**Running title:** Antioxidants from *in vivo* and *in vitro Coriandrum sativum*

# Comparative study of lipophilic and hydrophilic antioxidants from *in vivo* and *in vitro* grown *Coriandrum sativum*

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**Abbreviations**

| | |
|---|---|
| **CAE** | Chlorogenic acid equivalents |
| **DPPH** | 2,2-Diphenyl-1-picrylhydrazyl |
| **HPLC** | High-performance liquid chromatography |
| **IS** | Internal standard |
| **ME** | Malvidin 3-glucoside equivalents |
| **MS** | Mass spectrometry |
| **QE** | Quercetin equivalents |
| **RI** | Refraction index |
| **TBARS** | Thiobarbituric acid reactive substances |

**Abstract**

Coriander is commonly used for medicinal purposes, food applications, cosmetics and perfumes. Herein, the production of antioxidants in vegetative parts (leaves and stems) of *in vivo* and *in vitro* grown samples was compared. *In vitro* samples were clone A- with notorious purple pigmentation in stems and leaves and clone B- green. Seeds were also studied as they are used to obtain *in vivo* and *in vitro* vegetative parts. Lipophilic (tocopherols, carotenoids and chlorophylls) and hydrophilic (sugars, ascorbic acid, phenolics, flavonols and anthocyanins) compounds were quantified. The antioxidant activity was evaluated by radical scavenging activity, reducing power and lipid peroxidation inhibition. The *in vivo* sample showed the highest antioxidant activity mainly due to its highest levels of hydrophilic compounds. Otherwise, *in vitro* samples, mainly clone A, gave the highest concentration in lipophilic compounds but a different profile when compared to the *in vivo* sample. Clones A and B revealed a lack of β-carotene, β- and δ-tocopherols, a decrease in α-tocopherol, and an increase in γ-tocopherol and clorophylls in comparison to the *in vivo* sample. *In vitro* culture might be useful to explore the plants potentialities for industrial applications, controlling environmental conditions to produce higher amounts of some bioactive products.

**Keywords** Antioxidants; *Coriandrum sativum*; *In vitro* culture; Lipid peroxidation inhibition; Micropropagation; Radical scavenging activity

## Introduction

Antioxidant activity prevents the formation of free radicals, decreasing oxidative stress, and avoiding destruction of molecules like proteins, DNA and lipids. This activity has been related with anti-inflammatory processes and with the prevention of some human chronic diseases including Alzheimer, cancer, diabetes and cardiovascular disease [1-3]. The antioxidants obtained from herbs could play an important role in the prevention of those situations acting as reducing agents, free radical scavengers or synglet oxygen quenchers [4].

Coriander (*Coriandrum sativum* L.) is an annual herb that can be used to enhance food flavor and organoleptic quality, and also as source of natural antioxidants in order to substitute synthetic ones according to the global market demands [5-9]. Nowadays coriander is extensively cultivated in India, Russia, Morocco and Central Europe, mainly for its seeds that are subsequently transformed into powder to be used in food industry [3,9]. Besides food applications, its secondary metabolites including antioxidants can be used in cosmetics, perfumes and for medicinal purposes due to anti-endemic, anti-inflamatory, antidiabetic and antihypertensive properties [4,9].

*In vitro* culture of plant tissue might be very useful to explore the potentialities of an herb for industrial applications (such as for production of specific metabolites) to obtain numerous copies of the same sample. Nevertheless, to produce reliable results a sterile and controled environment must be mantained, and this biotechnological methodology might be a time-consuming process [10]. In the present study, the production of lipophilic and hydrophilic metabolites (e.g. antioxidants) by *in vivo* (commercial) and *in vitro* (Clones A and B) grown coriander samples was compared. Furthermore, seeds

were also studied since they constitute the starting material for *in vivo* or *in vitro* vegetative parts.

