

CALLUS FORMATION, PHENOLICS CONTENT AND RELATED ANTIOXIDANT ACTIVITIES IN TISSUE CULTURE OF A MEDICINAL PLANT COLOCYNTH (*Citrullus colocynthis*)

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Abstract: Callus cultures from stems, leaves and roots of colocynth were initiated on MS media supplemented with various combinations of 2,4 dichlorophenoxyacetic acid (2,4-D) with kinetin (KIN) and benzyladenine (BA) with α -naphthaleneacetic acid (NAA). The highest percentage of callus formation frequency (98.9%) was obtained from stem explants grown on MS media supplemented with (1.0 mg/L) 2,4-D + (1.0 mg/L) KIN. The total phenolics and flavonoid content of the colocynth callus cultures were measured. The results showed that the MS medium supplemented with 6.0 mg/L 2,4-D + 2.0 mg/L KIN (MD3) gave the highest content of total phenolics (19.2 mg/100g d.w.) in leaf-derived calli. The highest content of flavonoids (47.3 mg/100g d.w.) was obtained in stem derived calli grown on the same medium (MD3). Antioxidant activities of extracts were determined using different assays, including DPPH radical scavenging activity, hydrogen peroxide (H₂O₂) scavenging activity and ferric reducing power. Leaf-derived calli cultured on MS medium + 2.0 mg/L 2,4-D + 1.0 mg/L KIN (MD1) showed the highest DPPH radical scavenging activity (85.3%). The highest percentage of H₂O₂ scavenging activity (61.4%) was detected in leaf explant-derived calli growing on MD1. The leaf-derived calli growing on (MD3) gave the highest ferric reducing power (22.3 μ g/g d.w.), compared to the activities of stems, leaves and roots of *in vitro* grown seedlings (3.28, 12.9 and 2.85 μ g/g d.w.), which were used as controls. On the basis of the current findings, we conclude that MS media supplemented with different combinations of 2,4-D and KIN yields higher phenolics, flavonoids contents and antioxidant activities than MS media supplemented with BA and NAA.

Key words: callus culture, phenolics, flavonoids, H₂O₂ scavenging activity, plant growth regulators

1. Introduction

Plant cell cultures are an attractive alternative source to whole plant for the production of high value secondary metabolites (ALFERMANN and PETERSEN, 1995; DORNENBURG and KNORR, 1997). However, a considerable progress has been made to stimulate production and accumulation of secondary metabolites using plant cell cultures (KALIDASS *et al.*, 2010; ABOUZID *et al.*, 2010).

Several strategies have been adopted for the enhancement of bioactive metabolite production in *in vitro* cultures; one of them is using growth regulators which are often a crucial factor in secondary product accumulation (DUANGPORN and SIRIPONG, 2009). The type and concentration of auxin or cytokinin or the auxin/cytokinin ratio may alter dramatically both the growth and the product formation in cultured plant cells (MANTELL and SMITH, 1984). Auxin appears to be the primary factor controlling growth and morphology of roots, while the effects of cytokinin vary depending on secondary metabolites and species concerned (ROA and RAVISHANKAR, 2002). For example kinetin stimulated the production of anthocyanins in *Haplopappus gracilis* but inhibited the formation of anthocyanins in *Populus* cell cultures (SEITZ and HINDERER, 1988).

In recent years, with enhanced awareness of the importance of antioxidant compounds in health and disease, considerable attention has been devoted to medicinal plants with high antioxidant properties (KONG *et al.*, 2010; MOHAMED *et al.*, 2010). Colocynth or bitter melon (*Citrullus colocynthis*) is a medicinal plant species of Cucurbitaceae family. It grows fast in sandy soils and is widespread in different parts of South Eastern Desert of Egypt (HASSANANE *et al.*, 2001). A number of secondary metabolites have previously been reported in this plant including cucurbitacins, flavonoids, caffeic acid derivatives and terpenoids in addition to flavonoid glycosides and cucurbitacin glucosides (SEGER *et al.*, 2005; DELAZAR *et al.*, 2006). Moreover, higher content of total cucurbitacins and cucurbitacin-E have been attained in colocynth callus culture as a result of the impact of different combinations of growth regulators (HEGAZY *et al.*, 2010). Colocynth fruit extracts demonstrated activity against some bacteria and fungi prevalent in dermatology, so it can be considered as an effective antimicrobial agent to treat numerous diseases (ZIYYAT *et al.*, 1997). No information is available about the stimulation effects of plant growth regulators on antioxidant activity of colocynth callus cultures. The aim of this study was to develop conditions for callus cultures of *C. colocynthis* manipulating the concentrations and combinations of plant growth regulators, and determination of the effect of media composition on total phenolics and flavonoids content together with the related antioxidant activity of induced calli.

2. Materials and methods

2.1 Plant material

Mature seeds of *C. colocynthis* (L.) Schrad. were collected from naturally growing plant populations in Wadi Soule, Sinai, Egypt. The collected seed material was botanically authenticated by the Herbarium of the Botany Dept., Faculty of Science, Cairo University. Dehusked sterilized seeds were *in vitro* germinated on hormone-free MS medium (MURASHIGE and SKOOG, 1962).

