IJPSR (2017), Vol. 8, Issue 3 **(Research Article)**

INTERNATIONAL JOURNAL OF PHARMACEUTICAL SCIENCES AND RESEARCH

Received on 13 August, 2016; received in revised form, 25 October, 2016; accepted, 06 December, 2016; published 01 March, 2017

PHYTOCHEMICAL PROFILE OF *SEQUOIA SEMPERVIRENS* GROWN IN EGYPT

Kamilia F. Taha and Zeinab T. Abd El Shakour *

Laboratory of Phytochemistry, National Organization for Drug Control and Research, Cairo- Egypt.

Keywords:

S. sempervirens, Leaves, Immunomodulatory, Antiulcerogenic, Phenolics; Flavonoids, HPLC

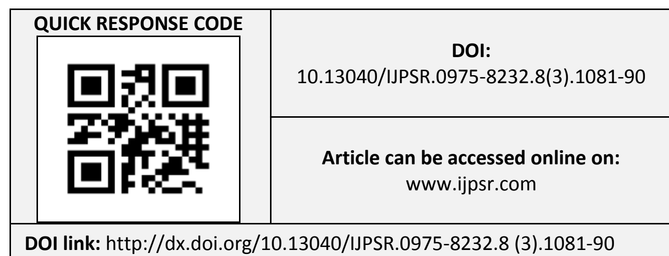
Correspondence to Author:
Zeinab T. Abd El Shakour

Laboratory of phytochemistry, National Organization for Drug Control and Research, Cairo11364, Egypt.

E-mail: zizishakour@yahoo.com

ABSTRACT: The immunomodulatory and antiulcerogenic activities of the chloroform, ethyl acetate and n-butanol fractions of *Sequoia sempervirens* (D. Don.) leaves were investigated. The ethyl acetate fraction showed the best results regarding to a high significant increase of RAW 264.7 macrophage cells and reduction of ulcers number and severity compaered with Ranitidine. HPLC technique was used to investigate the phenolic contents in the most bioactive fraction of *S. sempervirens* (D. Don.) The results allowed the identification and quantification of 12 phenolic acids and 15 flavonoids (in mg /g dry powder ± SD), protocatechuic acid was the predominant phenolic acid (10.94 ± 1.22), followed by salicylic acid (4.52 ± 1.23), coumaric acid (4.42 ± 0.21) and sinapic acid(3.57 ± 0. 63). Vitexin showed the highest content of identified flavonoids (6.67± 1.21) followed by orientin (5.96 ± 1.18) and quercetin-3,7-di-O-glucoside (4.92± 0.73). Ten flavonoids named as isoorientin (luteolin-6-C-glucopyarnoside) (1) isovitexin (apigenin-6-C -glucopyarnoside) (2) orientin (luteolin-8-C-glucopyarnoside) (3) vicenin-II (6, 8- di-C-glucosylapigenin) (4) mangiferin (5) vitexin (apigenin-8-C-glucopyarnoside) (6) protocatechuic acid (7) luteolin (8). apigenin (9) kaempferol (10) were isolated from the *S. sempervirens* (D. Don.) leaves extract using usual chromatographic techniques. The results constitute the first report on the phenolic contents in *S. sempervirens* (D. Don.).

INTRODUCTION: Family Cupressaceae, one of the largest and most widely distributed of all conifer families, have 28 genera with 142 species [1] (Earle, 2013). Many of Cupressaceae species produce valuable timber, and also many have major importance in the ornamental plantings and environmental forestry [2-5]. *Sequoia* is a genus of redwood coniferous trees in the subfamily Sequoioideae of the family Cupressaceae. The only extant species of the genus is *Sequoia sempervirens* in the Northern California coastal forests and Southwestern Oregon in the United States [6].



QUICK RESPONSE CODE

DOI: 10.13040/IJPSR.0975-8232.8(3).1081-90

Article can be accessed online on: www.ijpsr.com

DOI link: http://dx.doi.org/10.13040/IJPSR.0975-8232.8 (3).1081-90

The two other genera *Sequoia dendron* and *Metasequoia*, in the subfamily Sequoioideae are closely related to *Sequoia*. Traditionally, a poultice of the heated *S. sempervirens* (D. Don.) leaves have been used in the treatment of earaches; the gummy sap has been used as a stimulant and tonic in the treatment of rundown conditions [7].

A variety of compounds such as terpenoids, essential oils, lignans, flavonoids and flavonones have earlier been isolated from *S. sempervirens* (D. Don.) [8].

Some of these compounds showed antifungal, antibacterial and antitumor activity. There is only one *S. sempervirens* (D. Don.) tree in Egypt which had been threatened, never produces seeds, neither vegetative propagated, however, (Gad et al., 2006) [9], showed it was possible to propagate through tissue culture techniques.

In our study, we aimed to evaluate the immunomodulatory and antiulcerogenic activity of the chloroform, ethyl acetate and n-butanol fractions prepared from an ethanol extract of the leaves of *S. sempervirens* (D. Don.) also explore and isolate phenolic compounds in the most bioactive fraction which are important constituents of many plants with their identification and quantification by high performance liquid chromatography (HPLC).

Experimental:

General: The structure of the compounds was identified by spectroscopic methods including: UV/VIS (Ultraviolet and Visible Absorption Spectrometer, Labomed Inc.) for measuring UV spectral data of the isolated compounds, in the range of 200–500 nm in methanol and with different diagnostic shift reagents. NMR (Nuclear Magnetic Resonance Spectrophotometer, JEOL EX, 500 MHz for determination of ^{1}H NMR and 125 MHz for determination of ^{13}C NMR), ESI/MS (Electrospray Ionization Mass Spectrometer, Thermo Finnigan (ion trap)) were carried out for determination of molecular weight of compounds, CC was carried out on Polyamide 6S (Riedel-De-Haen AG, Seelze Haen AG, D-30926 Seelze Hanver, Germany) and Sephadex LH-20 (Pharmazia). Paper Chromatography, Whatman paper No. 1 and No. 3 (Whatman Ltd. Maidstone, Kent ,England using solvent systems 15% HOAc (H_2O–HOAc 85:15), BAW (n-BuOH: HOAc: H_2O 4:1:5, upper layer). Precoated silica gel 60 F254 plates (E. Merck) were used for TLC.

MATERIAL AND METHODS:

Plant material: *Sequoia sempervirens* (D. Don.) leaves were collected during October and May, respectively, from the El Orman Garden, Giza, Egypt (2012) The collected plant materials were botanically authenticated by Prof. Dr. Monir Mohamed Abd Elghany, The Herbarium, Botany department, Faculty of Science, Cairo University, Egypt, also Voucher specimen of the authenticated plant was deposited at Laboratory of phytochemistry, National Organization for Drug Control and Research, Cairo, Egypt.

