Phenolic Content and Related Antioxidant Activities in *in vitro* Cultures of *Cephalotaxus Harringtonia* L. Grown in Egypt

Sawsan S. Sayd[1] and Zeinab T. Abd El Shakour[2]

Ornamental Department Horticulture Research Institute[1]
Laboratory of phytochemistry, National Organization for Drug Control and Research, Cairo- Egypt[2].
zizishakour@yahoo.com

Abstract: Quantitative estimation of the total phenolics, total flavonoids, antioxidant activity and HPLC analysis of phenolic compounds in the mother plant, *in vitro* shooting and callus cultures of *Cephalotaxus harringtonia* L. grown in Egypt were measured. For shooting behaviour, using Woody plant medium (WPM) with 2 mg/l Benzyladinin (BA) in the second subculture gave the highest number of shootlet/explant (2.33 shootlet/explant) and shootlet length (2.67cm). It gave the highest content of total phenolics (67.916 mg/g d.w.) and the highest content of flavonoids (24.410 mg/g d.w.). For callus formation adding 6 mg/l naphthalene acetic acid (NAA) with 0.2 mg/l BA to WPM medium in the second subculture produced the highest weight of callus while MS medium supplemented with 2 mg/l NAA + 0.2 mg/l BA gave the highest content of total phenolics (34.060 mg/. g d.w.) and the highest content of flavonoids (4.368 mg/g d.w.). Antioxidant activity of all extracts was assayed using the 2,2'diphenylpicrylhydrazyl (DPPH) radical method, the results showed that *in vitro* shooting culture using WPM with 2 mg/l BA in the second subculture gave the lowest IC_{50}= 0.220 while callus cultured on WPM medium with 2 mg/l NAA + 0.2 mg/l BA showed the lowest IC_{50}= 0.432. Gallic, caffeic, chlorogenic acids, rutin, apigenin-7-glucoside, quercetin and apigenin were identified and measured in mother plant and shootlets extracts by HPLC analysis but only Gallic, caffeic, chlorogenic acids, rutin and quercetin were identified in callus extracts.

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1. Introduction

Family Cephalotaxaceae has two genera and about 11 species, most of them come from East Asia, from the Himalayas, through China to Japan and south to the Malay, Peninsula. The plants in this family have foliage that resembles those of the yews (*Taxus*) and they are sometimes lumped into the *Taxaceae* family. *Cephalotaxus* is a genus of conifers evergreen trees and shrubs, these plants are known as cowtail pines or plum yews; they are cultivated for timber, culinary purposes, and ornamental uses (Wang *et al*., 2004, Bocar *et al*., 2003 and Du *et al*., 1999). *Cephalotaxus harringtonia* L., commonly known as Japanese Plum Yew, is a coniferous bush or small tree in the plum yew family. It is native to Japan, but is occasionally utilised in western gardens. Japanese plum yew has been in cultivation in Europe since 1829. Traditionally, *C. harringtonia* L. is used against dyspepsia, ascariasis, inflammation and cough (Huang and Xue, 1984). Investigations of the chemical constituents of extracts of Cephalotaxus sps. resulted in the isolation of a number of anti-tumor alkaloids such as cephalotaxine, harringtonine, and homodeoxyharringtonine and their derivatives (Takano *et al*., 1996 and Morita *et al*.,2000).To the best of our knowledge, there are no reports on the phenolic content of *C. harringtonia* L. in the literature. The current study aimed at investigation of bioactive constituents in the mother plant, *in vitro* shooting and callus cultures of *C. harringtonia* L. grown in Egypt and evaluation of their antioxidant activities. *In vitro* culture study of the explants was carried out on WPM media using different kinds and concentrations of cytokinins (BA) for shootlets multiplication and auxins (NAA) for callus production, with a strategy employed to increase the bioactive constituents as compared with those of the mother plant.

