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Optimization of callus induction with enhancing production of phenolic compounds production and antioxidants activity in callus cultures of carob (*Ceratonia siliqua* L.)

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Abstract

Carob (*Ceratonia siliqua* L.) is an important Mediterranean plant species with worldwide commercial and medicinal uses. The establishment of a callus culture protocol as an alternative system to produce polyphenols of chemical and pharmaceutical interest was made in the present study for the first time in carob. Explant type and the light regime are two important factors that influence morphogenic responses and biochemical production. Maximal callus induction (100 %) and biomass accumulation were obtained in cotyledon explants under both tested light regimes (16-h photoperiod and darkness). However, leaf-derived callus produced higher amounts of polyphenols (TPC) and flavonoids (TFC) but a lower amount of total condensed tannins (TCT) as compared to cotyledon-derived callus. The light treatment has significantly increased TCT content but decreased the antioxidant activity in carob callus cultures. Strong and positive correlations were obtained between TPC, TFC, and the antioxidant activities with correlation coefficients in range 0.68 – 0.98. The obtained results indicated that calli of *C. siliqua* have the potential for enhanced production of phenolic compounds with antioxidant activity that is favored by cultivation under dark condition.

Introduction

Ceratonia siliqua L., commonly known as 'carob', is an important xerophytic plant belonging to the Fabaceae family. Due to its important economic and ecologic values, it has drawn much attention in recent years. The interest in carob as an inexpensive source of several products has been increasing over the last few years. Carob seeds are being used for carob bean gum extraction. Carob pods can also be exploited for the production of sugar syrups (Fidan *et al.* 2020), cocoa substitutes (Akdeniz *et al.* 2021), bioethanols (Germec *et al.* 2020), and secondary metabolites (Kyratzis *et al.* 2021). Carob pods and leaves are being in use since olden time to cure digestive disorders, asthma, pharyngitis, bronchitis, and anemia (Rtibi *et al.* 2017; Sargin and Büyükcengiz 2019). Furthermore,

many studies have been conducted on several carob-tree parts for their bioactive components, including total phenolics which exhibit potent cytotoxic, anticancer, anti-inflammatory, hepatoprotective, nephroprotective, antidiabetic, sedative, and antioxidative properties (Benchikh *et al.* 2014; Custódio *et al.* 2015; Aboura *et al.* 2017; Ghanemi *et al.* 2017; Othmen *et al.* 2020; Gurel *et al.* 2021). The major phenolic compounds in this plant are phenolic acids, flavonoids, and tannins (Stavrou *et al.* 2018). However, phenolic contents in carob field-grown plants are highly variable due to genetics and environmental factors (Custódio *et al.* 2007; El Hajaji *et al.* 2011; Korkmaz *et al.* 2020). *In vitro* culture techniques emerge as efficient biotechnological tools to produce commercially important medicinal compounds such as polyphenols, under aseptic and controlled conditions. These techniques would allow continuous, economical, viable, and sustainable production of pure bioactive compounds, regardless of the climatic and geographic conditions, during a shorter and more flexible production cycle (Dias *et al.* 2016; Gai *et al.* 2020). Different factors affect phenolic production in *in vitro* plant cultures, such as explants type and light conditions, although responses vary with the class of secondary metabolites and among species (Estell *et al.* 2016; Farhadi *et al.* 2017; Shehzad *et al.* 2021). It must be noted that while studies on callus culture of carob have been reported (Ksia *et al.* 2008; Lozzi *et al.* 2015; 2018; 2019; Zouari and El Mtili 2020), the antioxidant properties of their phenolic compounds have not been investigated. To our knowledge, this study represents the first attempt to demonstrate that the callus culture of carob is a reliable tool for polyphenols production. The objectives of the present work were to: (i) optimize methods for producing vigorous carob callus by monitoring the effects of explant type and light conditions, (ii) determine the content of the total phenolics, flavonoids, and condensed tannins and (iii) evaluate the antioxidant activity in *in vitro* calli to elucidate the medicinal potency of *in vitro* cultures of this plant.

