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In vitro production of some secondary metabolites from *Cupressus sempervirens*

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Abstract

Cupressus sempervirens L. has known medicinal activities, which are related to the presence of miscellaneous secondary metabolites, involved in patents for pharmaceuticals and/or cosmetics in the market. The aim of this study is to perform a comparative investigation of the main metabolites in the wild, cultivated and callus cultures of *C. sempervirens* using different treatments and the *in vitro* production of certain metabolites from *C. sempervirens* callus cultures in concentrations adequate for use commercially and also to develop a convenient chromatographic method for both the qualitative and quantitative determination of the most beneficial flavonoids (rutin and quercitrin), along with other metabolites (ferruginol, 2-furancarboxaldehyde,5 methyl, pentadecanoic acid, totarol, quinic acid and hinokiol) using HPLC/DAD and GC/MS, respectively.

Leaf extract of the wild plant induced reduction in cell viability of some tested cell lines as: human hepatocellular carcinoma cell (HEPG2), lung carcinoma cell (A549), human Caucasian breast adenocarcinoma (MCF7) and especially on human colon cancer cell (HCT116), which was higher than doxorubicin used as a standard. This study is a stepping stone for obtaining natural and renewable bioactive secondary metabolites from *C. sempervirens* through *in vitro* callus cultures.

Key words

Cupressus sempervirens, callus, metabolites, HPLC, GC

1. Introduction

The growing demand in today's market for natural, renewable phytochemical products has refocused attention on in vitro produced plant materials as potential factories for secondary metabolites (Shukor et al., 2013; Lodha et al., 2014). Trials for production of promising secondary metabolites through plant *in vitro* culture techniques are methods commercially adopted as a promising potential alternative source for the production of high-value secondary metabolites of industrial importance, which can also serve as starting materials for drug development programs. Among the *in vitro* culture techniques, callus culture is distinguished as an economically viable alternative that may be used for obtaining bioactive plant metabolites (Braga et al., 2015).

Cupressus sempervirens L. (Cupressaceae) is known as the Mediterranean cypress, mainly cultivated as an ornamental plant. The aerial parts of the plant are used in folk medicine as astringent, incorporated in preparations (ointments and suppositories) and used to treat haemorrhoids, varicose veins and venous circulation disorders. The Antidiabetic activity of saponins and phenolic extracts of the cones and seeds from *C. sempervirens* was reported by Boussoussa et al. (2018). The essential oil is used as antiseptic and antispasmodic for stubborn coughs (Rawat et al., 2010) and possesses antimicrobial, antioxidant and anticancer properties (Mothana et al., 2009; Elansary et al., 2012; Fayed, 2015), while the extracts possess antimicrobial and antibiofilm properties, and therefore, can be used as natural preservative ingredients in food and/or pharmaceuticals (Gomez-Estaca et al., 2010; Romeo et al., 2010; Selim et al., 2014).

This plant is also described as a deodorant, a diuretic, promoting venous circulation to the kidneys and bladder area, improving bladder tone and as a co-adjuvant in therapy of urinary incontinence and enuresis (Mahmood et al., 2013; Al-Snafi, 2016). Additionally, the dried leaves are used in treatment of stomach pain, diabetes, inflammation, toothache, laryngitis and as contraceptive. In addition, *C. sempervirens* can be a promising source of natural products with antiprotozoal activity. (Al-Musayeib et al., 2012).

Flavonoids and biflavonoids were isolated from *C. sempervirens*, where rutin and querictrin are present in concentrations of 0.01 and 0.18 mg g^{-1} fresh weight (Romani et al., 2002).

The anticancer research showed that rutin could exert significant and potentially beneficial effects on decreasing the amount of precancerous lesions and in inducing apoptosis in the large intestine cancer, and also as a potential therapeutic drug for neuroblastoma treatment (Chen et al., 2013). Quercitrin, on the other hand, acts as an antihistaminic, anti-inflammatory, antioxidant and anticancer substance. It helps in solving problems of cellular regeneration, hemorrhoids, menopausal symptoms and non-healing ulcers (Sandhar et al., 2011), and also for inhibiting human platelet aggregation (Mosawy, 2015).