2. Material and Methods

Plant material:

The aerial parts (leaves and twigs) of *C. harringtonia* (Cephalotaxaceae) were collected in EL-Orman Botanical Garden, Giza, Egypt (July, 2013). The collected plant materials were botanically authenticated in the Herbarium of the Botany Dept., Faculty of Science, Cairo University, also a voucher specimen (No. ch 11/211) of the plant materials were kept in Laboratory of phytochemistry, National Organization for Drug Control and Research. Tissue culture was carried out in Tissue Culture and Germplasm Conservation Research Laboratory, Horticulture Research Institute, Agriculture Research

Center. All the experiments have been done in NODCAR laboratories.

Standards and Reagents:

Woody plant medium (WPM) (Dutchiva Co.) cytokinin (BA) and auxin (NAA) (Sigma Co. USA). Gallic, caffeic, chlorogenic acids, rutin, apigenin-7-glucoside, quercetin and apigenin were used as a reference compounds from Sigma Co. USA.

Disinfection of explants:

The explant of *C. harringtonia* L. was surface sterilized with 70% ethanol solution for 1 min. After that immersed in 10% in Clorox with a few drops of tween-20 for 10 min, rinsed in sterile distilled water 3 times and immersed in 0.1 mercuric chloride solution with a few drops of tween-20 for 10 min then rinsed in sterile distilled water 3 times.

Culture media and conditions:

Using WPM (Lloyed and McCown, 1980) enriched with 25 g/l sucrose and 7g/l agar. For shooting stage, the medium supplemented with 0, 1, 2, or 3 mg/l BA (Benzyladinin) and 2 g/l AC (activated charcoal) for all treatments. For callus stage, adding to WPM 2, 4 or 6 mg/l NAA (Naphthalene acetic acid) without BA and with 0.2 mg/l BA. The medium was adjusted to PH 5.7±0.1, then poured at 25 ml in 200 ml capacity glass jars before autoclaving at 121° and 1.2 kg/cm^2 for 15 min. The cultures were incubated in growth chamber at 24±1° under 16 hr photoperiod (day light fluorescent tube) at 3 k lux. For shooting, the explant was cultured incubated for one month (twenty explants were cultured in five replicate). The shootlet were subcultured two times, after that shootlet number/explant and shootlet length (cm) was calculated. For callus fresh weight, callus incubated for one month (1g callus/jar for five replicate) and subculture two times.

Extraction of plant materials

The aerial parts, *in vitro* shooting (4 samples) and callus (6 samples) cultures of *C. harringtonia* L. were air dried at room temperature and ground in a mortar. The dried powder from each sample (10g) was extracted with 80% ethanol at 80°C for 3 h. The above procedure was repeated three times. The combined ethanol extracts for each powder were dried in a rotary evaporator at reduced pressure at 40ºC and subsequently stored at 4ºC. The residues were used for of phenolic, flavonoid contents, antioxidant activities and HPLC analysis.

Determination of total phenolics content:

The total phenolic content of the aerial parts, *in vitro* shooting (4 samples) and callus (6 samples) cutures of *C. harringtonia* L. extracts were determined by the Folin–Ciocalteu method (Meda *et al*., 2005). Briefly, aliquots of 0.1 g of each extract of plant samples were dissolved in 1 ml of deionized water. This solution (0.1 ml) was mixed with 2.8 ml of deionized water, 2 ml of 2% (w/v) sodium carbonate (Na_2CO_3), and 0.1 ml of 50% (v/v) Folin–Ciocalteau reagent. After incubation at room temperature for 30 min, the relative absorbance of the reaction mixture at 750 nm. was compared to deionized water with a spectrophotometer (Hitachi, Tokyo, Japan; Model 100 - 20). Gallic acid (GA) was chosen as a standard phenolic to construct a seven point standard curve (0–200 mg/l), the total phenolic contents in plant extracts were determined in triplicate. The data were expressed as milligram gallic acid equivalents (GAE)/g dry weight of extract. The data were then converted into GAE/g dry matter from plant samples.

