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CHEMICAL COMPOSITION, ANTIOXIDANT AND CYTOTOXIC ACTIVITIES OF *SEQUOIA SEMPERVIRENS* LEAVES AND *IN VITRO* CULTURES EXTRACTS

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ABSTRACT

Sequoia sempervirens plants belonging to family Cupressaceae produce many of pharmaceutical important secondary metabolites known to have cytotoxic and antioxidant properties. *In vitro* callus and shootlet cultures were established from shoot tips and nodal explants then the tissues were assessed phytochemically and biologically. Microcutting of *S. sempervirens* tree were carried out on Murashige and Skoog (MS) medium supplemented with 25 g/l sucrose only, or plus 2g/l activated charcoal and treated with 0.5 mg/l of BA or Kin in presence of 50 g/l sucrose for each to obtain shootlets culture, then repeated subculture for three times. Callus induction and growth were obtained when micro cutting were cultured on MS medium provided with different concentrations of NAA and/or 2, 4 -D. Gas chromatography/mass spectrometry (GC/MS) analysis of *n*-hexane extracts of calli (HC), shootlets (HS) and leaves (HL) have been done along with quantitative estimation of the phenolics, flavonoids and triterpenoids in ethanolic extracts of calli (EC), shootlets (ES) and leaves (EL).The highest survival significant percent were recorded when MS medium was provided by either 25 g/l sucrose + 2g/l activated charcoal or by adding 50 g/l sucrose + 0.5 mg/l KIN. The last treatment showed significant increase in means of shootlet length, fresh and dry weight of shootlet. In respect of callus, MS medium provided to 1.6 mg/l 2, 4-D + 0.8mg/l NAA produced highest callus induction and highest fresh and dry weight. While, the lowest

significant percent was recorded with control (free-MS) treatment which was modified with 25g/l sucrose only (where callus results). By GC/MS, aliphatic hydrocarbons were the major components in HC, whereas higher percentage of monoterpene hydrocarbons compounds were found in HS and HL. The phenolic, flavonoids and triterpenoids showed much higher contents in ES and EL than in EC. Antioxidant screening of extracts demonstrated that (ES) and (EL) were the most potent, followed by (EC). Only shootlets cultured on free MS medium and those provided with 0.5 mg/l Kin + 50 g /l sucrose showed inhibiting activity on human liver (HepG-2) and breast (MCF-7) tumor cell lines; conversely, (EC) and (EL) were inactive **.** Maximum phenolic, flavonoids and triterpenoids contents were obtained in shootlet cultures of *S. sempervirens* which exhibited powerful antioxidants and cytotoxic activities.

Key words: biological activities; callus culture; GC/MS; secondary metabolites; *Sequoia sempervirens*.

INTRODUCTION

An essential purpose for the scientific discipline of tissue culture is the study for producing biologically active secondary metabolites in high yields and reasonable time frame. The current progress in tissue culture techniques has made it possible to increase metabolites comparing to mother plant samples, however the demand to develop efficient and reliable methods for producing cytotoxic metabolites by tissue culture technique is on the rise, together with an increase in the legal requirements for safety and consistent levels of active compounds.

Family Cupressaceae, one of the largest and most widely distributed of all conifer families, comprising 28 genera with 142 species **(Earle,2013)**. Many of Cupressaceae members were well-known for its valuable timber and ornamental importance **(Pye,*et al*.,2009;Bonner *et al*.,2008; Miguel,2010;Joshi and Sati,2012 and Wink,2012)**. *Sequoia sempervirens,* coast redwood, is a long-lived evergreen tree which is endemic to the coastal regions of California and Oregon, USA and is the tallest known trees in the world with a high volume biomass **(Bajpai,*et al.*,2009).** It contains a plethora of phytochemicals including terpenoids, tannin, phenolic, flavonoids and glycosides which can be used in large scale in pharmaceutical

preparations, they have a wide spectrum of medicinal properties, including cytotoxic, antibacterial, anti-inflammatory, antioxidant, antiviral, and antifungal ones **(De Silva, *et al*.,2012 and Otto, *et al*.,2002).**

Previous phytochemical analyses have mostly focused on the volatile oil and phenolic contents in *S. sempervirens*, with less information on terpenoids although the letter have potential functions in cytotoxic effects against tumor cells at low activity toward normal cells **(Single and Pathak,1990)**. The major goal of the current study is to induce shooting and callus from *S. sempervirens* with considerable yield and metabolites production. Therefore, the *n*-hexane extracts of the tissue extracts with mother plant leaves examined for non-polar compounds and ethanolic extracts were assessed quantitatively for polar secondary metabolites (phenolics, flavonoids and triterpenoids). As a secondary goal, ethanol (70%) extracts of shooting, calli and mother plant leaves were investigated for antioxidant and cytotoxic activities.

MATERIALS AND METHODS

Plant material:

The leaves of *Sequoia sempervirens* (D.Don) Endl. (Cupressaceae) were collected from the unique tree cultivated in Orman Botanical Garden, Giza, Egypt (July, 2017). The plant was kindly identified and authenticated by Prof. Dr. Abdel Salam El Noiehy, Professor of Plant Taxonomy, Botany department, Faculty of Science, Ain Shams University. Voucher specimen (No. ch 11/211) of the plant materials were kept in Laboratory of phytochemistry, National Organization for Drug Control and Research.

Disinfection of explants:

Shoot tips and nodal explants were collected during the period of autumn 2016 as explants and washed in soapy water by using septol soap, then agitated in disinfectant solution of savlon (3%) for 30 min. and rinsed with running tap water for one hour. Thereafter, explants were treated under aseptic conditions for 30 sec. with 70% ethanol, then with 20%(v/v) Clorox for10 min., further sterilized for 15min. in 0.1 mercuric chloride solution (w/v), amended with a few drops of

tween-20 as emulsion, and gently rinsed in sterile distilled water for three times.

After culturing on MS medium **(Murashige and Skoog,1962)**, the *in vitro* obtained shootlets were aseptically microcut (2-5 mm) and were used as sources of the explants to serve in all tissue culture treatments following experiments.