Experimental

Plant materials and callus culture

Mature seeds were collected from selected *C. siliqua* trees growing in Beni Mellal, Morocco, treated with concentrated sulfuric acid (98 %) for 60 min, washed and then immersed for 48 h in sterile distilled water. Cotyledons were aseptically excised from seeds and segmented into portions of 4 – 6 mm in length. Leaf, hypocotyl, epicotyls, and root segments were cut from two-month-old seedlings, germinated on the carob culture medium (LAC) that we have developed and successfully applied in our previous research on carob micropropagation (Lozzi *et al.* 2019). All explants (cotyledons, leaves, hypocotyls, epicotyls, and roots) were inoculated on the LAC medium supplemented with 5 µM 2,4-dichlorophenoxyacetic acid (2,4-D), 3 % (w/v) sucrose and 0.7 % (w/v) agar. The cultures were maintained at 26 ± 2 °C, either in darkness or under a 16-h photoperiod (60 $\mu mol.m^{-2}.s^{-1}$) for five weeks.

Extraction

Callus cultures of known fresh weight were dried in a hot air oven at 60 °C to constant weight. Each finely ground dried sample (0.1 g) was mixed with 70 % (v/v) acetone (40 mL). The mixtures were then centrifuged at 4,000 rpm for 20 min. The supernatant was collected and maintained at 4 °C until used.

Total phenolic content (TPC)

Total phenolic content of the calli extracts was determined spectrometrically according to the Folin–Ciocalteu assay (Singleton and Rossi 1965) using the gallic acid as standard. The calli extract (0.1 mL) was mixed with 1.4 mL of deionized water and 0.1 mL of Folin-Ciocalteu reagent. After 6 min of incubation, 0.5 mL of Na_2CO_3 (20 %) was added and the reaction mixture was allowed to incubate in the dark for 30 min. Absorbance was measured at 750 nm and results were expressed as mg gallic acid equivalents (GAE) per gram of dry weight of the sample.

Total flavonoid content (TFC)

Total flavonoid content was evaluated in the extracts using the aluminum chloride ($AlCl_3$) assay (Lamaison *et al.* 1990). 1 mL of $AlCl_3 \cdot 6H_2O$ (2 %) was added to 1 mL of acetonic extract and the absorbance was read 10 min later at 430 nm. Quercetine was used to make the calibration curve, and the results were expressed as mg of Quercetine equivalents (QE) per g dry matter in extracts.

Total condensed tannin (TCT)

Condensed tannins were analyzed using the vanillin–HCl method (Broadhurst and Jones 1978). 0.1 mL of calli extract was added to 3 mL of vanillin (4 %) and 1.5 mL of highly concentrated HCl. The absorbance was measured at 500 nm after standing for 20 min in a dark room. The results were expressed as mg of catechin equivalent (CE) per g of dry weight.

DPPH radical-scavenging assay

The free radicals scavenging activity of the *in vitro* callus of carob was evaluated spectrophotometrically using the 2,2-diphenyl-1-picryl hydrazyl (DPPH) method (Brand-Williams *et al.* 1995). Briefly, 1 mL of 0.1 mM DPPH solution (in ethanol) was added to 0.5 mL of calli extract diluted in 1.4 mL of ethanol. The mixtures were vortexed and incubated for 30 min in darkness. The absorbance was measured at 515 nm and the ascorbic acid was used as a standard. The radical scavenging activity was calculated according to the following formula (Eq. 1):

$$\text{Radical scavening activity (\%)} = 100 \times \left(1 - \frac{A_1}{A_0}\right) \quad (1)$$

where A_0 is the absorbance of the negative control (ethanol) and A_1 is the absorbance of the reaction mixture containing the sample extract.