Determination of total flavonoid content:

The total flavonoid contents of the aerial parts, *in vitro* shooting (4 samples) and callus (6 samples) cultures of *C. harringtonia* L. extracts were determined by the aluminum chloride colorimetric method described by (Chang *et al*., 2002). Briefly, aliquots of 0.1 g of plant extracts were dissolved in 1 ml deionized water. This solution (0.5 ml) was mixed with 1.5 ml of 95% (v/v) alcohol, 0.1 ml of 10% (w/v) aluminum chloride hexahydrate ($AlCl_3$), 0.1 ml of 1 M potassium acetate (CH_3COOK), and 2.8 ml of deionized water. After incubation at room temperature for 40 min, the absorbance was measured at 415 nm compared to deionized water with a spectrophotometer (Hitachi, Model 100 - 20). Quercetin was chosen as a standard to construct a seven point standard curve (0 -50 mg/l). The total flavonoid contents in plant 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

Antioxidant activity

The antioxidant activity of the aerial parts,*in vitro* shooting (4 samples) and callus (6 samples) cultures of *C. harringtonia* L. were evaluated by using the 2, 2'diphenylpicrylhydrazyl (DPPH) assay (Cuendet *et al*., 1997; Burits and Bucar, 2000). Fifty microliters of the extracts were added to 5 ml of a 0.004% (w/v) of DPPH in methanol (100% v/v). 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). 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

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concentration. Determinations were carried out in triplicate.

High performance liquid chromatography analysis:

Sample preparation

The ethanol extracts of the aerial parts, *in vitro* shooting and callus samples of *C. harringtonia* L. (10 µl, each), were injected in the HPLC system for quantitative determination of phenolic compounds using HPLC, optimization of parameters in HPLC was done through investigating. The influence of the mobile phase and wavelength detection, these two parameters play a key role on resolution and sensitivity.

HPLC conditions:

Samples of extracts were analyzed separately with Agilent 1200HPLC with column BDS hypersil C18, Length 250mm x ID 4.6 mm, Particle size: 5um, UV Detector. At 330 nm, 0.5 AUFS. Flow rate1.0 ml / minute. Injection volume 10µl. Mobile phase: Isocratic elution with (methanol: water: phosphoric acid) (100:100: 1).

Experimental design and data analysis:

The lay-out experiments was designed in completely randomized design and the test of LSD (Least Significant Differences) at P≤0.05 was used for comparison among means according to (Steel and Torrie 1980).

3. Results

Shooting behavior:

The data in Table (1) revealed a significant fluctuation in the behavior shooting explants (shootlet number/explant and shootlet length cm). Adding 2 mg/l BA to WPM promoting shootlet number (2.33 shootlet/explant) and shootlet length (2.42 cm) in compared to control which gave (1.33 shootlet/explant) and (1.48cm). For subculture, the second subculture increased shootlet number and shootlet length to (1.92 shootlet/explant and 2.06 cm) than first subculture (1.58shootlet/explant and 1.73 cm).

Table (1): Effect of different concentrations of BA and subculture on shooting behavior of *C. harringtonia* L.on *in vitro* culture.

Plant samples	Shootlet number			Shootlet length		
	Sub1	Sub2	Mean B	Sub1	Sub2	Mean B
Control	1.00	1.33	1.17	1.40	1.57	1.48
1.0 mg/l BA	1.33	1.67	1.50	1.67	2.17	1.92
2.0 mg/l BA	2.33	2.33	2.33	2.17	2.67	2.42
3.0 mg/l BA	1.67	2.33	2.00	1.67	1.83	1.75
Mean A	1.58	1.92		1.73	2.06	
LSD	A: 0.274 B: 0.387 A×B: 0.548			A: 0.448 B: 0.634 A×B: 0.896		

Table (2): Effect of different concentrations of hormones on chemical composition of shooting *C. harringtonia* L. on *in vitro* culture in compared to mother shrub.