Culture media and conditions:

In both shooting proliferation and callus induction, basal salts of MS medium at full strength were used and solidified with Anchamia agar (7g/l). Different types of growth regulators and sucrose concentrations were used on shootlets growth and calli growth cultures across three subsequent of subcultures. Four treatments were tested at shooting growth stage; sucrose 25g /l as control, sucrose 25g / l + 2 g/l actavited charcoal (AC), sucrose 50 g/l + 0.5 mg/l benzylaminopurine (BA) and sucrose 50g/l + 0.5 mg/l kintien (kin). For callus induction treatments were MS medium combined with growth regulators as follows: Control , 0.4 or 0.8 mg/l naphthalene acetic acid (NAA) only, 0.4 or 0.8 or 1.6 mg/l 2,4-dichlorophenoxy acetic acid (2,4-D) only, 0.4 mg/l (NAA) + 0.8 mg/l (2,4-D) and 0.8mg/l (NAA) + 1.6 mg/l (2,4-D). The medium was adjusted to PH 5.7± 0.1 and autoclaved at 121°C and 1.2 kg/ cm² for 20 min. The shootlet explants were placed in 200 ml glass jar containing 25ml medium. Cultures were incubated at 25± 1°C under cool florescent light lamps with light intensity of 3 k lux at 16 hours photoperiod. The highest four callus induction percentage 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, shootlet length (mm) after each subculture, while fresh and dry weights at the last subculture were calculated. On the other hand, the percentage of callus induction was calculated, for callus growth two gram of callus were put in all jars with treatments of five replicates and subcultured on the highest four callus growth% medium for three times and fresh weight of callus were recorded. Tissue culture was carried out at Tissue Culture and Germplasm Conservation Research Laboratory, Horticulture Research Institute, Agricultural Research Center Ministry of Agriculture, Giza.

Experimental design and data analysis:

The lay-out of the experiment was designed in completely randomized design and test of Least Significant Difference (LSD) at $p \leq 0.05$ was used for comparison among means according to (**Steel and Torrie,1980)**

Preparation of extracts

Preparation of *n*-hexane extracts:

Shooting on 50 g/l sucrose + 0.5 mg/l kin (S4) medium and callus on1.6 mg/l 2, 4-D + 0.8 mg/ l NAA (C4) medium were chosen as they gave the highest fresh weight. The hexane-extracted volatiles were prepared from tree fresh leaves, callus (C4) and shootlets samples (S4) (3g, each), by cold percolation till exhaustion (HL, HC4 and HS4, respectively). The solvent was then removed by evaporation under reduced pressure at a temperature not exceeding 40 °C to yield a semi solid product. Percentage yields of the prepared samples were calculated on fresh weight basis, followed by storing in sealed glass vials, at -4 °C, until gas chromatographic analysis was adopted.

Preparation of ethanol extracts:

Ethanol extracts of dried powders leaves, *in vitro* shooting (4 samples; S1-S4) and callus (4 samples; C1-C4) cultures of *S. sempervirens* L. were prepared using defatted marc and ethanolic solution (70%). The ethanol extracts for each powder were dried in a rotary evaporator at reduced pressure at 40 °C and subsequently stored in sealed amber bottles at 4°C for phytochemical and biological investigation. The stock solution (1mg ml^{-1}) for each plant sample was prepared by dissolving 100mg of the prepared ethanolic extracts in 100 ml distilled water using sonication. The prepared stock solution was used for determination of the total phenolic, flavonoid contents.

Preparation of triterpenoids extracts:

To prepare samples, 3 g of each dried *in vitro* shooting (4samples; S1-S4), callus (4 samples; C1- C4) cell mass and powdered leaves samples were drenched separately in methanol (200 ml) for 48 hr and, thereafter, sonicated for 40 min. The methanolic mixture thus obtained was filtered, and the filtrate was centrifuged for 10 min. Supernatant was pooled, filtered, and dried in a rotary evaporator at 40°C. This methanolic extract was further fractionated into an organic (ethyl acetate) and aqueous fraction. The ethyl acetate

fraction was further dried under reduced pressure in a rotary evaporator at 40°C and used for further study.

Phytochemical investigation:

Folin-ciocalteu reagent and sodium carbonate (Sigma Chemicals, USA) were utilized for the assessment of the total phenolic content. For determination of flavonoids, sodium hydroxide and aluminum chloride (Sigma Chemicals, USA) were purchased. Vanillin-glacial acetic acid 5% were prepared by dissolving vanillin (500 mg) in acetic acid (10ml) for the assessment of the total triterpenoids content. Solvents of analytical grade were used: methanol, absolute ethanol, *n*-hexane and perchloric acid (Fisher chemical, UK). Reference standers: gallic acid, quercetin and oleanolic acid were obtained from Sigma Chemicals (USA). Distilled water was used in all experiments.

Biological investigation:

1,1-diphenyl-2-picrylhydrazyl (DPPH) was purchased from Sigma Chemicals (USA) for the assay of *in vitro* antioxidant activity. For cytotoxic activity**,** methylthiazolyl diphenyl-tetrazolium bromide (MTT) and Roswell Park Memorial Institute (RPMI-1640) medium (Sigma Chemicals, USA) were used to evaluate *in vitro* cytotoxic activity. HEPG2 (liver carcinoma) and MCF7 (breast cancer) cell lines, obtained frozen in liquid nitrogen (-80°C) from the American Type Culture Collection (ATCC, USA), were undertaken at the Regional Center for Mycology&Biotechnology, Al-Azhar University. The cells were maintained in (RPMI-1640) medium, containing 10% inactivated foetal calf serum and 50µg/ml gentamycin. The cells were incubated for 24 h at 37°C in an atmosphere containing 5% CO_2. Doxorubicin®: Sigma-Aldrich Co., USA.

GC/MS analysis:

n-hexane extracts of fresh *in vitro* shootlets, callus and leaves samples were analyzed on an Agilent 6890 Series GC System (CA, USA) coupled with an Agilent 5973 mass spectrometric detector. The column used was an HP-5MS capillary column (30 m× 320 µm × 0.25 µm film thickness). Helium was the carrier gas (1.0 ml/min). The volume injected was 1.0 µl. The Column temperature program was: 40°C (1 min), 7.5°C /min to 150°C, 1.2°C / min to 250°C. Injector and detector temperatures were 250°C and 280°C. The GC/MS instrument

was used in the ionization mode EI, under an ionization source 70 eV; the data of mass spectra were acquired in the scan mode in m/z range 40-500. Identification of the components was achieved by library search database (Wiley 7 Nist 05 Lib. and W8N08 Lib.) and by comparing their retention indices with reference to a homologous series of C8-C20 *n*-alkanes, and their mass spectra with published data **(Chen and Ho,1986 ;Kouokam *et al*.2002 and Adams,2007)**. The percentages of different constituents were determined by computerized measurement of peak areas.