2.2 *In vitro* culture and growth regulators treatments

Two weeks old *in vitro* germinated seedlings of *C. colocynthis* were used as a source of explants for initiation of callus cultures. Stems, leaves and roots were cut into small pieces and cultured on MS medium supplemented with different combinations of 2,4-dichlorophenoxyacetic acid (2,4-D) and Kinetin (KIN) as well as BA and NAA in different concentrations as presented in Table 1. The cultures were incubated for callus induction in the growth chamber at $24 \pm 2^\circ\text{C}$ under a fluorescent light ($80 \mu\text{E m}^{-2} \text{s}^{-1}$) at a 16-h photoperiod for three weeks. After that callus samples were collected for determination of callusing frequency as well as determination of total phenolics, total flavonoid and antioxidant activity by different methods.

2.3 Callusing frequency

The frequency of callus induction was calculated according to the following formula: Callus induction frequency (%) = [No. of seeds produced calli / No. of seeds cultured] x 100.

Table 1. List of MS media supplemented with different growth regulators used for colocynth callus cultures.

No.	Media codes	Growth regulators concentration (mg/L)
1	MD1	MS + 2.0 2,4-D + 1.0 KIN
2	MD2	MS + 1.0 2,4-D + 1.0 KIN
3	MD3	MS + 6.0 2,4-D + 2.0 KIN
4	MD4	MS + 2.0 2,4-D + 4.0 KIN
5	MB1	MS + 0.0 BA + 5.0 NAA
6	MB2	MS + 0.01 BA + 1.0 NAA
7	MB3	MS + 0.1 BA + 5.0 NAA
8	MB4	MS + 1.0 BA + 0.1 NAA

2.4 Preparation of plant extracts

Callus cultures derived from stems, leaves and roots as well as *in vitro* raised seedling materials (used as control) were air dried at room temperature and ground in a mortar. The extracts were prepared using the modified method of MATKOWSKI and PIOTROWSKA (2006). Briefly, 0.5 g of the dried powder from each sample was refluxed with methanol in a water bath at 45°C for 3 h. The extracts were filtered through Whatman filter paper No. 4. The collected filtrates were dried under vacuum at 40°C. The extraction was repeated twice. The resulting residue was re-dissolved in methanol and used for the determination of phenolics, flavonoid contents and antioxidant activities.

2.5 Determination of total phenolics content

The total phenolics content of the extracts was determined using the Folin-Ciocalteu reagent (KAUR and KAPOOR, 2002). Each extract solution (200 µL) was completed to 3 mL with distilled water then mixed thoroughly with 0.5 mL of Folin-Ciocalteu reagent. After mixing for 3 min, 2 mL of 20% (w/v) sodium carbonate was added and allowed to stand for a further 60 min in the dark. The absorbance of the reaction mixtures were measured at 650 nm. The total phenolics content was expressed as mg pyrogallol equivalents / 100 g dry weight (d.w.) of the extract.

2.6 Determination of total flavonoid content

The total flavonoid content was determined according to the method of KIM *et al.* (2003). In brief, 0.5 mL of sample solution was completed to 1 mL with methanol then mixed with 4 mL of distilled water and subsequently with 0.3 mL of 5% NaNO₂ solution. After 6 min of incubation, 0.3 mL of 10% AlCl₃ solution was added and then allowed to stand for 5 min, followed by adding 2 mL of 1 M NaOH solution to the mixture. Immediately after water was added to the sample to bring the final volume to 10 mL and the mixture was thoroughly mixed. The absorbance was determined at 510 nm. The total flavonoid content was expressed as mg rutin equivalents / 100 g d.w. of the extract.

2.7 Determination of antioxidant activity

2.7.1 DPPH free radical scavenging activity

Quantitative measurement of radical scavenging properties of colocynth was carried out according to the method of BLOIS (1958). Briefly, 0.1 mM solution of 1,1-diphenyl-2-picryl-hydrazyl (DPPH·) in methanol was prepared and 1 mL of this solution was added to 3 mL of each methanolic extract at one concentration (500 µg/mL). Butylated hydroxytoluene (BHT) was used as a positive control. Discoloration was measured at 517 nm after incubation for 30 min. Measurements were taken at least in triplicate. The capacity to scavenge the DPPH· radical was calculated using the following equation:

$$\text{DPPH}\cdot \text{ scavenging effect (\%)} = [A_{DPPH} - A_S / A_{DPPH}] \times 100$$

where, A_{DPPH} is the absorbance of the DPPH· solution and A_S is the absorbance of the solution when the sample extract is added. The extract concentration providing 50% inhibition of radical-scavenging activity (IC_{50}) was calculated and expressed as mg/mL, d.w.

2.7.2 Ferric reducing power determination

Ferric reducing power was determined following the method reported by ZHAO *et al.* (2008). Extracts at concentration of 500 µg/mL were mixed with phosphate buffer (2.5 mL, 200 mM, pH 6.6) and 1% potassium ferricyanide (2.5 mL). Then the mixtures were incubated at 50 °C for 20 min. The quantity 2.5 mL of 10% trichloroacetic acid was added and the mixture was centrifuged at 10000 rpm for 10 min. The upper layer of the solution (5 mL) was mixed with distilled water (5 mL) and 0.1% ferric chloride (1 mL). The absorbance of the reaction mixtures were measured at 700 nm. The final results were expressed as µg ascorbic acid equivalents / g based on dry weight of the extract.