## Material and Methods

### Plant material

Vegetative parts and seeds of coriander (**Fig. 1A** and **B**) were obtained in a local supermarket. For the *in vitro* culture, the seeds were sterilized with sodium hypochlorite containing Tween 20 (2 drops/10 ml of sodium hypochlorite) for 10 min with agitation. After being filtered and washed with sterilized water, the seeds were treated with ethanol 70% for 5 min, washed again with sterilized tap water and dried over sterile filter paper. The seeds were then inoculated in a basic germination medium with 0.9% agar and kept at 25°C, in darkness, for one week. The seedlings were transferred from the germination medium to a modified culture medium, MS/2 with 1/2 of Murashige and Skoog [11] macronutrients, l mg/l thiamine, 1 mg/l nicotinic acid, 1 mg/l pyridoxine, 2% sucrose, 0.1 mg/l IBA (indolbutiric acid) and 0.1 mg/l BAP (benzilaminopurine). The pH of the culture medium was adjusted to 5.7 before autoclaving. The culture conditions were $T_{min}$. [16-19] ºC, $T_{max}$ [23-26] ºC, photoperiod of 16/8 h (light/dark) supplied by light-bulbs Silvana day light (Phillips, Amsterdam, Netherlands). After 5 weeks, the shoots were excised from the young plants and inoculated in a modified culture medium, MS with 0.1 mg/l IBA and 0.1 mg/l BAP, pH 5.5. They were kept in the same culture conditions and subculture occurred every 4 weeks. After 6 months in culture, two clones were differentiated (with phenotypical

stability): clone A showing a notorious purple pigmentation on vegetative parts (leaves and stems), and clone B being just green (**Fig. 1C** and **D**).

Coriander seeds and *in vivo* as well as *in vitro* grown plants (clones A and B) were lyophilised and reduced to a fine dried powder (20 mesh) for further analyses.

## Standards and Reagents

Acetonitrile 99.9%, *n*-hexane 95%, and ethyl acetate 99.8% for HPLC grade were obtained from Lab-Scan (Lisbon, Portugal). Ascorbic acid, tocopherols and sugars standards, Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), chlorogenic acid, quercetin, and malvidin 3-glucoside were purchased from Sigma (St. Louis, USA). Racemic tocol, 50 mg/ml, was purchased from Matreya (Chalfont, USA). 2,2-Diphenyl-1-picrylhydrazyl (DPPH) was obtained from Alfa Aesar (Ward Hill, USA). All other chemicals and solvents were of analytical grade and purchased from common sources. Water was treated in a Milli-Q water purification system.

## Lipophilic compounds

Tocopherols content was determined by high performance liquid chromatography HPLC; Knauer, Berlin, Germany) coupled to a fluorescence detector, using *rac*-tocol as internal standard [12]. β-carotene, lycopene, chlorophylls a and b were determined measuring the absorbance at 453, 505, 645, 663 nm [13].

## Hydrophilic compounds

Free sugars were determined by HPLC coupled to a refraction index detector (RI) using melezitose as internal standard [12].

Ascorbic acid was determined by the 2,6-diclorophenolindophenol assay, on the basis of the calibration curve of authentic L-ascorbic acid, as previously described by the authors [14].

For phenolics determination, a fine dried powder (20 mesh; ~1 g) was extracted by stirring with 50 ml of methanol at 25 ºC at 150 rpm for 12 h and filtered through Whatman No. 4 paper. The residue was then extracted with one additional 50 ml portion of methanol. The combined methanolic extracts were evaporated at 35 ºC under reduced pressure, re-dissolved in methanol at a concentration of 40 mg/ml, and stored at 4 ºC for further use. The extract sample at a known concentration (250 µl) was mixed with HCl 0.1% in 95% ethanol (250 µl) and HCl 2% (4550 µl). After 15 min the absorbance was measured at 280, 360, and 520 nm. The absorbance at 280 nm was used to estimate total phenolic content (mg of chlorogenic acid equivalents (CAE) per g of extract), $A_{360\ nm}$ was used to estimate flavonols (mg of quercetin equivalents (QE) per g of extract), and $A_{520\ nm}$ was used to estimate anthocyanins (mg of malvidin 3-glucoside equivalents (ME) per g of extract) [15].

