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GC/MS Analysis and Secondary Metabolites Determination with Antioxidant and Anticancer Evaluation of *Sequoia sempervirens* (D. Don) Endl. through Micropropagated Cultures

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Abstract

Micro-cuttings of *Sequoia sempervirens* tree were carried out on Murashige and Skoog medium enriched with various concentrations of sucrose and/or growth regulators to induce callus or shootlet cultures. Gas chromatography/mass spectrometry (GC/MS) analysis of *n*-hexane extracts of the leaves of *S. sempervirens* mother plants, shootlets, and calli were investigated to identify nonpolar compounds. Likewise, their aqueous ethanol extracts were assessed quantitatively for polar secondary metabolites (phenolics, flavonoids, and triterpenoids) and then investigated to explore their antioxidant and cytotoxic activities. Maximum survival shootlets (100%) were recorded when the medium was provided by either (25 g/l sucrose + 2g/l activated charcoal) or (50 g/l sucrose + 0.5 mg/l kinetin) with an increase in means of shootlet length, fresh and dry weights. Moreover, GC/MS analysis of *n*-hexane extracts of the leaves of mother plants, shootlets, and calli revealed the identification of 43 (93.56 %), 42 (96.15 %), and 17 (95.05%) compounds, respectively. Aliphatic hydrocarbons were the major components in *n*-hexane extract of callus treated with 1.6 mg/l 2, 4-D + 0.8 mg/l NAA (62.53%), with high levels eicosane, 2-methyl (19.55%), and heptacosane (17.10 %) whereas a high percentage of monoterpene hydrocarbons compounds were found in *n*-hexane extract of shootlet treated with 50 g/l sucrose + 0.5 mg/l kinetin. The major compounds in *n*-hexane extract of shootlets and the leaves of mother plants were monoterpene hydrocarbons (79.35 and 69.23%), α-Phellandrene (36.02 and 35.93%) and α-pinene (31.41 and 10.57%), respectively. Furthermore, quantitative estimation of the total flavonoids, phenolics, and triterpenoids of the aqueous ethanol extracts showed that shootlet cultures treated with 50 g/l sucrose + 0.5 mg/l kinetin had the highest contents (2.514, 0.98, 172.640 µg mg^{-1}, d.w.) compared with those of leaves (2.061, 0.684, 172.672 µg mg^{-1}, d.w.), respectively. Moreover, the same shootlet cultures gave the most potent antioxidant activity and showed inhibition effects on the human liver (HepG-2) and breast (MCF-7) tumor cell lines compared to the other aqueous ethanolic extracts of the tested samples. Consequently, shootlet culture enriched with 50 g/l sucrose + 0.5 mg/l kinetin was the most biologically active medium and could be considered as an alternative source of bioactive metabolites.

Keywords:*Sequoia sempervirens*; *in vitro* cultures; phenolics; flavonoids; triterpenoids; hydrocarbons;biological activities.

1. Introduction

Cupressaceae, is one of the largest and most diverse conifer families, with 33 genera and 142 species [1]. Many Cupressaceae species yield valuable timber and play an important role in rnamental plantings and environmental forestry [2]. *Sequoia* Endle. is a genus of redwood coniferous trees in the Sequoioideae subfamily, and it contains many biochemical components like tannins, phenolics, flavonoids, and triterpenoids [3].

Sequoia sempervirens (D. Don.) Endl. is a great valuable tree not just for its decorative appeal, but also for its industrial importance, since it may be utilized in the paper, lumber (plywood), and pulp industries [4]. The tree is known for its richness with several

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bioactive constituents such as diterpene [5], lignans [6, 7], phenolic compounds [8, 9], essential oils [4, 8], resin acids, and flavonoids [9]. Additionally, *S. sempervirens* was found to possess anticancer, antifungal [7, 10], antibacterial [8], anti-hepatotoxicity [11] effects, and have activity toward cathepsin B [7].

Currently, *in vitro* cultures (shooting and callus cultures) are now commonly used as alternative sources for producing secondary metabolites in high yields and within reasonable timeframes. The exposure of the *in vitro* shootlets of *S. sempervirens* to different laser radiations resulted in an increase in shooting behavior, rooting percentage, contents of carotenoids, cellulose, carbohydrate, total phenolics, flavonoids, tannins, and antioxidant activity (DPPH) [12]. Also, the effect of L-phenylalanine, silver nitrate, UV radiation, and nano oxides on the flavonoids and phenolics production in the callus culture of *S. sempervirens* in comparison with the mother plant has been evaluated [3, 11]. More recently, the *in vitro* shootlets and callus cultures were examined to analyze the total phenols, flavonoids, and oil constituents as well as the photosynthetic pigments using various cytokinin and auxin types and concentrations [4]. However, more efforts are required to achieve efficient micropropagation of such vital medicinal tree.

The current study was carried out to induce shooting and callus cultures from *S. sempervirens* and to investigate their extracts compared to mother plant leaves using different growth regulators. Non-polar compounds were examined in *n*-hexane extracts, and polar secondary metabolites were quantified in aqueous ethanol extracts (phenolics, flavonoids, and triterpenoids) and were further investigated to explore their antioxidant and cytotoxic activities.