2.7.3 Hydrogen peroxide scavenging activity

The hydrogen peroxide scavenging ability of methanolic extracts was determined according to the method of SHON *et al.* (2007). A solution of H_2O_2 (43 mM) was prepared in phosphate buffer (0.1 M, pH 7.4). Extracts at concentration 50 µg/mL dissolved in 3.4 mL phosphate buffer were added to a H_2O_2 solution (600 µL). The absorbance of the reaction mixture was recorded at 230 nm. The percentage of H_2O_2 scavenging of each extract and BHT at concentration 50 µg/mL used as positive control was calculated as:

$$H_2O_2 \text{ scavenging effect (\%)} = [A_{Control} - A_{Sample} / A_{Control}] \times 100$$

where $A_{Control}$ is the absorbance of the control (blank, without extract), and A_{Sample} is the absorbance in the presence of the sample extract.

The extract concentration providing 50% of H₂O₂ scavenging activity (IC₅₀) was calculated and expressed as µg/mL based on sample dry weight.

2.8 Statistical analysis

The experiment was conducted using Completely Randomized Design (CRD). All tests were conducted in triplicate. Data are reported as means ± standard deviation (SD). Analysis of variance and significant differences among means were tested by one-way ANOVA using the COSTAT computer package (COHORT SOFTWARE, 1989). The least significant difference (LSD) at $P \leq 0.05$ level was calculated. Correlation coefficients (R^2) from regression analysis between total phenolic or total flavonoid contents and antioxidant activities were also calculated.

3. Results and discussion

Calli of colocynth began to appear on stems, leaves and roots grown on MS media supplemented with different concentrations and combinations of growth regulators (2,4-D/KIN and BA/NAA) within one week. The results presented in Table 2 shows that the stem explants grown on MD2 gave the highest frequency of callus formation (98.9 %). Generally, among all 2,4-D and KIN combination treatments, the medium MD2 showed significant superiority in frequencies of callus formation for the three studied explants. These results are in accord with HEGAZY *et al.* (2010) who reported that high concentration of KIN more than 2,4-D in the MS-media had a positive effects on the callus induction (fresh and dry weight) in both stem and root explants of colocynth. On the other hand, DABAUZA *et al.* (1997) found that the highest frequency (81.8 %) of *Citrullus colocynthis* cotyledon explants derived calli was obtained on a medium containing 25 µM 6-benzylaminopurine (6-BA).

Table 2. Callusing frequency of different explants derived from seedlings of colocynth cultured on MS media supplemented with different combinations of growth regulators.

Growth regulators treatments	Media codes	Callus formation frequency (%), mean ± SD		
		Origin of callus (explants)		
		Stems	Leaves	Roots
2,4-D + KIN	MD1	94.9 ± 1.80 ^e	68.6 ± 1.81 ^b	26.8 ± 1.41 ^a
	MD2	98.9 ± 1.10 ^g	86.4 ± 1.38 ^f	61.1 ± 0.95 ^d
	MD3	83.2 ± 2.17 ^b	70.5 ± 1.78 ^c	44.2 ± 0.96 ^b
	MD4	98.8 ± 1.20 ^g	47.8 ± 0.82 ^a	45.2 ± 1.29 ^c
BA + NAA	MB1	85.1 ± 1.73 ^c	85.0 ± 1.30 ^c	68.6 ± 1.58 ^e
	MB2	81.8 ± 1.42 ^a	74.3 ± 1.48 ^d	77.8 ± 1.62 ^f
	MB3	97.9 ± 1.38 ^f	98.2 ± 1.17 ^h	91.7 ± 1.80 ^g
	MB4	90.9 ± 1.90 ^d	92.6 ± 1.53 ^e	92.9 ± 1.50 ^h
LSD ≤ 0.05		0.699	0.324	0.281

Data represents mean of 10 replicates per treatment in three repeated experiments. Data with different superscript letters in the same column were significantly different ($P \leq 0.05$).

Fig. 1 and 2 show the total phenolics content in different colocynth callus cultures. One-way ANOVA analysis showed significant differences ($p \leq 0.05$) in total phenolics content among the eight studied media formulations. A wide range of total phenolics content was found in 2,4-D and KIN treatments as shown in Fig. 1. The high concentration of 2,4-D over KIN in MD3 media yielded the highest phenolics content (19.2 mg/100g d.w.) in the leaf derived calli. This content was higher than the content of phenolic compounds in stems, leaves and roots organs of seedlings (3.32, 17.1 and 4.49 mg/100g d.w.) which were used as controls.