Preparation of samples: Plant material: 1kg of leaves of *S. sempervirens,* were extracted with 70% ethanol. The extract was filtered through fresh cotton bed and finally with Whatman No. 1 filter paper, the filtrate was evaporated with a rotary evaporator at low temperature (40°-50°C) and reduced pressure to provide crude ethanol extract (150g). It was suspended in water and portioned successively with chloroform, ethyl acetate and *n*-butanol saturated with water to give 17.43 g (CFL - chloroform fraction of the leaves), 25.1 g (EAFL - ethyl acetate fraction of the leaves) and 13.2g (BFL - *n*-butanol fraction of the leaves) dry residues, respectively. The most active fraction will be subjected to further phytochemical investigation.

Toxicity: The median lethal dose (LD_{50}) values of chloroform, ethyl acetate and *n*-butanol fractions prepared from leaves of *S. sempervirens* (D. Don.) [10].

Immunomodulatory activity: The tested fractions (CFL, EAFL, BFL) of *S. sempervirens* (D. Don.) were evaluated on the growth of Raw macrophage 264.7 by the 3-(4, 5- dimethyl-2-thiazolyl)-2, 5-diphenyl-2H-tetrazolium bromide (MTT) assay[11]. The yellow tetrazolium salt of MTT is reduced by mitochondrial dehydrogenase in metabolically active cells to form insoluble purple formazan crystals, which are solubilized by the addition of a detergent. Cells (5×104 cells/ well), were incubated with 100 μg/ml of the tested frsctions at 37°C in a FBS-free medium, and before submitted to MTT assay. The relative cell viability was determined by the amount of MTT converted to the insoluble formazan salt. ELISA reader was used for measurment the absorbance at 570 nm.

The data were the mean percentage of viable cells as compared to DMSO-treated cells. The macrophages treated with 1000 U/ ml recombinant macrophage colony-stimulating factor (M-CSF, Pierce, USA) used positive control.

Anti-ulcerogenic activity: The anti-ulcerogenic activity of CFL, EAFL, BFL fractions of *S. sempervirens* (D. Don.) were evaluated by Ethanol-induced ulcer model [12]. Acute erosion of the gastric mucosa was induced in fasting rats (18 hours) by intragastric administration of 1 ml absolute ethanol. The rats were divided into 5 groups (each of 6 rats). Ranitidine (20 mg/kg, 60 min prior to ethanol) was used in one group as a reference drug; the control group was given vehicle

and the other groups were given were given ethanol and the tested fractions (100 mg kg^{-1} b. wt.) The animals were sacrificed one hour after giving ethanol and the gastric lesions were examined under an illuminated magnifier [13]. Petechial lesions were counted, and then each five petechial lesions were taken as 1 mm of ulcer [14]. To calculate the ulcer index (mm), the sum of the total length of long ulcers and petechial lesions in each group of rats was divided by its number. The curative ratio was determined according to the formula: curative ratio (%) = 100(CS)/C, where C is the ulcer index of the control and S is the ulcer index of the test compound.

Qualitative and Quantitative HPLC Analysis of Phenolic Acids: HPLC analysis of the EAFL of *S. sempervirens* (D. Don.) was performed using a Knauer HPLC system (Germany) with a model 64-00 pump, model 87-00 UV detector and model 7, 125 injection valve (Rheodine, Cotai, CA, USA), and chromatographic separation was performed on a Li Chrospher RP-18 (5 mm) column (250 × 4 mm i.d. Merck, Darmstadt, Germany). The solvent system used for these analyses consisted of a gradient of water and acetonitrile at a pH of 2, adjusted with phosphoric acid. The following gradient was used: 0–20 min, water/acetonitrile, 95:5 v/v; 20–40 min, water/acetonitrile, 75:25 v/v; 40–45 min, water/acetonitrile, 1:1 v/v; 45–60 min, water/acetonitrile, 25:75v/v.

The operating conditions were as follows: column temperature, 25 °C; injection volume, 20 µL; flow rate, 1.0 mL/min; and the UV spectra were recorded from 220 to 600 nm. The following standards were used: caffeic acid, gallic acid, sinapic acid, *trans*-cinnamic acid, ferulic acid, coumaric acid, protocatechuicacid, gentisic acid, chlorogenic acid, salicylic acid, Syringic acid and *p*-hydroxybenzoic acid, which were obtained from Sigma -Aldrich Co. (St. Louis, MO, USA). Each standard phenolic acid sample (2 mg) was dissolved in methanol/water (10 mL, 50:50 v/v), and 20 µL of each standard phenolic acid sample was injected for HPLC analysis under the same conditions. The spectrum of each standard was recorded and stored in the HPLC spectrum library. The criteria for the identification of phenolic compounds were established based on comparison of the retention time and spectrum of an unknown compound with library data on HPLC standards. Quantification was carried out using the external standard method. Solutions of each standard at various concentrations were injected into the HPLC system, and the peak areas were determined. Thus, the calibration curves and response factors were recorded under the same conditions as for the samples.

Qualitative and Quantitative HPLC/MS Analysis of flavonoid Contents: HPLC analysis of the EAFL of *S. sempervirens* (D. Don.) was performed using a Hewlett-Packard series 1100 system (Waldbronn, Germany) with a symmetry C18 column (250 mm × 4.6 mm, i.d. 4 µm) and a guard column (10 mm × 3.9 mm, i.d. 4 µm) from Waters (Barcelona, Spain), equipped with a vacuum degasser, a binary pump and a photodiode array detector (HP1050), connected to HP Chem Station software (Hewlett-Packard) and an APAL autosampler (CTC analytics) controlled by its own software. Elution was carried out with a gradient of acetonitrile (solvent B) in the form of a 0.05% TFA solution in water (solvent A), and the elution conditions applied were as follows: 0–60 min, linear gradient of 5–50% B; 60–70 min, linear gradient of 50–75% B; 70–80 min, 75–100% B; and 80–90 min, 100% B, isocratic. The flow rate was 1 mL/min, and the injection volume was 50 µL. The system was operated at room temperature. Identification of compounds was accomplished by comparing mass spectroscopic data on the identified compounds with computerized data and the available literature.

Quantitative estimation of each flavonoid component was achieved by preparing the ME in triplicate, and each preparation was analyzed in triplicate. Standards (luteolin-7-O-glucoside, vicenin-II, mangiferin, quercetin-3, 7-di-O-glucoside, isovitexin, kaempferol, vitexin, quercetin, luteolin, apigenin, orientin, isoorientin, rutin, apigenin-7-O-rutinoside and epicatchin) and solvents were purchased from Sigma–Aldrich GmbH (Steinheim, Germany).

Calibration curves were constructed for each flavonoid in the range of sample quantities of 0.02–0.5µg. HPLC grade acetonitrile was obtained from Merck (Darmstadt, Germany).