Antioxidant activity assays

The antioxidant activity of the methanolic extracts was evaluated by DPPH radical-scavenging activity, reducing power, inhibition of β-carotene bleaching in the presence of linoleic acid radicals and inhibition of lipid peroxidation using TBARS in brain

homogenates [12]. The extract concentrations providing 50% of antioxidant activity or 0.5 of absorbance ($EC_{50}$) were calculated from the graphs of antioxidant activity percentages (DPPH, β-carotene bleaching and TBARS assays) or absorbance at 690 nm (reducing power assay) against extract concentrations. Trolox was used as standard.

Statistical analysis

The results are expressed as mean values and standard deviation (SD). The results were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's HSD test with $\alpha = 0.05$. This treatment was carried out using SPSS v. 16.0 software.

## Results and Discussion

Composition in lipophilic compounds (tocopherols, carotenoids and chlorophylls) of coriander seeds and *in vivo* and *in vitro* grown samples is shown in **Table 1**. β- and δ-Tocopherols were not detected in *in vitro* samples. Otherwise, it was observed a significant decrease in α-tocopherol and a significant increase in γ-tocopherol levels in those samples, mostly in clone A (13 mg/100 g dry weight- dw). *In vivo* samples revealed the highest tocopherols concentration (26 mg/100 g dw), mainly due to the contribution of α-tocopherol (23 mg/100 g dw). Coriander from Malaysia revealed higher α-tocopherol levels (53 mg/100 g dw; data calculated from fresh weight (fw) and moisture content available), but lower δ-tocopherol (0.1 mg/100 g dw) and γ-tocopherols (0.7 mg/100 g dw) levels [1]. Otherwise, Marero et al. [16] reported almost no tocopherols in coriander. Tocopherols inhibit lipid peroxidation processes of polyunsaturated fatty acids and other compounds in cell membranes, mainly due to their lipid soluble antioxidant properties [15].

β-Carotene was only found *in vivo* samples (99 mg/100 g dw), while lycopene was quantified in all the samples. The highest levels were obtained for *in vitro* grown samples, mostly in clone A. Different authors reported lower β-carotene concentration in coriander from India (47 mg/100 g dw; data calculated from fw and moisture content available), estimated colorimetrically after chromatographic separation [17], Malaysia (56 mg/100 g dw; data calculated from fw and moisture content available) [1] and Israel (10 mg/100 g dw) [2], quantified by HPLC. Furthermore, Guerra et al. [18] also found β-carotene in lower levels using TLC (thin layer chromatography) analysis (33 mg/100 g dw). It has been proved that carotenoids are related to antioxidant effects, increasing provitamin A activity and immunomodulation being very important in the prevention of some human chronic diseases [2].

Chlorophylls were the major lipophilic compounds, and as expected they were found in higher concentration *in vivo* grown samples than in seeds. Once more, *in vitro* grown samples mainly clone A, revealed higher levels than *in vivo* samples. This might be due to controlled conditions related to light/dark and temperature of growth established for *in vitro* culture. It has been suggested a pro-oxidant activity of chlorophylls under light probably due to an energy transfer of singlet-excited chlorophyll to oxygen that would form reactive oxygen species. However, it was also reported that chlorophylls provide protection by preventing auto-oxidation of vegetable edible oils stored in the dark and suggested a hydrogen donating mechanism as radical chain reactions breaker. In addition, the authors stated that the intact chemical structure of porphyrin seems to be essential for antioxidant activity [19].

Data on hydrophilic compounds (sugars, ascorbic acid, phenolics, flavonols and anthocyanins) are also shown in **Table 1**. Reducing sugars such as glucose

(monosaccharide) and raffinose (trisaccharide) might act as antioxidants, but non reducing sugars such as trehalose (disaccharide) have been related to suppression of auto-oxidation of unsaturated fatty acids [20]. Raffinose was not detected neither *in vivo* sample nor in clone A. Coriander seeds revealed the lowest concentration in total sugars (2 g/100 g dw) due to the contribution of sucrose and raffinose. Otherwise, *in vivo* grown coriander presented the highest sugars concentration (15 g/100g dw), followed by the *in vitro* grown samples.