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Flavonoids; as rutin and querictrin have attained considerable attention because of their beneficial influence on human health. Their commercial importance necessitates the development of new technologies for their production from alternative sources for drug development. There are many reports on the production of flavonoids from plant callus cultures, but low productivity has been achieved (Jedinak et al., 2004). Flavonoids derived from plant callus cultures can be easily separated than those derived from intact plant tissues (Masoumian et al, 2011; Ong et al, 2011; Bandekar and Lele, 2014). Their concentrations can also be significantly enhanced in callus and cell cultures through control of *in vitro* conditions and elicitation or precursor feeding to stimulate metabolite production (Lila et al., 2005).

To the best of our knowledge there was relatively little or no information available on the production of secondary metabolites from *C. sempervirens* plant using *in vitro* callus culture technique. The aim of this study was is to optimize the production and accumulation of certain metabolites, especially rutin and quercitrin, from callus growth of *C. sempervirens* and to study the effect of gibberellic acid (GA_3) (as an elicitor) and phenylalanine (as a precursor) on the yield concentrations. Efficient, simple and convenient chromatographic techniques were applied to detect and quantify bioactive compounds, to facilitate their use commercially.

2. Materials and Methods

2.1. Plant material

Leaves of *C. sempervirens* were collected and used as explants for *in vitro* callus culture production from wild and cultivated plant sources. The wild species of *C. sempervirens* was obtained from Al-Almain north coast (CNC), kindly given and identified by Prof. Dr. Salama El Darier, Department of Botany and Microbiology, Faculty of Science, Alexandria University; whereas the cultivated species were from New Cairo public gardens, near Future University, Cairo, Egypt (CFU).

Samples were collected in summer (June). A voucher specimen was deposited at the Herbarium of the Department of Pharmacognosy, Faculty of Pharmacy, Minia University, and given the Code: Mn-Ph-Cog-020

2.2. Cytotoxic activity test

Cytotoxic activity testing was performed using human cell lines; human hepatocellular carcinoma cell (HePG2), human colon cancer cell (HCT116), human caucasian breast adenocarcinoma (MCF7) and lung carcinoma cell (A549) that were investigated on the extract of the wild plant leaves.

2.2.1. Preparation of plant samples for cytotoxicity testing

The leaves of the wild plant were air-dried, powdered and extracted with analytical grade methanol, for three successive times. The extracts were mixed and dried under vacuum, and then residue was directed to the four above mentioned cell lines for cytotoxic activity testing. This study was conducted at the Bioassay-Cell Culture Laboratory, National Research Centre, El-Tahrir St., Dokki, Cairo 12622, Egypt.

2.2.2. Cytotoxic activity test procedure

2.2.2.1. Cell viability assay

Cell viability was assessed by the mitochondrial dependent reduction of yellow MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) to purple formazan (Mosmann, 1983; Thabrew et al., 1997). The absorbance was measured using a microplate multi-well reader (Bio-Rad Laboratories Inc., model 3350, Hercules, California, USA) at 595 nm and a reference wavelength of 620 nm. Statistical significance was tested for samples and negative control (cells with vehicle) using independent *t*-test by SPSS 11 program. DMSO was the vehicle used for dissolution of plant extract and its final concentration on the cells was less than 0.2 %. The percentage of change in viability was calculated according to the formula: (Reading of extract / Reading of negative control) -1) x 100). A probit analysis was carried for IC_{50} determination using SPSS 11 program.

2.2.2.2. Cell lines and culture condition

All the following investigations were performed in a sterile area using a laminar flow cabinet biosafety class II level (Baker, SG403INT, Sanford, ME, USA). Cells were suspended in RPMI 1640 medium for HEPG2, HCT116 and MCF7, while DMEM medium was used for A549. The media were supplemented with 1% antibiotic-antimycotic mixture (10,000 U/ml Potassium Penicillin, 10,000 µg/ml Streptomycin Sulfate and 25 µg ml^{-1} Amphotericin B), 1 % L-glutamine and 10 % fetal bovine serum and kept at 37 ºC under 5 % CO_2. Cells were batch cultured for 10 days, then seeded at concentration of $10x10^3$ cells/well in fresh complete growth medium in 96-well microtiter plastic plates at 37 ºC for 24 h under 5 % CO_2 using a water jacketed carbon dioxide incubator (Sheldon, TC2323, Cornelius, OR, USA). Media were aspirated, fresh medium (without serum) was added and cells were incubated either alone (negative control) or with different concentrations of sample to give final concentrations of 100, 50, 25, 12.5, 6.25, 3.125, 0.78 and 1.56 µg ml^{-1}. After 48 h of incubation, medium was aspirated, 40 ul MTT salt (2.5 µg/ml) were added to each well and incubated for further four hours at 37 ºC under 5 % CO_2. Two hundred µl of 10 % sodium dodecyl sulphate (SDS) in deionized water were added to each well and incubated overnight at 37 ºC (to stop the reaction and dissolve the formed crystals). A positive control composed of 100 µg/ml Adrinamycin (Doxorubicin) [Mwt.= 579.99] was used as a known cytotoxic agent under the same conditions.