2. Experimental

2.1. General

Solvents of analytical grade were purchased from (Fisher chemical, UK). Reference standards: gallic acid, quercetin, and oleanolic acid were obtained from Sigma Chemicals (USA). Distilled water was used in all experiments. Folin-ciocalteu reagent and sodium carbonate (Sigma Chemicals, USA) for the total phenolics content. Sodium hydroxide and aluminum chloride (Sigma Chemicals, USA) for the total flavonoids. Vanillin-glacial acetic acid 5 % was prepared by dissolving vanillin (500 mg) in acetic acid (10 ml) for the total triterpenoids content. 1, 1-diphenyl-2-picrylhydrazyl (DPPH) (Sigma Chemicals, USA) for *in vitro* antioxidant activity. Roswell Park Memorial Institute (RPMI-1640) medium (Sigma Chemicals, USA) and Doxorubicin® (Sigma-Aldrich Co., USA) for the *in vitro* cytotoxic activity. HepG2 (liver carcinoma) and MCF7 (breast cancer) cell lines were obtained in liquid nitrogen (-80°C) from the American Type Culture Collection (ATCC, USA). GC/MS data were analyzed on an Agilent 6890 Series GC System (CA, USA) coupled with an Agilent 5973 mass spectrometric detector. The UV absorbance of the samples was measured using a UV spectrophotometer (Hitachi, Tokyo, Japan; Model 100 - 20). Tissue culture was carried out at the Tissue Culture and Germplasm Conservation Research Laboratory, Horticulture Research Institute, Agricultural Research Centre Ministry of Agric, Giza. Cytotoxic activity was assessed at the Regional Centre for Mycology and Biotechnology, Al-Azhar University**.**

2.2. Plant Material

The leaves of *S. sempervirens* (Cupressaceae) were collected through El-Orman Botanic Garden, Giza, Egypt (July 2019) and authenticated by Teriza Labib. A voucher specimen (No. ch 11/211) was kept in the Laboratory of Phytochemistry, National Organization for Drug Control and Research.

2.3. Disinfection of explants

Shoot tips and nodal explants were collected as explants, washed in soapy water by septol soap, stirred in a savlon (3%) disinfection solution for 30 min and then rinsed under running tap water for one hour. Afterward, explants were treated under aseptic conditions for 30 sec. with 70% ethanol, then with 20% (v/v) Clorox for 10 min., further sterilized for 15 min. in 0.1 mercuric chloride solution (w/v), amended by few drops of tween-20 as emulsion, and three times gently rinsed in sterile distilled water. After culturing on MS Medium (MSM) [13], the *in vitro* obtained shootlets were aseptically micro-cut (2-5 mm) and were used as sources of the explants to be used in all tissue culture treatments.

2.4. Culture media and conditions

For experiments of shooting proliferation and callus induction growth, full salts strength of MSM were used and solidified with Anchamia agar (7g/l). Different growth regulators and sucrose concentrations were used on shootlets and calli growth cultures across three subsequent of subcultures. For shooting growth four treatments were used: sucrose 25g /l as control (S1), sucrose 25g / l + 2 g/l activated charcoal (AC) (S2), sucrose 50 g/l + 0.5 mg/l benzyl adenine (BA) (S3) and sucrose 50g/l + 0.5 mg/l kinetin (kin) (S4) (Table 1). For Callus induction eight treatments were used consisted of MSM combined with growth regulators as follows: Control (zero growth regulators) (C1), 0.4 mg/l naphthalene acetic acid (NAA) (C2), 0.8 mg/l NAA (C3), 0.4 mg/l 2,4-Dichlorophenoxyacetic acid (2, 4-D) (C4), 0.8 mg/l 2, 4-D (C5), 1.6 mg/l 2, 4-D (C6), 0.4 mg/l NAA + 0.8

mg/l 2, 4-D (C7) and 0.8 mg/l NAA + 1.6 mg/l 2, 4-D (C8) (Table 2). The media pH were adjusted to 5.7± 0.1 and autoclaved for 20 min at 121° and 1.2 kg/ cm². The shootlet explants were placed in 200 ml glass jar containing 25 ml medium. Cultures were incubated at 25± 1° under cool florescent light lamps with the light intensity of 3 k lux at 16 hours photoperiod. The highest callus induction percentages (C5-C8) (Table 2) were subcultured on the same four optimum selected induction callus medium. In each treatment twenty-five explants with five replicates were cultured for one month and subcultured in the same treatments for three subcultures. Survival percentage, shootlet number per explant, and shootlet length (mm) after each subculture were calculated (Table 1), while fresh and dry weights were recorded at the last subculture. On the other hand, the percentage of callus induction was calculated, for callus growth two grams of callus were put in all jars with treatments of five replicates and subcultured on the highest four callus growth % media for three times and fresh weights of calli were recorded (Table3).

2.5. Data Analysis

The experiment was designed in a completely randomized design, and the Least Significant Difference (LSD) test at *p* 0.05 was used to compare means according to Steel and Torrie [14].

2.6. Preparation of extracts

Calli cultures (C5-C8) were selected as they gave significant percentage of calli induction and maximum fresh weights. The dried powdered leaves (L) of the mother tree, *in vitro* shooting cultures (S1-S4) and callus cultures (C5- C8) of *S. sempervirens* (9 g, each) were extracted with *n*-hexane through cold percolation until exhaustion. The solvent was evaporated under reduced pressure at (40°) temperature to yield their concentrated hexane extracts (HE); HEL, HEC5-HEC8, and HES1- HES4, respectively. The prepared samples' percentage yields were calculated on a fresh weight basis before being stored in sealed glass vials at -4 °C for gas chromatographic analysis. The remaining powdered samples were then extracted with 70 % ethanol. Separately, the aqueous ethanol extract for each sample was under reduced pressure at 40-60° yielding the concentrated aqueous ethanol extracts (AEEs); AEEL, AEEC5- AEEC8 and AEES1-AEES4, respectively. They are consequently stored insealed amber bottles at 4ºC for phytochemical, antioxidant, and cytotoxicity investigations.

