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Phenolic content and antioxidant activity of *in vitro* and *in vivo* grown carob (*Ceratonia siliqua* L.) plants

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Abstract

Carob (*Ceratonia siliqua* L.) is a plant with significant nutritional and medicinal properties that is widely used in the Mediterranean region. In this study, we investigated the phenolic content and antioxidant activity of extracts from *in vitro* and *in vivo* grown carob plant parts. We found that the LAC medium was more effective than MS medium in promoting the accumulation of phenolic compounds and antioxidant activity *in vitro* in leaves. Our comparative analysis of *in vivo* and *in vitro* plant samples revealed considerable variations in the levels of bioactive compounds in different parts of the carob tree. Notably, the *in vitro* leaf extracts showed a comparable production of total phenolic content compared to *in vivo* leaf extracts, indicating that *in vitro* culture techniques could be a promising alternative to produce bioactive compounds from carob. Overall, our findings provide new insights into the potential applications of plant tissue culture to produce valuable phytochemicals and highlight the need for further research to optimize the *in vitro* production of phenolics in carob.

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Introduction

Over the last few decades, the significance of plant secondary metabolites has been increasingly recognized across various scientific and industrial domains, such as pharmaceuticals, food industries, and agricultural management. These natural compounds are valued for their broad spectrum of biological activities, contributing to advancements in health, nutrition, and environmental sustainability (Meziani *et al.* 2019; Jeldi *et al.* 2022; Razzouk *et al.* 2022). This growing appreciation emphasizes their crucial role in fostering sustainable practices across industries, underscoring their importance in modern scientific and economic frameworks.

Carob (*Ceratonia siliqua* L.) is an important component of Mediterranean vegetation due to its economic and environmental significance. Its two major constituents are pulp (80 – 90 %) and seeds (10 – 20 % by weight) (Naghmouchi *et al.* 2009). Both components have a wide range of applications

in the food, pharmaceutical, and cosmetic industries (Stavrou *et al.* 2018). Carob pods and leaves are rich sources of bioactive compounds such as polyphenols, which have been linked with various health benefits (Ghanemi *et al.* 2017; Ben Othmen *et al.* 2020; Macho-González *et al.* 2020; Gregoriou *et al.* 2021; Basharat *et al.* 2023).

The integration of plant cell and organ culture technologies in recent years has revolutionized the production of these valuable secondary metabolites. This biotechnological approach not only facilitates the enhanced yield and quality of bioactive substances but also allows systematic studies comparing the biochemical profiles of tissue culture-derived and in vivo-derived plant extracts, such as those explored by Koufan *et al.* (2020).

Plant cell and organ culture technology has proven to be an efficient and attractive tool for producing valuable secondary metabolites under controlled conditions (Lozzi *et al.* 2021; Sreelekshmi *et al.* 2023; Thapa *et al.* 2023). This technology offers various benefits, such as shorter and more adaptable production cycles, independence from geographical and climatic limitations, improved extraction and purification processes, greater control over biosynthesis direction, and the ability to conserve and sustainably utilize biodiversity (Matkowski 2008; Smetanska 2018). Research suggests that changing the nutrient medium composition can significantly enhance the synthesis of phenolic metabolites (Knobloch and Berlin 1980; de Queiroz Braga *et al.* 2015; Smetanska 2018).

Comparative analyses reveal that in vitro culture technologies can achieve levels of phenolic compounds and antioxidant activity that are comparable to, or exceed, those found in intact plant tissues. This has been demonstrated in species such as *Agrania spinosa* (L.) (Koufan *et al.* 2020) and *Arnica montana* (Nikolova *et al.* 2013), highlighting the potential of these methods to replicate and enhance the natural biochemical profiles of plants.

