SUPPLEMENTARY MATERIAL

Eliciting callus culture for production of hepatoprotective flavonoids and phenolics from *Sequoia sempervirens* (D. Don Endl)

S. S. El-hawary[1], E. M. Abd El-Kader[2], M. A. Rabeh[1], G. A. Abdel Jaleel[3], M. A. Arafat[4], Tanja Schirmeister[5,*] and Usama Ramadan Abdelmohsen[6,*]

[1] Department of Pharmacognosy, Faculty of pharmacy, Cairo University, Cairo 11562, Egypt.

[2] Timber Trees Dept., Horticultural Research Institute, A.R.C., Giza, Egypt.

[3] Department of Pharmacology, National Research Center, Giza 12622, Egypt.

[4] Department of Pharmacognosy, Faculty of Pharmacy, Nahda University, Beni-Suef, Egypt.

[5] Institute of Pharmacy and Biochemistry, Johannes Gutenberg University, Staudingerweg 5, D. 55128 Mainz, Germany; schirmei@uni-mainz.de

[6] Department of Pharmacognosy, Faculty of Pharmacy, Minia University, 61519 Minia, Egypt;

SUPPLEMENTARY MATERIAL

Abstract

Hepatoprotective effects of the ethanolic extract of the leaves of *Sequoia sempervirens* was estimated by determination of liver biomarkers (ALT, AST, total bilirubin and albumin in serum) and by histopathological examinations. Concurrent administration of ethanolic extracts of *S. sempervirens* leaves improved the alterations in liver morphology where it was a potent protector of the liver. The potential of L-phenylalanine, silver nitrate as chemical elicitors and UV radiation as a physical elicitor on flavonoid production in callus culture of *S. sempervirens*. Murashige and Skoog's medium fortified with phenylalanine, silver nitrate produced enhanced production of flavonoids and phenolics per g dry weight. HPLC analysis was performed for qualitative and quantitative estimation of some flavonoid compounds in all of the calli produced in comparison with the mother plant. This finding highlights the use of *Sequoia sempervirens* its callus' phenolics and flavonoids in the treatment of liver diseases.

SUPPLEMENTARY MATERIAL

Experimental

Plant Material

Leaves were taken from a *Sequoia sempervirens* (D. Don Endl) tree grown in El-Orman Botanical Garden (voucher specimen no. 457-26/11/1961 and 12962-5-7-2015), Giza, Egypt to obtain the ethanolic extract. The plant identity was kindly authenticated by Dr. Reem Sameer Hamdi, associate professor of plant taxonomy, Department of Botany, Faculty of sciences, Cairo University herbarium.

Establishment of callus culture

Leaf segment explants of *Sequoia sempervirens* were rinsed in soapy water using septol soap with shaking for 20 min., and then washed with running tap water for one hour. Under aseptic conditions in a laminar air-flow cabinet, the explants were then immersed in 70% ethyl alcohol for 30 sec. followed by sterilization by 10% (v/v) Clorox (NaOCl 5.25%) for 10 minutes then 0.1% (w/v) mercuric chloride with a few drops of Tween-20 for 10 minutes. After acquiring the disinfection treatments, the explants were rinsed three times with sterile distilled water. The sterile explants (of about 2 cm length) were cultured on sterile MS medium. Cultures were incubated under the temperature of 26±2°C for 16 hrs. of fluorescent lighting lamps (2000-2500) lux per day and 8 hrs. darkness cycle for 4-6 weeks.

Hepatoprotective study

Experimental Animals

Swiss male mice of 20-30 g body weight and Wister albino male rats, with a weighing ranging from 200-225 g were used throughout the experiments. The mice were used for determination of the acute toxicity, while the rats were used for the main hepatoprotective biomarkers and histopathological estimation. The animals were obtained from the colony of animal house in the National Research Centre Giza, Egypt. The animals were accommodate in standard metal cages in an air-conditioned room at 22 ± 3˚C, 55 ± 5% humidity and supplied with standard laboratory diet and water ad libitum. Experiments were performed between 9:00 and 15:00 h. A group of 6 rats was used for this experiment. The experimental procedure were conducted in accordance with the guide for care and use of laboratory animals and in accordance with the animal procedures according to the Ethics Committee of the National Research Centre (NRC) and followed the recommendations of the National Institutes of Health Guide for Care and Use of Laboratory Animals (Publication No. 85-23, revised 1985).

Acute toxicity study

The 70% vacuum dried alcoholic extract of *S. sempervirens* leaves was dissolved in distilled water and was then given orally in graded doses to Swiss mice (1, 2, 3, 4 and 5 g kg^{-1}).The control group received the same volume of the vehicle and the mortality percentage was recorded three days later (Braca et al 2001).

Hepatoprotective activity

Twenty-four rats were randomly divided into four groups (n=6 per group): Group one (normal control group) was treated with 1 ml kg^{-1} distilled water as vehicles. Group two (positive control group) was treated with 200 mg kg^{-1} thioacetamide (TAA) dissolved in distilled water, (Sigma-Aldrich corp. St. Louis, USA) by intraperitoneal injection during a 24 hr interval before sacrificing. Group three was treated with *S. sempervirens* extract (200 mg kg^{-}

[1] dissolved in distilled water) by intragastric administration daily for two weeks followed by two doses of TAA (200 mg kg^{-1}, i.p.) during a 24 hr interval before sacrificing. Group four was treated with ascorbic acid (200 mg kg^{-1} dissolved in distilled water, Loba chemie, Pvt. Ltd. India) by intragastric administration daily for two weeks followed by two doses of TAA (200 mg kg^{-1}, i.p) during 24 hr intervals before sacrificing (Koblihová et al. 2014)**.** No animals died during the experiment. At the end of the 2nd week and 24 hr after the last dose of treatment and TAA administration, animals were weighted and then anesthetized with diethyl ether. Retro-orbital plexus blood was collected and centrifuged at 4,000 rpm for 15 min. The serum was stored at 8 °C for biochemical estimation with aspartate aminotransferase (AST) and alanine aminotransaminase (ALT) determined according to the method of Reitman and Frankel 1957. The absorbance was measured at 510 nm and the results were expressed as unites per liter of serum using the Quimica Clinica Aplicada (Spain) kit. Albumin levels were determined according to the method of Doumas et al. (1971). The absorbance was measured at 630 nm and the results were expressed as grams per decililter of serum. In addition, total bilirubin levels were measured in milligrams per decililter of serum according to the method of Walter and Gerade (1970) using (BioVision, California, USA) kits. Livers were fixed in 10% buffered neutral formalin. The tissues were embedded in liquid paraffin and stained with hematoxylin and eosin stain for histopathological findings (Sathyasaikumar et al. 2007).