2.7. Phytochemical investigation

2.7.1. Investigation of n-hexane extracts

The nine *n*- hexane extracts of *S. sempervirens* were analyzed using GC-MS assessment following the method [15]. The components were identified using a library search database (Wiley 7 Nist 05 Lib. and W8N08 Lib.) by comparing their retention indices with reference to a homologous series of C8-C20 n-alkanes, and their mass spectra with published data [15, 16].

2.7.2. Investigation of AEEs of S. sempervirens

2.7.2.1. Determination of total phenolics

The total phenolics of the aqueous ethanol extracts of *S. sempervirens* were determined by the Folin–Ciocalteu method according to [17], with slight modification. 0.1 g of each sample was dissolved in 1 ml of deionized water. An aliquot solution (0.1 ml) was mixed with 2.8 ml of deionized water, 2 ml of 2% (w/v) Na_2CO_3, and 0.1 ml of 50 % (v/v) Folin–Ciocalteu reagent. After 40 min incubation period at room temperature, the relative absorbance of the reaction mixture was measured at 750 nm against a blank solution (containing all the reagents except the test solution).

Gallic acid (GA) was selected as a standard phenolic to create a seven-point standard curve (4–16 μg ml^{-1}), and the total phenolics content in each extract was determined in triplicate. The data was expressed as milligram GA equivalents (GAE) g^{-1} dry weight of the extract. The data was then converted into GAE g^{-1} dry matter from plant samples using this equation: Y= 98.597X + 250.08, R^2 = 0.9909, where Y= the absorbance, X= concentration (μg ml^{-1}), R^2= the correlation coefficient.

2.7.2.2. Determination of total flavonoids

The total flavonoids of the aqueous ethanol extracts of *S. sempervirens* were determined by the $AlCl_3$ colorimetric method [18], with some modification. 10 mg of each extract was dissolved in 10 ml of deionized water. 0.5 ml of the solution was mixed with 1.5 ml of 95% (v/v) alcohol, 0.1 ml of 10 % (w/v) $AlCl_3$ hexahydrate, 0.1 ml of 1 M CH_3COOK, and 2.8 ml of deionized water. After 40 min incubation period at room temperature, the absorbance was measured at 415 nm against a blank (containing all the reagents except the test solution).

Quercetin was selected as a standard to create a four-point standard curve (10 -70 μg ml^{-1}). The total flavonoids of each extract were determined from triplicate assays. The data were expressed as mg quercetin equivalents (QE) g^{-1} dry weight of each extract using the equation: Y = 54.785X + 785.64, R^2 = 0.9985, where Y= the absorbance, X= the concentration (μg ml^{-1}), R^2= the correlation coefficient. The data were then converted from plant samples into QE g^{-1} dry matter.

2.7.2.3. Determination of total triterpenoids

A part of each AEE was dissolved in water, then fractionated using ethyl acetate till exhausted. Separately, each ethyl acetate fraction was dried under reduced pressure (40°C) and subjected to chromogenic method [19] with some modifications. This method is based on the reaction of triterpenoids with strong acids to form carbonium ions, which gave a stable visible color with 5 % of vanillin-glacial acetic acid and perchloric acid. An aliquot of 100 mg of each fraction was dissolved in 10 ml ethyl acetate. The color-developing agent (5% vanillin- glacial acetic acid solution + 2 mL of perchloric acid) was heated (65°, 20 min), then cooled in ice water and warmed up to room temperature after being shaken. An equal volume of each tested sample solution with the color-developing agent was heated (70°, 15 min), then cooled to room temperature in ice water after being shaken, 5 ml dilute acetic acid was added, and the absorbance was measured at 549 nm against a blank (containing all the reagents except the test solution). Oleanolic acid was selected as a standard to create a seven-points standard curve (10 -70 µg ml^{-1}). The total triterpenoids content in each sample was estimated in triplicates. The data were expressed as mg oleanolic acid equivalents (OAE) g^{-1} dry weight of each extract. The data were then converted from plant samples into OAE g^{-1} dry matter applying the equation $Y= 2.1511X + 0.0188$, $R^2 = 0.9967$, where Y= the absorbance, X= the corresponding concentration (µg ml^{-1}), R^2= the correlation coefficient.

2.7.3. Biological investigation of AEEs

2.7.3.1. In vitro Antioxidant activity assay

The antioxidant activity of the aqueous ethanol extracts of *S. sempervirens* were evaluated using $DPPH^{\cdot}$ assay [20], with some modifications. Succinctly, aliquots (1, 2, and 3 ml) of each stock methanol solutions (10 mg ml^{-1}) of the extracts were separately added to 5 ml of a 0.004 % (w/v) of DPPH and volume was completed to 10 ml with methanol. After, a 30 min incubation period at room temperature, the absorbance at 517 nm was compared to DPPH in ethanol (blank). All the experiments were carried out in triplicates. Free radical formation percent inhibition of (I %) was calculated as: $I\% = (A_{blank} - A_{sample} / A_{blank}) \times 100$, where A_{blank} is the control reaction absorbance (containing all reagents except the extract) and A_{sample} is the mixture absorbance containing the extract. The IC_{50} was calculated by plotting the percentage of inhibition against extract concentration in each case, the determinations were made in triplicate. GA was used as a positive control, with different concentrations (5-50 µg ml^{-1}) treated similarly to the tested extracts.