Plant samples	Total Phenolics (mg/g d.w.)	Total Flavonoids (mg/g d.w.)	Antioxidant IC_{50} (mg/ml)
Mother shrub	43.578± 0.41	7.284± 0.32	0.241±0. 01
Control (free hormone)	51.852± 0.51	8.545± 0.46	0.341±0.03
1.0 mg/l BA	61.080± 0.65	12.037± 0.60	0.285±0.02
2.0 mg/l BA	67.916± 1.03	24.410± 0.35	0.220±0.02
3.0 mg/l BA	55.484± 0.55	10.748± 0.43	0.271±0.01

Each value is expressed as mean ± standard deviation (n =3).

Growth of callus

Data in Table (3) indicated that culture callus on WPM supplemented with NAA with BA promote cell division than using NAA alone. Adding 6 mg/l NAA with 0.2 mg/l BA gave the highest weight (4.45 g/jar) than adding 6 mg/l NAA alone which gave (2.72 g/jar). For subculture, the second subculture produced higher weight (3.94 g/jar) than the first subculture (2.05 g/jar).

Total phenolic and flavonoid contents

Analysis of total phenolic and flavonoid contents in ethanol extracts of different shootlets cultures of *C. harringtonia* L. relative to the concentration of the reference sample found that full WPM exhibited a

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good increase in total phenolic and flavonoid contents (51.852 and 8.545 mg/g d.w., respectively) which was significantly increased after the addition of 2 mg/l BA to WPM (67.916 and 24.410 mg/g d.w., respectively) as compared with results of the mother plant (43.578 and 7.284 mg/g d.w., respectively) (Table 2).

Callus cultured on WPM treated with 2.0 mg/l NAA + 0.2 mg/l BA significantly increased its content of total phenolic and flavonoid contents to (34.060 and 4.368 mg/g d.w., respectively) followed by 4.0 mg/l NAA + 0.2 mg/l BA (33.782 and4.251 mg/g d.w., respectively) (Table 4).

Table (3): Effect of different concentration of NAA without and with BA and number of subculture on weight of callus of C. *harringtonia* L. on *in vitro* culture.

Plant samples	Callus fresh weight (g)		
	Sub1	Sub2	Mean B
2.0 mg/l NAA	1.37	2.83	2.10
4.0 mg/l NAA	1.27	2.83	2.05
6.0 mg/l NAA	1.20	4.23	2.72
2.0 mg/l NAA + 0.2 mg/l BA	3.13	5.00	4.07
4.0 mg/l NAA + 0.2 mg/l BA	3.40	3.93	3.67
6.0 mg/l NAA + 0.2 mg/l BA	4.10	4.80	4.45
Mean A	2.05	3.94	
LSD	A: 0.449 B: 0.779 A×B: 1.101		

DPPH radical scavenging activity

The aerial parts, *in vitro* shooting and callus samples of *C. harringtonia* L. extracts each showed a concentration dependent scavenging activity by quenching DPPH radicals (Table 3 and 4). As judged by this assay the IC_{50} values of shootlets culture of C. *harringtonia* L. ethanol extracts are ranged from (0.220 to 0.341 mg/ml), shootlet culture on WPM with 2 mg/l BA had the highest antioxidant activity and the lowest IC_{50} (0.220 mg/ml) followed by the mother plant IC_{50} (0.240 mg/ml). Callus cultures ethanol extracts showed moderate antioxidant activities, Callus cultured on WPM treated with 2.0 mg/l NAA + 0.2 mg/l BA showed the lowest IC_{50} (0.432mg/ml) among Callus cultures.

This high antioxidant capacity of the mother plant and shootlets cultures may be due to the high concentration of phenolics compounds, which are common in medicinal plants, spices, vegetables, fruits, grains, pulses and other seeds. These compounds are an important group of natural antioxidants with possible beneficial effects on human health (Meyers *et al*., 2002). They can participate in protection against the harmful action of reactive oxygen species, mainly oxygen free radicals.