Tissue culture study

Effect of different growth regulators on induction of callus and growth

To increase the biomass of calli, the callus culture of explants grown on MS medium was harvested after an incubation period of 6 weeks (approximately ultimate growth occurred) and was then dissected to uniformly sized pieces (about 2 cm length) which were subcultured on sterile MS medium supplemented with different plant growth regulators (2,4-dichlorophenoxy acetic acid (2,4-D), naphthalene acetic acid (NAA), trichlorophenoxy acetic acid and *p*-chlorophenoxy acetic acid) at 2.0, 4.0 and 6.0 mg L^{-1}. All treatments were incubated for 4-6 weeks.

Elicitation of callus for flavonoid and phenolic production

The proliferated callus culture was subcultured on MS medium supplemented with *p*-chlorophenoxy acetic acid at 4.0 mg L^{-1} (the most favorable medium for callus growth) of concentration which contained different chemical and/or physical elicitors including phenylalanine (100 and 200 mg L^{-1}), silver nitrate (5 and 10 ml L^{-1}) and UV-C light (15 and 30 watt for 1 hour). MS medium supplemented with *p*-chlorophenoxy acetic acid at 4.0 mg L^{-1} was used as the control. After four weeks, cultures were harvested, dried at 60°C in an oven and used for the estimation of total flavonoid and phenolic contents.

Estimation of total flavonoid and phenolic contents

One gram of the dried powdered non-flowering parts of *Sequoia sempervirens* was extracted twice, each for 15 min. with 10 ml 80% acetone containing 1% HCl by the help of sonication. The extract was then centrifugated at 3000 rpm for 10 min. The supernatant was filtered off. Then, the filtrate was transferred to a 25 ml volumetric flask and the volume was adjusted with distilled water. The extracts were filtered off before spectrophotometric determination of the total phenolic and flavonoid contents (Ivanova et al. 2010). The total flavonoid content was evaluated according to the colorimetric assay with aluminium chloride reagent (Zhishen et al. 1999) and total phenolic content was determined by the Folin–Ciocalteu method (Siger et al. 2008).

Determination of individual flavonoids by HPLC

HPLC analysis was performed for qualitative and quantitative estimation of the same flavonoid compounds (apigenin, luteolin, kaempferol and quercetin) in all of the calli produced in comparison with the mother plant (non-flowering parts). The working solutions of the dried powder of non-flowering parts (25 mg/10 ml) and authentic samples (1 mg/5 ml) were prepared in MeOH, membrane filtered and used for HPLC. HPLC Hewlett Packard

(series 1050) equipped with solvent degasser, ultraviolet (UV) detector set (at 280 nm for flavonoids determination) and a quaternary pump. The column temperature was uphold at 35 °C. The column used for separation was Phenomenex C18 (250 mm length). An isocratic separation was carried out using 30, 50 and 90% (v/v) of methanol with 0.5, 1.0 and 1.5% (v/v) acetic acid as a mobile phase at a flow rate of 1 ml/min. Authentic flavonoids were dissolved in the mobile phase and injected into the HPLC. The retention time and peak area were used to identify and calculate the flavonoids concentrations (Mattila et al. 2000).

Statistical analysis

All the experiments were performed with a minimum of five replicates per treatment. Significance of treatment effects was analyzed by one-way analysis of variance (ANOVA) using SPSS 20.0 software and *post hoc* Duncan test ($p<0.05$) to determine significant differences among treatment means.

References

Braca A, De Tommasi L, Di Bari C, Pizza M, Politi I, Morelli. 2001. Antioxidant principles from *bauhinia tarapotensis*." J Nat Prod. 64(7): 892-5.

Doumas BT, Watson WA, Biggs HG. 1971. Albumin standards and the measurement of serum albumin with bromcresol green. Clin. Chim. Acta. 31(1):87-96

Ivanova V, Stefova M, Chinnici F. 2010. Determination of the polyphenol contents in Macedonian grapes and wines by standardized spectrophotometric methods. J Serbian Chem Soc. 75(1): 45-59

Koblihová E, Mrázová I, Vernerová Z, Ryska M. 2014. Acute liver failure induced by thioacetamide: selection of optimal dosage in Wistar and Lewis rats. Physiol Res. 63: 491-503

Mattila P, Astola J, Kumpulainen J. 2000. Determination of flavonoids in plant material by HPLC with diode-array and electro-array detections. J Agricult Food Chem. 48(12): 5834-41

Reitman S , Frankel S. 1957. A colorimetric method for the determination of serum glutamic oxaloacetic and glutamic pyruvic transaminases. American J Clin Pathol. 28: 56-63.

Siger A, Nogala-Kalucka M, Lampart-Szczapa E. 2008. The content and antioxidant activity of phenolic compounds in cold-pressed plant oils. J Food Lipids. 15(2): 137-9.

Walter M, Gerade H. 1970. Bilirubin assay. Microchem J. 15, 231-6.

Zhishen J, Mengcheng T, Jianming W. 1999. The determination of flavonoid contents in mulberry and their scavenging effects on superoxide radicals. Food Chem. 64(4): 555-9

SUPPLEMENTARY MATERIAL

Fig. S1 Effect of *Sequoia sempervirens* (D. Don Endl) extract (200 mg kg^{-1}) on serum AST and ALT in thioacetamide-induced liver injury model.