2.7.3.2. Cytotoxic evaluation

HepG2 and MCF7 cell lines were maintained in (RPMI-1640) medium, containing 10 % inactivated foetal calf serum and 50 µg ml^{-1} gentamycin. The cells were incubated at 37°C for 24 h in an atmosphere containing 5% CO_2. The AEEs of *S. sempervirens* were tested for the cytotoxic activity using Sulphorhodamine B Assay (SRB) [21]. Tumor cell lines were plated in 96-multiwell plates (10^4cells/well) for 24 hrs before treatment with the extracts to allow attachment of the cells to the wall of the plate. Each extract with concentrations of 0, 1, 2.5, 5, and 10 µg ml^{-1} was introduced into the cell monolayer. Each individual dose was prepared in triplicate wells. The extracts were incubated with monolayer cells for 48 hours at 37 °C in a 5% CO_2 atmosphere. SRB stain was applied to the cells after they had been fixed and washed and acetic acid was used to remove the excess stain, and Tris EDTA buffer was used to recover the attached stain. The intensity of the colours was measured using an ELISA reader. The survival curve of each tumor cell line was obtained by plotting the relation between surviving fractions and extract concentrations. The IC_{50} dose of each extract was calculated and compared with that of the cytotoxic drug Doxorubicin.

3. Results and discussion

3.1. Shooting, callus induction, and biomass yield

For shooting, the effect of AC, BA, Kin, and sucrose concentrations on shootlets growth parameters of *S. sempervirens* including survival%, shootlets numbers/explant, and the average of shootlets length (mm) across three subcultures are represented in (Table 1). Survival percentages are expanding significantly by increasing the number of subcultures from (79.17, 93.75 to 100%) across the three successive subcultures, respectively. Through the first subculture, 100 % survival was recorded when the explants were treated with MSM provided either with 25 g/l sucrose + 2g/l AC (S2) or 50 g/l sucrose + 0.5 Kin (S4). However, the third subculture revealed 100 % survival with all treatments (Table 1). Nevertheless, the repeated subcultures caused significant decreases in shootlets number/ explant and shootlet length parameters. Hence, the main shootlets number declined from 2.35, 2.69 to 1.92 shootlet /explant, and the main shootlet length recorded (26.28, 20.80, and 23.08 mm) after three subcultures, respectively. Cultured explants on MSM provided with 50g/l sucrose + 0.5 g/l BA (S3) recorded the highest value of shootlets number (5.5/explant) after the second subculture. On the other hand, the highest value of shootlet length (43.42 mm) is recorded at S1 treatment through the first subculture (Fig.1). These results agree with Phulwaria et al., [22], who reported that repeated transferring of explants releases the meristem /bud to

proliferation to yield numerous shoot buds of *Salvadora persica* L. tree.

Table 1 Effect of activated charcoal (AC), benzyl adenine (BA), kinetin (Kin) and sucrose concentrations on shootlets growth parameters of *S. sempervirens* explants across three subcultures

Treatments	Explant Survival %			Number of shootlets /Explant			Shootlet Length (mm)		
	Sub 1	Sub 2	Sub 3	Sub 1	Sub 2	Sub 3	Sub 1	Sub 2	Sub 3
S1	50	75	100	1.11	1.35	1.22	43.42	25.96	14.44
S2	100	100	100	2.72	2.58	3.0	18.01	16.27	25.15
S3	66.67	100	100	2.83	5.52	2.27	10.17	8.69	31.38
S4	100	100	100	2.75	1.32	1.20	33.54	32.28	21.57
Mean of Subcultures	79.17	93.75	100	2.35	2.69	1.92	26.28	20.80	23.08
LSD (0.05)	A=16.21 B= 14.04 AB= 28.09			A= 0.45 B =0.39 AB =0.78			A=5.80 B =5.02 AB = 10.04		

S1; 25 g/l sucrose (control), S2; 25 g/l sucrose + 2g/l AC, S3; 50 g/l sucrose+ 0.5 mg/l BA, S4; 50 g/l sucrose + 0.5 mg/l kin, AB; A multiplied by B.

Moreover, the positive effect of increasing sucrose concentration was shown by Saikia et al., [23] on *Aquilaria malaccesis* Lam. tree, who mentioned that suitable concentrations of sucrose in a culture medium caused an improvement of biomass value. Furthermore, these findings matched with various reports [24-27] which concluded that the supplementation with BA promotes reinvigorations of old tissues and induces bud induction as a prerequisite for the cloning of mature trees. On the other hand, Wybouw& De Rybel, [28] showed that the use of high cytokinin levels reduced shoot and leaf growth and promoted the formation of meristematic clusters. As well, AC adsorbed toxic metabolites released from the tissue into a culture medium and allowed shoot growth [29, 30].

Fig. 1. Shootlets of *S. sempervirens* grown on control treatment

Figure 2 demonstrated that MSM supplemented with 50 g/l sucrose + 0.5 mg/l Kin recorded the highest values of fresh and dry weights (2.34 and 0.50 g /shootlet, respectively) compared to the control (1.55 and 0.34g/shootlet, respectively). These results could be attributed to cytokinins important physiological effects as stimulation of cell divisions, elongation to activate RNA synthesis, and stimulation of enzyme activity and protein synthesis [31].

The effect of both auxins NAA and 2, 4-D on callus induction cultured on MSM are shown in Table (2). The data cleared that the addition of 1.6 mg l^{-1}

2, 4-D only or plus 0.8 mg l^{-1} NAA into callus induction medium gave 100% of callus formation. However, the control medium without auxin failed to induce callus, while medium provided with NAA recorded the lowest values of callusing of *S. sempervirens* explant.