The current study aims to conduct a comparative analysis of the total phenolic content, flavonoids, condensed tannins, and antioxidant potential of extracts from *in vitro* (leaves) and *in vivo*-derived materials of *C. siliqua* (leaves from female and male trees, pulp, and seeds). To the best of our knowledge, this study is the first to conduct comparative analysis of the phenolic content of *in vivo* and *in vitro* materials of carob.

Experimental

Plant material

Fresh pods and leaves from male and female trees of *C. siliqua* were collected from the area of El Ksiba (32° 33′ 54″ N latitude, 6° 01′ 59″ W longitude), in the Middle-Atlas Mountains of Morocco.

Culture conditions

In vitro plants were obtained as previously described (Lozzi *et al.* 2019). Nodal segments, 10 mm in length, from two-month-old seedlings previously germinated in LA medium enriched with 3 % (w/v) sucrose, 0.7 % (w/v) agar, and 0.1 % (w/v) activated charcoal, were used for axillary shoot bud induction and proliferation. These nodal segments were then transferred and maintained every 4 weeks in a liquid culture system using Murashige and Skoog basal medium (MS, 1962) and carob culture medium (LAC, 2019) both supplemented with 4.44 µM BAP (6-benzyl aminopurine), 3 % (w/v) sucrose, and 0.1 % (w/v) activated charcoal. The cultures were then kept at 26 ± 2 °C under a 16 h photoperiod (60 $\mu mol.m^{-2}.s^{-1}$).

Extraction

To prepare extracts, plant samples *in vitro* (leaves) and *in vivo* (leaves from mature female and male trees, pulp, and seeds) were dried in an oven at 60 °C to a constant weight. 0.1 gram of each finely ground sample was extracted with 70 % (v/v) acetone (40 mL) and centrifuged at 4,000 rpm for 20 min. The supernatant was collected and stored at 4 °C until use.

Total phenolics

The total phenolic content (TPC) of the *C. siliqua* extracts was determined spectrophotometrically by the Folin–Ciocalteu method using gallic acid as a

standard (Singleton and Rossi 1965). Briefly, 0.1 mL of the extract was mixed with 0.1 mL of Folin-Ciocalteu reagent and 1.4 mL of deionized water. After 6 min, the reaction was neutralized with 0.5 mL of Na_2CO_3 (20 %) and then incubated in the dark for 30 min. The absorbance was read at 750 nm, and the amount of total phenolic content was expressed as mg gallic acid equivalents (GAE) per g dry weight of the sample.

Total flavonoid

The total flavonoid content (TFC) was measured using the aluminium chloride ($AlCl_3$) assay (Lamaison *et al.* 1990). One ml of acetonic extract was added to 1 mL of $AlCl_3 \cdot 6H_2O$ (2 %). After 10 min, the absorbance was spectrophotometrically recorded at 430 nm. The results were expressed as mg of quercetine equivalents (QE) per g of dry weight of the sample.

Total condensed tannin

The total condensed tannins (TCT) were analyzed using the vanillin–HCl method (Broadhurst and Jones 1978). Each plant extract (0.1 mL) was mixed with 1.5 mL of highly concentrated HCl and 3 mL of vanillin (4 %). The absorbance was read at 500 nm after incubation for 20 min in a dark room. The yield of total condensed tannin was expressed as mg of catechin equivalent (CE).

DPPH radical-scavenging assay

The free radical scavenging capacity was evaluated spectrophotometrically using the 2,2-diphenyl-1-picryl hydrazyl (DPPH) assay (Brand-Williams *et al.* 1995). Briefly, one ml of a 0.1 mM ethanolic solution of DPPH was mixed with 0.5 ml of carob extract diluted in 1.4 ml of ethanol and incubated for 30 min in the dark. Absorbance was measured at 515 nm. The percentage of DPPH scavenging activity was calculated according to the following equation: scavenging activity (%) = (Absorbance control-Absorbance sample)/ Absorbance control ×100.