Determination of total phenolic content:

The total phenolic content of *in vitro* shootlets, callus and leaves samples of *S. sempervirens* extracts were determined by the Folin–Ciocalteu method **(Meda *et al* 2005)**. Gallic acid (GA) was chosen as a standard phenolic to construct a seven-point standard curve (4–16 µg ml^{-1}), the total phenolics contents in plant extracts were determined in triplicate. The data were expressed as milligram gallic acid equivalents(GAE)/g dry weight of extract.

Determination of total flavonoid content:

The total flavonoids content of *in vitro* shootlets, callus and leaves samples of *S. sempervirens* extracts were determined by the aluminum chloride colorimetric method **(Chang *et al*. 2002)**. Quercetin was chosen as a standard to construct a seven points standard curve (10 -70 µg ml^{-1}). The total flavonoids content in sample extracts were determined from triplicated assays. The data were expressed as milligram quercetin equivalents (QE)/g dry weight of extract. The data were then converted into QE/1 g dry matter from plant samples.

Determination of total triterpenoid content:

Total triterpenoids content, *in vitro* shootlets, callus and leaves samples of *S. sempervirens* was determined by chromogenic method **(Skrzypek and Wysokinska,2003)** with some modifications.This method based on that triterpenoid can easily react with strong acids to form carbonium ion, which can form stable visible color with 5 % of vanillin-glacial acetic acid and perchloric acid agent. An aliquot of 100 mg of each plant extracts was dissolved in 10 ml ethyl acetate (10mg ml^{-1}). The color developing agent was prepared as follows, 5% vanillin-acetic acid solution plus 2mL of perchloric acid were heated

at 65℃ for 20min, then cooled in ice water and warmed up to room temperature after being shaken. Equal volume of each test solution with 5 % of vanillin-glacial acetic acid and perchloric acid agent, heat at 70℃ for 15min, then cooled to room temperature in ice water after being shaken, add 5 ml dilute acetic acid, the absorbance was measured at 549 nm against a blank containing all the above reagent except the sample using a UV spectrophotometer (Hitachi, Tokyo, Japan; Model 100 - 20). Oleanolic acid was chosen as a standard to construct a sevens point standard curve (10 -70 μg ml^{-1}). The estimation of total triterpenoids content in the extracts was carried out in triplicates. The data were expressed as milligram oleanolic acid equivalents (OAE)/g dry weight of extract. The data were then converted into OAE/g dry matter from plant samples by using this 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.

Evaluation of biological activities:

Shootlets cultures on MS medium supplemented with 25g/l sucrose (ES1) and 50 g/l sucrose + 0.5 mg/l KIN (ES4), callus cultured on MS media supplemented with 1.6 mg/l 2, 4 –D (EC2) and 1.6 mg/l 2, 4 -D + 0.8 mg/l NAA (EC4) were chosen for antioxidant and cytotoxic investigation as they contained the highest secondary metabolites comparing to the leaves ethanol extract.

***In vitro* antioxidant activity (DPPH assay):**

The antioxidant activity of *in vitro* shootlets (ES1 and ES4), callus (EC2 and EC4) and leaves (EL) of *S. sempervirens* were evaluated by using the 1, 1 diphenyl-2-picrylhydrazyl (DPPH) assay (Burits and Bucar,2000) with some modifications, aliquots (1, 2 and 3 ml) of each stock solution (10mg ml^{-1}) extract were separately added to 5 ml of a 0.004% (w/v) of DPPH and volume was completed to 10 ml methanol. After, a 30 min incubation period at room temperature, the absorbance at 517 nm was compared to DPPH in ethanol without an extract sample (blank) using a UV spectrophotometer (Hitachi, Tokyo, Japan; Model 100 - 20). All the experiments were carried out in triplicate. The percent inhibition of free radical formation (I %) was calculated as;

$$I\% = (A_{blank} - A_{sample} / A_{blank}) \times 100$$

Where; A_{blank} is the absorbance of the control reaction (containing all reagents except the extract) and A_{sample} is the absorbance of the mixture containing the extract. The IC_{50} (defined as the concentration of extract required to produce 50% of the maximum inhibition) was calculated from graphing inhibition percentage against extract concentration in each case. Determinations were carried out in triplicate. Gallic acid was used as a positive control, with different concentrations (5-50 μg ml^{-1}) were treated similarly as the tested extracts.

Investigation of cytotoxic activity:

The ethanol extracts of *in vitro* shootlets (ES1 and ES4), callus (EC2 and EC4) and leaves (EL) of *S. sempervirens* were tested for cytotoxic activity against HEPG2 (liver carcinoma cell line) and MCF7 (breast cancer cell line) using sulphorhodamine B Assay (SRB) by **(Skehan *et al*.,1990)**. Tumor cell lines were plated in 96-multiwell plate (104 cells/well) for 24 hrs before treatment with the extract to allow attachment of the cells to the wall of the plate. Different concentrations of the extracts under investigation (0, 1, 2.5, 5 and 10 (μg/ml) were added to the cell monolayer, triplicate wells were prepared for each individual doses. Monolayer cells were incubated with the extracts for 48 hrs at 37°C and in atmosphere of 5% CO_2. After 48 hrs, cells were fixed, washed and stained with sulphorhodamine B stain. Excess stain was washed with acetic acid and attached stain was recovered with Tris EDTA buffer. Colour intensity was measured in an ELISA reader. The relation between surviving fraction and extract concentration is plotted to get the survival curve of each tumor cell line after the specified extract. The IC_{50} dose of each extract was calculated and compared with that of the cytotoxic drug Doxorubicin.