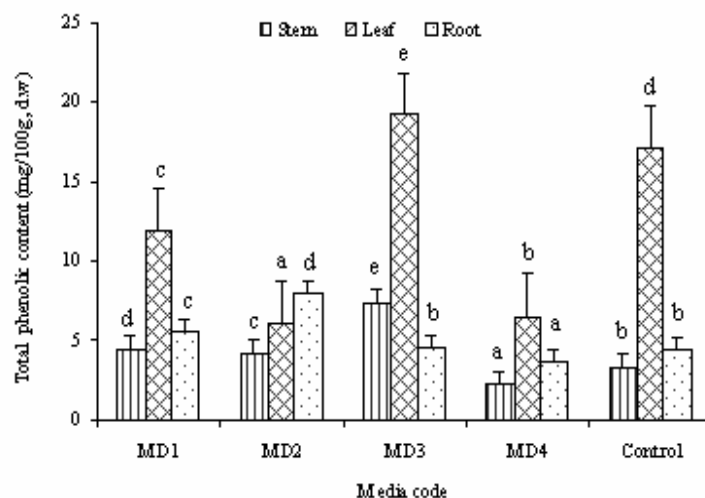


Fig. 1. Total phenolics content of colocynth callus cultures grown on MS media supplemented with different combinations and concentrations of 2,4-D and KIN. Data with different superscript letters in the same column were significantly different ($P \leq 0.05$). Bars on the columns represent the standard deviation.

Fig. 2 shows the total phenolics content in calli derived on BA and NAA containing media. Overall, the higher concentration of NAA over BA (MB2) gave the maximum amount of total phenolics (7.54 and 6.52 mg/100g d.w.) in the stem and root derived calli respectively. Unlike, the lowest value of phenolics content (2.71 mg/100g d.w.) was detected in the leaf-derived calli cultured on medium MB3. The effect of plant growth regulators on biosynthesis of phenolic compounds were studied in hairy roots of *Panax ginseng* by JEONG *et al.* (2007). They found that addition of benzylaminopurine and kinetin to the culture media led to increasing the phenolic compound biosynthesis. In addition, NIKOLAEVA *et al.* (2009) reported that growing transgenic tea callus tissue (strain IFR ChS-2) on nutrient medium containing NAA at concentration of 2×10^{-5} M stimulated total soluble phenolics more than total phenolics which presented in intact plants.

The total flavonoid content showed significant ($P \leq 0.05$) differences among the explant types and the different combinations of growth regulators as shown in Fig. 3. Dealing with 2,4-D and KIN media treatments, MD3 gave the maximum values of total flavonoid content (47.3 mg/100g d.w.) in the stem derived calli. This content is higher than those contents (11.8, 46.4 and 15.6 mg/100g d.w.) of *in vitro* raised

seedlings stems, leaves and roots respectively, which were used as a control. It is clear that 2,4-D was the favorable enhancer of flavonoids accumulation in colocynth callus cultures. These results are in agreement with MATKOWSKI (2004), who reported that medium containing 2,4-D was found to be optimum for isoflavone accumulation in callus cultures of *Pueraria lobata* and *Psoralea sp.* Similarly, SHINDE *et al.* (2009) confirmed that the content of isoflavones in root and leaf-derived callus cultures of *Psoralea corylifolia* was higher on medium containing 2,4-D and IAA than in intact plant roots and leaves.

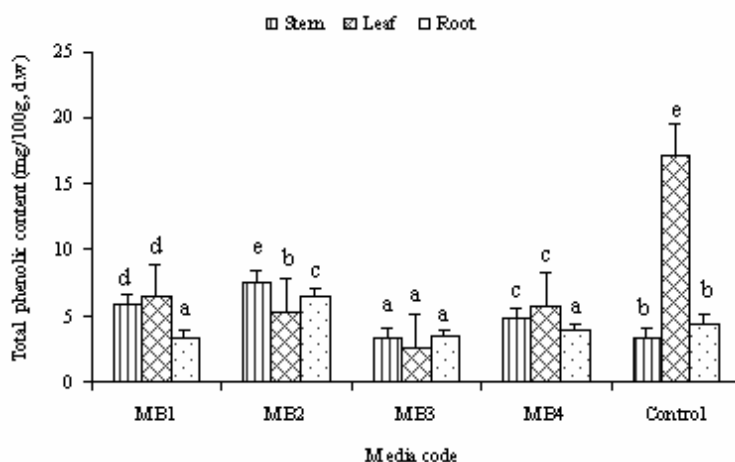


Fig. 2. Total phenolics content of colocynth callus cultures grown on MS media supplemented with different combinations of BA and NAA. Data with different superscript letters in the same column were significantly different ($P \leq 0.05$). Bars on the columns represent the standard deviation.