Ferric-reducing power (FRAP)

The reducing power of the extract was measured according to the method reported by (Oyaizu 1986). 0.22 mL of sample solution was added to 0.25 mL of potassium ferricyanide (1 %) and 0.25 ml of phosphate buffer (0.2 M, pH 6.6). After 20 min of dark incubation at 50 °C, 0.25 mL of trichloroacetic acid (10 %) was added. Finally, the above mixture was mixed with 0.25 mL of iron trichloride (0.1 %) and 1 mL of distilled water, and the absorbance was taken at 700 nm. An increased absorbance indicates an increased reducing power of the extract.

Statistical analysis

The results are expressed as mean ± standard deviation (replicated 3 times). Data were analyzed with the analysis of variance (ANOVA) method, and Duncan's multiple range test (DMRT) at 5 % was used to examine significant differences. Correlation analysis was performed by the Pearson correlation method.

Table 1. Effects of culture medium composition on phenolic compounds and antioxidant activity of *C. siliqua in vitro* leaves.

Mineral composition	TPC [mg.GAE.g^{-1} DW]	TFC [mg.QE.g^{-1} DW]	TCT [mg CE g^{-1} DW]	DPPH [%]	FRAP [OD]
LAC	42.31 ± 3.95^{a}	18.21 ± 1.04^{a}	0.22 ± 0.01	85.69 ± 0.98^{a}	0.89 ± 0.06^{a}
MS	28.81 ± 1.82^{b}	12.70 ± 0.91^{b}	0.20 ± 0.01	67.68 ± 3.38^{b}	0.74 ± 0.06^{b}
Significance of two-way ANOVA	**	*	NS	***	*

Values are mean ± SE (standard error). Mean values within a column followed by the same letter are not significantly different by Duncan's multiple range test (5 %). FW, fresh weight, DW, dry weight, OD, optical density, GAE gallic acid equivalents, QE, quercetine equivalents, CE, catechin equivalent, NS, non-significant, *, **, ***: significant at $P \leq 0.05$, $P \leq 0.01$, and $P \leq 0.001$, respectively (two-way ANOVA).

Results and discussion

Effects of culture medium composition on phenolic compounds and antioxidant activity of C. siliqua in vitro leaves

The nutrient composition of culture media is a crucial determinant in shoot growth and bioactive compounds accumulation (Espinosa-Leal *et al.* 2018; Smetanska 2018; Ríos-Ríos *et al.* 2019). In this experiment, we aimed to evaluate the effect of two different culture media on the accumulation of polyphenols and antioxidant activity in carob *in vitro* leaves. The two-culture media used were MS, which is the most used medium for carob micropropagation, and LAC medium, which we have previously developed and successfully applied in our research on carob (Lozzi *et al.* 2019). By comparing the results obtained from the two media, we aimed to determine the optimal nutrient composition for promoting shoot growth and bioactive compounds accumulation in carob *in vitro* leaves.

The obtained results indicate that LAC medium stimulates bioactive compounds accumulation in the *in vitro* leaves of *C. siliqua* compared to MS medium. The total phenolic (TPC) and flavonoid (TFC) production in grown leaves were significantly higher in LAC medium (42.31 mg GAE g-1 DW and 18.21 mg.QE.g^{-1} DW) compared to MS medium (28.81 ± 1.82 mg GAE.g^{-1} DW and 12.7 mg.QE.g^{-1} DW). The total condensed tannin content (TCT) was not found to be significantly different between the two-culture media. Additionally, the LAC medium showed significantly higher DPPH radical scavenging activity (85.69 %) and ferric reducing antioxidant power (0.89 OD) in grown leaves compared to MS medium (Table 1), indicating that LAC medium may promote higher antioxidant activity in carob *in vitro* leaves.

