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Biotechnological and chemical analysis of Egyptian *Diospyros kaki* L. cv. Costata grown in Egypt

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Abstract

Background: *Diospyros kaki* L. is considered one of the most important economical medicinal plant in Chinese herbal medicine belonging to family Ebenaceae as it contains varied secondary metabolites and used in treatment of many diseases. For there is an efficient and promising protocol for in vitro propagation of *Diospyros kaki* L. cv. Costata was established *Diospyros kaki* L. cv. Costata although it is difficult to initiate it in sterilized artificial media due to the browning of tissue caused by oxidation of phenolics. An efficient sterilizing method for leaf and internode explants was obtained by using 0.2% mercuric chloride (Hg_2Cl_2) for 5 min. Woody plant medium (WPM) supplemented with 2 mg/l zeatin (Zt) + 5 mg/l isopentenyl adenine (2 iP). Calli were induced on Murashige and Skoog medium (MS) augmented with 10 mg/l Zt + 10 mg/l indole-3-acetic acid (IAA) + 500 mg/l polyvinylpyrolidone (PVP) + 0.1 mg/l thiamine HCL from internode explants. However, the regeneration efficiency was obtained with ½ MS-media fortified with 1 mg/l Zt + 2 mg/l IAA + 4 mg/l benzylaminopurine (BAp) + 0.5 g/l PVP from internode calli explants.

Results: The highest amounts of scopoletin 57.08, 26.42, and 25.30 (μg/g DW) were detected using reversed phase of high-performance liquid chromatography (RP-HPLC) in leaves extract of intact plant followed by regenerated and calli cultures of internod explants, respectively.

Conclusion: This study is the first record for in vitro propagation and production of secondary metabolites from *Diospyros kaki* L. using biotechnology techniques. Chemical anaysis were carried out using HPLC technique.

Keywords: *Diospyros kaki* L. cv. Costata, Explants, Mercuric chloride, Zeatin, HPLC

Background

Plants that possess therapeutic properties or exert beneficial pharmacological effects on the human body are generally designated as medicinal plants. They naturally synthesize and accumulate some secondary metabolites, like alkaloids, sterols, terpenes, flavonoids, saponins, cyanogenic glycosides, tannins, resins, lactones, quinines, and volatile oils (Ramachandra & Ravishankar, 2002). Medicinal plants have been used for the treatment of diseases, since the dawn of the time (Motaleb et al., 2011). Family Ebenaceae include four genera *Diospyros*, *Euclea*, *Lissocarpa*, and *Royena*. It consists of woody shrubs and trees distributed in the tropical, sub-tropical, and temperate areas and are known worldwide for its biological activities. The largest, important, and economically genus of Ebenaceae is *Diospyros* with approximately 300 species which occurs in Asia and Pacific area (De Vera & Santiago, 2014; Matsushita et al., 2010). *Diospyros kaki* L. cv. Costata is well known in Chinese herbal medicine and used for prevention and treatment of hypertension, cancer, diabetes, and atherosclerosis. Moreover, it contains tannins, phenols, and flavonoids which are the most active constituents of this plant (Tang & Eisenbrand, 1992).

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Diospyros kaki L. cv. Costata is the main persimmon variety progressively consumed in the Egyptian market and exportation (Fathi et al., 2011). Further, the in vitro culture and propagation of this plant is more important; however, it is very difficult to be initiated in sterilized artificial media due to the browning of tissue, which is caused by oxidation of polyphenols (Monaco et al., 1977) , and it may be the first record on in vitro calli production and regeneration induction from *Diospyros kaki* L. cv. Costata growing in Egypt.

This study aimed to obtain calli and regeneration from leaf and internode explants of *Diospyros kaki* L. cv. Costata. Further, carrying out of chemical analysis using RP-HPLC technique, to figure out the secondary metabolites accumulated in calli and regenerated shootlets compared to leaves of *Diospyros kaki* L. cv. Costata intact plant.