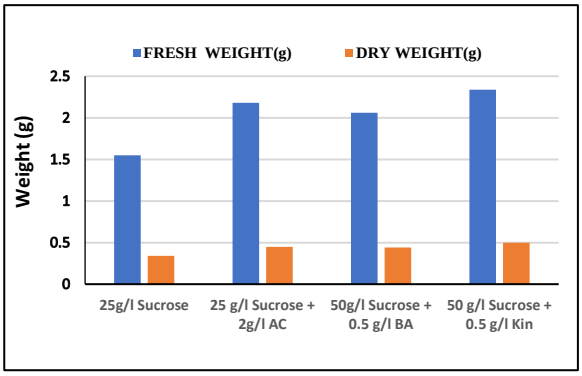

Fig.2. Effect of activated charcoal (AC), benzyl adenine (BA), kinetin (Kin) and different sucrose concentrations on shootlets fresh and dry weight (g/shootlet) of *S. sempervirens* after the last subculture.

Data summarized in Table 3 indicated that MSM supplemented with 1.6 mg l^{-1} 2, 4-D only or plus 0.8 mg l^{-1} NAA gave the highest significant callus fresh weight of means recorded 3.21 g and 3.23 g, respectively. However, raising the number of subcultures had no significant effect on callus fresh weight.

The addition of NAA at 0.4 mg l^{-1} in presence of 0.8 mg l^{-1} 2, 4-D recorded the lowest fresh weight (2.46 g) compared with (2.55 g) in presence of 0.8 mg l^{-1} 2, 4-D only. The interaction effect of the treatment on the fresh weight of callus cultured on the medium provided with 1.6 mg l^{-1} 2, 4-D+ 0.8 mg l^{-1} NAA resulted in the highest fresh weight (3.62g) after the second subculture. The lowest fresh weight (2.27g) is observed when the callus was cultured two times on a medium provided 0.8 mg l^{-1} 2, 4-D +0.4 mg l^{-1} NAA. In the same manner, the highest values of dry weight (0.318 and 0.329 g) were recorded on medium supplemented with 1.6 mg l^{-1} 2, 4-D only or combined with 0.8 mg l^{-1} NAA, respectively. These findings were confirmed through the micropropagation of *Aconitum violaceum* Jacq. [32], *A. malaccesis*[33] and *Dillenia indica* [34] trees. Moreover, callus induction and growth were affected by different types and concentrations of auxins. Their promotive effect may be attributed to the fact that auxins promote the biosynthesis of ethylene by increasing the activity of 1-aminocylopropane-1-carboxylic acid syntheses [35].

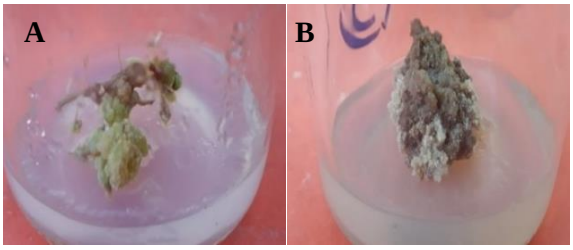

Fig. 3. (A) Callus induction of *S. sempervirens* explants, (B) Growth of callus after three subcultures

Table 2 Effect of NAA and/or 2, 4-D on percentage of callus induction of *S. sempervirens*

Treatment	Callus induction (%)
C1	0.00
C2	18.33
C3	18.36
C4	36.32
C5	73.35*
C6	100.00*
C7	91.64*
C8	100.00*
LSD (0.05)	1.36

C1; Control (free auxin), C2; 0.4 mg/l NAA, C3; 0.8 mg/l NAA, C4; 0.4 mg/l 2, 4- D, C5; 0.8 mg/ l 2, 4- D, C6; 1.6 mg/l 2, 4- D, C7; 0.8 mg/l 2, 4- D + 0.4 mg/l NAA, C8; 1.6 mg/l 2, 4- D + 0.8 mg/l NAA, *; significant callus induction

Table 3 Effect of 2, 4-Dichlorophenoxyacetic acid and different combinations with naphthalene acetic acid on callus growth across three subcultures as fresh and dry weights of the last subculture of *S. semperivrens*

Treatments	Callus fresh weight (g)				Callus Dry Weight (g)
	Sub1	Sub2	Sub3	Mean Treatment (A)	
C5	2.54	2.59	2.51	2.55	0.294
C6	3.39	3.29	2.94	3.21	0.318
C7	2.38	2.27	2.72	2.46	0.209
C8	3.14	3.62	2.92	3.23	0.329
Mean subculture	2.86	2.94	2.77		

C5; 0.8 mg/ l 2, 4 D, C6; 1.6 mg/l 2, 4 D, C7; 0.8 mg/l 2, 4 D + 0.4 mg/l NAA, C8; 1.6 mg/l 2, 4 D + 0.8 mg/l NAA

3.2. Investigation of n-hexane extracts

The HEC5 and HEC6 showed non-significant residues. The residues (0.2 and 0.6 % w/w) were produced from *n*-hexane extracts of HEC7 and HEC8, respectively. Whereas *n*- hexane extracts of both leaves of the mother plant (HEL) and the shootlets (HES1-HES4) afforded residues with characteristic aromatic and pleasant odors (2.4, 3, 2.5, 1.9, and 2.9%, respectively). Results of GC/MS analysis showed qualitative and quantitative variability in the chemical profiles of the samples under consideration (Table 4, Fig. S1). A total of 44 compounds were identified in all extracts and distributed as 8 and 17 in HEC7 and HEC8, respectively, 29, 34, 38, and 42 compounds in HES1-4, respectively and 43 in HEL. The total percentages of the identified components are calculated as 93.56, 57.58, 95.05, 46.19, 48.96, 57.33, and 96.15% of HEL, HEC7, HEC8, HES1, HES2, HES3, and HES4, respectively. These compounds included non-oxygenated hydrocarbons (aliphatics, monoterpenes, and sesquiterpenes) and oxygenated compounds (alcohols, ketone, phenols, and esters). All classes were abundant in shootlets and mother plant extracts. Whereas, HEC7 included only aliphatic non-oxygenated hydrocarbons. The absence of monoterpene and sesquiterpene hydrocarbons in the HEC7 could partly explain its odorless, where these compounds are responsible for the strong odor [36].