RESULTS AND DISCUSSION

Shooting and callus induction and biomass yield:

For shooting, the effect of activated charcoal, BAP, KIN and sucrose concentrations on shootlets growth parameters of *S. sempervirens* (survival %, shootlets number per explant and average of shootlets length (mm) across three subcultures are represented in **Table (1)**. Survival percentage are raising significantly by increasing

number of subculture (79.17, 93.75 to 100%) across the three subcultures. The highest significant percent was recorded when explant was treated with MS medium provided either with 25 g/l sucrose + 2 g/l activated charcoal or by adding 50 g/l sucrose + 0.5 KIN. However, the lowest significant percent (50%) was recorded with control (free-MS) treatment in first subculture which has 25 g/l sucrose only. For shootlets number formed / explant and shootlet length the data showed that, repeated subcultures caused significant decreasing in both parameters hence, shootlet no. is declined (2.35, 2.69 to 1.92 shootlet /explant) after three subcultures. Also, average of shootlet length was recorded (26.28,20.80 and 23.08mm). However, in the same way, cultured explants on MS medium provided with 50g/l sucrose + 0.5 g/l BAP recoded the highest significant value of shootlets no. after the second subculture. On the other hand, the highest value of shootlet length (43.42mm) is occurred at control treatment through the first subculture **(Fig.1)**. Data presented in **(Fig.2)** demonstrated that, supplemented the culture MS media with 50g/l sucrose + 0.5 mg/l KIN recorded the highest value (2.34 and 0.50 g /shootlet) fresh and dry weight compared to (1.55 and 0.34 g/shootlet) with control but, with significant effect on dry weight only.

Table 1: Effect of activated charcoal, BAP, KIN and sucrose concentrations on shootlets growth parameters of *S. sempervirens* explants across three subcultures.

Subcultures Number / Tretments	Explant Survival %			
	Sub1	Sub2	Sub3	Mean (A)
25 g/l sucrose (control) (S1)	50	75	100	75
25 g/l sucrose + 2g/l activated charcoal (S2)	100	100	100	100
50 g/l sucrose+ 0.5 mg/l BAP (S3)	66.67	100	100	88.0
50 g/l sucrose + 0.5 mg/l kin (S4)	100	100	100	100
Mean of Subcultures (B)	79.17	93.75	100	
LSD (0.05)	A=16.21	B= 14.04		AB= 28.09
	Number of Shootlets /Explant			
25 g/l sucrose (control) (S1)	1.11	1.35	1.22	1.23
25 g/l sucrose + 2g/l activated charcoal (S2)	2.72	2.58	3.0	2.77
50 g/l sucrose+ 0.5 mg/l BAP (S3)	2.83	5.52	2.27	3.54
50 g/l sucrose + 0.5 mg/l kin (S4)	2.75	1.32	1.20	1.76
Mean of Subcultures (B)	2.35	2.69	1.92	
LSD (0.05)	A= 0.45	B =0.39		AB =0.78
	Shootlet Length (mm)			
25 g/l sucrose (control) (S1)	43.42	25.96	14.44	27.86
25 g/l sucrose + 2g/l activated charcoal (S2)	18.01	16.27	25.15	19.81
50 g/l sucrose+ 0.5 mg/l BAP (S3)	10.17	8.69	31.38	16.75
50 g/l sucrose + 0.5 mg/l kin (S4)	33.54	32.28	21.57	29.12
Mean of Subcultures (B)	26.28	20.80	23.08	
LSD (0.05)	A=5.80	B =5.02		AB = 10.04

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

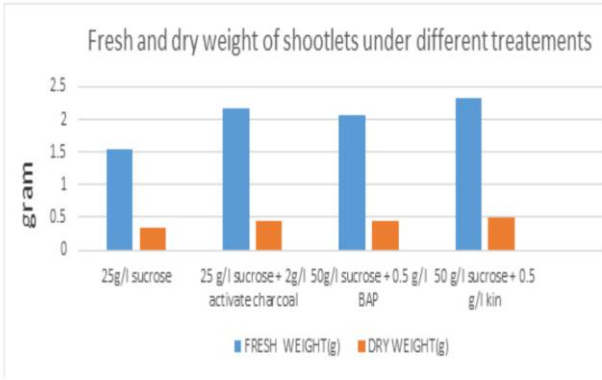

Fig.2. Effect of activated charcoal, BAP, KIN and different sucrose concentrations on shootletes fresh and dry weight (g/shootlet) of *S. sempervirens* after the last subculture.

The effect of both auxins NAA and 2,4-D concentrations on callus induction cultured on MS medium are shown in **Table (2)**. The data in this table cleared that, the addition of1.6 mg/l 2, 4 -D only or plus 0.8 mg/l NAA into callus induction medium gave the highest percent of callusing. However, the control medium with no auxin failed to induce callus, while medium provided with NAA recorded the lowest values of callusing of *S. semperivrens* explant (**Fig.3 and4).**

Table 2: Effect of different combinations of NAA and 2, 4 -D on percentage of callus induction of *S. semperivrens*.

Treatment	Callus % induction
Control (free auxin)	0.00
0.4 mg/l NAA	18.33
0.8 mg/l NAA	18.36
0.4 mg/l 2,4 D	36.32
0.8 mg/ l 2,4 D	73.35
1.6 mg/l 2,4 D	100.00
0.8 mg/l 2,4 D + 0.4 mg/l NAA	91.64
1.6 mg/l 2,4 D + 0.8 mg/l NAA	100.00
LSD (0.05)	1.36

The results indicated that, raising the number of subcultures had no significant effect on callus fresh weight. The treatment 0.8 mg/l 2, 4-D NAA at 0.4 mg/l recorded the lowest fresh weight (2.46 g) compared with 0.8 mg/l 2, 4-D only. The interaction effect of the treatment on fresh weight of callus cultured on the medium provided with 1.6 mg /l 2, 4-D +0.8 mg/l NAA resulted in the highest fresh weight after the second subculture. The lowest fresh weight is observed when callus was cultured for two times on medium provided 0.8 mg/l 2, 4-D + 0.4mg/l NAA. **Table (3)**. In the same manner, dry weight recorded the highest values on medium supplemented with 1.6 mg/l 2, 4-D only and1.6mg/l 2, 4-D +0.8 mg/l NAA.

Fig. 3: Callus induction of *S. semperivrens* explants

Fig.4: Callus growth after three subcultures.