The observed enhancement of phenols accumulation and antioxidant activity in plants grown in LAC medium can be attributed to the specific composition of ions present in the medium. The higher concentrations of Mg^{2+}, Ca^{2+}, K^{+}, a higher NO_3^-/NH_4^+ ratio, and a lower concentration of nitrogen in LAC medium in comparison to MS medium likely played a role in this effect. This is supported by previous studies that have demonstrated that changes in the concentration of these ions in the medium can affect the biosynthesis of phenolic compounds in different plant species (Dodds and Roberts 1985; Ngadze *et al.* 2014; Farzadfar *et al.* 2017; Magangana *et al.* 2018; Marlin *et al.* 2022).

Comparative analysis of the in vivo and in vitro materials

Bioactive compounds are known to exhibit a non-uniform distribution across various plant tissues or organs. Notably, substantial variations in the levels of these molecules are observed among different parts of the carob tree, including leaves, pods, seeds, and sapwood (Stavrou *et al.* 2018). Fig. 1 presents the results of a comparative analysis of the antioxidant compounds present in different *in vivo* plant samples (leaves, pulp, and seeds) and *in vitro* leaves of *C. siliqua*.

The results indicate statistically significant differences in the phenolic compounds present in the different plant samples. Carob extracts were revealed to be good sources of phenolic compounds, with a complex profile based on TPC, TFC, and TCT. Total phenolic content of plant extracts varied from 22.53 to 47.17 mg.GAE.g^{-1} which was highest in male tree leaf extracts, followed by *in vitro* leaf and *in vivo* female leaf extracts (42.31 and 37.72, respectively), and lowest in seed extracts. In the case of TFC, values ranged from 6.30 to 32.89 mg.QE.g^{-1} of sample. *In vivo* leaf extracts showed the highest phenolic content, followed by *in vitro* leaf extracts (21.54 QE.g^{-1}). The lowest results were shown in both seed and pulp extracts (8.5 and 6.3 QE g^{-1} respectively). Nevertheless, seed extract exhibited a significantly higher amount of TCT (0.45 mg.CE.g^{-1}) which was about 2 – 2.5-fold than all other plant extracts except male tree leaf extract, which accumulated 0.3 mg.CE.g^{-1} of sample. Interestingly, in the present study *in vitro*, leaf extract accumulated about 2 – 6 and folds and 4 – 6 folds more TPC and TFC, respectively, compared to the results of our previous study using callus cultures of carob (Lozzi *et al.* 2021).