The aliphatic hydrocarbons are the major components in HEC8 (62.53%), with the frequency of eicosane, 2-methyl (18.20 %), and heptacosane (17.10 %). While, monoterpene hydrocarbons are predominant in HEL and HES4 (69.23 and 79.35%, respectively) with the prevalence of α-Phellandrene (35.93 and 36.02 %, respectively) with its pleasant aroma followed by α-Pinene and (10.57 and 31.41 %, respectively). Consequently, HES4 may be used as an approachable source for such biologically active components that reported antioxidant [37], anti-inflammatory [38], and cytotoxic activities [39] comparable with the mother plant [7, 8]. Moreover, HEL gave percentage monoterpene hydrocarbon contents (71.23%) higher than those reported for the leaves of mother plant (69.23%) [8]. The same for α-phellandrene, limonene, and α-pinene which were also described as the key compounds for *S. sempervirens* [4, 8]. This variation in the results could be directly correlated to the extraction method, as the prior report used hydro-distillation, but the present study used *n*-hexane extraction method. These results emphasize the fact that *n*-hexane extraction, which has been proposed as an alternative method provides a high recovery of volatile components as well as a high content of bioactive compounds in contrary to conventional extraction methods [40]. Furthermore, the HEC8 contained the highest percent of alcohols (30.1 %) compared to the rest cultures, where terpinene-4-ol (13.49) and 1-heptacosanol (10.29 %) were the predominant alcohol constituents. These bioactive compounds have been reported before to have several biological activities [41]. This variation could be attributed to the importance of differentiation in callus cultures to produce secondary metabolites [36]. Finally, a more recent study by Youssef et al., [4] revealed the detection of only 18 mono-/sesquiterpenes and alcohols. In addition, no information was mentioned about the aliphatic hydrocarbons, phenols, or esters. This may be attributed to the time of collection of the plant samples in addition to the method of extraction. The only ketone identified in these constituents is fenchone with a proportion less than that of the HEL content. Esters are present in all shootlet cultures in different proportions, the HES3 and HES2 (4.62 and 3.01%, respectively) produced more than those in HEL (2.65%). α-Terpinyl acetate is the major constituent in HES1-4 (1.55, 1.24, 2.43, 2.10 %, respectively), while thymol acetate is the major ester in the HEL. The calli are devoid of any esters.

Table 4 GC/MS analysis of *n*-hexane extracts of leaves, *in vitro* shootlets, and callus cultures of *S. sempervirens*

No.	RI	Name of compounds	MF (MW)	Relative percentage (%)						
				HEL	HES1	HES2	HES3	HES4	HEC7	HEC8
A) Non-oxygenated compounds										
		Aliphatic hydrocarbons								
11	1100	Dodecane	$C_{12}H_{26}$ (170)	1.52	7.54	9.11	2.02	2.14	9.43	11.99
33	1655	Cyclotetradecane	$C_{14}H_{28}$ (169)	0.10	-	0.32	0.22	0.03	1.22	0.03
36	2010	Eicosane	$C_{20}H_{42}$ (282)	0.32	0.33	0.55	0.45	0.51	0.54	0.42
37	2077	Eicosane,2-methyl	$C_{21}H_{44}$ (296)	-	16.4	9.43	0.12	0.13	19.55	18.20
38	2100	Heneicosane	$C_{21}H_{44}$ (296)	1.01	3.43	4.55	3.34	0.91	7.43	6.62
39	2300	1-Tricosene	$C_{23}H_{46}$ (322)	0.01	-	-	-	-	0.43	0.84
40	2490	Tetracosane	$C_{27}H_{50}$ (338)	0.21	0.31	0.34	0.43	0.14	-	0.41
41	2690	Heptacosane	$C_{27}H_{56}$ (380)	0.04	1.37	1.66	-	-	15.55	17.10
44	3100	Triacontane	$C_{30}H_{62}$ (422)	0.18	3.55	4.32	0.04	0.01	3.43	6.92
		Monoterpene hydrocarbons								
3	948	α-Pinene	$C_{10}H_{16}$ (136)	10.57	1.22	2.23	5.66	31.41	-	0.42
5	969	α-Phellandrene	$C_{10}H_{16}$ (136)	35.93	-	0.21	23.64	36.02	-	1.92
1	897	Thujene	$C_{10}H_{16}$ (136)	0.28	-	-	0.21	0.62	-	-
2	943	β-Pinene	$C_{10}H_{16}$ (136)	1.30	0.34	0.44	0.56	1.55	-	0.08
4	958	β-Myrcene	$C_{10}H_{16}$ (136)	3.72	-	-	1.53	1.53	-	-
8	1042	p-Cymene	$C_{10}H_{14}$ (134)	1.14	0.31	0.32	0.65	0.92	-	-
6	998	ɤ-Terpinen	$C_{10}H_{16}$ (136)	6.00	0.43	0.54	0.32	0.35	-	-
7	1018	Limonene	$C_{10}H_{16}$ (136)	10.29	1.54	3.87	3.32	6.95	-	-
9	1052	Terpinolene	$C_{10}H_{16}$ (136)	1.03	-	1.02	1.01	1.04	-	-
27	1465	ɤ-Elemene	$C_{15}H_{24}$ (204)	0.97	0.05	-	-	0.13	-	-
24	1399	β-Caryophyllene	$C_{15}H_{24}$ (204)	0.55	-	0.12	0.21	0.14	-	-
25	1452	trans-α-Bergamotene	$C_{15}H_{24}$ (204)	0.74	-	-	0.43	0.61	-	-
26	1463	Germacrene D	$C_{15}H_{24}$ (204)	0.51	0.22	0.32	0.65	0.62	-	-
28	1486	α-Humulene	$C_{15}H_{24}$ (204)	1.47	-	-	-	0.02	-	-
30	1493	Trans-β-Farnesene	$C_{15}H_{24}$ (204)	0.41	-	0.23	0.54	0.51	-	-
31	1500	E,E-α-Farnesene	$C_{15}H_{24}$ (204)	0.52	-	-	0.02	0.03	-	-