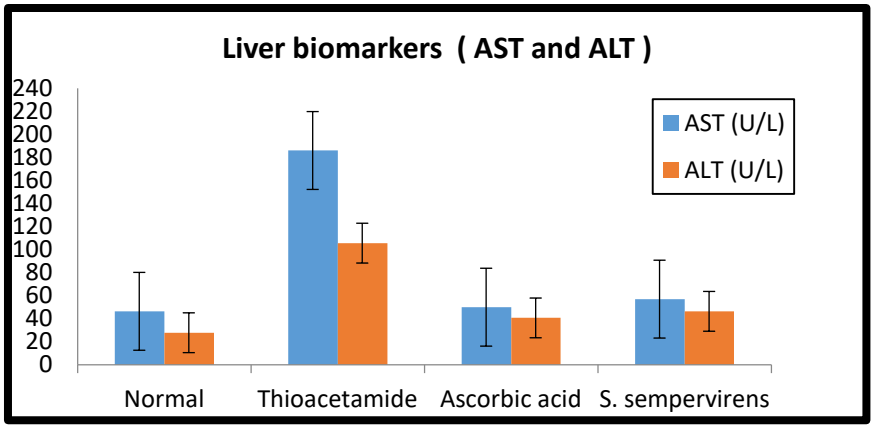
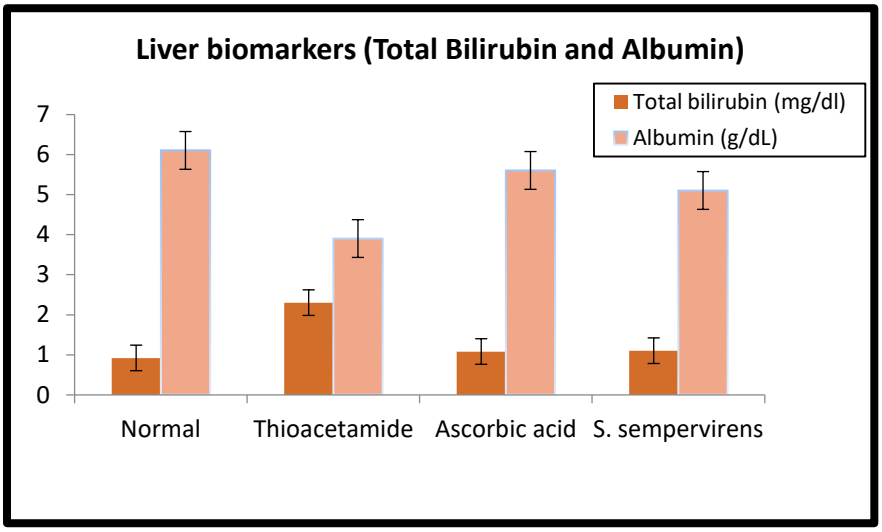


Fig. S2 Effect of *Sequoia sempervirens* (D. Don Endl) extract (200 mg kg^{-1}) on serum total bilirubin and albumin in thioacetamide-induced liver injury model.



SUPPLEMENTARY MATERIAL

Fig. S3 Photomicrographs of liver sections from (A) normal control rat (H & E, 400X), normal histological structure of hepatic lobule from central vein and concentrically arranged hepatocytes. (B, C & D) Thioacetamide treated group. (B) Fatty change of hepatocytes (small arrow) and focal hepatic necrosis associated with inflammatory cells infiltration (large arrow), (C) Steatosis of hepatocytes (small arrow), oval cells proliferation (large arrow), (D) congestion of hepatic sinusoids (large arrow) and Kupffer cells activation (arrow head). Photomicrographs of liver sections from (E , F) *S. sempervirens* (D. Don Endl) extract treated group (H & E, 400X), slight hydropic degeneration of hepatocytes (small arrow) and few inflammatory cells infiltration in the portal triad (large arrow).

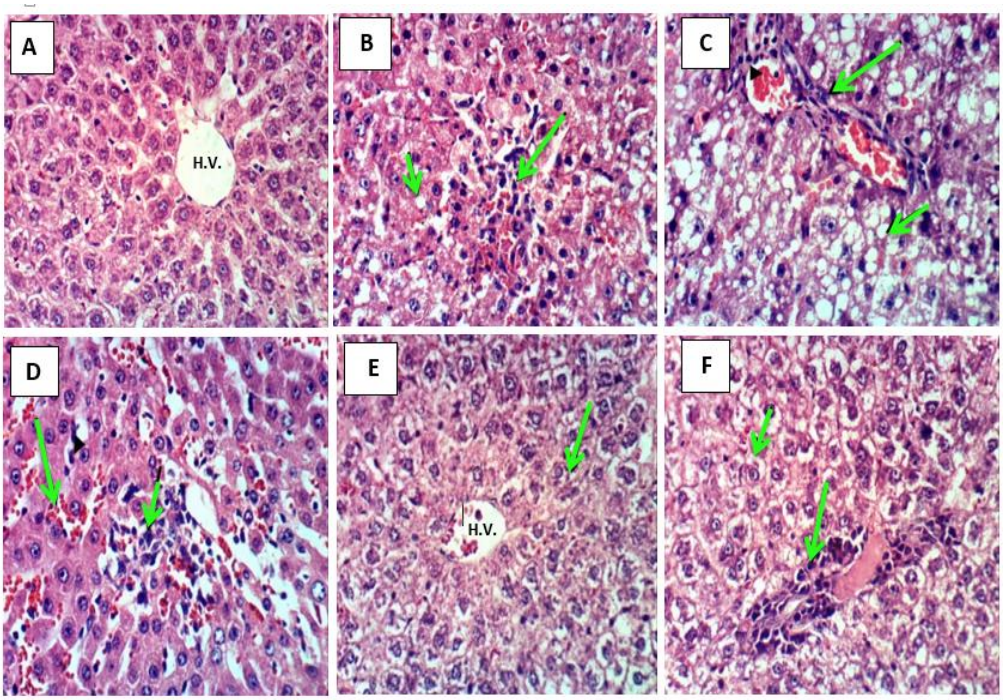


Fig. S4 *Sequoia sempervirens* (D. Don Endl) callus grown on MS medium

SUPPLEMENTARY MATERIAL

Fig. S5 *Sequoia sempervirens* (D. Don Endl) callus fresh weight increments in two successive subcultures