Ascorbic acid was found in all the samples, presenting coriander seeds the highest concentration (55 mg/100g dw); clone B revealed higher concentration than clone A. Other authors have previously reported ascorbic acid contents in coriander from Malaysia (35.2 mg/g fw) [1] and India (98.1 mg/100 g fw) [17] determined by HPLC and indophenol assay, respectively.

Vegetative parts (*in vivo or in vitro* grown samples) revealed higher concentration in phenolics and flavonols than seeds. Other authors quantified total phenolics in coriander from Malaysia using Folin-Ciocalteu method (4 mg gallic acid equivalents/g fw) [1]. Furthermore the phenolic profile (flavones, flavonols, and flavanones) of a sample from Denmark was also obtained by HPLC-MS (high performance liquid chromatography-mass spectrometry) [21]. Phenolic compounds are related to oxidative stress, having the ability to quench radical reactions ensuring the stability and nutritional characteristics of the plant product [4]. Phenolics and flavonols levels were found in similar amounts in coriander grown *in vivo* and *in vitro*, indicating that both environments conduct to their biosynthesis. Concerning anthocyanins, it was observed a significant difference from *in vivo* samples (70 mg ME/g extract) to *in vitro* grown samples. Furthermore, clone A

revealed higher levels (48 mg ME/g extract) than clone B (37 mg ME/g extract), which can be related to the purple pigmentation found in this sample (**Fig. 1C**).

Due to the complexity of oxidative process, the antioxidant capacity was acessed by DPPH radical scavenging activity, reducing power, β- carotene bleaching inhibition and lipid peroxidation inhibition assays, and data related to those analyses are shown in **Table 1** and **Fig. 2**.

Polar (methanolic) extracts of *in vivo* samples presented the highest antioxidant activity, with the lowest $EC_{50}$ values for DPPH scavenging activity (0.18 mg/ml), reducing power (1.21 mg/ml) and TBARS inhibition (0.11 mg/ml), which is in agreement with its highest contents in sugars, phenolics, flavonols and anthocyanins. Among *in vitro* samples, clone B showed higher antioxidant potential than clone A. Nevertheless, the latter sample revealed the highest β-carotene bleaching inhibition capacity ($EC_{50}$ value 0.30 mg/ml). The methanolic extracts of *in vivo* samples revealed higher DPPH scavenging activity than ethanolic extracts of leaves from Norway (0.4 mg/ml) [8]. Nevertheless, the results obtained for seeds were similar (0.5 mg/ml) [8].

Overall, the profiles of lipophilic compounds, mainly antioxidants, found in *in vivo* and *in vitro* grown samples were different. Clone A and B revealed a lack of β-carotene, β- and δ-tocopherols, a decrease in α-tocopherol, but an increase in γ-tocopherol and clorophylls in comparison to *in vivo* samples. Curiously, in previous works we also observed an increase in γ-tocopherol levels in ectomycorrhizal fungi mycelia produced *in vitro* when compared to their fuiting bodies [22]. Globally, the concentration of lipophilic compounds was higher in *in vitro* grown samples, mostly in clone A.

*In vivo* sample proved to have the highest antioxidant activity, maybe due to its highest concentration of hydrophilic compounds, mainly antioxidants, such as sugars, phenolics, flavonols and anthocyanins. Moreover clone A is richer in colourfol pigments such as anthocyanins and lycopene, than clone B. Besides the differences herein reported, further studies must be performed in order to understand the conditions that conduct to different phenotipical development of *in vitro* grown samples.