No.	RI	Name of compounds	MF (MW)	Relative percentage (%)						
				HEL	HES1	HES2	HES3	HES4	HEC7	HEC8
B) Oxygenated compounds										
	Alcohols									
42	2351	1-Henicosanol	$C_{21}H_{44}O$ (312)	0.73	0.43	0.62	0.65	0.75	-	1.99
43	2948	1-Heptacosanol	$C_{27}H_{56}O$ (396)	3.90	1.43	1.89	1.78	1.51	-	10.29
10	1086	Borneol	$C_{10}H_{18}O$ (154)	0.01	0.32	0.21	0.23	0.32	-	-
13	1190	Terpinene-4-ol	$C_{10}H_{18}O$ (154)	1.02	0.98	1.02	1.02	1.03	-	13.49
14	1196	p-Cymen-8-ol	$C_{10}H_{14}O$ (150)	0.71	0.43	0.41	0.44	0.63	-	-
15	1230	α-Terpineol	$C_{10}H_{18}O$ (154)	0.52	-	-	0.02	0.02	-	-
16	1268	cis-Piperitol	$C_{10}H_{18}O$ (154)	0.21	0.24	0.22	0.32	0.37	-	-
17	1276	trans-Piperitol	$C_{10}H_{18}O$ (154)	0.11	-	-	-	0.01	-	-
18	1288	*β*-Citronellol	$C_{10}H_{20}O$ (156)	0.20	0.04	0.03	0.02	0.03	-	-
19	1290	Geraniol	$C_{10}H_{18}O$ (154)	0.72	-	-	-	0.11	-	-
29	1489	Spathulenol	$C_{15}H_{24}O$ (220)	0.82	0.21	0.23	0.22	0.13	-	-
34	1680	α–Cadinol	$C_{15}H_{26}O$ (222)	0.68	0.16	0.15	0.14	0.17	-	4.22
35	1950	Phytol	$C_{20}H_{40}O$ (296)	1.72	1.47	1.45	1.43	1.36	-	0.11
	Phenols									
20	1300	Thymol	$C_{10}H_{14}O$ (150)	1.01	0.24	0.23	0.65	0.41	-	-
23	1389	Eugenol	$C_{10}H_{12}O_2$ (164)	0.22	0.15	0.13	0.21	0.20	-	-
	Ketone									
12	1103	Fenchone	$C_{10}H_{16}O$ (152)	1.51	-	1.12	1.22	1.13	-	-
	esters									
32	1578	Citronellyl pentanoate	$C_{15}H_{28}O_2$ (240)	0.50	0.23	0.54	0.65	0.71	-	-
21	1.02	α-Terpinyl acetate	$C_{12}H_{20}O_2$ (196)		1.55	1.24	2.43	2.10		
22	1.13	Thymol acetate	$C_{12}H_{16}O_2$ (192)		1.32	1.23	1.54	0.01		
Total identified aliphatic hydrocarbons				3.39	32.93	30.28	6.62	3.87	57.58	62.53
Total identified monoterpene hydrocarbons				69.23	3.84	7.29	35.89	79.35	-	2.42
Total identified sesquiterpene hydrocarbons				4.20	0.22	0.67	1.85	1.93	-	
Total identified alcohols				11.35	5.71	6.23	6.27	6.44	-	30.1
Total identified phenols				1.23	0.39	0.36	0.86	0.61	-	-
Totalidentified esters				2.65	3.1	3.01	4.62	2.82	-	-
Total % identified compounds				93.56	46.19	48.96	57.33	96.15	57.58	95.05
Number of identified compounds				43	29	34	38	42	8	17

RI; retention index values relative to CC n- alkanes calculated using a non-polar HP-5MS capillary column, MF; molecular formula, MW; molecular weight, HE; *n*-hexane extract, S1; 25 g/l sucrose (control), S2; 25 g/l sucrose + 2g/l AC, S3; 50 g/l sucrose+ 0.5 mg/l BA, S4; 50 g/l sucrose + 0.5 mg/l kin,C7; 0.8 mg/l 2, 4-D + 0.4 mg/l NAA, C8; 1.6 mg/l 2, 4-D + 0.8 mg/l NAA

3.3. Evaluation of secondary metabolites in the AEEs

Calli (AEEC1- AEEC4), shootlets (AEES1- AEES4), and tree leaves (AEEL) were yellowish brown in color, with yield ranges of (1.01-1.23), (8.20-9.87) and 11.21 %, respectively (calculated on a dry weight basis). Among all the investigated samples, the AEES4 exhibited the highest content of flavonoids, phenols, and triterpenoids (2.514, 0.98, and 172.64 µg mg^{-1}, respectively) in comparison with AEEL (2.061, 0.684, and 172.672 µg mg^{-1}, respectively) (Table 5). These results agree with some previous studies [42, 43], which suggested that the differentiation of any tissue is associated with the enhanced biosynthesis of such compounds under *in vitro* conditions that are caused by the appearance of complex tissues and cells that are more metabolically competent. Subsequently, this may explain the deficiency of the secondary metabolites in the non-differentiated calli. To the best of our knowledge, this is the first investigation of total triterpenoids in leaves of *S. sempervirens* and their *in vitro* calli and shootlets. Quercetin was selected as a standard to create a four- point standard curve (10 -70 µg ml^{-1}). The total flavonoids of each extract were determined from triplicate assays.