Material and methods

Plant material

Diospyros kaki L. cv. Costata explants (leaves and internods) were obtained from Aga Center, Fisha Village, Dakahlia Governorate, Egypt. It was kindly authenticated by Dr. Abd El-Halim Abd El-Mogali Mohamed, senior researcher in Flora and Phytotaxonomy Researches Department, Agricultural Museum, Dokki, Giza, Egypt.

Chemical materials

Benzyl aminopurine (BAp) (Santa Cruz Biotechnology, USA), indole-3-acetic acid (IAA) (Science Lab. USA), Mc Cown's woody plant medium with vitamins (WPM) (Melford Laboratories Ltd.), Mercuric chloride (Hg_2Cl_2) (Mallinckrodt. Inc. Paris), Murashige, and Skoog with vitamins (MS) (Caisson Laboratories, USA), Polyvinyl pyrrolidone (PVP) (Alfa Aesar, USA), 6-(γ, γ-dimethyl allylamino purine (2iP), naphthaleneacetic acid (NAA), kinetin (KN), (Santa Cruz Biotechnology, USA), and zeatin (ZT) (Science Lab., USA).

Sterilization of explants

Internodes (stem cuttings) 1–1.5 cm length and leaf explants 3–5 mm were excised from a shrub tree of *Diospyros kaki* L. cv. Costata. Further, these explants were washed with tap water for 1 hr. using detergent solution. Then, they were surface sterilized by immersion in 70% ethanol for 5 s followed by four washes using sterile distilled water. Subsequently, they were immersed in solution of Hg_2Cl_2 at two concentrations (0.1 or 0.2%) for 5 min to study their effects on sterilization process. Explants were cultured under aseptic condition on half MS nutrient medium.

Development media

The disinfected internodes and leaves explants were cultured on different solidified and modified nutrient media. These types of nutrient media were designed to study its effects on development characters (calli induction or primordial shootlets regeneration). All used media were augmented with 30 (w/v) sucrose and agar 0.7 %. The pH of all used media was adjusted to 5.8 with 0.1 N each of KOH (potassium hydroxide) or HCl (hydrocholric acid). The different type of nutrient media were distributed into 300 ml glass jars where each jar contained 50 ml and sterilized by autoclaving for 23 min at 121 °C. The cultures media were incubated in complete darkness for one week. Then, they were exposed to illumination light (2000 lux) with fluorescent lamp and kept at 26 ± 1 °C 16 h photoperiod for 4 weeks. The composition of modified nutrient media was structured as follows:

1	½ MS-free growth regulators
2	WPM + 2 mg/l Zt + 5 mg/l 2iP
3	MS + 2 mg/l BAp + 0.2 mg/l NAA
4	MS + 5 mg/l BAp + 1.1 mg/l KN

Each treatment consisted of 10 replicates (jars) and each replicate contained one explant (leaf or internode)

Calli induction

Sterilized internode explants were excised from previously in vitro grown shootlets on WPM supplemented with 2 mg/l ZT + 5 mg/l 2iP. Further, cut and cultured on MS-medium (30 w/v sucrose and 7 g agar) or WPM supplemented with different combinations of growth regulators as follows:

1	MS-free growth regulators
2	WPM + 2 mg/l Zt + 5 mg/l 2 iP
3	MS + 10 mg/l Zt + 10 mg/l IAA + 500 mg/l PVP + 0.1 mg/l thiamine HCL
4	½ MS + 1 mg/l BAp + 0.1 mg/l NAA + 1 mg/l IAA + 0.1 mg/l thiamine HCL
5	½ MS + 0.5 mg/l BAp + 5 mg/l 2,4-D + 1 mg/l IAA + 0.1 mg/l thiamine HCl

Regeneration induction

In this experiment, obtained calli from MS supplemented with 10 mg/l Zt + 10 mg/l IAA + 500 mg/l PVP + 0.1 mg/l thiamine HCL were subjected to the following nutrient media to induce shootlets regeneration as follows:

1	½ MS + 0.22 mg/l thidiazuron (TDZ) + 1 mg/l IAA + 500 mg/l PVP + 40 mg/l adenine sulfate + 0.1 mg/l thiamine HCL
2	½ MS + 1 mg/l Zt + 2 mg/l IAA + 4 mg/l BAp + 0.5 g/l PVP