Table (4): Effect of different concentrations of hormones on chemical composition (mg/g dry weight) of callus *C. harringtonia* L. on *in vitro* culture in compared to mother shrub.

Plant samples	Total Phenolics (mg/g d.w.)	Total Flavonoids (mg/g d.w.)	Antioxidant IC_{50} (mg/ml)
Mother shrub	43.578± 0.41	7.284± 0.32	0.241±0. 01
2.0 mg/l NAA	25.831± 0.03	3.143± 0.55	0.604± 0.04
4.0 mg/l NAA	27.029± 0.06	3.098± 0.69	0.577± 0.03
6.0 mg/l NAA	29.860± 0.04	3.869± 0.26	0.523± 0.02
2.0 mg/l NAA + 0.2 mg/l BA	34.060± 0.01	4.368± 0.32	0.432± 0.05
4.0 mg/l NAA + 0.2 mg/l BA	33.782± 0.02	4.251± 0.38	0.455± 0.06
6.0 mg/l NAA + 0.2 mg/l BA	25.660± 0.04	3.533± 0.27	0.608± 0.05

Each value is expressed as mean ± standard deviation (n =3).

HPLC analysis:

The components, gallic, caffeic, chlorogenic acids, rutin, apigenin-7-glucoside, quercetin and apigenin were identified in the mother plant aerial parts and shootlets cultures by comparison with the retention time, and the UV spectra of authentic standards and quantitative data were calculated on basis of the peak area of respective compound. The content of gallic, caffeic, chlorogenic acids, rutin, apigenin-7-glucoside, quercetin and apigenin for the mother plant aerial parts were (2.38, 0.12, 0.15, 4.47, 5.56, 2.02 and 1.43mg/g d.w. respectively). Whereas,

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in *in vitro* shooting, shootlet culture on WPM with 2 mg/l BA had the highest contents, they were present respectively at (3.76, 0. 42, 0.17, 17.56, 6.40, 2.85 and 7.79 mg/g d.w. respectively). It indicates the presence of high amount of gallic, caffeic, chlorogenic acids, rutin, apigenin-7-glucoside, quercetin and apigenin in *in vitro* regenerated plantlets than that of the mother plant. It may be explained that the hardened plantlets even after proper acclimatization have some environment sensitivity, and to adopt they produced certain variety of secondary metabolites vigorously by defense mechanism to develop resistance (Santarem *et al.,* 2003). Only gallic, caffeic, chlorogenic acids, rutin, and quercetin were identified in callus cultures. WPM culture treated with 2.0 mg/l NAA + 0.2 mg/l BA showed the highest content of these components (0.62, 0.96, 0.54, 3.89 and 2.05mg/g d.w. respectively).

Table (5): Results of HPLC analysis for ethanol extracts of *C. harringtonia* L. mother plant and *in vitro* shooting cultures.

Plant samples	Gallic (mg/g d.w.)	Caffeic (mg/g d.w.)	Chlorogenic (mg/g d.w.)	Rutin (mg/g d.w.)	Apigenin-7-glucoside (mg/g d.w.)	Quercetin (mg/g d.w.)	Apigenin (mg/g d.w.)
Mother shrub	2.38± 0.023	0.12± 0.001	0.15± 0.003	4.47± 0.001	5.56± 0.003	2.02± 0.002	1.43± 0.062
Control (free hormone)	1.89± 0.012	0. 19± 0.003	0.10± 0.410	3.11± 0.010	3.65± 0.012	1.31± 0.023	6.30± 0.001
1.0 mg/l BA	3.52± 0.041	0.20± 0.000	0.02± 0.014	1.74± 0.013	1.38± 0.002	0.71± 0.032	2.82± 0.021
2.0 mg/l BA	3.76± 0.034	0. 42± 0.003	0.17± 0.100	17.56± 0.002	6.40± 0.021	2.85± 0.003	7.79± 0.133
3.0 mg/l BA	2.51± 0.004	0.18± 0.002	0.15± 0.023	3.11± 0.025	3.94± 0.013	1.20± 0.005	5.60± 0.124

Each value is expressed as mean ± standard deviation (n =3).