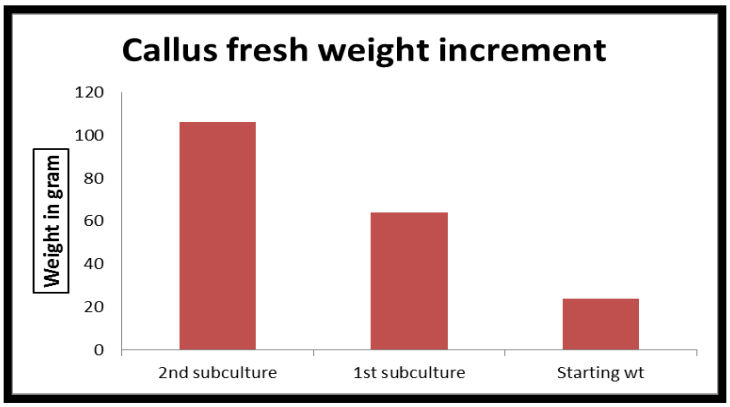


Fig. S6 HPLC chromatogram of callus control sample.

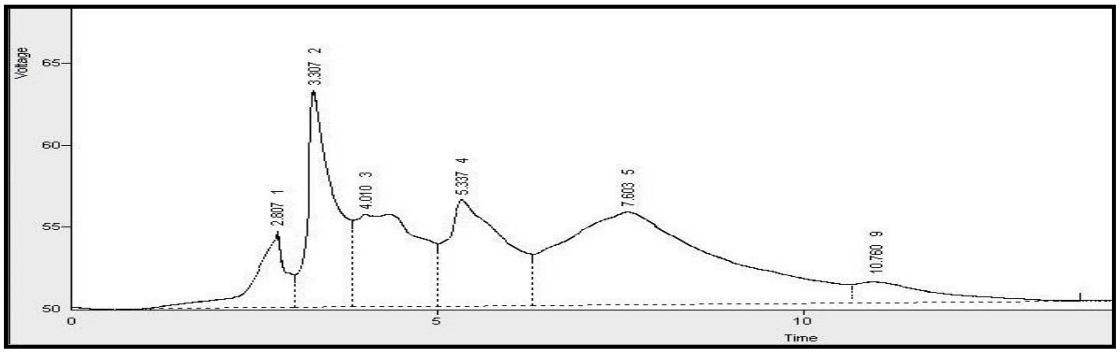
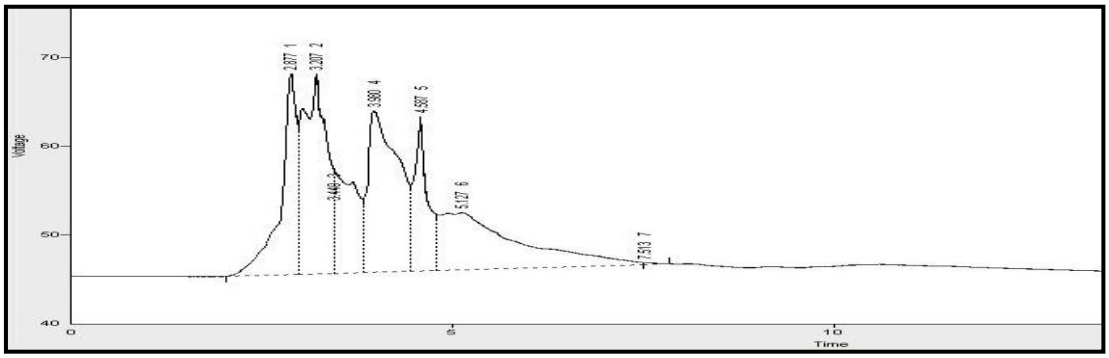


Fig. S7 HPLC chromatogram of callus sample from medium containing phenylalanine 100 mg L^{-1}



SUPPLEMENTARY MATERIAL

Fig. S8 HPLC chromatogram of callus sample from medium containing phenylalanine 200 mg L^{-1}

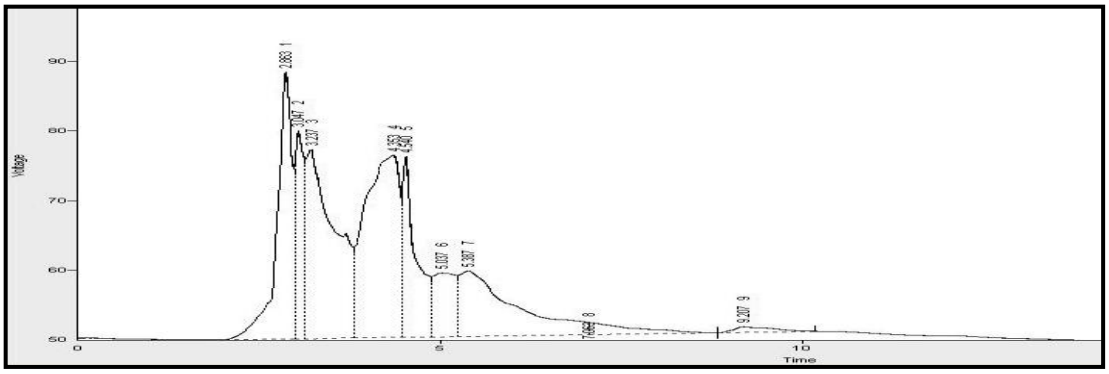


Fig. S9 HPLC chromatogram of callus sample from medium containing silver nitrate 5 ml L^{-1}