Where all cultures were incubated under darkness for 3 days then, incubated under light condition 16/18 h (2000 lux) for 4 weeks at 26 ± 1 °C

Chemical analysis

The present study was performed for the qualitative and quantitative determination of flavonoid and other phenolic contents in leaves-derived extracts of intact

Table 1 Effect of different type of nutrient media on development[a] percentage of leaf and internode explants of *Diospyros kaki* L. cv. Costata

No.	Type of nutrient media[b]	Explants development percentage			
		Leaf		Internode	
		Light	Dark	Light	Dark
1	½ MS-free growth regulators	–	–	–	–
2	WPM + 2 mg/l Zt + 5 mg/l 2iP	–	–	**50 ± 5.65**	**50 ± 4.25**
3	MS + 2 mg/l BAp + 0.2 mg/l NAA	–	–	**20 ± 3.15**	**20 ± 2.25**
4	MS + 5 mg/l BAp + 1.1 mg/l KN	–	–	–	–

[a]Explants were developed to either calli or shootlets regeneration
[b]Each treatment was the mean of 10 replicates ± SE

plant; calli and regenerated shootlets of *Diospyros kaki* L. cv. Costata. Samples were prepared as 1 g of dried powdered material, further, extracted with aqueous methanol (80%); filtered and evaporated. Before quantization by HPLC, the samples were filtered through a 0.4 μm membrane filter into vial for injection.

Specification of RP-HPLC instrument

Hewlett-Packard (series 1050) equipped with auto-sampling injector, solvent degasser, ultraviolet (UV) detector set at 280 nm and 330 nm for phenolics determination, quaternary HP pump (series 1050), and a lichrosorb RP C_{18} column (4.0 mm i.d. × 250 mm; particle size 5 μm) (Merck, Darmstadt) was used. The column temperature was maintained at room temperature. Isocratic separation was carried out using methanol and acetonitrile (2:1) as a mobile phase at flow rate of 1 ml/min. Authentic phenolics were dissolved in mobile phase and injected into RP-HPLC. The retention time and peak area were used to calculate phenolics concentrations by the data analysis of Hewlett-Packard software (Goupy et al., 1999; Mattila et al., 2000).

Statistical analysis

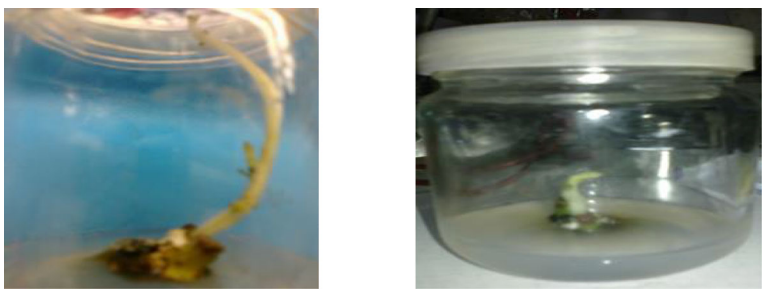
The design of all experiments was completely randomized and the obtained data were statistically analyzed using standard error (±SE) according to the method described by Snedecor and Cochran (Snedecor & Cohchran, 1967).

Results

Sterilization experiment

Regarding this experiment, leaf explants were treated with 0.1% of Hg_2Cl_2 for 5 min resulted in 37% contamination and 63% survival explants. However, treated leaf explants with 0.2% of Hg_2Cl_2 for 5 min resulted in 45% contamination and 55% survival explants, respectively. On the other hand, treated internode explants with 0.1% of Hg_2Cl_2 for 5 min recorded 65% and 35% of contaminated and survival explants, respectively. However, treated internode explants with 0.2% of Hg_2Cl_2 for 5 min recorded the lowest percentage of contamination (20%) in correlation with the highest percentage of survival explants (80%).