Phosphoric acid (J.T. Baker, Phillipsburg, NY, USA) and redistilled water were used; after preparation of the mobile phases, they were filtered through a 0.49 nm filter. All other chemicals used were of analytical grade.

Statistical analysis: Results are presented as means ± standard deviation. The statistical analyses of the experimental results were based on the analysis of variance method. Differences were considered significant at the $p < 0.001$ level.

Extraction, Fractionation, and Isolation: The most active fraction EAFL (20g) was then subjected to Sephadex LH-20 column chromatography (250g, 40 × 500 mm) and eluted with water followed by different ratios of water/ethanol (1L each eluent) to give rise to five fractions, which were further purified by a series of fractionations on a Sephadex LH-20 column and (PPC). Compounds (1, 25 mg and 2, 27 mg) were separated from fraction I by fractionation over Sephadex LH-20 column using MeOH/H_2O (decreasing polarity) for elution then PPC to the sub-fractions using (AcHO: H_2O; 6:94). Compounds (3, 78 mg and 4, 44 mg) were isolated as pure compounds from fraction II by using Sephadex LH-20 column and *n*-BuOH saturated with H_2O as developing system. Applying the third fraction on Sephadex LH-20 column (100 g, 20 × 250 mm) and eluted with ethanol to obtain the pure natural compounds (5, 55 mg and 6, 42 mg). From the fourth fraction, compound (7, 43mg) was separated in a pure form by applying on the Sephadex LH-20 column and eluted by 40% EtOH. Finally, the pure aglycones 8 (25 mg), 9 (23 mg), and 10 (29 mg) were obtained in a pure form from a cellulose column chromatography of fraction V using ethanol as eluent.

Compound 1: Isoorientin (luteolin-6-*C*-glucopyarnoside.): Yellow amorphous powder and it gave a dark purple spot on PC under UV light with HR_f 33 (BAW), 40 (%15 HOAc) and 9 (H_2O). UV λ_{max} nm(MeOH) 350, 271; NaOMe 400, 330, 281; $AlCl_3$ 430, 330, 281; $AlCl_3$/HCl 390, 349, 290 sh, 285; NaOAc 383, 319, 280, NaOAc/H_3BO_3 429 sh, 369, 270; ^{1}H-NMR δ (DMSO *d6*, ppm) 7.4 (d, *J*= 2.5 Hz, H-2\`), 6.83 (d, *J*= 8.5 Hz, H-5\`),7.29 (dd, *J*= 2.5 Hz and 8.5 Hz, H-6\`), 6.49 (s, H-3), 6.39 (s, H-8), 4.7 (d, *J*= 10 Hz, H-1' glucose), 3.0-4.2 (m, sugar protons). ^{13}C-NMR δ (DMSO *d6*, ppm): 164.4 (C-2), 101.57(C-3), 182.55 (C-4), 160. 88 (C-5), 105.00 (C-6) 170.62 (C-7),100.63 (C-8), 156.51 (C-9), 104.48 (C-10), 125.46 (C-1'),114.42 (C-2'), 136.33 (C-3'), 146.14 (C-4'), 116.17 (C-5'),119.89 (C-6'), 63.86 (C-1''), 75.66 (C-2''), 79.10 (C-3''), 70.19 (C-4''), 90.36 (C-5''), 61.51 (C-6'').

Compound 2: Isovitexin (apigenin- 6-*C*-glucopyarnoside): Yellow amorphous powder, it appeared as a dark purple spot on PC under UV light with HR_f 70 (BAW), 49(%15 HOAc) and 39 (H_2O). UV λ_{max} nm (MeOH): 332, 272; NaOMe 388,329 sh, 279; $AlCl_3$ 390, 337, 314, 282; $AlCl_3$/HCl 389, 308 sh, 278 sh,272; NaOAc 393, 309 sh, 286; NaOAc/H_3BO_3 400 sh, 341, 280. ^{1}H-NMR (δ ppm DMSO-*d6*,at room temperature) 7.72 (2H, d, *J*= 8 Hz, H-2\`, H-6\`), 6.92 (2H, d, *J*= 8 Hz, H-3\`, H-5\`),6.77 (1H, s, H-3), 6.47 (1H, s, H-8), 4.7 (1H, d, *J*= 8 Hz, H-1 glucose), 3.0-3.9 (m, sugar protons). ^{13}C NMR δ (DMSO *d6*, ppm): 156.11 (C-2), 111.62 (C-3), 170.78 (C-4), 159. 50 (C-5), 105.22 (C-6) 162.35 (C-7),93.97 (C-8), 156.12 (C-9), 102.49 (C-10), 120.97 (C-1'),128.30(C-2'), 110.80 (C-3'), 161.11 (C-4'), 114.87 (C-5'),130.29 (C-6'), 72.94 (C-1''), 70.06 (C-2''), 77.66 (C-3''), 74.81 (C-4''), 92.36 (C-5''), 66.11 (C-6'').

Compound 3: Orientin (luteolin - 8 – *C* - glucopyarnoside): Yellow amorphous powder gave a dark purple spot on PC under UV light, HR_f 32 (BAW), 20 (15% HOAc) and 6 (H_2O λ_{max} nm (MeOH) 350, 270. Other UV shifting reagents NaOMe 391, 332 sh, 281; $AlCl_3$ 387, 340, 305, 278; $AlCl_3$/HCl 389, 300 sh, 279 sh, 276; NaOAc 389, 300 sh,281; NaOAc/ H_3BO_3 400 sh, 340, ^{1}HNMR(δ ppm DMSO-*d6*, at room temperature) 7.49 (d, *J*= 2.5 Hz, H-2\`), 7.02 (d, *J*= 8.5 Hz, H-5\`), 7.3 (dd, *J*= 2.5Hz and 8.5 Hz, H-6\`), 6.53 (s, H-3), 6.20 (s, H-6), 4.7 (d, *J*= 10 Hz, H-1\` glucose), 3.2-3.9 (m, sugar protons).

Compound 4: Vicenin-II (6, 8-*di*-*C*-glucosylapigenin): Yellow amorphous powder gave a dark purple spot on PC under UV light with HR_f 31 (BAW) and 50 (15% HOAc). λ_{max} nm (MeOH) 272, 297, 330; $AlCl_3$, 282, 305, 317, 350, 398; $AlCl_3$/HCl, 280, 303, 343, 381; NaOMe, 279, 322, 390; NaOAc 278, 297 sh, 390; NaOAc/H_3BO_3 281, 300 sh, 355. ESI/MS (neg.) gave m/z: 593 $[M+1]^-$ indicated $C_{27}H_{30}O_{15}$.^{1}H-NMR (δ ppm

DMSO-*d6*, at room temperature) 7.8 (2H, d, *J*= 9 Hz, H-2`, H-6`), 6.8 (2H, d, J= 9 Hz, H-3`, H-5`), 6.5 (1H, s, H-3), 4.6 (2H, m, H-1``and H-1```), 3.7-2.9 (m, sugar protons).