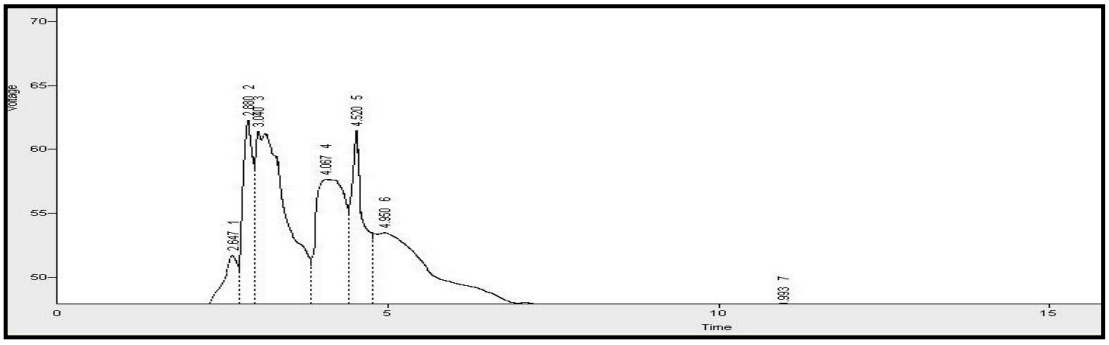
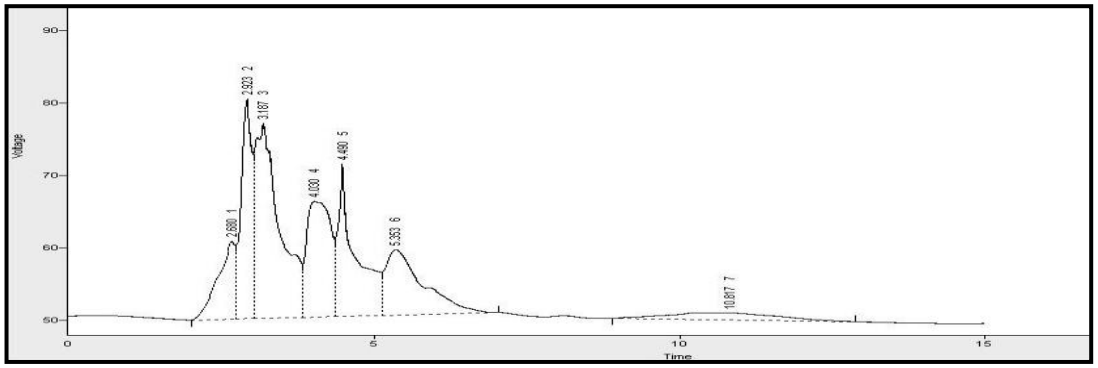


Fig. S10 HPLC chromatogram of callus sample from medium containing silver nitrate 10 ml L^{-1}



SUPPLEMENTARY MATERIAL

Fig. S11 HPLC chromatogram of callus sample from medium containing phenylalanine 100 mg L^{-1} and UV at 15 watt.

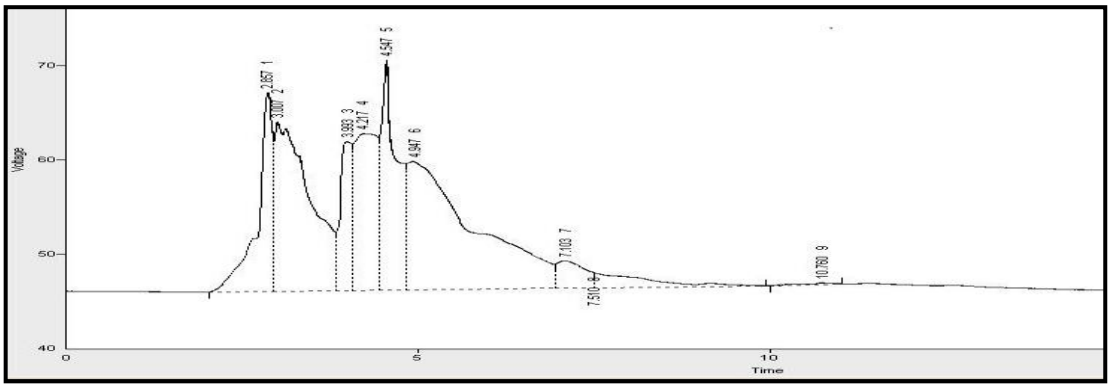


Fig. S12 HPLC chromatogram of callus sample from medium containing phenylalanine 100 mg L^{-1} and UV at 30 watt.

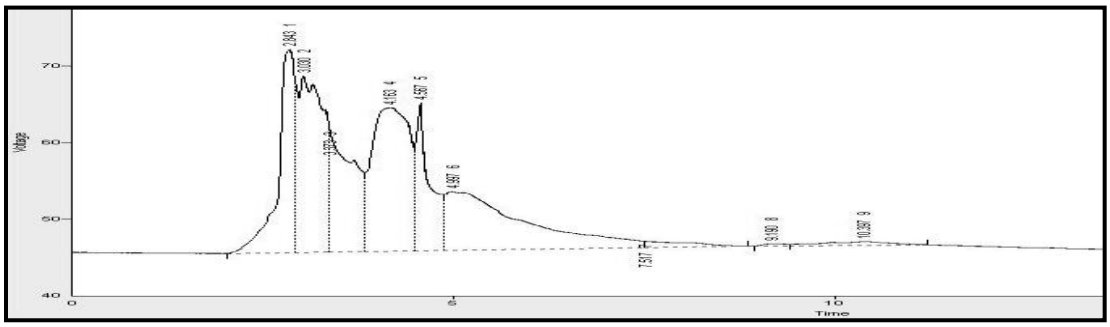
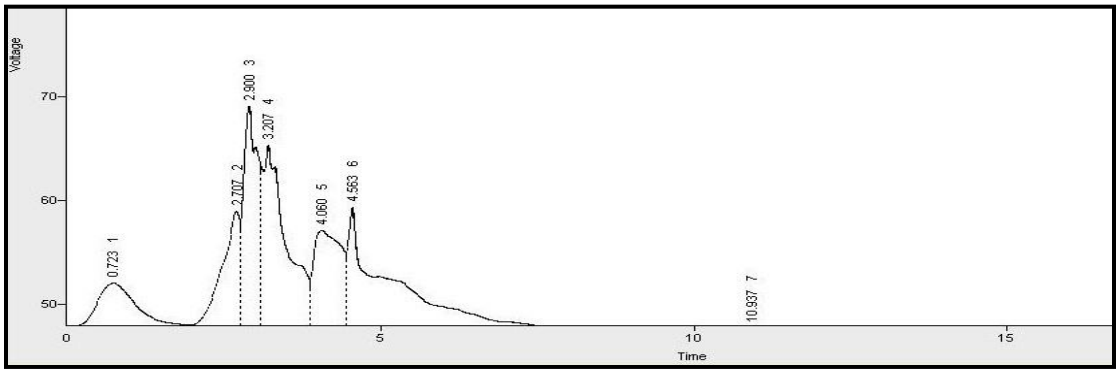