2.3. *In vitro* production of secondary metabolites

2.3.1. Culture medium and conditions

Fresh leaves were used as explants, and were thoroughly washed with running tap water for about 30 min, then surface

sterilized under complete aseptic conditions by immersing in 30 % (v/v) commercial Clorox solution (a disinfectant containing 2.5 % sodium hypochlorite) for 25 min with gentle agitation. Afterwards, leaves were washed three times with sterile distilled water. Then, they were immersed in 0.1 % (w/v) mercuric chloride solution. After shaking for 7 min, leaves were rinsed five times with sterile distilled water.

Leaf explants were cut into small pieces (0.5-1.0 cm) and cultured aseptically on solid full-strength basal Murashige and Skoog medium (MS; Murashige and Skoog, 1962) (Duchefa, Haarlem, Netherlands) with 30 g l^{-1} sucrose and 0.1 mg l^{-1} myoinositol. The pH of the medium was adjusted to 5.7-5.8 before being solidified with 3.0 g l^{-1} phytagel (Duchefa, Haarlem, Netherlands). Prepared media were autoclaved at a pressure of 1.06 kg cm^{-2} and 121 °C for 15 min (Harvey Sterilemax autoclave, Thermo Scientific, USA). The incubation conditions were 25 ± 2 °C at 16 h day^{-1} photoperiod (2000-2500 lux) under cool white florescent tubes (F140t9d/38, Toshiba).

2.3.2. Callus induction

Leaf explants of *C. sempervirens* were cultured on MS medium supplemented with plant growth regulators (PGRs) (Sigma Cell Culture, min. 90 %, St. Louis, USA); 0.5 mg l^{-1} 6-benzyladenin (BA) as a cytokinin and each of the concentrations of 0.5,1.0, 2.0 and 4.0 mg l^{-1} of *β*-naphthalene acetic acid (NAA) as an auxin. Percentage of callus induction (%) and mean fresh weight of callus (g) were recorded after six weeks from culturing.

2.3.3. Elicitation and precursor feeding

The best medium achieving the highest biomass was chosen (MS medium supplemented with 0.5 mg l^{-1} BA and 4 mg l^{-1} NAA) and supplemented with different concentrations of an elicitor or a precursor for the enhancement of rutin and quercitrin production. Gibberellic acid (GA_3) as an elicitor at concentrations of 1, 2, 4 and 8 mg l^{-1} and phenylalanine as a precursor at concentrations of 5, 10, 20 and 40 mg l^{-1} were added to the culture medium. Callus was subcultured using two grams of fresh callus in 40 ml of medium. Mean fresh and dry weights of callus (g) were recorded after six weeks from culturing.

2.3.4. Determination of flavonoids

2.3.4.1. Preparation of extracts for HPLC and GC/MS analyses

Samples were prepared for HPLC and GC/MS analyses of the tested plant leaves and the *in vitro* produced callus from each treatment. One gram of the leaves of both plant samples under investigation (CNC and CFU) were separately air-dried and ground to a fine homogenous powder, and then each was extracted with methanol HPLC grade for thrice. The obtained extracts were dried and the residues weighed. Also, the callus replicates obtained from leaf explants of *C. Sempervirens* subjected to different treatments were weighed (referred to as callus fresh weight), and completely dried in an oven with a temperature not exceeding 45ºC, and weighed (referred to as callus dry weight), homogenized in a mortar and extracted successively three times with methanol HPLC grade. These extracts were dried under vacuum and weighed. All samples prepared were kept refrigerated in dark vials until used. An amount of 1mg ml^{-1} of methanol extracts of the above mentioned dried extracts was prepared with methanol HPLC grade, and analyzed using HPLC-DAD for detection of rutin and quercetrin. For analysis of the plant volatiles, a sample of the above extracts (plant extracts as well as produced callus) were subjected to GC/MS investigation using GC/MS.