Table (6): Results of HPLC analysis for ethanol extracts of *C. harringtonia* L. mother plant and callus cultures.
Each value is

Plant samples	Gallic acid (mg/g d.w.)	Caffeic acid (mg/g d.w.)	Chlorogenic (mg/g d.w.)	Rutin (mg/g d.w.)	Apigenin-7-glucoside (mg/g d.w.)	Quercetin (mg/g d.w.)	Apigenin (mg/g d.w.)
Mother shrub	2.38± 0.023	0.12± 0.001	0.15± 0.003	4.47± 0.001	5.56± 0.003	2.02± 0.002	1.43± 0.062
2.0 mg/l NAA	0.26±0.003	0.78±0.023	0.12±0.034	1.52±0.031	-	0.62 ± 0.000	-
4.0 mg/l NAA	0.32±0.000	0.85±0.011	0.20±0.021	2.24±0.062	-	0.79± 0.000	-
6.0 mg/l NAA	0.51±0.001	0.82±0.021	0.41±0.081	2.65±0.032	-	1.15± 0.003	-
2.0 mg/l NAA + 0.2 mg/l BA	0.62± 0.002	0.96±0.004	0.54± 0.032	3.89 ±0.054	-	2.05± 0.000	-
4.0 mg/l NAA + 0.2 mg/l BA	0.59± 0.003	0.89±0.013	0.49± 0.023	1.83±0.081	-	1.82± 0.021	-
6.0 mg/l NAA + 0.2 mg/l BA	0.25± 0.004	0.74± 0.014	0.12± 0.045	2.74± 0.043	-	0.59± 0.133	-

expressed as mean ± standard deviation (n =3).