Ferric-reducing power (FRAP)

The reducing power of the extract was evaluated spectrophotometrically using the assay of Oyaizu (1986). 0.22 mL of the extract was added to 0.25 mL of phosphate buffer (0.2 M, pH 6.6) and 0.25 mL of potassium ferricyanide (1 %). The mixture was then incubated at 50 °C for 20 min. Afterward, 0.25 mL of 10 % trichloroacetic acid was added, and the mixture was mixed with 1 mL of distilled water and 0.25 mL of iron trichloride (0.1 %). The absorbance was read at 700 nm. The increased absorbance of the reaction mixture meant increased reducing power of the calli extract.

Statistical analysis

All extractions and assays were carried out in triplicate. The results were expressed as mean ± standard deviation. The experimental data were processed with the analysis of variance (ANOVA) followed by Duncan's multiple range test (DMRT) at 5 % to determine significant differences among the mean values. Pearson's correlation test was used to identify the correlation between total phenolics and antioxidant activity.

Results and discussion

Effect of explant type and light conditions on induction

The percentage of callus induction and the dry weights (DW) of calli were significantly ($P<0.001$) affected by explant type, confirming the results of previous works (Gourguillon *et al.* 2018; Rameshkumar *et al.* 2018; Wu *et al.* 2020). A successful callus formation was achieved in both cotyledon and leaf explants (Fig. 1A, B). However, the cotyledon explants showed 100 % callus induction and a much better callus dry weight than leaf explants (Table 1). Attempts to induce callus from hypocotyls and epicotyls were completely ineffective (Fig. 1C, D). The root explants exhibited poor callus formation, turned brown and died within 5 weeks (Fig. 1E). The difference of response between the explants could be related to the difference in endogenous hormone level and the competence of cells to respond to the external signals (Jiménez and Bangerth 2001a, 2001b; Wu *et al.* 2020; Saeedpour *et al.* 2021). Besides of the explant type, light is another factor that may influence competence by modifying the concentration of endogenous hormone in explant tissues (Jiménez and Bangerth 2001a; Su *et al.*

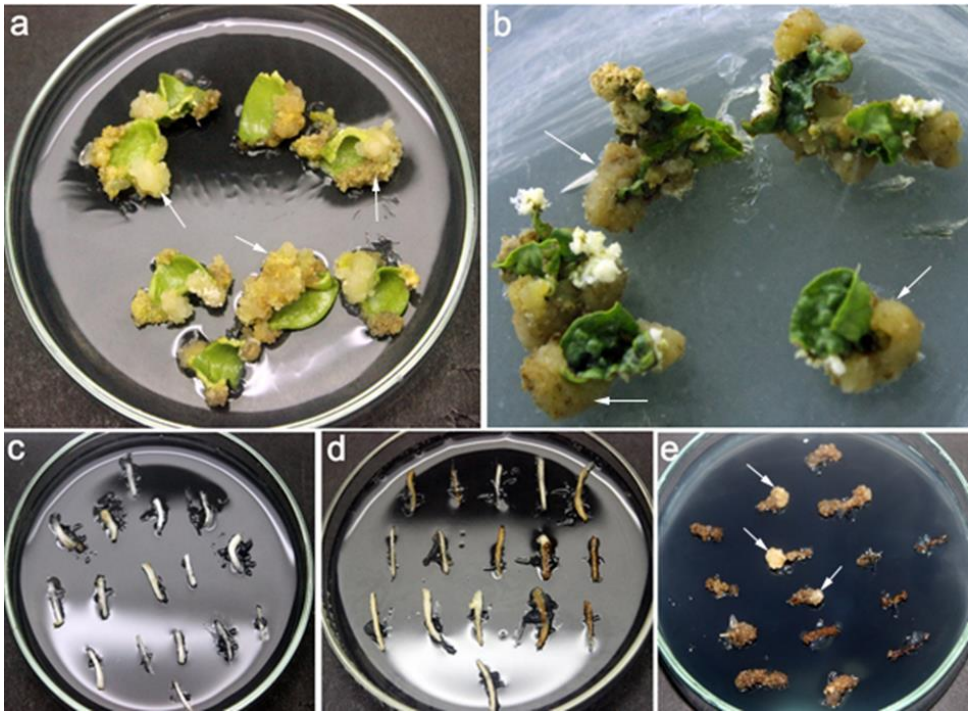
2014; Vinterhalter *et al.* 2020). Callus development of *C. siliqua* in response to light conditions is missing in the cited literature. In the present investigation, callus growth (DW) on cotyledon explants was similar under both tested light regimes (16-h photoperiod and darkness). The maximum dry weight (53.9 ± 4.9 mg.L^{-1}) being achieved in darkness. Note that the present result is considerably higher than our previous studies (Lozzi *et al.* 2015, 2018) on callus induction from callus cotyledons using other basal culture media: MS (Murashige and Skoog 1962), B5 (Gamborg *et al.* 1968), WPM (Lloyd and McCown 1980) and DKW (Driver and Kuniyuki 1984), indicating that LAC medium is more suitable for callus induction from carob cotyledons. In the case of leaf explants, the biomass accumulation (DW) in callus formed in the dark was significantly higher than that in the light. Previous studies revealed a close relationship between light regimes and callus biomass accumulation (Ju *et al.* 2014; Shehzad *et al.* 2021) which indicate the need for optimal light conditions for optimum biomass accumulation.