Compound 5: Mangiferin: Yellow amorphous powder, which appeared as intense yellowish orange colour on PC under short UV light of HR_f values 38 (BAW) and 24 (15%HOAc). λ_{max} nm (MeOH) 363, 315, 258, 241 suggesting a xanthones derivative. It exhibited a molecular ion at: m/z 423 $[M+H]^+$ corresponding to $C_{19}H_{18}O_{11}$. ^{1}H-NMR (δ ppm DMSO-*d6*, at room temperature) 6.4 (s, H-4), 6.78 (s, H-5), 7.35 (s, H-8), 4.6 (d, *J*= 9.5 Hz, H-1` glucose), 3.2-3.9 (m, sugar protons). $^{13}$C-NMRδ (DMSO *d6*, ppm): 161.79 (C-1), 107.42 (C-2), 163.83 (C-3), 93.27 (C-4), 101.63 (C-5), 155.309 (C-6),144.11 (C-7), 106.23 (C-8), 178.91 (C-9),156.16 (C-4a), 149.11 (C-4b), 110.98 (C-8a),101.21 (C-8b), 73.09 (C-1`), 70.65 (C-2`),79.02 (C-3`), 69.33 (C-4`), 79.51 (C-5`), 59.49(C-6`).

Compound 6: Vitexin (apigenin – 8 -C - glucopyarnoside): Yellow amorphous powder gave a dark purple spot on PC under UV light, HR_f 52 (BAW), 29(15%HOAc) and 35 (H_2O). λ_{max} nm (MeOH)334, 272; NaOMe 391, 332 sh, 281; $AlCl_3$ 378,339, 305, 278; $AlCl_3$/HCl 389, 300 sh, 279 sh,269; NaOAc 388, 305 sh, 277; NaOAc/ $H_3BO_3$400 sh, 339, 278. ^{1}H-NMR (δ ppm, DMSO-*d6*, at room temperature) 8.00 (2H, d, *J*= 8 Hz, H-2`,H-6`), 7.13 (2H, d, *J*= 8 Hz, H-3`, H-5`),6.77 (1H, s, H-3), 6.17 (1H, s, H-6), 4.54 (1H,d, *J*= 8 Hz, H-1 glucose), 3.1-3.9 (m, sugar protons).

Compound 7: Protocatechuic acid: Obtained as off-white amorphous powder; it appeared as an intense blue spot on PC under short UV light. HR_f 90 and 94 values using 15% acetic acid and BAW (4:1:5) as solvent system respectively. UV λ_{max} nm (MeOH): 293.40 and 259.40; ESI/MS: (negative ion): m/z $[M-H]^-$:153 indicating $C_7H_6O_4$. ^{1}HNMR (δ ppm DMSO-*d6*, at room temperature) 7.35 (d, *J*= 2.5 Hz, H-2), 6.80 (d, *J*= 7 Hz, H-5) and 7.28 (dd, *J*= 7 Hz & *J*= 2.5 Hz, H-6). ^{13}CNMRδ (DMSO *d6*, ppm): 121.63 (C-1), 116.84 (C-2), 145.0 (C-3), 149.0 (C-4), 110.17 (C-5), 129.63 (C-6), 167.96 (COOH).

Compound 8: Luteolin: It gave a major dark purple spot on PC under UV light with HR_f 78 (BAW), and 69(PhOH) under UV light. On crystallization from hot methanol it gave a yellow amorphous powder of compound 8. UV λ_{max} nm (MeOH) 350, 267; NaOMe 400, 329sh, 272; $AlCl_3$ 428, 354, 328, 305, 278; $AlCl_3$/HCl 383, 354,292 sh, 276; NaOAc 385, 328 sh, 269; NaOAc/H_3BO_3 430 sh, 370, 306 sh, 260.

Compound 9: Apigenin: Yellow powder appeared as deep purple under UV light on PC with HR_f 89 (BAW) and 90 (PhOH).Crystallization from hot methanol afforded yellow powder (7 mg), UV λ_{max} nm (MeOH): 359, 271; NaOMe 390, 319, 275; $AlCl_3$ 382, 346, 304, 271; $AlCl_3$/HCl 379, 340, 301, 272; NaOAc 373, 300, 272 and NaOAc/H_3BO_3 340, 300 sh, 272.

Compound10: Kaempferol: Amorphous yellow powder, UV λ_{max} nm (MeOH): 254 sh, 270, 330 sh, 365; NaOMe: 283, 324, 423; $AlCl_3$: 262 sh, 270, 352,426; $AlCl_3$/HCl 260, 271, 350, 426; NaOAc: 275, 307, 379; NaOAc/H_3BO_3: 268, 298 sh, 323 sh, 374.

RESULTS AND DISCUSSION:

Biochemical analysis: In the present study, immunomodulatory and antiulcerogenic activities were chosen as a guide to show the most active fractions prepared from a 70% ethanol extract of *S. sempervirens* (D. Don.) leaves, the incubation of CFL, EAFL, BFL fractions on the RAW 264.7 macrophage cells which resulted in a high significant increase ($P<0.05$) with mean viable cell percent ± SD values in the rate of cells proliferation at dose of 100 µg/ ml by 158± 2.91, 145± 1.8, 178± 1.45, 160± 1.84 folds respectively in comparison to the control (**Fig. 1**), indicating an immunomodulatory activity [15].

The tested fractions at doses 100 mg/kg showed antiulcerogenic effects comparable with Ranitidine (20 mg/kg; $p<0.05$) (**Table 1**). The most active, in this respect, was the ethyl acetate fraction followed by the butanol and chloroform fractions. They reduced ulcers number and severity by 70.21, 42.05 and 45.81%, respectively. No research on the immunemodulatory and antiulcerogenic properites of active fractions of *S. sempervirens* (D. Don.) has previously been carried out. There is abundant literature regarding medicinal plants establishing a relation between immunomodulatory and

antiulcerogenic activities and their phenol/ flavonoid content [16, 17]. For this reason, our chromatographic investigation was directed towards the highly bioactive the ethyl acetate fraction.