3.4. Evaluation of the biological activities of the AEEs

The antioxidants were assessed based on DPPH free radical scavenging activity method (Table 6). According to IC_{50} results, AEEC1-AEEC4 as well as AEES2 and AEES3 exerted no activity at all. The shootlet culture AEES1 and AEES4 showed the IC_{50} (5407.74 and 6017.61 µg ml^{-1}) higher than that of AEEL (6420.24 µg ml^{-1}), compared with that of gallic acid (32.85 µg ml^{-1}). The antioxidant activity could be attributed to the presence of higher amounts of antioxidant metabolites, such as terpenoids, phenolic, and flavonoids [42]. Moreover, screening of the cytotoxic activity of the tested extracts was evaluated using two different types of human carcinoma cell lines (HepG-2 and MCF7). Results show that only shootlets cultures extracts exhibited cytotoxic activity against the tested cancer cell lines; in contrast, callus culture extracts and leaves mother plant displayed no activity under the tested conditions. The highest IC_{50} was achieved by AEES1 (39.1 ± 0.8 and 61.2 ±1.4 µg/ml), followed by AEES4 (97.6±3.2 and 123±1.9 µg/ml) against HepG-2 and MCF-7, compared to those of doxorubicin (25 µg ml^{-1}) (0.36±0.04 and 0.35±0.03), respectively. These activities could be attributed to the presence of higher terpenoid contents [42, 44]. As far as we know, this is the first report on researching the impact of mother leaves, calli and shootlet cultures of *S. sempervirens* on MCF7 and HepG2 cells.

Table 5 Evaluation of secondary metabolites in ethanol extracts of *S. sempervirens* mother plant, *in vitro* shootlet and calli cultures

Secondary metabolites (µgmg^{-1}, d.w.)	**Tested extracts**								
	AEEL	**AEES1**	**AEES2**	**AEES3**	**AEES4**	**AEEC5**	**AEEC6**	**AEEC7**	**AEEC8**
Total Flavonoids	2.061	2.121	1.098	1.313	2.514	0.116	0.091	0.167	0.136
Total phenolics	0.684	0.886	0.820	0.752	0.98	0.169	0.172	0.257	0.216
Total triterpenoids	172.672	106.912	60.864	79.872	172.640	5.632	3.008	10.976	26.816

AEE; Aqueous ethanol extract, L; mother tree leaves, S1; shootlets treated with 25g/l sucrose, S2; shootlets treated with 50g/l sucrose + 2g/l AC, S3; shootlets treated with 25g/l sucrose +0.5 mg/l BA, S4; shootlets treated with 50g/l sucrose+ 0.5mg/l Kin,C5; calli treated with 0.8 mg/l 2,4-D, C6; calli treated with 1.6 mg/l 2,4-D, C7; calli treated with 0.8 mg/l 2,4-D + 0.4 mg/l NAA, C8; calli treated with 1.6 mg/l 2,4-D + 0.8 mg/ l NAA

Table 6 Antioxidant activity of ethanol extracts of *S. sempervirens* mother plant and *in vitro* shootlet culture.

Radical-scavenging activity (%)					
Tested samples				**Gallic acid**	
Concentration (μgml^{-1})	**AEEL**	**AAES1**	**AAES4**	**Concentration (μgml^{-1})**	**Radical-scavenging activity (%)**
1000	90.77 ± 0.33	88.53 ± 0.30	89.63 ± 0.32	**100**	90.8 ± 1.52
500	78.73 ±0.54	68.42 ± 0.24	71.10 ± 0.41	**50**	83.7 ± 0.61
250	73.41 ±0.21	60.31 ± 0.25	61.36 ± 0.12	**25**	76.3 ± 0.22
IC_{50} (μgml^{-1})	6420.24	5407.74	6017.61	**IC_{50} (μgml^{-1})**	32.85

AEEL; Aqueous ethanol extract the mother tree leaves; AAES1, Aqueous ethanol extract of *in vitro* shootlets treated with 25g/l sucrose; AEES4: Aqueous ethanol extract of *in vitro* shootlets treated with 50g/l sucrose+ 0.5mg/l Kin

4. Conclusion

This study provides an important basis for further investigation of shootlets arising from *S. sempervirens* tissue cultures, identification of metabolites responsible for cytotoxic activity, and further screening of the plant's *in vivo* pharmacological activity. Furthermore, from the perspective of tissue culture, different growth factors and techniques can be evaluated to increase the levels of bioactive metabolites

List of Abbreviations

GC-MS: Gas chromatography-mass spectrometry; MS medium: Murashige and Skoog medium; BA: benzyl adenine; NAA: 1-Naphthaleneacetic acid; 2, 4-D: 2, 4-Dichlorophenoxyacetic acid; kin: kinetin; AC: activated charcoal; AEEL: Aqueous ethanol extract of the mother tree leaves; AEEC: Aqueous ethanol extract of *in vitro* calli; AEES: Aqueous ethanol extract of *in vitro* shootlets; HepG-2: hepatocellular carcinoma cell line; MCF-7: breast carcinoma cell line

Conflicts of interest

The authors declare that they have no conflict of interests.

Author's contributions

NMS: Work proposal, supervision, data validation, writing-editing. MMM: Work proposal, writing-editing, Methodology. MMF: data validation, writing-editing, Methodology, data analysis. HMF: Methodology, data analysis, writing first draft. ZTA: Methodology, data analysis, writing first draft.

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