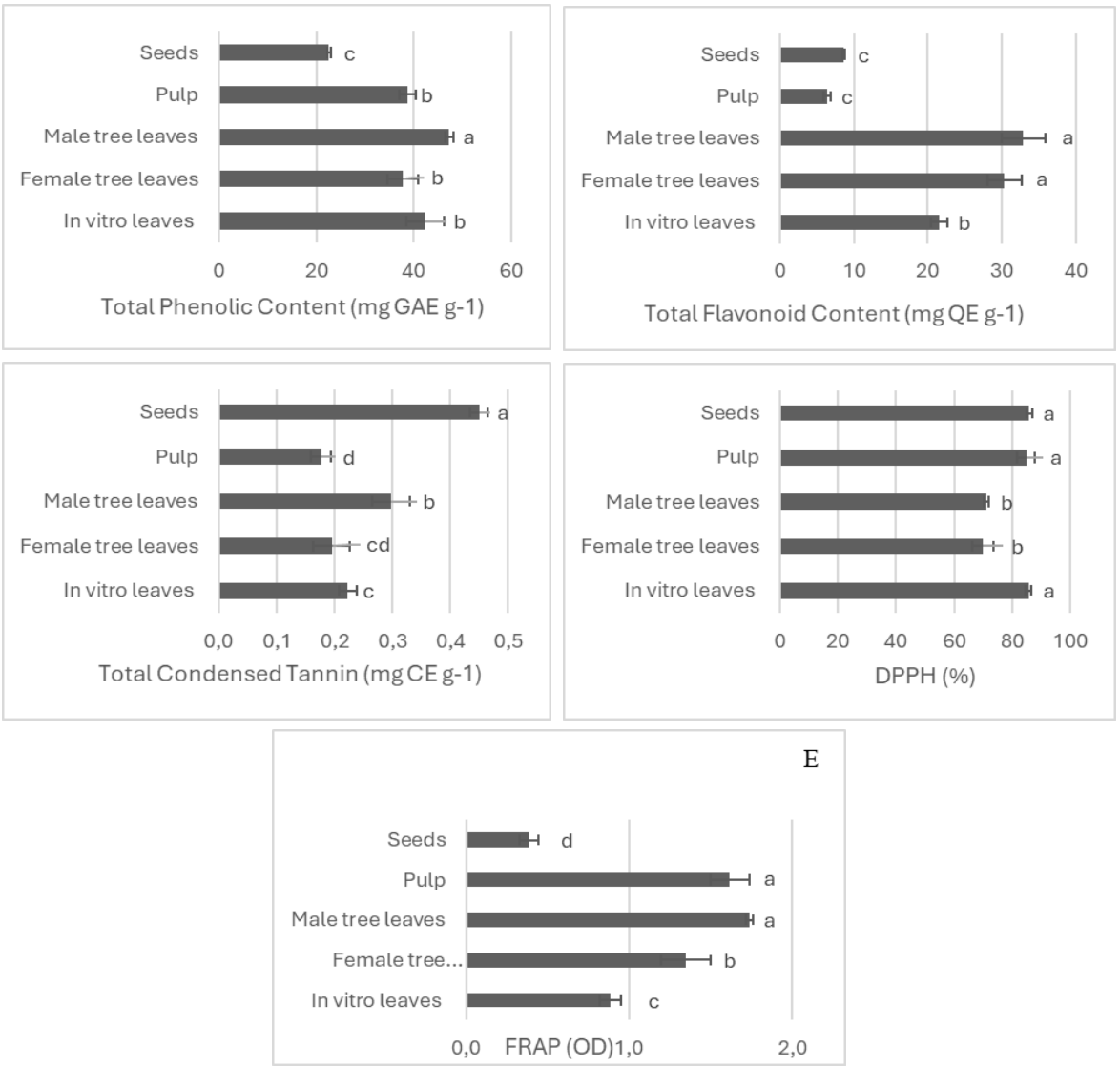

Fig 1. Antioxidant compounds of acetonic extracts materials from *in vitro* and *in vivo* plant samples of *C. siliqua;* **A** – total phenolic content; **B** – total flavonoid content; **C** – total condensed tannin; **D** – DPPH radical-scavenging assay; **E** – ferric-reducing power (FRAP). Mean values within a column followed by the same letter are not significantly different by Duncan's multiple range test (5 %). OD, optical density, GAE, gallic acid equivalents, QE, quercetin equivalents, CE, catechin equivalent.

High phenolics production by micropropagated plants compared to calluses might be due to the dedifferentiation process in callus cultures, which could potentially lead to a decline in their production capacity (George 2008). Additionally, isolated parts of the plant may not have the capability to synthesize certain compounds that can only be produced by the whole plant (Grzegorczyk *et al.* 2007).

Generally, *in vitro* leaf extracts showed comparable production of total phenolic content to *in vivo* leaf extracts, suggesting that *in vitro* culture techniques may offer a promising alternative to produce bioactive compounds from carob. However, distinct differences in secondary metabolite profiles across plant parts were observed: seeds displayed markedly higher levels of condensed tannins, and although the pulp had lower flavonoid content, it still contributed significantly to the overall phenolic profile of the plant. This suggests variability in the biosynthesis of these compounds, reflecting the unique biochemical environments of each plant part. On the other hand, the total flavonoid content and condensed tannin levels were notably lower in *in vitro* leaves compared to those extracted from *in vivo* leaves. Similar behaviour was observed in *in*

vitro culture extracts of *Arnica montana* (Nikolova *et al.* 2013) and *Trifolium pretense* (Esmaeili *et al.* 2015) where low rates of phenolic compounds were detected.