Table 3: Effect of different combination of NAA and 2,4-D on callus growth across three subcultures as fresh weight and dry weight (g) of the last subculture of *S. semperivrens.*

Subcultures number of fresh weight / Treatments	Sub1	Sub2	Sub3	Mean Treatment (A)	Callus Dry Weight (g)
0.8 mg/l 2,4D(C1)	2.54	2.59	2.51	2.55	0.294
1.6 mg/l 2,4 D(C2)	3.39	3.29	2.94	3.21	0.318
0.8 mg/l 2,4D + 0.4 mg/l NAA(C3)	2.38	2.27	2.72	2.46	0.209
1.6 mg/l 2,4-D + 0.8 mg/ l NAA(C4)	3.14	3.62	2.92	3.23	0.329
Mean subculture(B)	2.86	2.94	2.77		
LSD (0.05)	A=0.49		B=(NS.)	AB=0.85	0.12

GC/MS analysis of *n*-hexane extracts of calli, shootlets cultures and leaves:

A white odorless semi-solid residue (0.6 % w/w) was produced from *n*-hexane extracts of calli (HC), whereas *n*- hexane extracts of both mother plant (HL) and shootlet (HS) afforded a semi-solid residue with characteristic aromatic odour (3% and 2.9%). Results of chromatography/mass spectroscopy (GC/MS) analysis revealed qualitative and quantitative variability for the chemical profiles of the investigated samples (HC, HS and HL). A total of 43 compounds (17 in HC, 41 in HS and 42 in HL) were identified in each all extract, with 40 compounds being common between HS and HL and only 15 compounds present in HC and the two other samples; **Table (4)**.

These compounds included variable classes viz. non-oxygenated compounds such as hydrocarbons (aliphatic, monoterpene and sesquiterpene) and oxygenated compounds (alcohols, ketones, fatty acid esters). Aliphatic hydrocarbons were the major components in HC, but monoterpene hydrocarbons compounds were predominant in HS and HL.

Table 4: GC/MS components analysis of *n*-hexane extracts of leaves (HL), *in vitro* shootlets (HS) and callus cultures (HC) of *S. sempervirens*.

No	RI	Name of compound	MF(MW)	Relative percentage (%)			ID
				HC	HS	HL	
A) Non-oxygenated compounds Aliphatic hydrocarbons							
1	1100	Dodecane	$C_{12}H_{26}$(170)	11.99	2.14	1.52	MS,RI
2	1655	Cyclotetradecane	$C_{14}H_{28}$(169)	0.03	0.03	0.10	MS,RI
3	2010	Eicosane	$C_{20}H_{42}$(282)	0.42	0.51	0.32	MS,RI
4	2077	Eicosane,2-methyl	$C_{21}H_{44}$(296)	18.20	0.13	-	MS,RI
5	2100	Heneicosane	$C_{21}H_{44}$(296)	6.62	0.91	1.01	MS,RI
6	2300	1-Tricosene	$C_{23}H_{46}$(322)	0.84	-	0.01	MS,RI
7	2490	Tetracosane	$C_{27}H_{50}$(338)	0.41	0.14	0.21	MS,RI
8	2690	Heptacosane	$C_{27}H_{56}$(380)	17.10	-	0.04	MS,RI
9	3100	Triacontane	$C_{30}H_{62}$(422)	6.92	0.01	0.18	MS,RI
Total				62.53	3.87	3.39	
Monoterpene hydrocarbons							
10	948	alpha.-Pinene	$C_{10}H_{16}$(136)	1.42	31.41	10.57	MS,RI
11	969	alpha.-Phellandrene	$C_{10}H_{16}$(136)	1.92	36.02	35.93	MS,RI
12	897	Thujene	$C_{10}H_{16}$(136)	-	0.62	0.28	MS,RI
13	943	beta.-Pinene	$C_{10}H_{16}$(136)	0.08	1.55	1.30	MS,RI
14	958	beta.-Myrcene	$C_{10}H_{16}$(136)	-	1.53	3.72	MS,RI
15	1042	*p*-Cymene	$C_{10}H_{14}$(134)	-	0.92	1.14	MS,RI
16	998	gamma.-Terpinen	$C_{10}H_{16}$(136)	-	0.35	6.00	MS,RI
17	1018	Limonene	$C_{10}H_{16}$(136)	-	6.95	10.29	MS,RI
18	1052	Terpinolene	$C_{10}H_{16}$(136)	-	1.04	1.03	MS,RI
19	1465	gamma.-Elemene	$C_{15}H_{24}$(204)	-	0.13	0.97	MS,RI
Total				3.42	80.52	71.23	

Continue Table (4)

No	RI	Name of compound	MF(MW)	Relative percentage (%)			ID
				HC	HS	HL	
Sesquiterpene hydrocarbons							
20	1399	*β*-Caryophyllene	$C_{15}H_{24}$(204)	-	0.14	0.55	MS,RI
21	1452	trans-*α*-Bergamotene	$C_{15}H_{24}$(204)	-	0.61	0.74	MS,RI
22	1463	Germacrene D	$C_{15}H_{24}$(204)	-	0.62	0.51	MS,RI
23	1486	*α*-Humulene	$C_{15}H_{24}$(204)	-	0.02	1.47	MS,RI
24	1493	Trans-*β*-Farnesene	$C_{15}H_{24}$(204)	-	0.51	0.41	MS,RI
25	1500	E,E- *α*- Farnesene	$C_{15}H_{24}$(204)	-	0.03	0.52	MS,RI
Total					1.93	4.20	
B) Oxygenated compounds							
Alcohols							
26	2351	1-Henicosanol	$C_{21}H_{44}O$(312)	1.99	0.75	0.73	
27	2948	1-Heptacosanol	$C_{27}H_{56}O$(396)	10.29	1.51	3.90	MS,RI
28	1086	Borneol	$C_{10}H_{18}O$(154)	-	0.32	0.01	MS,RI
29	1190	Terpinene-4-ol	$C_{10}H_{18}O$(154)	13.49	1.03	1.02	MS,RI
30	1196	*p*-Cymen-8-ol	$C_{10}H_{14}O$(150)	-	0.63	0.71	MS,RI
31	1230	*α*-Terpineol	$C_{10}H_{18}O$(154)	-	0.02	0.52	MS,RI
32	1268	cis-Piperitol	$C_{10}H_{18}O$(154)	-	0.37	0.21	MS,RI
33	1276	trans-Piperitol	$C_{10}H_{18}O$(154)	-	0.01	0.11	MS,RI
34	1288	*β*-Citronellol	$C_{10}H_{20}O$(156)	-	0.03	0.20	MS,RI
35	1290	Geraniol	$C_{10}H_{18}O$(154)	-	0.11	0.72	MS,RI
36	1300	Thymol	$C_{10}H_{14}O$(150)	-	0.41	1.01	MS,RI
37	1389	Eugenol	$C_{10}H_{12}O_2$(164)	-	0.20	0.22	MS,RI
38	1489	Spathulenol	$C_{15}H_{24}O$(220)	-	0.13	0.82	MS,RI
39	1680	α–Cadinol	$C_{15}H_{26}O$(222)	4.22	0.17	0.68	MS,RI
40	1950	Phytol	$C_{20}H_{40}O$(296)	0.11	1.36	1.72	MS,RI
Total				30.1	7.05	12.58	MS,RI
Ketones							
	1103	Fenchone	$C_{10}H_{16}O$(152)	-	1.13	1.51	MS,RI
Fatty acids esters							
41	1578	Citronellylpentanoate	$C_{15}H_{28}O_2$(240)	-	0.71	0.50	MS,RI
42	1345	α-Terpinyl acetate	$C_{12}H_{20}O_2$(196)	-	2.10	1.02	MS,RI
43	1355	Thymol acetate	$C_{12}H_{16}O_2$(192)	-	0.01	1.13	MS,RI
Total				-	2.82	2.65	
		Total % identified compounds		96.05	96.19	94.05	
		Number of identified compounds		17	42	43	