Therefore, it can be concluded that, treated internode explants of *Diospyros kaki* L. cv. Costata with 0.2% of



A -After 4 weeks (Dark) **B -After 4 weeks (Light)**

Fig. 1 a, **b** Effect of supplementation of WPM with 2 mg/l Zt and 5 mg/l 2iP on development stage (calli initiation or shootlet regeneration) from internode explant of *Diospyros kaki* L. cv. Costata

Table 2 Effect of different type of nutrient media on percentage of calli induction calli fresh, and dry weights (g/jar) induced from internode explants of *Diospyros kaki* L. cv. Costata[a]

No.	Media[b]	% of callus induction	Calli fresh weight (g/jar)	Calli dry weight (g/jar)
1	MS-free growth regulators	———	———	———
2	WPM + 2 mg/l Zt + 5 mg/l 2iP	30 ± 4.15	0.68 ± 0.32	0.11 ± 0.06
3	MS + 10 mg/l Zt + 10 mg/l IAA + 500 mg/l PVP + 0.1 mg/l thiamine HCL	60 ± 5.25	6.69 ± 0.79	0.38 ± 0.11
4	½ MS + 1 mg/l BAp + 0.1 mg/l NAA + 1 mg/l IAA + 0.1 mg/l thiamine HCL	10 ± 2.33	———	———
5	½ MS + 0.5 mg/l BAp + 5 mg/l 2,4-D + 1 mg/l IAA + 0.1 mg/l thiamine HCl	40 ± 4.25	3.40 ± 0.75	0.13 ± 0.05

[a]Cultures were incubated under dark condition for four weeks at 26 ± 1 °C
[b]Each treatment was the mean of 10 replicates ± SE

Hg_2Cl_2 for 5 min was the best to obtain lowest percentage of contamination in correlation of highest percentage of sterilized survival explants.

Development media

Data in Table 1 and Fig. 1a, b clearly show that the effect of different type of nutrient media on leaf and internode explants development percentage. The highest percentage of explants development (50%) was recorded with internode explants cultured on WPM supplemented with 2 mg/l Zt + 5 mg/l 2iP and incubated under either dark or light conditions. Whereas, leaf explants were completely failed on development using different nutrient media. This result may be attributed to the high accumulation and release of phenolic compounds from leaf explants to the culture media upon blacking which make toxicity. Further, the internode explants will be subjected to calli and regeneration experiments.

Callus induction experiment

Data tabulated in Table 2 show that the highest percentage of calli induction (60%) was recorded with supplementation of full MS medium with 10 mg/l Zt + 10 mg/l IAA + 500 mg/l PVP + 0.1 mg/l thiamine HCL. While the lowest percentage of calli induction (10%) was recorded with augmentation of ½ MS medium with 1 mg/l BAp + 0.1 mg/l NAA + 1 mg/l IAA + 0.1 mg/l thiamine HCL. However, culturing of internode explants on MS medium-free growth regulators failed completely on callus induction (Fig. 2).

On the other hand, and regarding calli fresh and dry weights, the maximum calli fresh and dry weights (6.69, 0.38 g/jar) were recorded with culturing of internode explants on MS medium supplemented with 10 mg/l Zt + 10 mg/l IAA + 500 mg/l PVP + 0.1 mg/l thiamine HCL, respectively. While the minimum calli fresh and dry weights (0.68, 0.11 g/jar) were recorded with supplementation of WPM with 2 mg/l Zt + 5 mg/l 2iP, respectively. However, no positive results were observed with culturing of internode explants on MS medium-free growth regulators.

Regeneration induction experiment

Data tabulated in Table 3 and Fig. 3 clearly show that the highest percentage of regenerated shootlets (50%) was recorded with supplementation of half MS medium with 1 mg/l Zt + 2 mg/l IAA + 4 mg/l BAp + 0.5 g/l PVP. While the lowest percentage of regenerated shootlets (10%) was recorded with fortified of half MS medium with 0.22 mg/l TDZ + 1 mg/l IAA + 500 mg/l PVP + 40 mg/l Adenine sulfate + 0.1 mg/l thiamine HCL.

Regarding the number of regenerated shootlets, numbers of leaves/shoot, and length of shoot (cm), the maximum number of shoots/explant (3.4), number of leaves/shoot (3.8), and longest shoot (5 cm) were recorded with fortified of ½ MS with 1 mg/l Zt + 2 mg/l IAA + 4 mg/l BA + 0.5 g/l PVP compared with other used medium.