The observed variation in secondary metabolite content is likely attributable to the differences in growth conditions and developmental stages of the plant tissues. Nonetheless, the production rates of secondary metabolites in *in vitro* cultures can be effectively improved through the modulation of various factors that influence synthesis in plant tissues, including the adjustment of environmental conditions, the use of precursors and plant growth regulators, and the selection of high-yielding materials (Biswal *et al.* 2022; Hazrati *et al.* 2022; Aksenova *et al.* 2023). On the other hand, in the present investigation, micropropagated plants produced a significantly higher amount of TPC and TFC compared to others studied *in vivo* plant samples (pulps and seeds).

Another important aspect observed in this study was the influence of the gender of the tree on phenolic compounds production. We found that TPC and TCT were markedly higher in the male tree. While there was no significant difference in TFC between extracts from female and male trees. Working with the same species, (Custódio *et al.* 2009) observed different levels of phenolic compounds between extracts from different Portuguese carob tree genders and different cultivars. Similar results were found in other plant species such as *Pistacia chinensis* (Zhang *et al.* 2016), *Pistacia atlantica* (Ben Ahmed *et al.* 2017) and *Populus cathayana* (He *et al.* 2021).

In addition to the determination of phenolic contents, we analyzed the antioxidant activity of the extracts using DPPH and FRAP assays scavenging activity of the extracts. Based on the data presented in Fig. 1, it is evident that all extracts exhibit a significant level of DPPH scavenging activity, which ranged from 70 to 86 %. This suggests that they have the potential to scavenge free radicals and may be effective in preventing the initiation of chain reactions mediated by free radicals. The best antiradical efficiency was detected in extracts from *in vitro* leaves, pulp, and seeds (85.7, 85, and 86 % respectively), while *in vivo* leaves showed significantly lower results (70 – 71.1%).

In contrast, the FRAP assay results showed a different trend, with the male tree leaf extract exhibiting the highest antioxidant activity (1.74 OD), followed by the pulp extract (1.62 OD). The female tree leaf extract also showed a high FRAP value (1.35 OD), while the *in vitro* leaf and seed extracts had lower FRAP values of 0.89 and 0.38 OD, respectively. These results suggest that different parts of the carob tree possess varying levels of antioxidant activity, which may be attributed to the presence of different phytochemicals in each extract.

Correlation between phenolics content and antioxidant activities

Table 2 presents the correlation between TPC, TFC, TCT, and antioxidant activity for the four types of carob extract: *in vitro* leaves, *in vivo* leaves, pulp, and seeds. The results showed a strong positive correlation between TPC, TFC, and antioxidant activity measured by DPPH and FRAP assays in *in vitro* leaf extracts ($r = 0.947^{**}$ and 0.785, for TPC; $r = 0.931^{**}$ and 0.939^{**} for TFC, respectively), in agreement with our previous study where significant correlations were obtained in carob callus extracts (Lozzi *et al.* 2021).

Table 2. Correlation coefficient between TPC, TFC, TCT and the antioxidant activity of carob extract.

	***In vitro* leaves**		***In vivo* leaves**		**Pulp**		**Seeds**	
	DPPH	**FRAP**	**DPPH**	**FRAP**	**DPPH**	**FRAP**	**DPPH**	**FRAP**
TPC	0.947^{**}	0.787	0.533	0.909^{*}	-0.337	0.476	0.992^{**}	-0.883
TFC	0.931^{**}	0.939^{**}	0.611	0.333	-0.815	0.718	-0.941^{*}	0.956
TCT	0.770	0.533	0.099	0.704	-0.442	0.573	0.954	-0.984

∗, ∗∗: significant at $P \leq 0.05$ and $P \leq 0.01$, respectively (two-way ANOVA).