RI: retention index values relative to CC *n*- alkanes calculated using a non-polar HP-5MS capillary column; MS: mass spectra; ID: identification method

Evaluation of different secondary metabolites in ethanol extracts of *S. sempervirens* mother plant and *in vitro* calli and shooting cultures:

Ethanol extracts of calli (EC1- EC4), shootlets (ES1- S4) and tree leaves (EL) were yellowish brown in colour, with yield ranges of (1.01-1.23, 8.20-9.87 and 11.21 % calculated on a dry weight basis), respectively. According to the data shown in **Tables (4and 5)**, among all the investigated metabolites, EC exhibited lower content of phenols, flavonoids and triterpenoids than ES and EL, however callus cultured on MS medium supplemented with 1.6 mg/l 2, 4 -D (EC2) showed the greatest phenolics and flavonoids contents **Table (5,6)**. The phenolic and flavonoids contents were approximately fourfold higher in ES and EL than in EC; shootlets cultured on MS medium with 50 g/l sucrose + 0.5 mg/l KIN (ES4) showed higher total phenolics and flavonoids content as compared with results of the mother plant leaves (EL)as shown in, **Tables (5 and 6)**.

Table 5: Evaluation of secondary metabolites in ethanol extracts of *S. sempervirens* mother plant and calli cultures *in vitro* after the last subcultures.

Secondary metabolites (µg mg^{-1}, d.w.)	EC1	EC2	EC3	EC4	EL
Total Flavonoids	0.116	0.167	.091	0.136	0.684
Total phenolic content	0.169	0.257	0.172	0.216	2.061
Total triterpenoid content	26.816	3.008	10.976	5.632	172.672

EC1:0.8 mg/l 2,4D, EC2:1.6 mg/l 2,4 D, EC3: 0.8 mg/l 2,4D + 0.4 mg/l NAA, EC4: 1.6 mg/l 2,4- D + 0.8 mg/ l NAA, EL: Mother tree leaves.

Table 6: Evaluation of secondary metabolites in ethanol extracts of *S. sempervirens* mother plant and *in vitro* shootlets after the last subcultures.

Secondary metabolites (µg mg^{-1}, d.w.)	ES1	ES2	ES3	ES4	EL
Total Flavonoids	0.752	0.820	0.816	0.982	0.684
Total phenolic content	1.313	1.098	2.514	2.121	2.061
Total triterpenoid content	106.912	60.864	172.640	79.872	172.672

ES1:25 g/l sucrose+ 3g/l activated charcoal, ES2: 50 g/l sucrose +0.5 mg/l BAP, ES3: 25 g/l sucrose, ES4: 50 g/l sucrose + 0.5mg/l KIN, EL: Mother tree leaves.

Evaluation of the biological activities of *S. sempervirens* mother plant and *in vitro* calli and shooting cultures:

The antioxidants were assessed **Table (7)** based on free radical scavenging activity (1, 1 diphenyl-2-picrylhydrazyl; DPPH) method. According to IC_{50} results, EC2 and EC4 exerted no activity compared to ES1, ES4 and EL. In addition, shootlets (ES4) cultures on MS with 50 g/l sucrose + 0.5 mg/l KIN showed the highest antioxidant activity followed by shootlets (ES1) cultures on MS with 25g/l sucrose and leaves of mother plant.

Screening of the cytotoxic activity of the tested extracts was evaluated using two different types of human carcinoma cell lines (HepG-2 and MCF7). The results represented in **Table (8)** 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} by the investigated extracts was achieved by shootlets cultured on MS medium with 25g/l sucrose (ES1) against HepG-2and MCF-7, followed by shooting on culture MS media with 50 g/l sucrose + 0.5 mg/l KIN (ES4) which showed IC_{50} against HepG-2 and MCF-7.

Table 7: Antioxidant activity of ethanol extract of *S. sempervirens* mother plant and *in vitro* calli and shooting cultures.

Concentration ($\mu g\ ml^{-1}$)	Radical-scavenging activity (%)					Gallic acid	
	EC2	EC4	ES1	ES4	EL	Concentration ($\mu g\ ml^{-1}$)	Radical-scavenging activity (%)
1000	-	-	88.53±0.30	89.63± 0.32	90.77±	100	90.8 ± 1.52
500	-	-	68.42 ± 0.24	71.10 ± 0.41	78.73±	50	83.7 ± 0.61
250	-	-	60.31 ± 0.25	61.36± 0.12	73.41±	25	76.3 ± 0.22
IC_{50} ($\mu g\ ml^{-1}$)							
	-	-	5407.74	6017.61	6420.24	32.85	

EC2: 1.6 mg/l 2-4, D + 0.8 mg/l NAA, EC4: 1.6 mg/l 2-4, D, ES1: 25 g/l sucrose, ES4: 50g/l sucrose+ 0.5 mg/l KIN, EL: Mother leaves

Table 8 Cytotoxic activity of *S. sempervirens* mother plant and *in vitro* calli and shooting cultures.