Chemical analysis

The chemical analysis of phenolic compounds in leaves of intact plant; calli of internode explants and regenerated shootletes extracts of *Diospyros kaki* L. cv. Costata were carried out using RP-HPLC. Phenolic compounds appeared at λ_{max} 280 nm using HPLC analysis are shown in Table 4.

Fig. 2 Callus induction from internode explant cultured on MS-medium supplementation with 10 mg/l Zt + 10 mg/l IAA + 500 mg/l PVP + 0.1 mg/l thiamine HCL (cultures were incubated under dark condition for 4 weeks at 26 ± 1 °C)

Table 3 Effect of supplementation of half MS nutrient medium with two concentrations of growth regulators on percentage, number of regenerated shoots/explant, number of leaves/shoot, and length of shoot (cm) induced from internode calli explants of *Diospyros kaki* L. cv. Costata[a]

	1/2 MS medium supplemented with[b]	% of regenerated shootlets	Number of regenerated shoots/explant	Number of leaves/ shoot	Length of shoot (cm)
1	0.22 mg/l TDZ + 1 mg/l IAA + 500 mg/l PVP + 40 g/l adenine sulfate + 0.1 mg/l thiamine HCL	10 ± 2.15	1.3 ± 0.3	2.3 ± 0.3	1.2
2	1 mg/l Zt + 2 mg/l IAA + 4 mg/l BAp + 0.5 g/l PVP	50 ± 5.25	3.4 ± 0.5	3.8 ± 0.3	5

[a]Cultures were incubated under light condition for four weeks at 26 ± 1 °C
[b]Each treatment was the mean of 10 replicates ± SE

From the result above, scopoletin was found to be the highest compound recorded (μg/g DW) in leaves of intact plants, calli, and regenerated shootlets extracts 57.08, 25.30, and 26.42, respectively. However, pyrogallol (8.8 μg/g DW) was the lowest amount recorded in leaves extract while resveratrol and coumarin were recorded as the lowest amount in calli extracts (0.5 μg/g DW) and ellagic (1.03 μg/g DW) in regenerated shootlets.

As well as the chemical analysis of phenolic compounds in leaves of intact plant; calli of internode explants and regenerated shootletes extracts of *Diospyros kaki* L. cv. Costata were carried out using RP-HPLC. Phenolic compounds were appeared at λ_{max} 330 nm as shown in Table 5.

From the previous data, it can be concluded that kaempferol was the major compound in leaves extract (80.9 μg/g DW), while luteo.6-arbinose 8-glucose recorded the highest amount in calli extract and regenerated shootlets (65.1, 70.4 μg/g DW), respectively. Luteolin was detected only in leaves extract (62.59 μg/g DW). However, quercetin-3-O-glucoside (1.5 μg/g DW) was detected only in calli extract; this variation may be due to changes in metabolic pathways.

Fig. 3 Shootlets regenerated from internode calli explants of *Diospyros kaki* L. cv. Costata cultured on ½ MS-media supplemented with 1 mg/l Zt + 2 mg/l IAA + 4 mg/l BAp + 0.5 g/l PVP (cultured was incubated under light condition (2000 lux) at 26 ± 1 °C for 8 weeks)

Discussion

May be it is the first protocol for in vitro propagation of *Diospyros kaki* L. cv. Costata. Moreover, there is no reliable method was defined for culturing calli and shootlets regneration from *Diospyros kaki* L. cv. Costata grown in Egypt.

The production of secondary metabolites in plant calli or cell cultures has been reported from various medicinal plants. Thus, and in agreement with our previous results (Nigra et al., 1987), (Toppei et al., 1987), (Jha et al., 1988), Shirley 2000, Phillipson, 2005, Taha et al., 2009) reported that many phenolic compounds in plants are synthesized by the phenylpropanoid pathway which is typically initiated by phenyl alanine. Further, Monaco et al., 1977) reported that browning of tissue is caused by the oxidation of tannin and polyphenols and the formation of quinones which are highly reactive and toxic to the plant tissues.