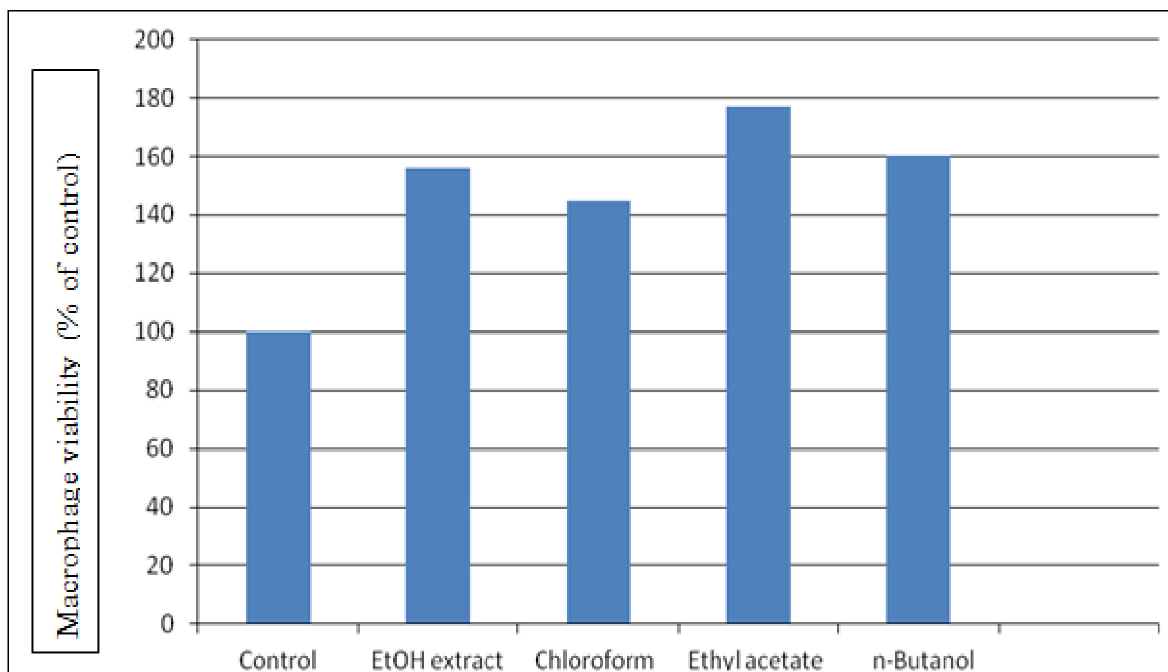


FIG. 1: CELL VIABILITY PERCENTAGE OF RAW 264.7 CELL TREATED GROUPS OF CFL, EAFL, AND BFL OF *S. SEMPERVIRENS* (D. DON.) LEAVES COMPARED TO CONTROL (UNTREATED GROUP). EACH VALUE REPRESENTS THE MEAN PERCENT ± S.D.*SIGNIFICANTLY DIFFERENT VERSUS CONTROL GROUP, P ≤ 0.05

TABLE 1: ANTI-ULCEROGENIC EFFECT OF CFL, EAFL, AND BFL OF *S. SEMPERVIRENS (D. DON.)* LEAVES ON ETHANOL –INDUCED –GASTRIC RATS (MEAN±SD, N=5)

Group Dose (mg/kg)	Score	No. of ulcers	Ulcer index	%Protection
Control	5.00	14.00 ± 2.31	11.00 ± 1.63	0.00
Ranitidine	2.64	7.60b±3.05	8.00^{a}±2.24	33.30
Chloroform extract	2.88	8.20^{b}±3.96	7.94 ± 3.29	45.81
Ethyl acetate extract	1.42	4.20^{c}±3.27	5.60^{b}±2.74	70.21
Butanol extract	1.92	6.60^{b}±2.07	6.60^{a}±3.21	42.05

Data are expressed as mean ± SD, n = 5. a p ≤ 0.05 b p ≤ 0.01. c p ≤ 0.001.

Phenolics analysis:

HPLC analysis of phenolic acids and flavonoids: HPLC analysis of the phenolic acids in the ethyl acetate fraction of *S. sempervirens* leaves (**Table 2**) showed the presence of 12 phenolic acids. Protocatechuic acid was the predominant phenolic acid (10.94 ± 1.22 mg/ g.d.w.), followed by salicylic acid (4.52 ± 1.23 mg/ g.d.w.), coumaric acid (4.42 ± 0.21 mg/ g.d.w.) and sinapic acid (3.57 ± 0. 63 mg/ g.d.w.). Fifteen flavonids compounds were identified by HPLC (**Table 3**) named as (Luteolin-7-*O*-glucoside, vicenin-II, mangiferin, quercetin-3, 7-di-O-glucoside, isovitexin, kaempferol, vitexin, quercetin, luteolin, apigenin, orientin, isoorientin, rutin, apigenin-7-O-rutinoside and epicatchin). Results showed that, the highest content of vitexin (6.67± 1.21 mg/ g.d.w.) followed by orientin (5.96 ± 1.18 mg/ g.d.w.) and quercetin-3,7-di-O-glucoside (4.92± 0.73 mg/ g.d.w.). The presence of phenolic compounds in *S. sempervirens* (D. Don.) leaves isn't relatively well documented but much more attention is paid to the terpenoids and essential oils, and their applications [3, 4]. To our knowledge this is the first HPLC analysis of phenolic acids and flavonoids in the genus Sequoia.