Table 1. Comparative response of callus induction in *Ceratonia siliqua* from mature cotyledons and leaves in photoperiod and darkness.

Type of explant	**Light *conditions***	**Callus induction [%]**	**Callus DW [mg.L^{-1}]**
Mature cotyledons	16-h Photoperiod	100.0^{a}	53.9 ± 4.9^{a}
	Darkness	100.0^{a}	52.5 ± 7.0^{a}
Leaves	16-h Photoperiod	96.3^{a}	30.8 ± 5.0^{b}
	Darkness	73.6^{b}	24.4 ± 1.1^{c}
Significance of two-way ANOVA			
Type of explant (A)		***	***
Light conditions (B)		**	*
A×B		**	NS

Values are mean ± SE. Mean values within a column followed by the same letter are not significantly different by Duncan's multiple range test (5 %). DW – dry weight; ∗, ∗∗, ∗∗∗ – significance at P≤0.05, P≤0.01, and P≤0.001, respectively (two-way ANOVA).

Fig. 1. Callus induction from different explants of *Ceratonia siliqua* L.

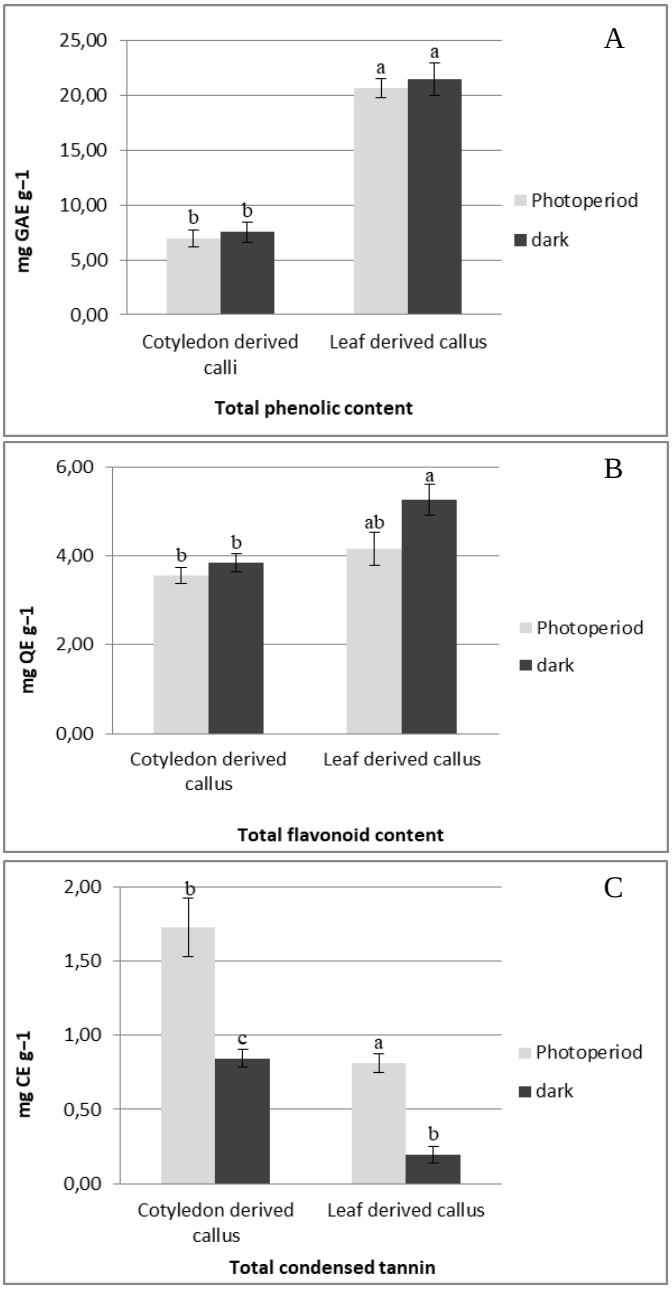


a – mature cultyledons; **b** – leaf explants; **c** – hypocotyl explants; **d** – epicotyls explants; **e** – root explants. Arrows indicate callus tissues.

Effects of explant type and light conditions on antioxidant compounds

The total phenolic contents of the extracts are shown in Fig. 2. In contrast to the finding on callus induction and biomass accumulation, the TPC, TFC, and TCT contents in leaf derived calli extracts were significantly higher when compared to cotyledon-derived calli extracts. Leaf-derived callus produced the highest amounts of phenolic compounds (21.4 ± 1.4 mg GAE.g^{-1} in darkness and 20.6 ± 0.9 in light) which were about three-fold higher than those of cotyledon callus. Similarly, the leaf-derived calli produced a higher amount of flavonoids than