Fig. S13 HPLC chromatogram of callus sample from medium containing phenylalanine 200 mg L^{-1} and UV at 15 watt



SUPPLEMENTARY MATERIAL

Fig. S14 HPLC chromatogram of callus sample from medium containing phenylalanine 200 mg L^{-1} and UV at 30 watt.

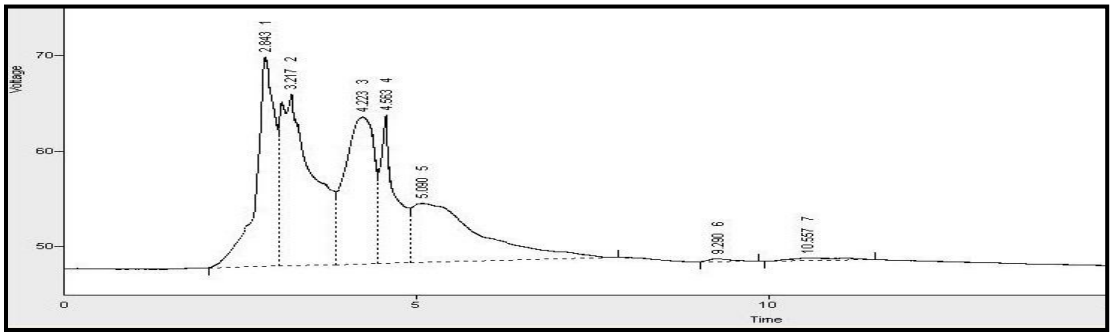


Fig. S15 HPLC chromatogram of callus sample from medium containing silver nitrate 5 ml L^{-1} and UV at 15 watt.

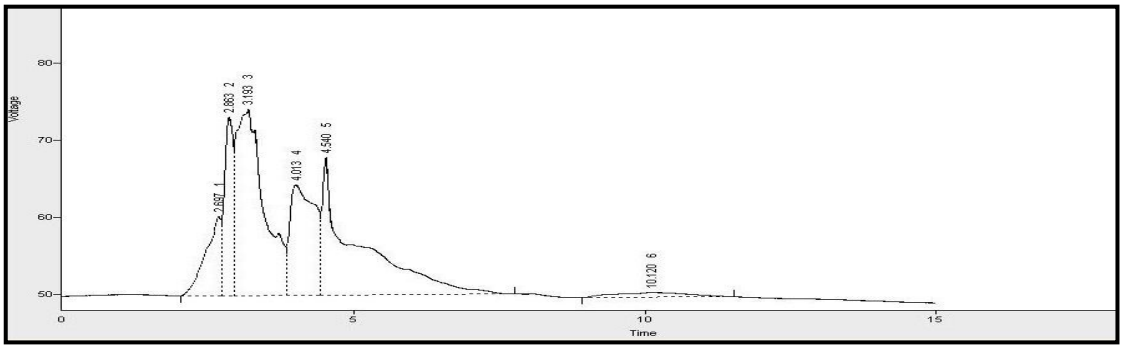
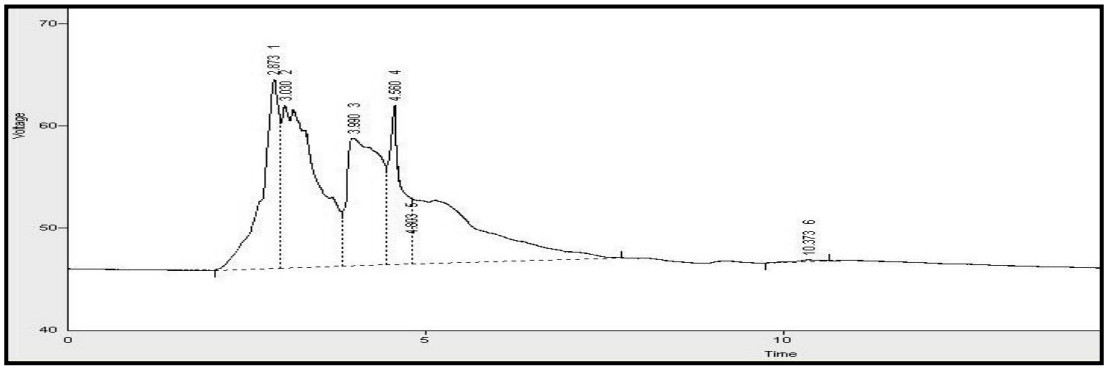


Fig. S16 HPLC chromatogram of callus sample from medium containing silver nitrate 5 ml L^{-1} and UV at 30 watt.



SUPPLEMENTARY MATERIAL

Fig. S17 HPLC chromatogram of callus sample from medium containing silver nitrate 10 ml L^{-1} and UV at 15 watt.