2.3.4.2. Identification of flavonoids (rutin and quercitrin)

The most suitable HPLC method was developed for qualification and quantification of the two most beneficial flavonoids (rutin and querictrin) present in the methanol extract of the plant samples and the callus obtained from the different treatments using a diode array detector. The detection was performed using calibration curves of both flavonoids.

Calibration curves were performed for both flavonoids by the use of pure authentic standards. Rutin (Pharco. Company) and quercitrin **(**quercetin-3-rhamnoside) (Sigma, Aldrich). The two flavonoids were quantified by the use of four point regression curve; $r^2 = 0.9954$ for rutin and $r^2 = 0.9992$ for quercitrin.

R ---y = 53.272x + 271.47 Q----y = 130.59x - 300.69

Under the following operating conditions:

Agilent Eclipse 1200- diode array detector, Germany.

Column: Eclipse XDB-C18, 4.6 mm x 150 mm (5 µm) 80 A. Injection (sample) volume: 20 µl. UV-Visible: 190-450 nm (240, 280, 330 and 350), temperature: ambient, concentrations prepared: 0.01/10 ml--------- 1mg/ml. Rutin R_t (7.16 minutes) while quercetrin R_t (8.874 minutes), mobile phase: 4 stepped gradient elution, water (adjusted to pH 3.2 with phosphoric acid buffer) and acetonitrile.

2.3.4.3. GC/MS analysis (head-space)

The methanol HPLC grade extracts of all the samples examined were analyzed using GC/MS. Shimadzu GC-MS Model (QP-2010 ultra-equipped with head space AOC-5000 auto injector and a Rtx-5 MS fused-silica column (30 meter length, 0.25 mm ID.; 0.25 µm film thickness).

2.4. Experimental design and statistical analysis of data

Experiments were subjected to a completely randomized design. Analysis of variance (ANOVA) and Duncan's multiple range test (Duncan, 1955), as modified by Snedecor and Cochran (1998), were used to analyze the obtained data. Each experiment included at least 20 replicates and was repeated twice. The means followed by the same letter are not significantly different at $P \leq 0.05$.

3. Results and Discussion

3.1. Cytotoxic activity test

The half inhibitory concentration IC50 of the extract samples ranged between 33.6 and 66 µg/ml while that of the standard (Doxorubicin) [M.wt= 579.99] ranged from 21.6 and 37.6 µg/ml. The results are quite promising for human colon cancer cell lines, the extract IC50 is 33.6 µg/ml which is more potent than Doxorubicin which has an IC50 of 37.6 µg/ml, whereas IC50 of the extract is 56.8, 64.6 & 66.0 µg/ml for human Caucasian breast adenocarcinoma, lung cancer & liver cancer cell lines compared to Doxorubicin which has IC50 of 26.1, 28.3 & 21.6 µg/ml, respectively **(Table 1)**.

The medicinal value of *C. sempervirens* is due to its components, flavonoids ; as rutin and quercitrin, showing high antioxidant, anti-inflammatory, antimicrobial and antitumor properties (Kumar and Pandey, 2013; Mosawy, 2015). Also the activity on human colon, breast, and lung cell lines is probably due to the presence of ferruginol, -which is a natural phenol and a meroterpene- that is supported by Son et al. (2005).

3.2. *In vitro* production of secondary metabolites

3.2.1. Callus induction

Callus induction of *C. sempervirens* was observed on all media containing NAA and BA. There was no callus formation in the absence of PGRs (control treatment). Hundred percent of leaf explants induced callus on MS medium supplemented with 1, 2 and 4 mg l^{-1} NAA with 0.5 mg l^{-1}BA, whereas the lowest callus induction percentage (77.77 %) occurred on MS medium supplemented with 0.5 mg l^{-1} of both NAA and BA **(Table 2)**. Results demonstrate the differential effect of the concentration of auxin used. They also highlight the importance of auxin and cytokinin for the establishment of callus cultures of *C. sempervirens*. According to Su et al. (2011), auxins and cytokinins control cell division in undifferentiated cells and interaction efficiency depends on the plant species and tissue. Callus was produced from the entire cut end of leaf explants and was friable and varied between green and yellow colours **(Figure 1)**. In this concern, Mustafa et al. (2011) reported that friable callus is mostly used for the cultivation of cells in suspension and for the study of metabolites production, because the cells rapidly divide and disperse in culture medium.