Cancer cell line	IC_{50} (µg ml^{-1})					
	Tested extracts					Doxorubicin (25 µg ml^{-1})
	EC2	EC4	ES1	ES4	EL	
MCF-7	>500	>500	61.2 ±1.4	123 ±1.9	>500	0.35±0.03
HepG-2	>500	>500	39.1 ± 0.8	97.6 ± 3.2	>500	0.36± 0.04

EC2: 1.6 mg/l 2-4, D + 0.8 mg/l NAA, EC4: 1.6 mg/l 2-4, D, ES1: 25g/l sucrose, ES4: 50 g/l sucrose+ 0.5 mg/l KIN, EL: Mother leaves

Cultured explants on MS medium provided with 50g/l sucrose + 0.5 g/l BAP recoded the highest significant value of shootlets no. after the second subculture, in agreement with **Shekhawat *et al*.,(1993) and Phulwaria *et al*.,(2011),** who stated that, repeated transferring of explants releases the meristem /bud to proliferation to yield numerous shoot buds of *Salvadora persica* tree. The positive effect of raising sucrose concentration was showed also by **Moitreye *et al*.,(2012**) on *Aquilaria malaccesis* tree, who mentioned that, suitable concentrations of sucrose in culture medium caused an improvement of biomass value.

Concerning to the role of cytokinines **Zhang *et al*. (2010)**, while studying the metabolism of BAP treated mature pine plant suggested that, BAP causes reinvigorations of mature/old tissues and induces bud induction a prerequisite for cloning of mature trees. Also, these trends were in agreement with, **Jun *et al*.,(2011)**; **Khater and Elashtorkhy,(2015) and Sami *et al*., (2016)**. On the other hand, **Ziv ,(1991)** showed that, the use of high cytokinin levels was one of the most effective methods to reduce shoot and leaf growth and promote the formation of meristematic clusters, as well the addition of activated charcoal has an ability to adsorb toxic metabolites released from tissue into culture medium and allowed shoot growth **(Kummar and Thorpe,(1991); Gad,(2011) and Hwida,(2017).**

In general, these results could be attributed to the important physiological effect of cytokinins as they stimulate cell division as well as elongation to activate RNA synthesis and to stimulate protein

synthesis and enzyme activity as were reviewed by **Kulaeva and Skoog ,(1980).**

The addition of 2, 4-D to callusing medium is recommended to obtain good quality of callus mass with high production percentage. The results were confirmed by those of, **Rawat *et al*., (2013**)on *Aconitum violaceum*, **Moitreye *et al*.,(2013)** on *Aquilaria malaccesis* and **Abd EL kadder *et al.*, (2014)** on *Dillenia indica*. Moreover, callus induction was affected by different types of auxins and concentrations. The promotive effect of auxins (IBA, NAA and 2,4-D) on callus induction and growth might be attributed to that auxins promote the biosynthesis of ethylene by increasing the activity of 1-aminocylopropane-1-carboxylic acid (ACC) syntheses, as was suggested by **Kende ,(1989)**.

Hydrocarbons (aliphatic, monoterpene and sesquiterpene) and oxygenated compounds (alcohols, ketones, fatty acid esters) were abundant in HS and HL. On contrast, HC was devoid of sesquiterpene hydrocarbon, ketones and fatty acids esters. α -Phellandrene and α -Pinene were major compounds in HS and HL respectively, whereas eicosane, 2-methyl and heptacosane were found in high levels in HC. This variation may be attributed to the fact that differentiation in callus cultures is essential for the production of secondary metabolites was stated by **(Hagimori *et al*.,(1982) and Dornenburg,(2008)** and could partly explain the absence of monoterpene and sesquiterpene hydrocarbons, such those responsible for the strong odour of HS and HM. Monoterpene hydrocarbons were predominant in HS and HL, among which α-phellandrene and α-pinene were the main components that present in higher amount in HS than in HL, this indicates that shootlets may be an accessible source for these biologically active components that have antioxidant **(Dar *et al*.,2011)** , anti-inflammatory **(Lin *et al*.,2008)**and cytotoxic activities **(Gould,(1997)**. Additionally, terpinene-4-ol the bioactive compound, which is reported to have antimicrobial and antifungal activities **(McMahon *et al*.,2007)**, could be easily supplied by HC.

The results of GC/MS analysis of HL in this study are in agreement with, **(Taha and Shakour, (2016)** regarding the content of monoterpene hydrocarbons (63.39 and 71.23%), additionally, α-phellandrene (29.60 and 35.93%) dl-limonene (15.60 and10.29%), α-pinene (8.65 and10.57%) and terpinene-4-ol (3.5 and1.02%) were the major compounds. This variation in the results could be directly

related to the extraction method, as the previous report used distillation, but in this study was used *n*- hexane extraction method. These results emphasis that *n*- hexane extraction, which is proposed as an alternative method to conventional extraction, provides high recovery of volatile constituents and a high content of bioactive compounds **(Liauw *et al*.,2008)**.

Due to their medicinal importance, we sought to assess the amount of triterpenoids in EC and ES compared to EL. It is known that they exhibit cytotoxic activity against human tumor cell lines, including HepG2, (hepatocellular carcinoma), HL-60 (leukemia) and MCF-7 (breast cancer) by (**Lu *et al*., 2012**). They are well-known for their antioxidant and anti-inflammatory activities (**Pensec *et al*., 2014**). Shootlets cultured on MS medium with 25g/l sucrose (ES1) has similar triterpenoids content (172.640 µg mg^{-1}, d.w.) as in leaves (EL) (172.672 µg mg-1, d.w.); they both were approximately sixfold higher than callus cultured on MS medium with 1.6 mg/l 2, 4 -D + 0.8 mg/l NAA (EC4) (26.816 µg mg^{-1}, d.w.), which is in accordance with **Santarem and Astaria ,(2003)** who found that shootlets showed similar secondary metabolites levels to those found in leaves of the field-grown plants. They suggested that the accumulation of these compounds is related to leaf differentiation, so this can explain the lower triterpenoids content in non-differentiated callus. It's the first time to investigate the triterpenoids content in leaves of *S. sempervirens* and their*in vitro* callus and shootlets cultures.