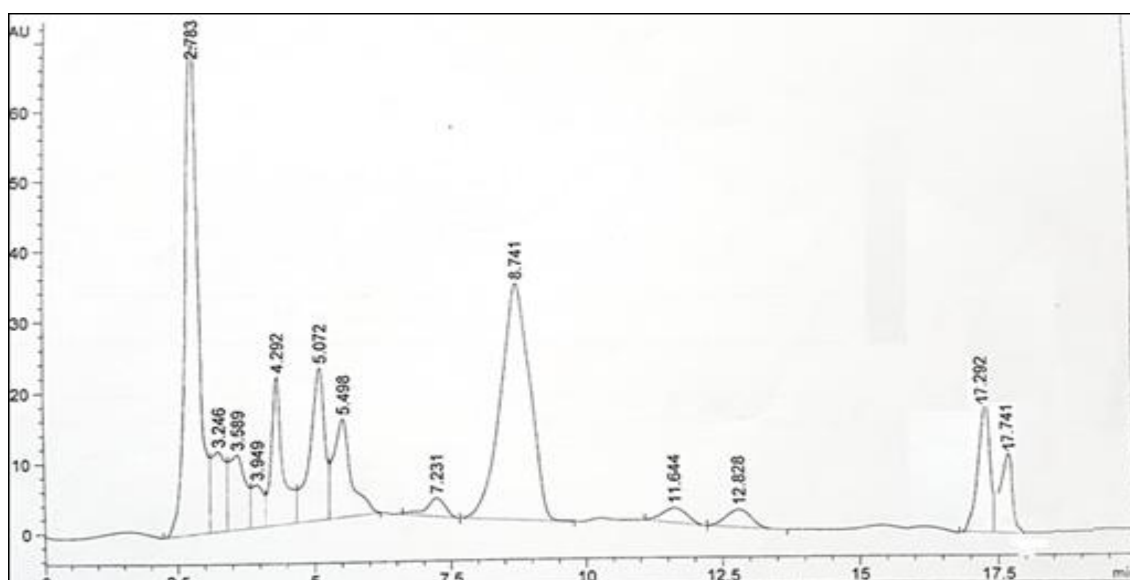


FIG. 2: HPLC CHROMATOGRAM OF PHENOLIC ACIDS IN OF ETHYL ACETATE FRACTION OF *S. SEMPERVIRENS* (D. DON.) LEAVES

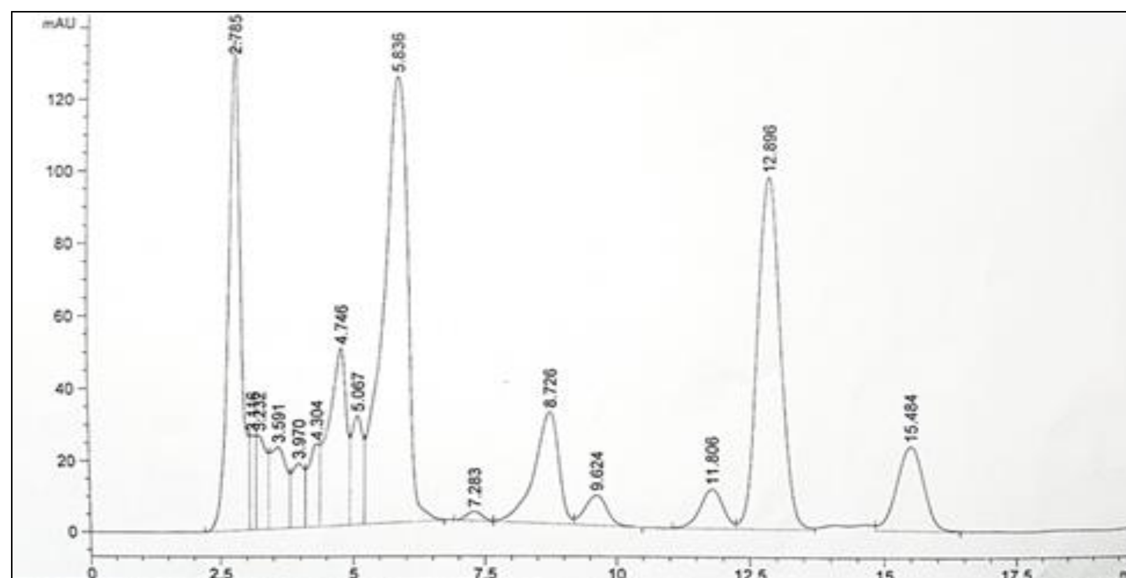


FIG. 3: HPLC CHROMATOGRAM OF FLAVONOIDS IN OF ETHYL ACETATE FRACTION OF *S. SEMPERVIRENS* (D. DON.) LEAVES

TABLE 2: PHENOLIC ACIDS IDENTIFIED BY HPLC ANALYSIS OF ETHYL ACETATE FRACTION OF *S. SEMPERVIRENS* LEAVES (mg/ g.d.w.).

Peak	Rt (min)	Concentration (mg/ g.d.w.)	Compound name
1	2.7	3.26 ± 1.23	Caffeic acid
2	3.2	1.33 ± 1.08	Gallic acid
3	3.5	3.57 ± 0.63	Sinapic acid
4	3.9	2.12 ± 1.02	Cinnamic acid
5	4.3	0.57 ± 1.83	Ferulic acid
6	5.1	4.42 ± 0.21	Coumaric acid
7	5.5	10.94 ± 1.22	Protocatechuic acid
8	7.2	0.37 ± 0.34	Gentisic acid
9	8.7	0.75 ± 1.72	Chlorogenic acid
10	11.6	4.52 ± 1.23	Salicylic acid
11	12.8	0.82 ± 1.38	Syringic acid
12	17.3	0.91 ± 0.65	Hydroxybenzoic acid

Values are the mean ± SD (n = 3).

Identification of the isolated compounds: As described in the experimental section, from the ethyl acetate fraction of *S. Semperverins* D. leaves, the flavonoids isoorientin (luteolin-6-*C*-glucopyarnoside) (1), isovitexin (apigenin-6-*C*-glucopyarnoside) (2), orientin (luteolin-8-*C*-glucopyarnoside) (3), vicenin-II (6, 8-*di*-*C*-glucosylapigenin) (4), mangiferin (5), vitexin (apigenin-8-C-glucopyarnoside) (6), protocatechuic acid (7), luteolin (8), apigenin(9) and kaempferol (10) were isolated. The structural identification of the isolates was elucidated by acid hydrolysis, UV, ^{1}H and ^{13}CNMR spectroscopic analysis and/or comparison with published data; **Fig. 4.**

Isoorientin (luteolin-6-*C* -glucopyarnoside) (1): Acid hydrolysis of compound 1 resulted in two flavonoidal spots as appeared on PC indicating, according to Wessely-Moser rearrangement, the *C*-glycoside nature of compound 1.The singlet at δ 6.47 (1H, H-8) in the ^{1}H NMR spectrum, and the quaternary carbon signal at δ 109.28 were attributed to H-6/C-6 of the 5,6,7-trisubstituted A-ring system of a flavonoid. The comparison of the NMR data of compound 1 with the literature confirmed this compound as being the luteolin-6-*C*-glycoside, isoorientin (**1**) [18] (Mabry, 1970).

TABLE 3: FLAVONOIDS IDENTIFIED BY HPLC ANALYSIS OF ETHYL ACETATE FRACTION OF *S. SEMPERVIRENS* LEAVES (mg/ g.d.w.)

Peak	Rt (min)	Concentration (mg/ g.d.w.)	Compound name
1	2.8	2.62± 1.05	Luteolin-7-O-glucoside
2	3.1	1.74± 0.86	Vicenin-II
3	3.2	3.4± 1.32	Mangiferin
4	3.6	4.92± 0.73	Quercetin-3,7-di-O-glucoside
5	3.9	2.52± 0.38	Isovitexin
6	4.3	1.84± 1.52	Kaempferol
7	4.7	6.67± 1.21	Vitexin
8	5.1	1.25 ± 0.35	Quercetin
9	5.8	3.51± 0.57	Luteolin
10	7.3	2.74± 1.52	Apigenin
11	8.7	5.96 ± 1.18	Orientin
12	9.6	2.30 ± 0.56	Isoorientin
13	11.8	2.48 ± 1.06	Rutin
14	12.8	2.53± 1.42	Apigenin-7-O-rutinoside
15	15.5	1.2± 1.22	Epicatchin

Values are the mean ± SD (n = 3).

Isovitexin (apigenin-6-*C*-glucopyarnoside) (2) Acid hydrolysis of compound 2 resulted in two flavonoidal spots as appeared on PC indicating, according to Wessely-Moser rearrangement, the *C*-glycoside nature of compound 2. In addition, ^{1}HNMR δ (ppm) indicated the absence of signal due to H-6 and appearance of singlet at δ 6.47 (H-8). The identity of compound 2 as isovitexin was achieved by comparison of its R_f values with those of authentic apigenin- 6-*C*-glucopyranoside (iso-vitexin) as well as comparison of its spectral data with those reported in the literature [18].