Regarding sterilization experiment, the optimum concentration of $Hg_2Cl_2$was 0.2% for 5 min recorded the lowest percentage of contamination and the highest survival rate in internode explants of *Diospyros kaki* L. cv. Costata. The obtained results are in agreement with Wang et al., 2010 who indicated that 0.1% of Hg_2Cl_2 for 15 min was more sufficient to obtain the highest survival rate of the explants in the dormant period of Boaibayuehuang *Diospyros kaki.*

In addition, the most suitable media for explants development was WPM medium supplemented with 30 g/l sucrose in combination with growth regulators as 2 mg/l ZT + 5 mg/l 2iP in dark condition, then transferred to light which gives the highest percentage of shoots development from the used explants. The extracted results were in contrast with Giordani et al., 2013 who used half MS supplemented with 20 g/l sucrose + 2.2 mg/l ZT + 5 mg/l BA for in vitro establishment of shoot induction of *Diospyros kaki* cultivars (Hiratanenashi, jiro, kaki Tip, and Fuyu).

Concerning calli induction experiment, it was found that MS medium supplemented with 10 mg/l Zt + 10 mg/

Table 4 Results of phenolic compounds identified by RP-HPLC in different extracts (80% MeOH) of leaves, calli, and regenerated shoots of *Diospyros kaki* L. cv. Costata that appear at λ_{max} 280 nm

No.	Phenolic compounds	Phenolic (μg/g D.W)			
		Rt.*	Leaves	Calli	Regenerated shoots
1	Gallic	7.041	33.88	2.7	7.31
2	Pyrogallol	7.243	8.81	5.95	9.5
3	4-amin-benzoic	7.842	14.83	0.9	3.88
4	Protocatechuic	8.52	19.09	2.9	4.49
5	Catechin	8.63	21.46	1.9	4.33
6	Chlorogenic	9.071	47.99	6.2	9.37
7	Catechol	9.541	24.07	7.2	7.85
8	Epicatechin	9.661	15.84	4.1	7.1
9	Caffeine	9.743	22.68	1.1	1.7
10	P-hydroxybenzoic	9.988	49.48	15.5	16.55
11	Caffeic	10.297	0	1.4	3.4
12	Vanillic	10.386	19.67	1.7	1.99
13	P-coumaric	11.716	28.66	15.3	17.55
14	Ferulic	11.928	13.2	4.6	10.35
15	Iso-ferulic	12.322	0	4.2	6.42
16	Resveratrol	12.813	13.8	0.5	1.73
17	Ellagic	13.076	12.09	11.7	1.03
18	E-vanillic	13.187	20.29	0	11.55
19	α-coumaric	13.533	17.55	0.9	1.54
20	Benzoic	13.729	40.52	18.2	21.75
21	3,4,5-methoxy-cinnamic	14.14	28.56	1.4	1.71
22	Coumarin	14.307	54.1	0.5	3.73
23	Salicylic	14.628	22.92	29.6	23.1
24	Cinnamic	15.542	91	1.3	5.62
25	Scopoletin	16.192	57.08	25.30	26.42

Table 5 Results of phenolic compounds identified by RP-HPLC in different extracts (80 % MeOH) of leaves, calli, and regenerated shoots of *Diospyros kaki* L. cv. Costata that appeared at λ_{max} 330 nm