The results showed a strong positive correlation between TPC, TFC, and antioxidant activity measured by DPPH and FRAP assays in *in vitro* leaf extracts (r = 0.947** and 0.785, for TPC; r = 0.931** and 0.939** for TFC, respectively), in agreement with our previous study where significant correlations were obtained in carob callus extracts (Lozzi *et al.* 2021). Whereas TCT exhibited a moderately positive correlation with DPPH scavenging activity (r = 0.77) and FRAP activity (r = 0.533). On the other hand, *in vivo* leaf extracts showed a strong positive correlation between TPC and the FRAP assay (r = 0.909**) but only a moderate correlation with DPPH (r = 0.533). In contrast, TFC showed a moderate correlation with DPPH (r = 0.611) and a weaker link with FRAP (r = 0.333). Pulp extracts exhibited no significant correlation between TPC and both DPPH and FRAP, with a mild positive correlation for FRAP (r = 0.476) and a weak negative correlation with DPPH (r = -0.337). However, TFC displayed a strong negative correlation with DPPH (r = -0.815) and a positive correlation with FRAP (r = 0.718). In carob pulp extracts, total phenolic content (TPC) shows limited influence on radical scavenging, with a slight negative correlation with DPPH (r = -0.337) and a mild positive correlation with FRAP (r = 0.476). Total Flavonoid Content (TFC) and Total Condensed Tannins (TCT) both negatively correlate with DPPH (r = -0.815 for TFC, r = -0.442 for TCT), indicating less effectiveness in radical scavenging, yet both exhibit positive correlations with FRAP (r = 0.718 for TFC, r = 0.573 for TCT). A high correlation between TFC and FRAP assay was also reported by Ydjedd *et al.* (2017) in the case of ripe (r = 0.941*) and unripe (r = 0.954**) carob pulp.

Seeds displayed contrasting antioxidant responses. TPC correlated strongly positively with DPPH (r = 0.992**) and negatively with FRAP (r = -0.883). Both TFC and TCT exhibited similar behaviors, showing negative correlations with DPPH (r = -0.941* for TFC and r = -0.984 for TCT) and positive correlations with FRAP (r = 0.956 for TFC and r = 0.954 for TCT).

These findings suggest that phenolic compounds significantly contribute to the antioxidant activity observed in carob extracts. However, instances of weak correlation, particularly noted in the pulp extracts, could be attributed to the presence of other nonphenolic compounds that also enhance the antioxidant capacity (Ydjedd *et al.* 2017). The contrasting responses in seed extracts could be influenced by genetic and environmental factors (Fidrianny *et al.* 2018) or by the specific molecular structures of the phenolic compounds, which variably affect their antioxidant activities (Rangani *et al.* 2023). These compounds might not act independently but could interact synergistically or antagonistically with other compounds within the seeds, thereby affecting their overall antioxidant capacity (Kandasamy *et al.* 2023). For instance, flavonoids in carob extracts can act as proton or electron donors, which is conducive to reducing power, as observed in the FRAP assay. However, their ability to stabilize radicals as required in the DPPH assay might be limited by the presence of other compounds or by the specific configuration of the flavonoids (Figueroa *et al.* 2014).

Conclusions

Our study highlights the role of culture medium composition in the accumulation of bioactive compounds in *in vitro* carob extracts. Our findings indicate that LAC medium is more effective than MS medium in promoting the accumulation of phenolic compounds and antioxidant activity. Additionally, our comparative analysis of *in vivo* and *in vitro* plant samples showed significant variations in the levels of bioactive compounds across different parts of the carob tree. Notably, *in vitro* leaf extracts demonstrated comparable production of total phenolic content to *in vivo* leaf extracts, indicating the potential of micropropagation to produce plant-based bioactive compounds in carob, which could be exploited for various industrial and pharmaceutical applications. Future research should focus on characterizing individual groups of phenolics from *in vitro* culture samples.

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