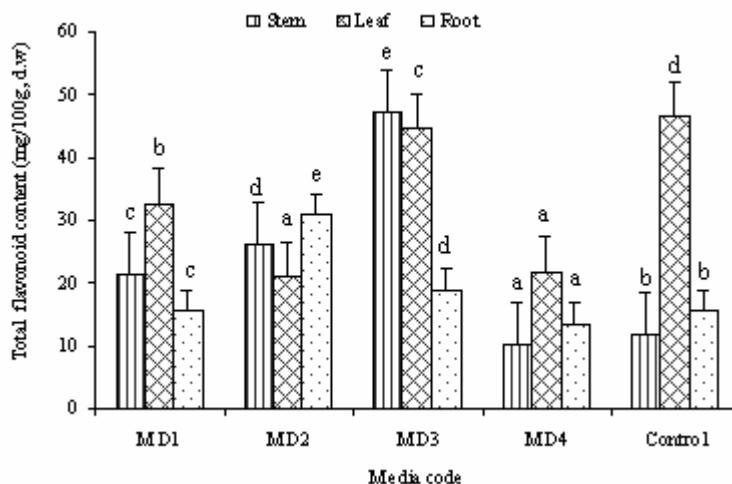


Fig. 3. Total flavonoid content of colocynth callus cultures grown on MS media supplemented with different combinations of 2,4-D and KIN. Data with different superscript letters in the same column were significantly different ($P \leq 0.05$). Bars on the columns represent the standard deviation.

The effect of BA and NAA treatments on stimulation of total flavonoid content is presented in Fig. 4. The high concentration of BA over NAA (MB4 media) gave the highest flavonoid content (34.0 mg/100g d.w.) in the stem derived calli. However, this content is lower than the flavonoid content (46.4 mg/100g d.w.) detected in the leaves of the *in vitro* raised seedlings. The present results are in agreement with findings of AGARWAL and KAMAL (2007). The authors confirmed that the maximum flavonoid content was observed in 2 weeks old callus culture of *Momordica charantia* cultured on MS medium supplemented with NAA (2 mg/L) and BAP (0.5 mg/L).

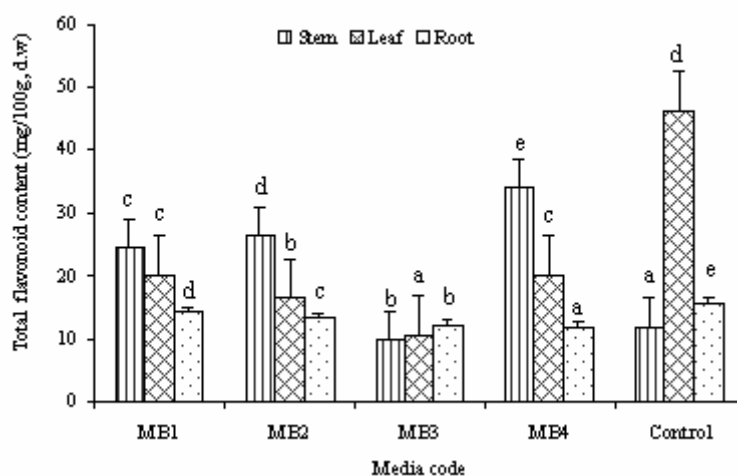


Fig. 4. Total flavonoid content of colocynth callus cultures grown on MS media supplemented with different combinations of BA and NAA. Data with different superscript letters in the same column were significantly different ($P \leq 0.05$). Bars on the columns represent the standard deviation.

It is well known that, the concentrations of phenolics content usually higher than the concentrations of flavonoid in most cases, but in this study we found an inverse trend. It can be explained by the fact that differences in the polarity of the extracting solvents could result in a wide variation in polyphenolic contents of the extract. So, the low content of phenolics in our extracts may be possibly due to the fact that extraction with methanol does not release bound phenolics from the callus cells. On the other side, the Folin-Ciocalteu method is a rapid and widely-used assay, to detect the total phenolic content but it is known that different phenolic compounds have different responses in the Folin-Ciocalteu method (KÄHKONEN *et al.*, 1999). Moreover, different authors found a higher concentrations of flavonoid more than phenolics such as SULTANA *et al.* (2009) found a higher concentration of total flavonoids (1.68 g/100g, d.w.) in absolute methanolic extract of *Moringa oleifera* root more than the total phenolics (0.22 g/100g, d.w.). Also, the aqueous extract of *Momordica charantia* yielded higher content of total flavonoid (62 mg/g, d.w.) than total phenolics (51.6 mg/g, d.w.) as reported by (WU and Ng, 2008). The variation in the total flavonoid production may be attributed to the activation of key enzyme phenylalanine ammonia-lyase (PAL) which is involved in the flavonoid biosynthesis pathway (GUO *et al.*,

2007). The knowledge of biosynthetic pathway and mechanisms for stimulation of flavonoids production in callus culture remains unclear. Whether phytohormones induce different biosynthetic enzymes responsible for the biosynthesis of flavonoids is yet to be clarified.

The antioxidant activities of methanolic extracts of colocynth callus cultures and of the standard antioxidant BHT were determined using the DPPH method. This method is based on the reduction of alcoholic DPPH solution in the presence of a hydrogen-donating antioxidant due to the formation of the non-radical form DPPH-H by the reaction (GÜLÇİN, 2006). The DPPH radical scavenging activity of methanolic extracts of colocynth callus cultures at concentration 500 µg/mL is presented in Table 3. The leaf-derived calli cultured on MD1 showed relatively higher radical scavenging activity (85.3 %) with IC₅₀ value (0.209 mg/mL) as presented in Table 4. This activity was higher than the activity of stem, leaf and root organs of seedling which were used as control (51.6, 80.9 and 27.9 %) with IC₅₀ values (0.485, 0.241 and 0.915 mg/mL) respectively. The scavenging activity of BHT which was used as positive control showed higher activity (87.5 %) with IC₅₀ value (0.086 mg/mL) than our methanolic extracts of colocynth callus cultures. These results are consistent with those of TADHANI *et al.* (2007) who found a relatively higher DPPH radical scavenging activity (56.8%) with IC₅₀ value (0.528 mg/mL) of extract of *Stevia rebaudiana* leaf-derived callus cultured on MS media supplemented with 2.0 mg/L NAA and 0.3 mg/L BA than the activity (33.2 %) with IC₅₀ value (0.904 mg/mL) of intact plant leaves.