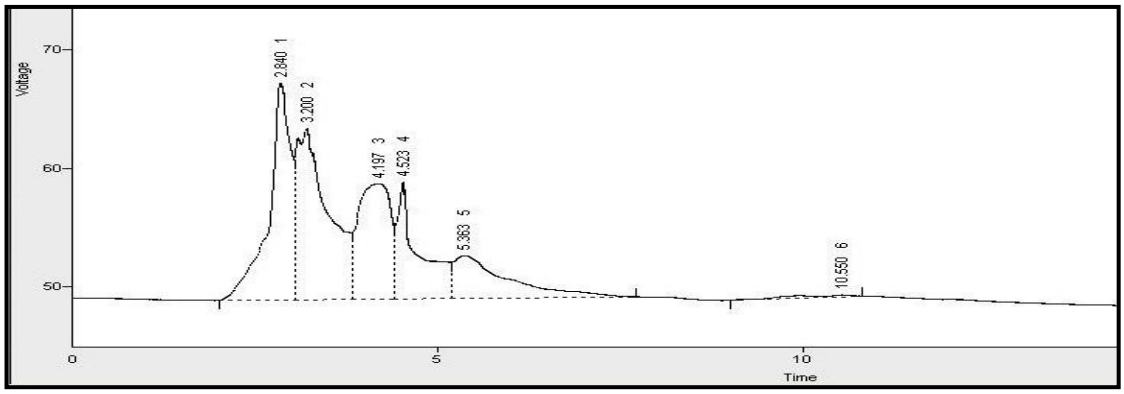


Fig. S18 HPLC chromatogram of callus sample from medium containing silver nitrate 10 ml L^{-1} and UV at 30 watt.

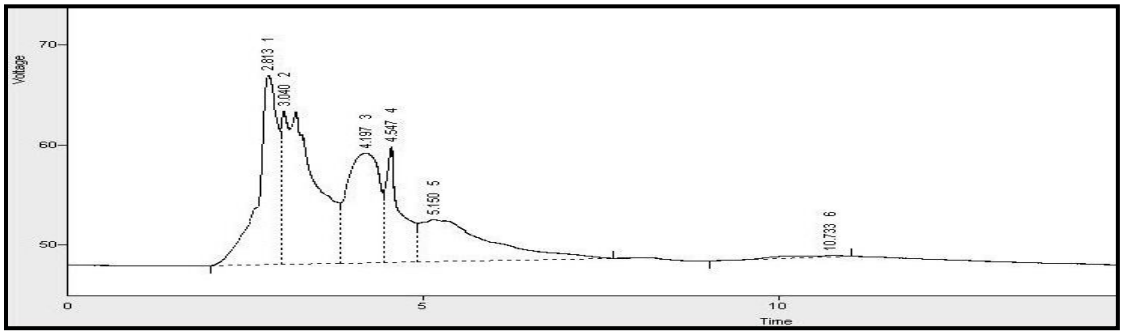
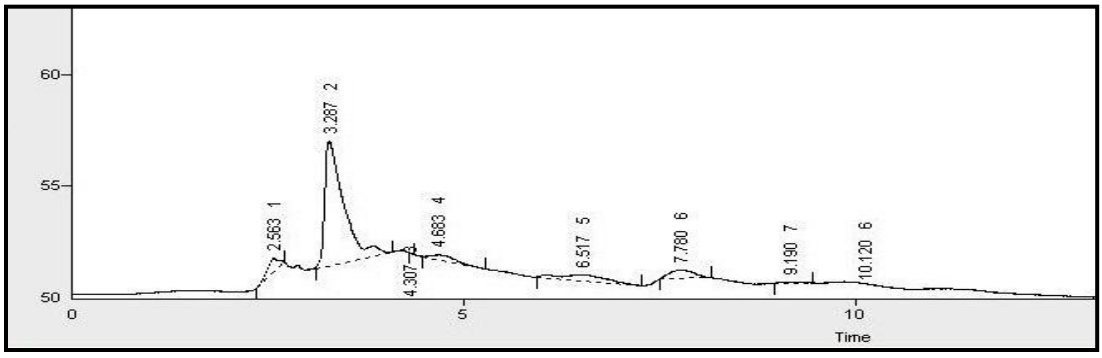


Fig. S19 HPLC chromatogram of mother plant of *Sequioia sempervirens* (D.Don Endl)



SUPPLEMENTARY MATERIAL

Table S1 Effect of *Sequoia sempervirens* (D. Don Endl) extract on serum liver biomarkers in thioacetamide-induced liver injury.

Groups / Biomarkers	AST ($U\ L^{-1}$)	ALT ($U\ L^{-1}$)	Total bilirubin ($mg\ dl^{-1}$)	Albumin ($g\ dl^{-1}$)
Normal	46.4 ± 2. 9	27.8 ± 2.37	0.92 ± 0.03	6.1 ± 0.54
Thioacetamide	186 ± 10.42 [a]	105.6 ± 8.8 [a]	2.3 ± 0.22 [a]	3.9 ± 0.34 [a]
Ascorbic acid ($200\ mg\ kg^{-1}$)	50 ± 3. 6*	40.7 ± 2.6*	1.08 ± 0.07*	5.6 ± 0.48*
S. sempervirens extract ($200\ mg\ kg^{-1}$)	57 ± 3.7*	46.4 ± 3.4*	1.1 ± 0.09*	5.1 ± 0.36*

Data are mean ± SD (n = 6). AST: Aspartate aminotransferase ALT: Alanine aminotransferase [a] Significant versus control ($P \leq 0.05$). *****Significant versus TAA group ($P \leq 0.05$) using thioacetamide-induced (TAA) liver injury model.

Table S2 Different plant growth regulators used for *Sequoia sempervirens* (D. Don Endl) callus growth.