those of cotyledon explant (7.5 ± 0.9 mg.QE.g^{-1} in darkness and 7.0 ± 0.7 in light). Among calli derived from cotyledon explants, regardless of the light conditions, the level of TPC was not significantly different (P<0.05). Similar behavior was observed among calli derived from leaf explants, indicating that the light regime tested did not influence the accumulation of these compounds. Similar results were documented by Fazal *et al.* (2016) in callus culture of *Prunella vulgaris* where TPC production was not influenced by light conditions. Opposite results were however, obtained with calli from *Tecoma stans* and *Cnidium officinale* when illumination induced a strong accumulation of polyphenols (Rameshkumar *et al.* 2018; Adil *et al.* 2019). In contrast, the TFC level was significantly (P<0.01) influenced by the light regime. The total flavonoid content was higher in darkness (3.8 ± 0.2 mg.QE.g^{-1} in cotyledon-derived callus and 5.3 ± 0.3 in leaf-derived callus) than in periodic light. In contrast to the finding on TPC and TFC accumulation in carob calli extracts, the cotyledon-derived callus accumulated higher amounts of TCT, which were about two-fold higher than those of leaf-derived callus; the difference being significant (P<0.001). Furthermore, callus grown under periodic light enhanced the accumulation of TCT (1.7 ± 0.2 mg.CE.g^{-1} in cotyledon-derived callus and 0.84 ± 0.07 in leaf-derived callus) as compared to dark incubation. Similarly, (Castro *et al.* 2016) found a positive response of light on the TCT in *Byrsonima verbascifolia* callus cultures, whereas Favre *et al.* (1991) found no effect in *Quercus* callus.