Table 3. DPPH radical scavenging activity of colocynth callus cultures grown on MS media supplemented with different combinations of growth regulators.

Growth regulators treatments	Media codes	DPPH scavenging activity (% , mean ± SD)		
		Origin of callus (explants)		
		Stems	Leaves	Roots
2,4-D + KIN	MD1	15.23 ± 0.046 ^e	85.31 ± 0.685 ⁱ	38.73 ± 0.158 ^h
	MD2	11.62 ± 0.016 ^e	56.32 ± 0.213 ^f	27.55 ± 0.110 ^d
	MD3	8.45 ± 0.028 ^a	70.89 ± 0.223 ^g	22.80 ± 0.073 ^c
	MD4	10.02 ± 0.016 ^b	23.01 ± 0.084 ^a	28.17 ± 0.081 ^f
BA + NAA	MB1	14.70 ± 0.770 ^f	44.63 ± 0.276 ^c	32.13 ± 0.097 ^e
	MB2	13.83 ± 0.012 ^e	46.78 ± 0.014 ^d	19.56 ± 0.035 ^b
	MB3	12.59 ± 0.013 ^d	41.47 ± 0.066 ^b	25.44 ± 0.047 ^a
	MB4	16.60 ± 0.073 ^h	49.32 ± 0.110 ^e	59.42 ± 0.204 ⁱ
	Control	51.60 ± 0.445 ⁱ	80.90 ± 0.748 ^h	27.91 ± 0.081 ^c
	LSD ≤ 0.05	0.442	0.705	0.299
	BHT (150µg/mL)		87.50 ± 0.099	

Note: Media formulations are presented in Table 1.

Each value is expressed as mean ± standard deviation (n =3). Data with different superscript letters in the same column were significantly different (P ≤ 0.05).

In addition, GRZEGORCZYK *et al.* (2007) reported that the methanolic extracts (50 µg/mL) of *Salvia officinalis* shoot cultures grown on MS media supplemented with

0.1 mg/L IAA and 0.45 mg/L BAP possessed a high DPPH radical scavenging activity (81.4 %) comparable to that (72.3 %) of shoots from field collected plants. With respect to these results, it is possible to consider the callus cultures as a potential source of natural antioxidants. Although, which constituents of colocynth callus methanolic extracts, show the free radical scavenging action is still unclear. It is possible that the antioxidative properties of colocynth extracts are caused, at least in part, by the presence of polyphenols and flavonoids and other yet to be discovered antioxidant compounds.

Table 4. IC₅₀ for inhibition of DPPH radical formation of colocynth callus cultures grown on MS media supplemented with different combinations of growth regulators

Growth regulators treatments	Media codes	IC ₅₀ values (mg/mL, d.w.) of DPPH scavenging activity		
		origin of callus (explants)		
		Stems	Leaves	Roots
2,4-D + KIN	MD1	1.670	0.209	0.654
	MD2	2.190	0.449	0.919
	MD3	2.970	0.354	1.118
	MD4	2.500	1.090	0.898
BA + NAA	MB1	1.730	0.569	0.794
	MB2	1.860	0.540	1.325
	MB3	2.030	0.613	1.000
	MB4	1.520	0.511	0.428
	Control	0.485	0.241	0.915
	BHT (150µg/mL)		0.086	

The presence of reductants such as antioxidant substances in samples causes the reduction of the Fe³⁺ ferricyanide complex to the ferrous form. The transformation of iron (III) to iron (II)-reducing activity in the colocynth callus methanolic extracts was expressed as µg ascorbic acid equivalent/g sample based on dry weight. The results showed statistically significant (P≤0.05) differences among the explant types and growth regulator treatments. Fig. 5 shows that the highest reducing power activity (22.3 µg/g d.w.) was found in leaf-derived calli cultured on MD3 comparing to the activities (3.28, 12.9 and 2.85 µg/g d.w.) of stems, leaves and roots of the *in vitro* seedlings which were used as controls, respectively.

Among the BA and NAA treatments, Fig. 6 indicated that high concentration of NAA in the medium (MB1) has notable effect on total reductive activity. It gave the highest activity (7.52 and 6.61 µg/g d.w.) in the callus cultures of stem and leaf explants respectively. However, these activities are lower than those of leaves of *in vitro* seedlings (12.9 µg/g d.w.). In this concern, PARSAEIMEHR *et al.* (2010) reported that *Ephedra strobilacea* stem callus cultures grown on MS media supplemented with 1.5 mg/L NAA and 1 mg/L KIN gave ferric reducing antioxidant power (0.28 mmol quercetin/g). This value was lower than in intact plants (1.61 mmol quercetin /g).