Media composition	Callusing
MS + 2,4-D 2 mg L^{-1}	+
MS + 2,4-D 4 mg L^{-1}	+
MS + 2,4-D 6 mg L^{-1}	+
MS + NAA 2 mg L^{-1}	+
MS + NAA 4 mg L^{-1}	+
MS + NAA 6 mg L^{-1}	+
MS + trichlorophenoxy acetic acid 2 mg L^{-1}	+
MS + trichlorophenoxy acetic acid 4 mg L^{-1}	+
MS + trichlorophenoxy acetic acid 6 mg L^{-1}	+
MS + *p*-chlorophenoxy acetic acid 2 mg L^{-1}	+
MS + *p*-chlorophenoxy acetic acid 4 mg L^{-1}	+++
MS + *p*-chlorophenoxy acetic acid 6 mg L^{-1}	++

SUPPLEMENTARY MATERIAL

Table S3 Effect of phenylalanine, silver nitrate and ultraviolet irradiation treatments on total phenolics and flavonoids content of *Sequoia sempervirens* (D. Don Endl) calli and mother plant

Treatment	Total phenolics (mg GAE/100 g)	Total flavonoids (mg KE/100 g)
Control	593.2 (±13.5) [a]	118.6 (±1.3) [a]
Phenylalanine 100 mg L^{-1}	910.2 (±54.6)*	182.0 (±2.5)*
Phenylalanine 200 mg L^{-1}	1186.4 (±45.6)*, [a]	237.3 (±4.1)*, [a]
Silver nitrate 5 ml L^{-1}	552.3 (±19.9)	110.5 (±1.2)
Silver nitrate 10 ml L^{-1}	613.6 (±26.7)*	122.7 (±1.4)*
Phenylalanine 100 mg L^{-1} + 15 W UV for 60 min.	1033 (±36.3)*	206.6 (±3.2)*
Phenylalanine 100 mg L^{-1} + 30 W UV for 60 min.	736.4 (±45.8)*	147.2 (±1.9)*
Phenylalanine 200 mg L^{-1} + 15 W UV for 60 min.	787.5 (±35.7)*	157.5 (±2.4)*
Phenylalanine 200 mg L^{-1} + 30 W UV for 60 min.	664.3 (±18.3)*	128.9 (±2.6)*
Silver nitrate 5 ml L^{-1} + 15 W UV for 60 min.	613.6 (±14.9)*	122.7 (±1.9)*
Silver nitrate 5 ml L^{-1} + 30 W UV for 60 min.	900.0 (±44.3)*	180.0 (±2.9)*
Silver nitrate 10 ml L^{-1} + 15 W UV for 60 min.	675.0 (±22.4)*	135.0 (±1.8)*
Silver nitrate 10 ml L^{-1} + 30 W UV for 60 min.	521.6 (±11.7)	104.3 (± 1.1)
Mother plant	1074.3 (±52.1)*	214.9 (±2.5)*

The data represent average values taken from three replicates ± standard deviation.
Results are significant at $p \leq 0.05$ by one way ANOVA statistical analysis.
* Significant difference as compared to control, [a] Significant difference as compared to mother plant

SUPPLEMENTARY MATERIAL

Table S4 Concentrations of identified flavonoids expressed as mg/100 g dry powder of different calli of *Sequoia sempervirens* (D. Don Endl)

Treatment	Flavonoids (mg/100 g dry weight)			
	Luteolin	**Quercetin**	**Kaempferol**	**Apigenin**
R_t (min.)	**3.29 ± 0.5**	**5.0± 0.5**	**7.78± 0.5**	**10.5± 0.5**
Control	0.327 (±0.011) [a]	0.248 (±0.007) [a]	0.955 (±0.013) [a]	0.01 (±0.004) [a]
Phenylalanine 100 mg L^{-1}	0.232 (±0.006)* [a]	0.331 (±0.011)* [a]	0.002 (±0.001)	-
Phenylalanine 200 mg L^{-1}	0.756 (±0.008)* [a]	0.136 (±0.008)* [a]	0.074 (±0.004)* [a]	0.02 (±0.002)* [a]
Silver nitrate 5 ml L^{-1}	0.505 (±0.005)* [a]	0.345 (±0.005)* [a]	-	0.01 (±0.003)* [a]
Silver nitrate 10 ml L^{-1}	0.757 (±0.009)* [a]	0.269 (±0.004)* [a]	-	**0.063 (±0.009)*** [a]
Phenylalanine 100 mg L^{-1} + 15W UV for 60 min.	0.18 (±0.008)* [a]	**0.64 (±0.010)*** [a]	**0.1 (±0.007)*** [a]	0.003 (±0.001)* [a]
Phenylalanine 100 mg L^{-1} + 30W UV for 60 min.	0.48 (±0.007)* [a]	0.42 (±0.004)* [a]	-	0.05 (±0.002)* [a]
Phenylalanine 200 mg L^{-1} + 15W UV for 60 min.	0.55 (±0.010)* [a]	0.4 (±0.009)* [a]	0.04 (±0.003)* [a]	0.02 (±0.001)* [a]
Phenylalanine 200 mg L^{-1} + 30W UV for 60 min.	0.58 (±0.012)* [a]	0.3 (±0.002)* [a]	-	0.01 (±0.003)* [a]
Silver nitrate 5 ml L^{-1} + 15W UV for 60 min.	**0.8 (±0.011)*** [a]	0.51 (±0.003)* [a]	-	0.03 (±0.002)* [a]
Silver nitrate 5 ml L^{-1} + 30W UV for 60 min.	0.4 (±0.008)* [a]	0.32 (±0.007)* [a]	-	0.002 (±0.001)* [a]
Silver nitrate 10 ml L^{-1} + 15W UV for 60 min.	0.44 (±0.007)* [a]	0.13 (±0.008)* [a]	-	0.01 (±0.001)* [a]
Silver nitrate 10 ml L^{-1} + 30W UV for 60 min.	0.511 (±0.009)* [a]	0.2 (±0.003)* [a]	-	0.01 (±0.001)* [a]
Mother plant	0.1 (±0.007)*	0.004 (±0.001)*	0.01 (±0.002)*	0.001 (±0.001)*

The data represent average values taken from three replicates ± standard deviation.

Results are significant at $p \leq 0.05$ by one way ANOVA statistical analysis.

[a] Significant difference as compared to mother plant * Significant difference as compared to control

+: 25-40% of explants formed callus with poor growth; **++:** 50-70% of explants formed callus with moderate growth; **+++:** 100% of explants formed callus with good growth.