38 ± 0.038 mg gallic equivalent per mg dry extract) and the lowest one was chloroform extract (0.87 ± 0.015 mg gallic equivalent per mg dry extract).

The brine shrimp lethality assay is considered a useful tool for preliminary assessment of toxicity. The brine shrimp bioassay can be employed for this purpose as it appears to be a convenient, rapid and inexpensive. All extracts used showed significant lethality against brine shrimp. The methanol extract was found to be the most active extract which has LC_{50} value about 140 mg /mg followed by ethyl acetate 165 mg /mg and chloroform 180 mg /mg.

Antioxidative and radical scavenging properties of compounds (**1-3**) were evaluated using 1, 1-diphenyl-2-picrylhydrazyl (DPPH) radical and phosphomolybdenum method (Table 4). The antioxidant results showed that compound **1** is a highest scavenger of the artificial radical. Compound **1** is phenylethanoid (*E*-isomer) glycoside and identified as strong antioxidant (34). It is suggested that the 3, 4-dihydroxyphenethyl alcohol group might be more responsible for their activities than the caffeoyl group (35).

In conclusion, we have clearly shown that different extracts *of Vitex trifolia* displays cytotoxic activity on the brine shrimp lethality bioassay. Also the methanol extract has the highest value of total phenolic content due to the presence of flavonoids followed by chloroform and ethyl acetate extracts, and the antioxidant results showed that compound **1** is the strongest antioxidant.

Table 1: ^{1}H, ^{13}CNMR spectral data of 1 (300/75 MHz, DMSO-d_6)

No.	δ_{H1}	δ_{C1}
1	…….	131.25
2	6.77 *d* (1.6)	116.87
3	……..	144.25
4	……..	145.7
5	6.73 *d* (8.1)	115.95
6	6.57 *dd* (8.1, 18)	121.05
7	2.78 *t*-like (7.5)	36.24
8	3.84 *t*-like (7.5)	72.17
1'	4.43 *d* (7.8)	103.78
2'	4.01*	79.65
3'	……	73.94
4'	4.903 *t-like* (10)	72.17
5'	……	76.27
6'	4.02 *brd* (12)	62.36
6'	3.37 *	62.36
1"	5.29 *d* (1.5)	101.85
2"	3.89 *brs*	71.53
3"	……	70.28
4"	……..	71.98
5"	3.71 *m*	69.47
6"	1.11 *d* (6)	18.54
1"'	……	127.62
2"'	7.17 *d* (2.1)	115.26
3"'	…….	148.89
4"'	……	146.25
5"'	6.87 *d* (8.1)	116.36
6"'	7.05 *dd* (8.1, 2.1)	122.77
7"'	7.59 *d* (15.9)	147.76
8"'	6.29 *d* (15.9)	115.15
9"'	…….	169

δ in ppm and *J* values in H_Z, are given in parentheses, *Unresolved protons

Table 2 ^{1}H, ^{13}CNMR spectral data of 2, 3 (300/75 MHz, DMSO-d_6)

No.	δ_{H2}	δc_2	δ_{H3}	δc_3
2	…….	163.9	……..	163.23
3	6.55 *s*	103.4	6.50 *s*	102.6
4	……	182.0	…….	181.83
5	……..	160.4	……...	160.7
6	6.45 *s*	98.4		109.1
7		161.2		161.43
8		104.6	6.44 *s*	94.02
9		156		156.46
10		104.5		102.6
1'		121.6		121.09
2'	7.95 *d* (8.3)	128.9	7.84 *d* (8.1)	128.36
3'	6.9 *d* (8.5)	115.9	6.92 *d* (8.1)	116.11
4'		161.01		161.43
5'	6.9 *d* (8.5)	115.9	6.92 *d* (8.1)	116.11
6'	7.95 *d* (8.3)	128.9	7.84 *d* (8.1)	128.36
1''	4.7 *d* (9)	73.5	4.98 *d* (8.1)	73.32
2''		71		70.54
3''		78.7		79.1
4''	4.11 *d* (7.8)	70.6	4.11 *d* (7.8)	70.27
5''	3.79- 3.34		3.79- 3.34	
6''	3.79- 3.34		3.79- 3.34	

δ in ppm and *J* values in H_Z, are given in parentheses.