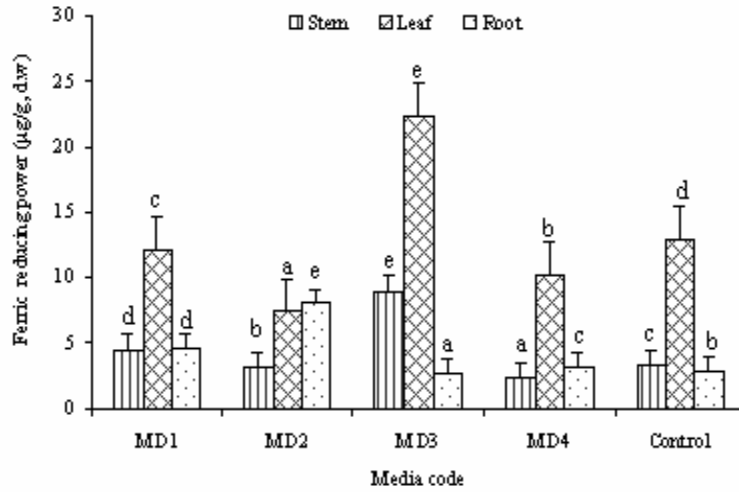


Fig. 5. Ferric reducing power of colocyth callus cultures grown on MS media supplemented with different combinations of 2,4-D and KIN. Data with different superscript letters in the same column were significantly different ($P \leq 0.05$). Bars on the columns represent the standard deviation.

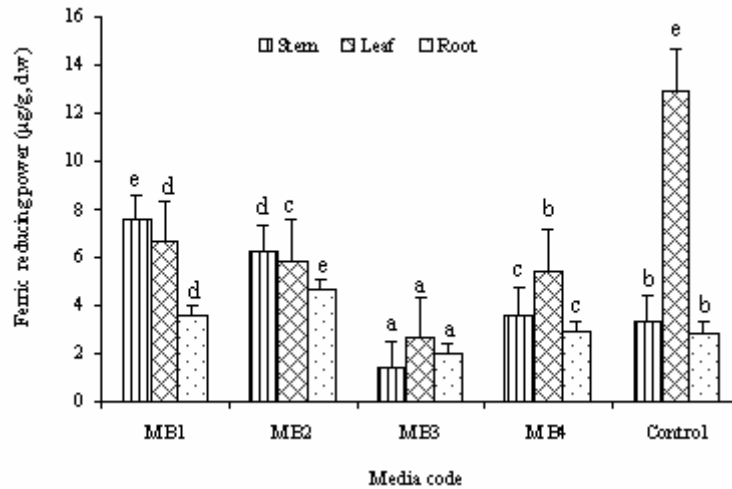


Fig 6. Ferric reducing power of colocyth callus cultures grown on MS media supplemented with different combinations of BA and NAA. Data with different superscript letters in the same column were significantly different ($P \leq 0.05$). Bars on the columns represent the standard deviation.

Hydrogen peroxide can cross membranes and may slowly oxidize a number of compounds. Thus, removing of hydrogen peroxide as well as superoxide anion is very important for protection of food systems (GÜLÇİN *et al.*, 2007). Table 5 points out that H_2O_2 scavenging activity of colocyth callus methanolic extracts at concentration 150 µg/mL differed significantly ($P \leq 0.05$) among the different concentrations of

growth regulators. Generally, high ratio of 2,4-D over KIN in MD1 exhibited remarkable scavenging activity on hydrogen peroxide, it gave the highest H₂O₂ scavenging activity (61.4 %) in leaf-derived calli with IC₅₀ value 40.74 µg/mL as presented in Table 6. This activity value was higher than the activity of the *in vitro* grown seedlings stems, leaves and roots organs (used as controls) which showed H₂O₂ scavenging activities of 26.2, 27.3 and 26.4 % with IC₅₀ values 95.4, 91.7 and 94.7 µg/mL, respectively. The BHT which was used as a positive control gave H₂O₂ scavenging activity of 90.5 % with IC₅₀ value 27.64 µg/mL, this value was higher than in colocynth callus cultures. Moreover, high concentration of BA in MB4 media gave the highest hydrogen peroxide scavenging activity in the root derived calli (51.8 %).