Callus showed higher values of fresh weight in media supplemented with higher concentrations of NAA. The fresh weight of callus increased proportionally with the increase in NAA concentration **(Table 2)**. It is well known that auxins initiate cell division and control the processes of growth and cell elongation, because they induce the transcription of messenger RNA molecules capable of encoding proteins important for growth (George et al., 2008). In general, plant growth regulators are important factors that affect cell growth, differentiation and formation of metabolites in plant cells and tissues. Auxins in the culture medium are related to the formation of adventitious roots and calogenic tissues, while cytokinins cause cell division and growth of shoots. The interaction of auxins and cytokinins is widely used for the induction and maintenance of callus (Mustafa et al., 2011).

Table 1: Cytotoxic activity test of *Cupressus sempervirens.*

	IC_{50} µg/ml		IC_{50} µM		At 100 ppm of extract (%)
	Extract	**Doxorubicin**	**Extract**	**Doxorubicin**	
HEPG2 (liver cancer cell line)	66.0	21.6	108.4	37.8	78.5
HCT116 (human colon cancer cell line)	33.6	37.6	63.8	65.1	97.9
A549 (lung cancer cell line)	64.6	28.3	104.4	48.8	82.2
MCF7 (Human Caucasian breast adenocarcinoma)	56.8	26.1	94.2	45.2	88.5
DMSO	---		---		5.0
Negative control 0%	---		---		0.0

Table 2: Callus induction from leaf explants of *Cuperssus sempervirens* and biomass production on MS medium supplemented with 0.5 mg l^{-1} BA in addition to different concentration of NAA after six weeks from culturing.

BA concentration (mg l^{-1})	NAA concentration (mg l^{-1})	Callus induction (%)	Fresh weight of callus (g)
0.0	0	0.000	0.000
0.5	0.5	77.770^{b}	1.700^{c}
0.5	1.0	100.00^{a}	3.428^{b}
0.5	2.0	100.00^{a}	4.123^{a}
0.5	4.0	100.00^{a}	4.247^{a}

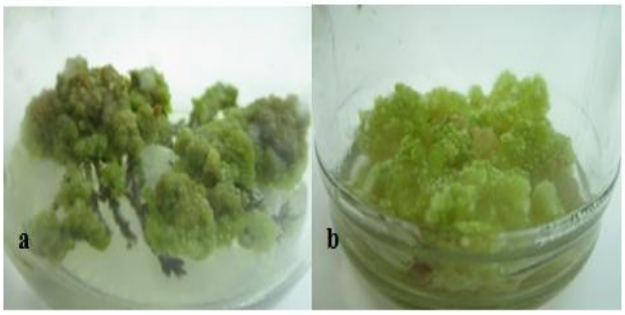

Figure 1: Callus induction from leaf explants of *Cuperssus sempervirens* on MS medium supplemented with 0.5 mg l^{-1} BA and 4.0 mg l^{-1} NAA.
a. Callus induction after six weeks, **b.** Proliferated callus.

3.2.2. Effect of GA_3 and phenylalanine on callus biomass and flavonoids content

When compared to the control treatment, the biomass of callus increased when callus was grown on media with various concentrations of GA_3 and phenylalanine, except for the highest concentration of GA_3 (8 mg l^{-1}) and the two concentrations of 10 and 20 mg l^{-1} of phenylalanine **(Table 3)**. The highest biomass of callus was obtained on the medium supplemented with 2 mg l^{-1} GA_3.

All calli of *C. Sempervirens* produced significant amounts of rutin and quercitrin compounds, regardless of the type and concentration of the additive, whether GA_3 or phenylalanine **(Table 3)**. The results of the present study indicate that *in vitro* culture both promote and increase the production of rutin and to a certain extent, quercitrin compounds in callus of *C. sempervirens.* This confirms that supplementing elicitors, precursors and growth promoters have been shown to increase secondary metabolite content in cell cultures (Preetpal and Bains, 2012).