Fig. 2. Effects of explant type and light conditions on antioxidant compounds in callus cultures of carob (*Ceratonia siliqua* L.).



A – total phenolic content; **B** – total flavonoid content; **C** – total condensed tannin. Mean values within a column followed by the same letter are not significantly different by Duncan's multiple range test (5 %).

The levels detected in the present study were found to be higher than those reported in different aerial parts of Moroccan carob, which ranged from 0.45 to 2.64 and from 15.8 to 18.08 mg.GAE.g^{-1} of phenolic content in intact carob leaves and pulp (Hajaji *et al.* 2010) and from 0.25 to 1.02 mg.CE.g^{-1} of condensed tannins in carob pulp (El Bouzdoudi *et al.* 2016). While in the aceton extract

of carob seed peel, total phenolics, flavonoids, and condensed tannins were found to be 0.16 µg.GAE.g^{-1}, 0.5 µg.QE.g^{-1} and 0.5 µg.CE.g^{-1}, respectively (Lakkab *et al.* 2019). Other studies on Algerian, Tunisian, Portuguese, and Cypriot carob showed different levels of total phenolic content that ranged from 1.8 to 307 and 6 to 336 mg.GAE.g^{-1} of leaves and pods (Papagiannopoulos *et al.* 2004; Custódio *et al.* 2009; Ortega *et al.* 2009; Meziani *et al.* 2015; Correia *et al.* 2018; Ayache *et al.* 2020; Kyriacou *et al.* 2021).These authors attributed the differences not only on genetic, geographical, environmental, and cultural conditions, but also on technological factors such as the extraction methodologies and the used solvent system.

Effects of explant type and light conditions on antioxidant activity

Total antioxidant capacity may vary depending on the assay used (Tabart *et al.* 2009). Therefore, using more than one method is recommended to evaluate this property (Tabart *et al.* 2014). In the present study, the antioxidant activities of carob calli extracts were determinate using two widely known assays, based on electron transfer reaction, the DPPH free radical-scavenging activity (DPPH) and Ferric-reducing power (FRAP). However, the two methods clearly showed that carob calli extracts contain considerable antiradical and antioxidant activities (Table 2). DPPH assay showed that all extracts were able to reduce the purple-coloured radical DPPH into pale, yellow-coloured DPPH-H. Free radical scavenging activity in different treatments ranged from 60.92 ± 4.98 to 88.82 ± 0.10 %, which levels were the highest in leaf-derived callus followed by cotyledon-derived callus. Cultivation in the light or darkness had no apparent effect on scavenging activity in leaf-derived callus. In contrast, light treatment induced a significant decrease of the scavenging activity in cotyledon-derived callus. Similar behavior was observed for the FRAP test that measures the ability of the extract to reduce Fe^{3+} to Fe^{2+} and may serve as a significant indicator of its antioxidant potential (Govindan and Muthukrishnan 2013). These findings are in line with the results reported by Ali and Abbasi (2014) in the cell culture of *Artemisia absinthium*, where dark-grown cultures showed maximum TPC, TFC, and antioxidant activity compared to light treatments. Weremczuk-Jeżyna *et al.* (2013) also reported that the hairy roots of *Dracocephalum moldavica* cultivated in the darkness showed the highest antioxidant activity compared to those cultivated under photoperiod. Very few studies about the effect of light and dark on secondary metabolites accumulation and the antioxidant activity in callus tissue culture are reported. The increased antioxidant activity under darkness in the present study could be attributed to the stressed callus culture conditions. Indeed, there is increasing evidence that some light conditions such as UV, high-intensity light, and continuous dark may act as a stressful factor requiring a change in the oxidative balance in plants which produce a large amount of scavenging enzymes and antioxidant molecules as an answer to the increased generation of reactive oxygen species (ROS), and thereby minimise potential oxidative damage (Gogorcena *et al.* 1997; Close and McArthur 2002; Ali *et al.* 2005; Pashkovskiy *et al.* 2018). Dark stress was reported to induce structural and oxidative damage in other *Fabaceae* species such as *Soybean and Phaseolus vulgaris* (Gogorcena *et al.* 1997; Wang *et al.* 2021). Our results are in good agreement with a previous report considering the antioxidant activity in intact carob leaves, pulp, and seeds (Hajaji *et al.* 2010; Lakkab *et al.* 2019; Ayache *et al.* 2020). The DPPH scavenging activity reached 61.17 %, 80.9 % and 79.4 % respectively. This antioxidant activity may be attributed to the levels of phenolic content, flavonoids, and condensed tannin.