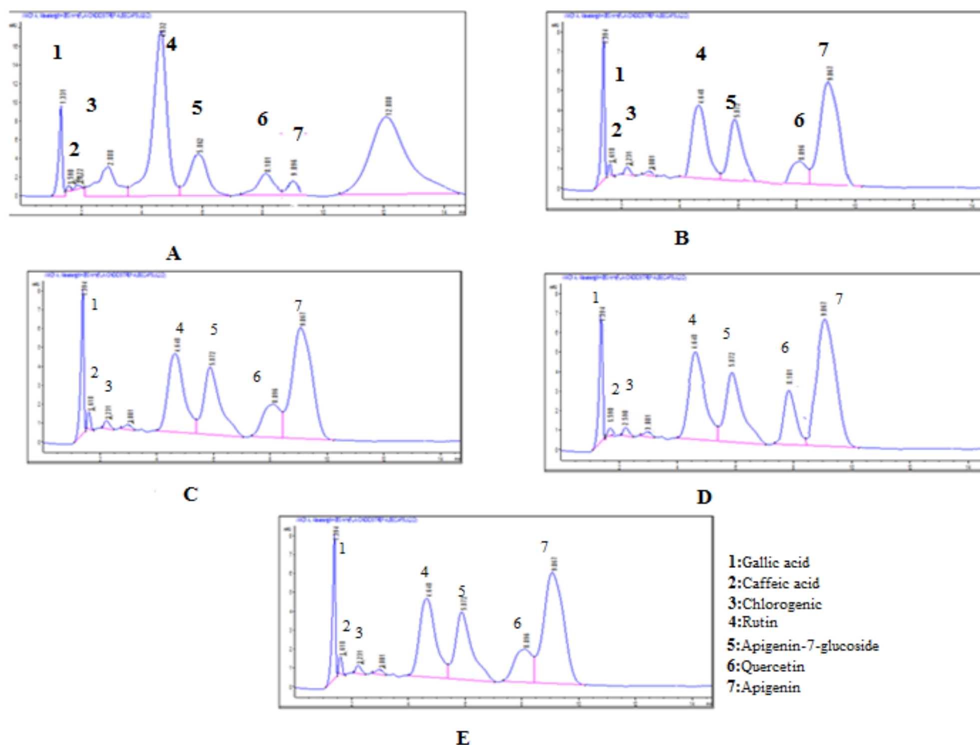

Fig 1: HPLC chromatograms of *C. harringtonia* L; (A): Extract from arial parts, (B): Control extract from *in vitro* shooting cultures, (C, D and E): Extracts from *in vitro* shooting cultures with (1, 2 and 3 mg/l BA).

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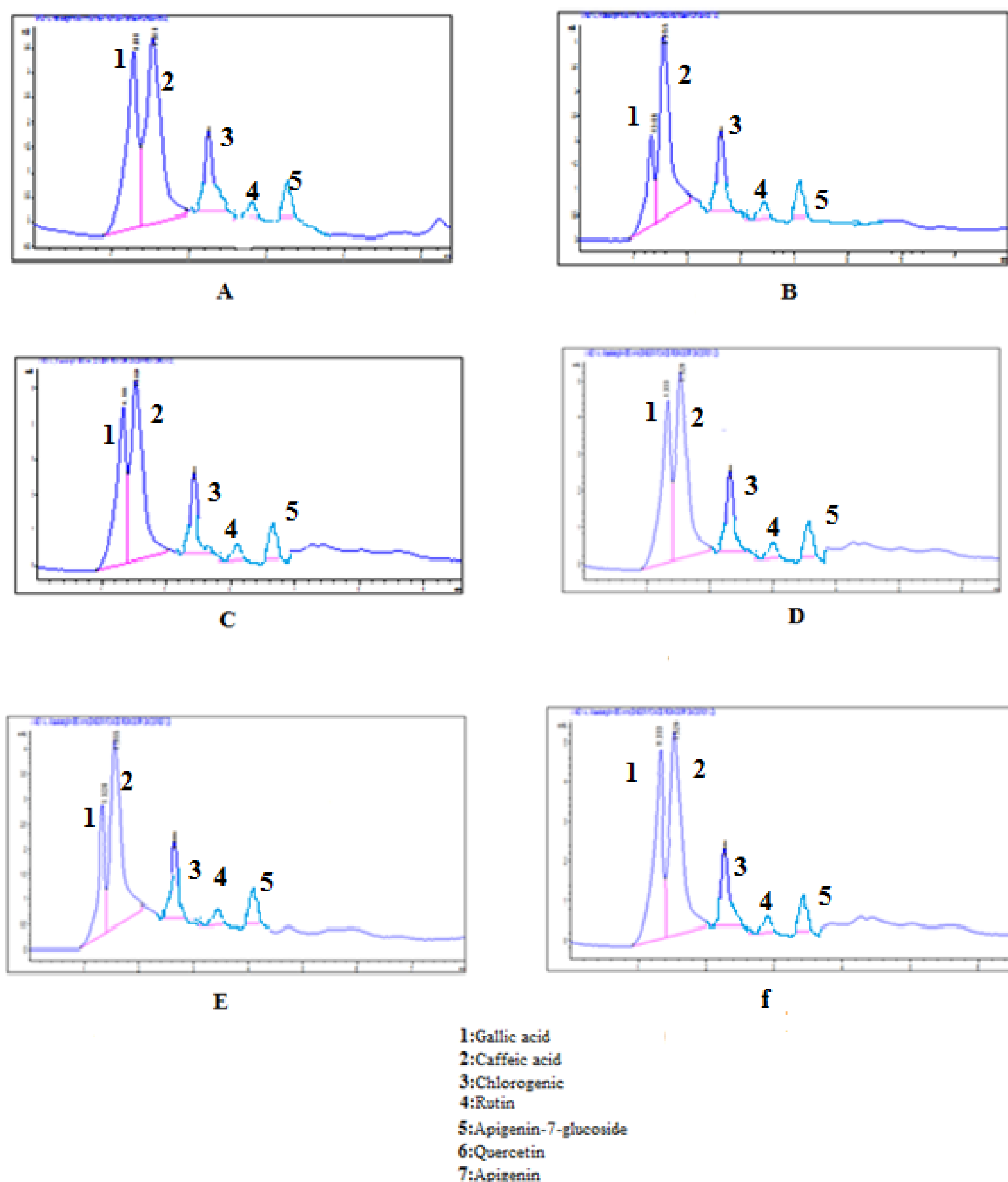