Orientin (luteolin-8-*C*--glucopyarnoside) (3): UV shifting reagents indicated the presence of free *ortho*-dihydroxy group at 3`, 4` and a free hydroxyl group at 5-position. Acid hydrolysis resulted in the absence of the sugar and the appearance of two flavonoidal spots on PC (Wessely-Moser interconversion) indicating the C-glycoside nature of compound 3. The ^{1}H and ^{13}C NMR spectra of the compound 3 exhibited characteristic signals of the aromatic systems (between δ 7.57-6.32) and of aglucopyranose moiety (between δ 4.07-3.51). In the ^{1}H NMR spectrum, the singlet at δ 6.32 (1H, H-6) was attributed to the A-ring aromatic proton of a 5, 7, 8- trisubstituted flavonoid. The doublets at δ 7.57 (*J*=8.0 Hz) and at δ 6.96 (*J*=8.0 Hz) were attributed to H-6`and H-5`, respectively, of the B-ring. The anomeric proton signal at relatively low field region, at δ 4.94 (1H, d, *J*=10.0 Hz, H-1``) as well as the coupling constant value was attributed to the occurrence of a *C*-glycoside flavonoid. The ^{13}C-NMRspectrum showed a quaternary carbon signal at δ 105.95,which is the characteristic of *C*-8 of the aglycon moiety.

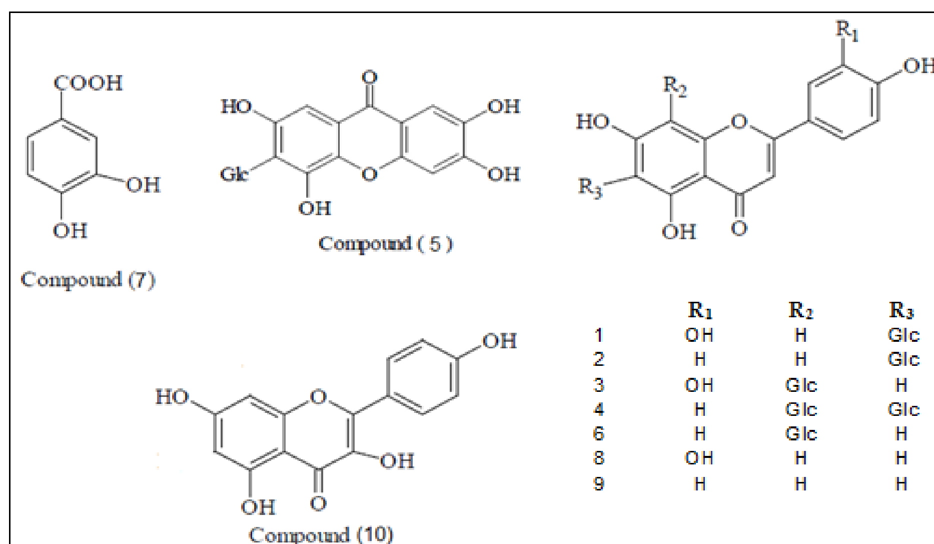
The comparison of the spectroscopic data of **3** with the literature confirmed the identity of this compound as being luteolin-8-*C*-glucoside, orientin (3) [19].

Vicenin-II (6, 8-*di*-*C*-glucosylapigenin) (4): UV shifting reagents indicated apigenin-type with a free 7-OH.The ^{1}H-NMR confirmed the apigenin B-ring, exhibiting a singlet at δ 6.3 for H-3 and no signals for H-6 or H-8. The presence of two *C*-linked glycosyl H-1s and also signals for two sugars was observed in the ^{1}H-NMR, indicating C-linked sugars at 6 and 8 positions respectively [20]. ESI/MS (neg.) gave m/z: 593 $[M+1]^-$.The retention time of compound 4 in HPLC is consistent with the retention time for vicenin-II (6, 8-*di*-*C*-glucosylapigenin) standard.

Mangiferin (5): λ_{max} nm (MeOH) 352, 309, 267, 250 suggesting a xanthones derivative. It exhibited in (negative) ESI/MS, molecular ion at: m/z 423 $[M+H]^+$ corresponding to $C_{19}H_{18}O_{11}$. ^{1}H-NMR revealed the presence of three downshift aromatic singlet signals at δ 6.4 for H-4, 6.78 for H-5, 7.35 for H-8, in addition, the characteristic chemical shift of sugar moiety at 4.6 (d, *J*= 9.5 Hz, H-1` glucose).The ^{13}C-NMRspectrum revealed the presence of 19 carbons, the aglycone had 13 carbons including 10 aromatic carbons at δ 161.79 (C-1), 107.42 (C-2), 163.83 (C-3), 93.27 (C-4), 101.63 (C-5), 155.309 (C-6),144.11 (C-7), 106.23 (C-8), 178.91 (C-9),156.16 (C-4a) and three methines at δ149.11 (C-4b), 110.98 (C-8a),101.21 (C-8b), the anomeric carbon at δ73.09 (C-1`) indicating C-linked sugar [20]. The interpretation of the data indicated the presence of mangiferin.

Vitexin (apigenin-8-*C*-glucopyarnoside) (6): by acid hydrolysis compound 6 gave two spots on PC indicating, according to Wessely-Moser rearrangement the *C*-glycoside nature of this compound. In addition, ^{1}H-NMR δ (ppm) indicated the absence of signal due to H-8 and appearance of signal at δ 6.11 (H-6). The UV and NMR spectral data indicated the presence of vitexin [20]. The identity of this compound was also confirmed by co-chromatography with vitexin standard. Thus, compound 6 was unequivocally identified as (vitexin), apigenin-8-*C*-glucopyarnoside.

Protocatechuic acid (7): white powder, gave reddish color with aniline/xylose reagent, specific for carboxylic acid. UV λ_{max} nm (MeOH): 282.30 and 250.49; ESI/MS: m/z $[M-H]^-$ 153 indicating $C_7H_6O_4$. The above data were in complete accordance with those published regarding the protocatechuic acid. The flavonoids luteolin (8), apigenin (9) and kaempferol (10) were characterized by comparison of their spectral data with the literature values [21]. *Sequoia* is the only genus of family Taxodiaceae which contains large amounts of C-glycosyl flavones [22]. Iso-orientin, vitexin and isovitexin was previously isolated from *S. Semperverins* D. leaves [23]. In the case of orientin, vicenin-II, mangiferin, protocatechuic acid, luteolin, apigenin and kaempferol to our knowledge this is the first reported occurrence of these compounds in the *S. Semperverins* D. leaves.