The concentrations of rutin and quercitrin in CNC were 0.669 and 1.073 mg g^{-1}, while in CFU were 2.356 and 1.023 mg g^{-1}, respectively. In all treatments, the tremendous increase of rutin content was detected when compared to the wild and cultivated plants. This increase varied but reached its peak with more than 164 times for rutin obtained from 4 mg l^{-1} GA_3 (109.887 mg g^{-1} dry weight). For quercitrin, on the other hand, the results were variable , a slight increase or decrease was observed, except for concentration obtained from sample CG3 with 4 mg l^{-1} GA_3 (33.441 mg g^{-1} dry weight), which represents 31.2 and 32.7 times that obtained from CNC and CFU, respectively. Also, it is clear that GA_3 had a greater effect, especially the case with 4 mg l^{-1} GA_3 supplementation. This is supported by Abbasi et al. (2012) and Naik and Al-Khayri (2016), who reported that GA_3 is well known as an effective elicitor for production of secondary metabolites and has a variety of growth effects; cell elongation; the modulation of enzymes activities such as phenylalanine ammonia lyase (PAL), chlorophyllase, and peroxidase; and also changes in the metabolism and accumulation of anthocyanins, glutathione, flavonoids, lignin, proteins and starch. Also, it is known that GA_3 speeds growth of cells and significantly induced flavonoids biosynthesis in a diversity of plant species (Jamwal et al., 2018)

Phenylalanine, as one of the known precursors of flavonoids, when supplemented into the MS medium, it is expected to produce elevated level of target compounds (Shinde et al., 2009). Its application as a precursor for flavonoids production was demonstrated in a large number of plants, such as *Balanites aegyptiaca* (Bedawat, 2006), *Ailanthus excelsa* (Rao, 2007), *Cocculus pendulus* and *Tinospora cordifolia* (Yadav, 2010), *Maytenus emarginata* (Mathur and Goswami, 2014),*Citrullus colocynthis* (Meena et al., 2014) and *Abutilon indicum* (Sajjalaguddam and Paladugu, 2015).

These results indicate that *in vitro* culture both promote and increase the production of rutin and to a certain extent, quercitrin compounds in callus of *C. sempervirens.*

3.2.3. GC/MS analysis of the methanol extracts

The GC/MS analyses were performed for/on the same extracts used for HPLC/DAD analysis previously **(Table 4)**. The methanol extract of the CNC and CFU produced 56 and 51 components, respectively, concomitantly some treatments produced up to 107 components, some of which were not present in the source explants (as Hinokiol, a multifunctional antiangiogenic and antitumor agent and totardiol) or were present as another derivative (tetradecanoic acid, also called myristic acid).

Compounds chosen for their value and were present in most of the methanol extracts were ferruginol, 2-furancarboxaldehyde,5-methyl furfural , pentadecanoic acid, totarol, quinic acid and hinokiol.

By comparison with source plants, some of these compounds increased greatly under the effect of GA_3 and phenylalanine application. Ferruginol was present as 1 % and 1.87 % in CNC and CFU extracts, respectively, but increased to more than 22 times (42.68 %) with CF2 treatment. The compound 2 furancarboxaldehyde, 5 methyl was present as 1.61 % in the CFU, and remarkably increased to 38.92 % in CF2, which represents 37 folds. Pentadecanoic acid was present as 1.3 and 2.02 % in CFU and CNC, respectively, where it increased to 15.02, 13.11 and 12.07 % in CF1, CG2 and CF4 media, respectively. Totarol was present as 0.63 in CNC, and no encouraging improvement was observed. Quinic acid was present as 0.88 % in CFU extract only (absent in CNC extract), but increased to 2.02 % in both CG4 and CF4 media. Hinokiol a compound absent in source explants was present as 2.73 % on MS media (without any additives) and all other treatments, except CF2 and CF3.

On the other hand, ethyl lineolate, the fragrant and flavouring agent was present in source explants as 0.49 and 1.84 % in CFU and CNC, respectively, and as 1.98 % in the callus extract of control MS medium and was not detected in all other treatments. Loliolide, an antirepellent compound, was present in CFU explant source as 1.19 %, but only detected in lower amounts in some treatments (CG1, CF1 and CF2).