Fig 2: HPLC chromatograms of callus of *C. harringtonia* L. on *in vitro* culture (A to F) with 2.0 mg/l NAA, 4.0 mg/l NAA, 6.0 mg/l NAA, 2.0 mg/l NAA + 2.0 mg/l BA, 4.0 mg/l NAA + 2.0 mg/l BA and 6.0 mg/l NAA + 2.0 mg/l BA, respectively.

4. Discussion

In this investigation, different concentrations of BA affected on shooting behavior of *C. harringtonia* L. adding 2.0 mg/l BA to WPM medium increased shootlet number and length. This data was agreement with Sawsan *et al* (2010) on *Gardenia jasminoides* which found that adding 2mg/l BA promoting shooting number to maximum value. The results could be attributed to the important physiological effect of cytokinins as they stimulate cell division as well as elongation to active RNA synthesis and to stimulate protein synthesis and enzyme activity as were reviewed by Kulaeva (1980). For callus, adding different concentrations of NAA alone and with BA affected on fresh weight. Maximum fresh weight was recorded when adding 6.0 mg/l NAA with 0.2 mg/l BA. The promotive effect of auxins on callus induction and growth might be attributed to auxin as it promotes the biosynthesis of ethylene by increasing the activity of 1-aminocyclopropane-1-carboxylic acid (ACC) syntheses Kende (1989).

This study elucidated for the first time the total phenolic and flavonoid contents in ethanol extracts of the mother plant aerial parts, shootlets and callus

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cultures of *C. harringtonia* L. and their antioxidant activity. Furthermore, we analysed the phenolic components in plant samples by using HPLC method. *In vitro* regenerated plantlets have been reported to produce higher yields of active compounds (Fowke *et al*., 1994, Han *et al*., 1999, Mahagamasekera and Doran 1998). Results of our study concluded that shootlet culture on WPM media supplemented with 2 mg/l BA produced the highest contents of total phenolics, total flavonoids and antioxidant activities. The HPLC analysis of the ethanol extracts of the mother plant aerial parts, and shootlets cultures callus cultures of *C. harringtonia* L. revealed the identification of seven phenolic compounds (gallic, caffeic, chlorogenic acids, rutin, apigenin-7-glucoside, quercetin and apigenin) in the mother plant aerial parts and shootlets cultures but only five phenolic compounds were identified in callus cultures. The data generated in the present study are expected to support micropropagation of *C. harringtonia* L. However, there is a need to carry out more advanced phytochemical studies by using different protocols of micropropagation.

Conclusion

Cephalotaxus harringtonia L. growing in Egypt contains phenolic compounds in the aerial parts, shootlets and callus cultures. The alcoholic extracts of plant samples showed significant antioxidant so *C. harringtonia* L. could be considered as a potential source of a natural antioxidant. All results on C. *harringtonia* L. are presented here for the first time; as a trial to introduce a valuable medicinal plant. Therefore, this report is a step towards exploration of other phenolic compounds in different extracts of *C. harringtonia* L. which can further be isolated and studied for their important pharmacological properties.

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