Table 5. Hydrogen peroxide (H₂O₂) scavenging activity of colocynth callus cultures grown on MS media supplemented with different combinations of growth regulators

Growth regulators treatments	Media codes	H ₂ O ₂ scavenging activity (% mean ±SD)		
		Origin of callus (explants)		
		Stems	Leaves	Roots
2,4-D + KIN	MD1	23.59 ± 0.117 ^b	61.37 ± 0.089 ^e	35.97 ± 0.056 ^h
	MD2	24.26 ± 0.139 ^{bc}	33.44 ± 0.062 ^d	15.19 ± 0.023 ^a
	MD3	21.36 ± 0.110 ^a	39.22 ± 0.407 ^c	17.47 ± 0.027 ^d
	MD4	39.37 ± 0.121 ^c	24.42 ± 0.048 ^a	18.83 ± 0.051 ^c
BA + NAA	MB1	25.83 ± 0.094 ^{cd}	40.38 ± 0.455 ^f	33.03 ± 0.053 ^e
	MB2	22.32 ± 0.049 ^{ab}	30.00 ± 0.070 ^c	16.72 ± 0.026 ^b
	MB3	27.66 ± 0.091 ^d	33.48 ± 0.039 ^d	16.75 ± 0.053 ^c
	MB4	21.48 ± 0.058 ^{ab}	38.44 ± 0.062 ^c	51.77 ± 0.079 ⁱ
	Control	26.21 ± 0.235 ^d	27.27 ± 0.623 ^b	26.39 ± 0.051 ^f
	LSD≤0.05	1.572	1.088	0.022
	BHT (50µg/mL)		90.46±0.011	

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

Data with different superscript letters in the same column were significantly different (P ≤ 0.05).

In this concern, RAKOTOARISON *et al.* (1997) found that both callus and cell suspension cultures of *Crataegus monogyna* showed high scavenging activities against H₂O₂. The decomposition of hydrogen peroxide into water may occur according to the presence and character of antioxidant compounds. Since antioxidant compounds present in the extract are good electron donors, they may accelerate the conversion of H₂O₂ into H₂O.

Phenolic and flavonoid compounds have been reported to be responsible for the antioxidant activities of medicinal plants and other botanical materials (MOHAMED *et al.*, 2010). Table 7 shows that the total phenolic compounds gave a positive correlation (R² = 0.511 and R² = 0.667) with DPPH radical scavenging activity and ferric reducing power respectively. These data could indicate that phenolic compounds in colocynth calli were the major contributors to DPPH radical scavenging activity and ferric reducing power. Polyphenolic compounds in extracts of colocynth

callus cultures might play a role as electron and hydrogen donors. The antiradical activity of flavonoids and phenolics is principally based on the redox properties of their hydroxy groups and the structural relationships between different parts of their chemical structure (BURDA and OLESZEK, 2001). In this concern, SHINDE *et al.* (2009) indicated that the content of phenolics is partially correlated with DPPH radical scavenging ($R^2 = 0.466$) in callus cultures of *Psoralea corylifolia*.

Table 6. IC₅₀ values of hydrogen peroxide (H₂O₂) scavenging activity of colocynth callus cultures grown on MS media supplemented with different combinations of growth regulators.

Growth regulators treatments	Media codes	IC ₅₀ values (µg/mL, d.w.) of H ₂ O ₂ scavenging activity		
		Origin of callus (explants)		
		Stems	Leaves	Roots
2,4-D + KIN	MD1	105.98	40.74	69.5
	MD2	103.05	74.76	164.58
	MD3	117.04	63.43	143.1
	MD4	63.50	102.38	132.77
BA + NAA	MB1	96.79	61.91	75.69
	MB2	112.01	83.33	149.52
	MB3	90.38	74.67	149.7
	MB4	116.39	65.04	48.29
	Control	95.38	91.68	94.73
	BHT (50µg/mL)		27.64	

Table 7. Correlation analysis (R^2)^{*} between antioxidant activities and antioxidant content (total phenolics and total flavonoids).

Antioxidant activities	Antioxidant contents	
	Total phenolics	Total flavonoids
DPPH radical scavenging activity	0.511	0.048
H ₂ O ₂ scavenging activity	0.017	0.026
Ferric reducing power	0.667	0.358

^{*} Correlation coefficient (R^2)

However, the poor correlations of H₂O₂ scavenging activity ($R^2 = 0.017$) with the total phenolic compounds as well as the poor correlations of DPPH radical scavenging activity, H₂O₂ scavenging activity and ferric reducing power ($R^2 = 0.048$, $R^2 = 0.026$ and $R^2 = 0.358$) with total flavonoids respectively in this study suggested that flavonoid compounds in colocynth calli might be a weak scavenger of DPPH radical and H₂O₂ and they do not contribute to ferric reducing power.

In conclusion, the development of *in vitro* cell lines with high antioxidant capacities provides a possibility of generating uniform material cultured under conditions independent of environmental factors. The results of the present study concluded that MS media supplemented with 2,4-D and kinetin produced higher contents of total phenolics, total flavonoids and antioxidant activities more than MS

media supplemented with BA and NAA in colocynth callus cultures. Stimulation of antioxidant activities in *C. colocynthis* will help to select callus culture type as a source of natural antioxidants and nutraceuticals to enhance health benefits. Moreover, more scientific work needs to be done regarding the characterization of effective antioxidant compounds contained in this plant species in order to further verify their antioxidant effects in *in vitro* conditions.

Acknowledgments: We are grateful to Dr. Mahmoud M. Saker, Professor of Plant Biotechnology, National Research Centre, where the practical part of tissue culture was carried out in his lab. We are also grateful to Dr. Ahmed K. Hegazy, Professor of Plant Ecology, Faculty of Science, Cairo University, for his critical reading of the manuscript.

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