Based on the correlation coefficient values calculated for the total phenolic components and the antioxidant assays, it could be suggested that both the scavenging activity and the reducing power of the analyzed extracts are strongly and positively correlated with the phenolic (r^2 = 0.84 and 0.98, respectively) and flavonoid accumulation (r^2 = 0.71 and 0.68, respectively) (Table 3). These results agree with the earlier reports on carob pulp extract (Benchikh *et al.* 2014; Benchikh and Louailèche 2014). In contrast, significant ($P<0.01$) negative relationships were observed of TCT tannins with TPC, TFC, DPPH, and FRAP, with correlation coefficients ranging between -0.86 and -0.73. Furthermore, the FRAP was significantly

P<0.01) correlated with DPPH ($r^2 = 0.88$) which rises the hypothesis that compounds which express reducing activity meet the criteria for exhibiting antiradical effect.

Table 2. Effects of explant type and light conditions on antioxidant compounds in callus cultures of carob (*Ceratonia siliqua* L.).

Type of explant	Light *conditions*	DPPH [%]	FRAP [OD]
Mature cotyledons	16-h Photoperiod	60.92 ± 4.98^{c}	0.29 ± 0.04^{c}
	Darkness	76.60 ± 7.14^{b}	0.38 ± 0.01^{b}
Leaves	16-h Photoperiod	86.93 ± 1.91^{a}	0.65 ± 0.02^{a}
	Darkness	88.82 ± 0.10^{a}	0.61 ± 0.01^{a}
Significance of two-way ANOVA			
Type of explants (A)		***	***
Light conditions (B)		**	NS
A×B		*	**

Values are mean ± SE. Mean values within a column followed by the same letter are not significantly different by Duncan's multiple range test (5m%). FW – fresh weight; DW – dry weight; OD – optical density; GAE – gallic acid equivalents; QE – Quercetine equivalents; CE – catechin equivalent; NS – nonsignificant; ∗, ∗∗, ∗∗∗ – significant at P≤0.05, P≤0.01, and P≤0.001, respectively (two-way ANOVA).

Table 3. Pearson's correlation coefficients of different biochemical parameters of carob calli extracts.

	TPC	TFC	TCT	DPPH
TPC	-	-	-	-
TFC	0.78**	-	-	-
TCT	-0.73**	-0.80**	-	-
DPPH	0.84**	0.71**	-0.86**	-
FRAP	0.98**	0.68*	-0.78**	0.88**

* Correlation is significant at the 0.05 level, ** Correlation is significant at the 0.01 level.

Conclusion

The present study demonstrates, for the first time, the potential of callus cultures of *Ceratonia siliqua* as a new source of phenolic compounds which possesses an important antioxidant activity. Leaves-derived calli were found to produce significantly higher levels of phenolics and showed higher antioxidant activity than the cotyledon-derived callus. However, cotyledon-derived callus accumulated more TCT. The light treatment has significantly increased total condensed tannins content, but significantly decreased the antioxidant activity in callus cultures. Moreover, a positive correlation between antioxidant activity and total phenolics and flavonoids contents were found in carob callus cultures. Further works on improving protocols for *in vitro* cultivation of carob and characterization of individual groups of bioactive components responsible for the biological activity are needed.

Conflict of Interest

The authors declare that they have no conflict of interest.

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