Guanosine (antiviral similar to acyclovir) was absent in source explants, but produced in CG4 and CF2 only as 5.35 and 1.1 %, respectively. Qinghaosu (artemisinin, a sesquiterpene antimalarial and antischistosomal drug) was present as 0.62 and

Table 3: Callus biomass and flavonoids (rutin and quercitrin) content in callus of *Cupressus sempervirens* (using HPLC/DAD), subjected to different concentrations of GA_3 as an elicitor and phenylalanine as a precursor, on MS medium supplemented with 4.0 mg l^{-1} NAA+0.5 mg l^{-1} BA, after six weeks from culturing.

Sample	GA_3 (mg l^{-1})	Phenyl-alanine (mg l^{-1})	Fresh weight of callus (g)	Dry weight of callus (g)	Conc. of rutin (mg/g dry weight)	Conc. of quercitrin (mg/g dry weight)	Extract dry wt (mg)
C	0.0	0.0	5.723^{c}	0.298^{de}	53.429	1.425	160
CG1	1.0	0.0	12.978^{a}	0.475^{ab}	27.767	1.957	270
CG2	2.0	0.0	14.329^{a}	0.481^{ab}	16.795	2.178	230
CG3	4.0	0.0	9.862^{b}	0.406^{bc}	109.887	33.441	260
CG4	8.0	0.0	4.169^{c}	0.243^{e}	8.976	0.709	50
CF1	0.0	5.0	8.151^{b}	0.359^{cd}	4.644	0.665	70
CF2	0.0	10.0	3.410^{c}	0.267^{e}	5.019	1.164	80
CF3	0.0	20.0	3.304^{c}	0.246^{e}	6.889	1.723	120
CF4	0.0	40.0	12.891^{a}	0.509^{a}	5.461	1.119	150

1 g dried CNC leaves, gave 110 mg extract, with the concentration of rutin and quercitrin as 0.669 and 1.073 mg g^{-1}, while 1 g CFU gave 100 mg extract with 2.356 and 1.023 mg g^{-1}, respectively.

Table 4: GC/MS analysis results of the source plants and callus of *Cupressus sempervirens* subjected to different concentrations of GA_3 as an elicitor and phenylalanine as a precursor, on MS medium supplemented with 4.0 mg l^{-1} NAA + 0.5 mg l^{-1} BA, after six weeks from culturing.

Methanol extract of samples	Metabolites (%)					
	Ferruginol		Penta-decanoic acid	Totarol	Quinic acid	Hinokiol
CNC	1.00	Nd	2.02	0.63	Nd	Nd
CFU	1.87	1.61	1.30	Nd	0.88	Nd
C	2.80	16.20	5.55	Nd	Nd	2.73
CG1	6.51	5.94	8.64	0.44	0.93	1.61
CG2	7.29	4.82	13.11	0.14	Nd	1.38
CG3	5.33	3.87	Nd	0.72	1.16	1.76
CG4	5.57	Nd	2.80	0.33	2.02	0.75
CF1	10.82	2.38	15.02	0.40	0.93	0.77
CF2	42.68	38.92	Nd	Nd	Nd	Nd
CF3	4.52	5.94	1.68	Nd	Nd	Nd
CF4	4.97	Nd	12.07	Nd	2.02	0.90

Nd: not detected

3.37 % in CNC and CF3 only. The presence or absence of some compounds in the plant tissue may be due to some effect on their metabolic pathway, which needs further investigation.

Several studies also reported that cells/tissues/organs grown under *in vitro* conditions showed higher production of biologically active secondary metabolites than the field-grown plants (Collin, 2001; Shekhawat and Shekhawat, 2011; Lodha et al., 2014).

4. Conclusion

The present study ascertains the potential of *C. sempervirens* callus to synthesize rutin and quercitrin, along with many other metabolites. These observations clearly point out that it is possible to control secondary metabolites production from callus cultures of *C. sempervirens*, with the prospect that specific compounds may be obtained in relatively much higher concentrations than source plants, according to conditions employed. These findings were detected and compared by efficient simple chromatographic techniques as HPLC/DAD and GC/MS for accuracy. Other metabolites were absent or produced as other derivatives possibly due to modification of biosynthetic pathways.

Results encourage additional studies for the future development of new protocols to obtain *in vitro* cell suspension cultures, with higher concentrations of these bioactive compounds for large scale production being medicinally useful.

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