	R1	R2	R3
1	OH	H	Glc
2	H	H	Glc
3	OH	Glc	H
4	H	Glc	Glc
6	H	Glc	H
8	OH	H	H
9	H	H	H

FIG. 4: STRUCTURES OF THE PHENOLIC COMPOUNDS ISOLATED FROM THE ETHYL ACETATE FRACTION OF *S. SEMPERVIRENS* (D. DON.) LEAVES

CONCLUSION: The ethanol extract of air-dried powdered leaves was fractionated with chloroform, ethyl acetate and *n*-butanol. The fractions were examined for their immunomodulatory and antiulcerogenic activities. The most active fraction EAFL could be recommended, with more laboratory and clinical trials, as immunomodulatory and antiulcerogenic agents. Ten known phenolic compounds were isolated from ethyl acetate fraction, seven of them were isolated for the first time from of *S. sempervirens* (D. Don.) leaves, also the fraction subjected to HPLC analysis; resulted in the identification and quantification of 12 phenolic acids and 15 flavonoids. The result supports the use of the plant as a natural source of phenolic compounds and plant fractions should be considered as good sources for drug discovery.

CONFLICTS OF INTEREST: The authors declare that they have no competing interests.

AUTHOR'S CONTRIBUTIONS: ZT wrote the manuscript, planed the work and carried out chemical analysis and interpreted data. KF revised the manuscript and supervised work.

ACKNOWLEDGEMENTS: The authors are thankful to the National Organization for Drug Control and Research, Cairo- Egypt, for providing the facilities.

REFERENCES:

1. Earle, CJ: The Gymnosperm Database. ed. 2013
2. Bonner FT, Karrfalt RP (EDS): The Woody Plant Seed Manual, USDA Forest Service's Agriculture Handbook. United States. 2008; 727.
3. Miguel MG: Antioxidant and anti-inflammatory activities of essential oils: A short review, Molecules. 2010; 15: 9252-9287.
4. Joshi S and Sati SC: Antifungal Potential of Gymnosperms: A Review. In Microbes: Diversity and Biotechnology" (Eds. S. C. Sati and M. Belwal). 2012; 333-345.
5. Wink M: Medicinal plants: a source of anti-parasitic secondary metabolites. Molecules. 2012; 17: 12771-12791.
6. Essam MA and Hwida MF: Production of Indole Acetic Acid from Sequoia sempervirens and its Reuse Under In Vitro Culture Research Journal of Agriculture and Biological Sciences, 2012;8(2): 167-173
7. Moerman D: Eriogonum nudum. In: Native American Ethnobotany Database [Online]. Univ. of Michigan, Dearborn 2012.
8. Nicholas TD, Hsin FW and Clemens MA: The chemistry and bioactivity of various heartwood extracts from redwood (*Sequoia sempervirens*) against two species of fungi; New Zealand Journal of Forestry Science 2014; 44:17.
9. Gad MMA, Salem MA and Hewida EFM: Clonal propagation and mass production of *Sequoia sempervirens* (D. Don) Endl. Through tissue culture techniques. In: The first international conference on "Strategy of botanic gardens", Agriculture Museum, Dokki, Giza, Egypt, 2006.
10. Theophine CO, Peter A A, Adaobi CE, Maureen OO, Collins AO, Frankline N and Lovelyn: Evaluation of the acute and sub-acute toxicity of *Annona senegalensis* root bark extracts Asian Pacific Journal of Tropical Medicine 2012, 5(4): 277-282.
11. Alet T , Annie MJ and Duncan AC: Limitations of the 3-(4,5-dimethylthiazol-2-yl)- 2,5-diphenyl-2H-tetrazolium bromide (MTT) assay when compared to three commonly used cell enumeration assays; BMC Research Notes 2015; 8:47.
12. Subramanian S, Sathish KD, Arulselvan P, Senthilkumar GP and Mahadeva RUS: Evaluation of Anti-ulcerogenic Potential of *Aloe vera* Leaf Gel Extract Studied in Experimental Rats. Journal of Pharmacology and Toxicology 2007; 2: 85-97.
13. Martin MJ, Marhuenda E, Perez-Guerrero C and Franco JM: Antiulcer effect of naringin on gastric lesions induced by ethanol in rats. Pharmacology 1994; 49(3): 144-50.
14. La Casa C, Villegas I, de la Lastra CA, Motilva V, Martín Calero MJ: Evidence for protective and antioxidant properties of rutin, a natural flavone, against ethanol induced gastric lesions. J Ethnopharmacol 2000; 71(1): 45-53.
15. Smith PK, Krohn RI, Hermanson GT, Mallia AK, Gartner FH, Provenzano MD, Fujimoto EK, Goeke NM, Olson BJ and Klenk DC: Measurement of protein using bicinchoninic acid, Anal. Biochem.1985; 150:76–85.
16. Lu J, Papp LV, Fang J, Rodriguez-Nieto S, Zhivotovsky B and Holmgren A: Inhibition of mammalian thioredoxin reductase by some flavonoids: implications for myricetin and quercetin anticancer activity,Cancer Res. 2006; 8: 4410–4418.
17. Sumbul S, Ahmad MF and Mohd A: Role of phenolic compounds in peptic ulcer: An overview, J. Pharm. Bioallied. Sci 2011; 3:361-367.
18. Mabry TJ, Markham KR and Thomas MB: The Systematic Identification of Flavonoids. Springer-Verlag, Berlin 1970.
19. Zhou X, Peng JG, Fan G and Wu Y: Isolation and purification of flavonoid glycosides from Trollius ledebouri using high-speed counter-current chromatography by stepwise increasing the flow-rate of the mobile phase, J Chromatogr B 2005; 32: 216-221.
20. Hilsenbeck AR, Sharon, JW and Mabry, TJ: C-Glycosylflavones from Yeatesia viridiflora, J Nat Prod, 1984; 47, 312-315.
21. Umikalsom Y, Grayer B, Harborne RJ: A comparison of the flavonoids in Athyriaceae and Aspleniaceae, Biochem Syst Ecol. 1994; 22: 587–594.
22. Niemann GJ, Genderen HH: Chemical relationship between Pinaceae, Biochem Syst Ecol. 1980; 3: 237 – 240.
23. El-Hela AA and Ismail LD: Flavone - C- Glucosides from Sequoia Semperverins Leaves Cultivated In Egypt, J Biomed Sci. 2004; 14: 1-14.

How to cite this article:
Taha KF and Abd El Shakour ZT: Phytochemical profile of *Sequoia sempervirens* grown in Egypt. Int J Pharm Sci Res 2017; 8(3): 1081-91.doi: 10.13040/IJPSR.0975-8232.8(3): 1081-90.

All © 2013 are reserved by International Journal of Pharmaceutical Sciences and Research. This Journal licensed under a Creative Commons Attribution-NonCommercial-ShareAlike 3.0 Unported License.

This article can be downloaded to **ANDROID OS** based mobile. Scan QR Code using Code/Bar Scanner from your mobile. (Scanners are available on Google Playstore)