Table 3 Total phenolic content TPC (mg Gallic equivalent/ mg dry extract) and Cytotoxicity expressed as % mortality and LC_{50} of *Vitex trifolia* aerial parts different extracts. Values of mortality and total phenolic content given are mean ± SE. (N=6). (Mean±SD).

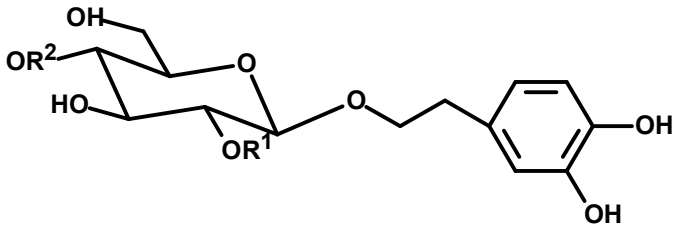
Extracts	Concentration mg ml^{-1} (Mean±SD)							TPC
	10	100	200	400	600	800	1000	
MeOH	9.33± 0.21	34.67±0.23	74.33±0.22	85.00±0.37	93.67±0.22	98.67±0.42	99.33±0.21	3.5 ± 0.054
$CHCl_3$	10.0± 0.36	34.00±0.37	58.66±0.56	79.66±0.20	91.33±0.21	96.67±0.21	99.67±0.22	1.38 ± 0.038
EtOAc	8.33± 0.23	26.00±0.43	57.67±0.22	67.66±0.21	79.32±0.20	94.00±0.37	99.32±0.42	0.87± 0.015

TPC = Total phenolic content

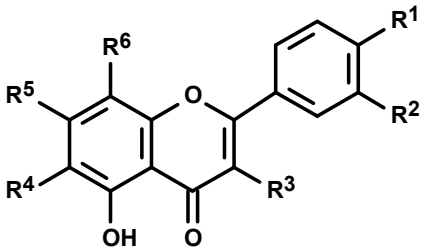
Table 4 DPPH as indicated by the low SC50 value and total antioxidant capacity (mg of ascorbic acid equivalent / g of extract). (Mean±SD)

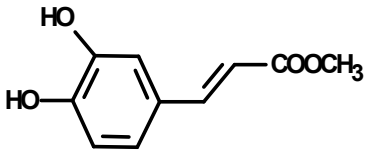
sample	$DPPHSC_{50}$ (µg/ ml)	Total antioxidant capacity (mg AAE/ g extract)
Compound 1	4.70 ± 0.12	900.21 ± 0.36
Compound 2	15.36 ± 0.07	315.35 ± 13.55
Compound 3	30.48 ±0.47	355.71 ± 1.36
Ascorbic acid	7.90 ± 0.20	----------

Results are (means ± S.D. (standard deviation) (n = 3); AAE (ascorbic equivalent).



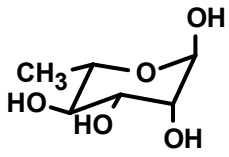
1- R^1 = α-L- rhamnopyranoside, R^2 = *E*- caffeoyl



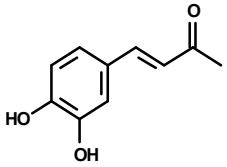


6

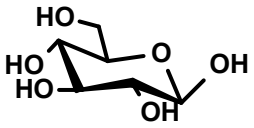
2- $R^2 = R^3 = R^4$ = H, $R^1 = R^5$ = OH, R^6 = C- glucopyranoside
3- $R^2 = R^3 = R^6$ = H, $R^1 = R^5$ = OH, R^4 = C- glucopyranoside
4- $R^3 = R^4 = R^6$ = H, $R^1 = R^2$ = OH, R^5 = O- glucuronopyranoside
5- $R^4 = R^6$ = H, $R^1 = R^2 = R^5$ = OH, R^3 = O- α-L- rhamnopyranoside



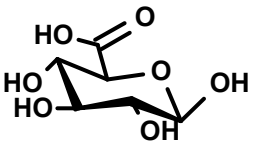
α-L- rhamnopyranoside



E- Caffeoyl



glucopyranoside



Glucuronopyranoside

Figure 1. Chemical structures of compounds isolated from *Vitex trifolia* aerial parts

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