No.	Phenolic compounds	Phenolic (μg/g D.W)			
		Rt.[a]	Leaves	Calli	Regenerated shoots
1	Luteo.6-arbinose 8-glucose	9.352	7.83	65.1	70.4
2	Luteo.6-glucose 8-arbinose	10.551	39.28	4.6	8.57
3	Apig. 6-arbinose 8-glactose	11.563	31.86	5.4	10.09
4	Apig. 6-rhamnose 8-glucose	11.909	57.8	7.6	14.99
5	Apig. 6-glucose 8-rhamnose	12.071	19.33	12.9	14.83
6	Luteolin	12.193	62.59	0	0
7	Luteol. 7-glucose	12.322	9.42	2.3	0
8	Narengin	12.4	50.18	12.8	16.26
9	Rutin	12.467	41.98	4.6	7.94
10	Quercetin-3-O-glucoside	12.506	0	1.5	0
11	Hisperidin	12.636	33.26	5.8	0
12	Kam.3,7-dirhamoside	12.907	0	0	3.68
13	Rosmarinic	12.923	38.29	1.1	5.66
14	Apig.7-O-neohespiroside	13.063	6.86	2.1	7.51
1154	Apig.7-*O*-glucose	13.257	13.93	1.5	7.54
16	Quercetrin	13.409	23.84	0.9	1.89
17	Narengenin	14.954	0	0	5.04
18	Quercetin	14.979	10.21	1.1	4.86
19	Kamp.3,(2-p-coumaroyl) glucose	15.129	21.96	0	0
20	Hespertin	15.616	42.38	4.7	0
21	Kaempferol	16.38	80.9	0.7	5.48
22	Rhamnetin	16.551	15.9	0.7	2.23
23	Apigenin	16.715	11.09	1.6	2.01
24	Acacetin	18.874	12.30	3.48	3.81

[a]Rt.(retention time)

l IAA + 500 mg/l PVP + 0.1 mg/l thiamine HCL was the best for calli induction from internode explants. This obtained result is in contrast with Gondo et al., 1999 who induced calli from axillary buds of Persimmon (*Diospyros kaki* Thunb.) on half MS medium supplemented with 1 mg/l IAA + 0.1 mg/l BA .

However, the best recorded regenerated media was ½ MS + 1 mg/l Zt + 2 mg/l IAA + 4 mg/l BA + 500 mg/l PVP. This obtained results are in agreement with Wang et al., 2010 who used MS medium for shoots regeneration from shoot tip of Boaibayuehuang *Diospyros kaki*.

Regarding identification of extracted and isolated phenolic compounds from leaves of intact plant or calli and regenerated shoots of *Diospyros kaki* L. cv. Costata growing in Egypt; the RP-HPLC technique was performed. The extracted results indicated that methanolic extract showed rutin, gallic acid, caffeic acid, chlorogenic acid, kaempferol, and quercetin. The obtained results were in agreement with Quezon and Ysrael (Quezon & Ysrael, 2014) who indicated that rutin was isolated from ethanolic extract of *Disopyros pilosanthera* intact plant. Moreover and in close with our obtained results Cho et al., **(Cho et al.,** 2015**)** identified gallic acid, caffeic acid, myricitrin, chlorogenic acid, kaempferol, and quercetin in methanolic extract of *Diospyros Lotus.*

Conclusions

From the obtained results of biotechnological and HPLC analysis on Egyptian *Diospyros kaki* L. cv. Costata, it indicates a promising protocol for sterilization, development,

calli induction, and shootlet regeneration of Egyptian *Diospyros kaki* L. In addition to qualitative and quantitative estimation of secondary metabolites.

Abbreviations

Hg_2Cl_2: Mercuric chloride; WPM: Woody plant medium; Zt: Zeatin; 2iP: Isopentenyl adenine; MS: Murashige and Skoog medium; IAA: Indole-3-acetic acid; PVP: Polyvinylpyrrolidone; BAp: Benzylaminopurine; RP-HPLC: High-performance liquid chromatography; KOH: Potassium hydroxide; HCl: Hydrochloric acid

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Authors' contributions

Seham S. El Hawary: Supervised the experimental work and reviewed the manuscript. Soad Hanna Tadros: Supervised the experimental work and reviewed the manuscript. Hussein Taha: Designed the experimental work, participated in analysis and interpretation of the data, and reviewed the manuscript. Mona Abdelmohsen: Participated in analysis and interpretation of the data and reviewed the manuscript. Naglaa Mohamed Nazif: Supervised the experimental work and reviewed the manuscript. Iman El Sheikh: Carried out the experimental work of the manuscript, participated in analysis and interpretation of the data, and was a major contributor in writing the manuscript. Medhat Seif El- Nasr: Supervised the experimental work and reviewed the manuscript. The author(s) read and approved the final manuscript.

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Availability of data and materials

The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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