These results are in agreement with those of **(Abyari *et al*.,2016 and Collin, 2001)** who stated that the differentiation of any tissue is associated with the increased synthesis of secondary metabolites under *in vitro* conditions, which could be due to the appearance of complex cells and tissues that are more metabolically competent.

The valuable biological activities of *in vitro* shootless could be attributed to the presence of higher amounts of antioxidant and cytotoxic metabolites, such as phenolic, flavonoids and triterpenoids (**Chun *et al*.,2003**).

This work provides an important basis for further investigation of shootlets cultures of *S. sempervirens* with regard to the isolation and identification of metabolites that could be responsible for the cytotoxic activity and further screening of the *in vivo* pharmacological activity of the plant. Moreover, from the tissue culture point of view, different growth factors and techniques can be assessed to increase the

levels of bioactive metabolites. To the best of our knowledge, this is the first report on callus and shootlets induction using nodal explant of *S. sempervirens* and evaluation of their secondary metabolites.

Conclusion

This is study providing a clear, simple and reliable protocol for successful callus and shootlets production from shoot tips explants of *S. sempervirens*, however the present work confirmed that we can obtain effective components have cytotoxic activity against tumor cells of human liver and breast by using the extraction of shootlets of explant when cultured on free MS medium provided with 25g/l sucrose or supplemented with 50g/l sucrose plus 0.5 mg/l KIN., comparing to callus cultures and mother plant tree leaves under investigation. The study could be considered as a starting point for further investigations and for enhancing the production of secondary metabolites from *in vitro* callus and shootlets cultures for future use in pharmaceutical applications as antioxidants and cytotoxic preparations. Additionally, the establishment of shootlets cultures is recommended as they could be a potential source of high amounts of cytotoxic metabolites.

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التركيب الكيماوى و نشاط مضادات الاكسده و السميه لمستخلص كلامن أوراق وناتج زراعة الانسجه لاشجار السكويا سمبرفيرنس

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1قسم بحوث الأشجار الخشبيه والغابات –معهد بحوث البساتين – مركز البحوث الزراعيه – جيزة - مصر

2معمل كيمياء العقاقير النباتيه –الهيئه القوميه لبحوث ا لرقابه الدوائية-جيزة - مصر

تعتبرنبات السكويا سمبرفيرنس *sequoia sempervirens* من النباتات التي تنتمى الى العائله السرويه والتي تعرف بخصائصها الهامه لمنتجاتها الثانويه كمضادات للاكسده والسميه.اجريت الدراسه المعمليه لانتاج لكلا من خلايا الكالس والفريعات النباتيه الناتجه من زراعة الانسجه لتقييم قدرتها الحيويه والدوائيه . تم استخدام العقد النباتيه وزراعتها علي البيئه الغذائيه مورشيجى وسكوج والمضاف اليها 25جرام/لتر من السكروز فقط اومضاف اليها 2جم/لتر فحم نباتى نشط وكذلك معاملة البيئه اما بالكينتين او البنزيل ادنين بمقدار 0.5ملجم/لتر لكلامنهما وذلك في وجود تركيز من السكروز مقداره 50جم/لتر ثم تكرر الزراعه بنقل الأجزاء النباتيه ثلاث مرات من النقلات الزراعيه.كما نم الحصول علي الكالس النباتى علي بيئه الزراعيه مورشيج وسكوج في وجود تركيزات مختلفة من نفثالين حمض الخليك وكذلك داى فينوكسى حمض الخليك. كما اجرى اختبار التحليل الكروموتجرافى على مستخلص الهكسان لكلامن الكالس والاجزاء النباتيه الناتجه من زراعة الانسجه و كذلك مقارنتها بمستخلص الأوراق لشجره الام .اضافه الى المقارنه لمستخلص الايثانول لثلاث أجزاء المختلفه من حيث محتوها من التقدير الكمى للفينولات والفلافونويد والتريتربينويدات .

أوضحت النتائج ان اعلى نسبه لبقاء الأجزاء النباتيه حيه كان عند إضافة 25جم/لتر سكروز مضافا اليها 2 جم/ لتر فحم نباتى نشط وكذلك عند إضافة الكينتين5، ملجم/لتر وفي وجود 50جم/لتر سكروز وقد أعطت المعامله بالكنيتبن زيادة معنويه لكلامن متوسط اطوال الفريعات وكذلك كلا من الوزن الطازج والجاف للاجزاء النباتيه. وقد أعطت معامله البيئه النباتيه مورشيجى وسكوج بإضافة1.6مجم /لتر من دى فينوكسى حامض الخليك في وجودمجم0.8 مجم/لتر نفثالين حمض الخليك أعطت اعلى نسبه من الدفع لتكوين الكالس وكذلك النمو من حيث الوزن الطازج والجاف بينما كانت اقل نسبه للكالس عند المعامله الكنترول .

وأوضح التحليل الكروماتوجرفى ان المكون الرئيسى لمستخلص الهكسان للكالس هو الهدروكربونات الاليفاتيه بينما المنوتربينات كانت في المستخلص الهكسانى للفريعات الناتجه

من زراعة الانسجه واوراق النبات الام. بينما كانت نسبة كلامن الفينولات والفلافونيدات والتريتربينودات اعلى تكوين في مستخلص كلامن الأجزاء النباتيه والأوراق الام مقارنة بالموجودة في مستخلص الكالس.وقد وجد ان اعلى نسبه من مضادت الاكسدة كانت في الفريعات النباتيه الناتجه من زراعة الانسجه يليها الأوراق من النبات الام ثم اقلها الناتجه من الكالس .

وقد اوضح اختبار مضادات السميه لخلايا الأورام لانسجة الكبد والثدى ان اعلي نسبه لتثبيط هذه الخلايا كان مقصورا على المستخلص الناتج بمعاملة الفريعات النباتيه بمعاملة الكنترول والمعاملة بالكنيتين 0.5مجم / لتر مضافا اليها 50 مجم/لتر سكروز مقارنتا بمستخلص الكالس اواوراق نبات الام . حيث وجد انه للحصول اعلى نسبه من النشاط المثبط لسميه الخلايا ومضادات الاكسدة والفينولات والفولافوندات وكذلك الترتربينودات كان من الفريعات الناتجه من زراعه الانسجه .