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**Table 1** Lipophilic and hydrophylic compounds, and antioxidant activity $EC_{50}$ values of coriander: seeds, *in vivo* and *in vitro* grown samples.

| | Seeds | *In vivo* | *In vitro* | |
|---|---|---|---|---|
| | | | clone A | clone B |
| Lipophilic compounds | | | | |
| α-tocopherol (mg/100 g dw) | 0.09 ± 0.00 c | 22.57 ± 0.98 a | 1.47 ± 0.05 b | 1.25 ± 0.02 cb |
| β-tocopherol (mg/100 g dw) | nd | 0.25 ± 0.01 | nd | nd |
| γ-tocopherol (mg/100 g dw) | 0.42 ± 0.09 d | 2.79 ± 0.43 c | 12.96 ± 0.42 a | 8.84 ± 0.03 b |
| δ-tocopherol (mg/100 g dw) | 0.68 ± 0.09 a | 0.64 ± 0.19 a | nd | nd |
| Total tocopherols (mg/100 g dw) | 1.19 ± 0.00 d | 26.25 ± 1.21 a | 14.43 ± 0.47 b | 10.09 ± 0.05 c |
| β-carotene (mg/100 g dw) | nd | 98.74 ± 17.57 | nd | nd |
| Lycopene (mg/100 g dw) | 5.11 ± 1.16 d | 38.84 ± 4.18 c | 145.64 ± 4.73 a | 79.97 ± 3.64 b |
| Chlorophyll a (mg/100 g dw) | 9.97 ± 2.15 d | 508.51 ± 5.64 c | 519.94 ± 3.53 b | 534.14 ± 1.57 a |
| Chlorophyll b (mg/100 g dw) | 13.87 ± 3.18 d | 233.91 ± 17.33 c | 770.42 ± 21.33 a | 422.49 ± 28.58 b |
| Hydrophilic compounds | | | | |
| Fructose (g/100 g dw) | nd | 4.33 ± 0.24 a | 3.64 ± 0.45 a | 3.54 ± 0.37 a |
| Glucose (g/100 g dw) | nd | 5.22 ± 0.23 b | 6.67 ± 0.92 a | 6.32 ± 0.29 ba |
| Raffinose (g/100 g dw) | 1.32 ± 0.06 a | nd | nd | 0.86 ± 0.14 b |
| Sucrose (g/100 g dw) | 0.35 ± 0.00 c | 4.03 ± 0.08 a | 0.56 ± 0.01 b | 0.18 ± 0.02 d |
| Trehalose (g/100 g dw) | nd | 0.88 ± 0.03 b | 1.39 ± 0.01 a | 0.35 ± 0.00 c |
| Total sugars (g/100 g dw) | 1.67 ± 0.06 c | 14.46 ± 0.35 a | 12.26 ± 1.37 b | 11.25 ± 0.83 b |
| Ascorbic acid (mg/100 g dw) | 54.78 ± 0.46 a | 15.91 ± 2.20 d | 20.22 ± 2.37 c | 52.35 ± 0.70 b |
| Phenolics (mg CAE/g extract) | 82.81 ± 1.85 c | 223.07 ± 6.77 ba | 231.33 ± 6.64 a | 214.65 ± 10.98 b |
| Flavonols (mg QE/g extract) | 44.60 ± 1.73 b | 90.70 ± 2.10 a | 90.50 ± 3.96 a | 89.14 ± 2.49 a |
| Anthocyanins (mg ME/g extract) | 16.63 ± 2.28 d | 70.04 ± 1.59 a | 48.04 ± 6.86 b | 36.93 ± 4.21 c |
| Antioxidant activity | | | | |
| DPPH scavenging activity (mg/ml) | 0.51 ± 0.02 c | 0.18 ± 0.01 d | 1.10 ± 0.01 a | 0.98 ± 0.00 b |
| Reducing power (mg/ml) | 3.44 ± 0.20 a | 1.21 ± 0.02 c | 2.10 ± 0.19 b | 2.05 ± 0.26 b |
| β-carotene bleaching inhibition (mg/ml) | 0.54 ± 0.03 b | 0.47 ± 0.08 c | 0.30 ± 0.01 d | 1.06 ± 0.07 a |
| TBARS inhibition (mg/ml) | 0.10 ± 0.03 c | 0.11 ± 0.05 c | 0.29 ± 0.01 a | 0.20 ± 0.03 b |

dw- dry weight; nd- not detected. In each row, different letters mean significant differences ($p$<0.05); Mean ± SD; n=9.

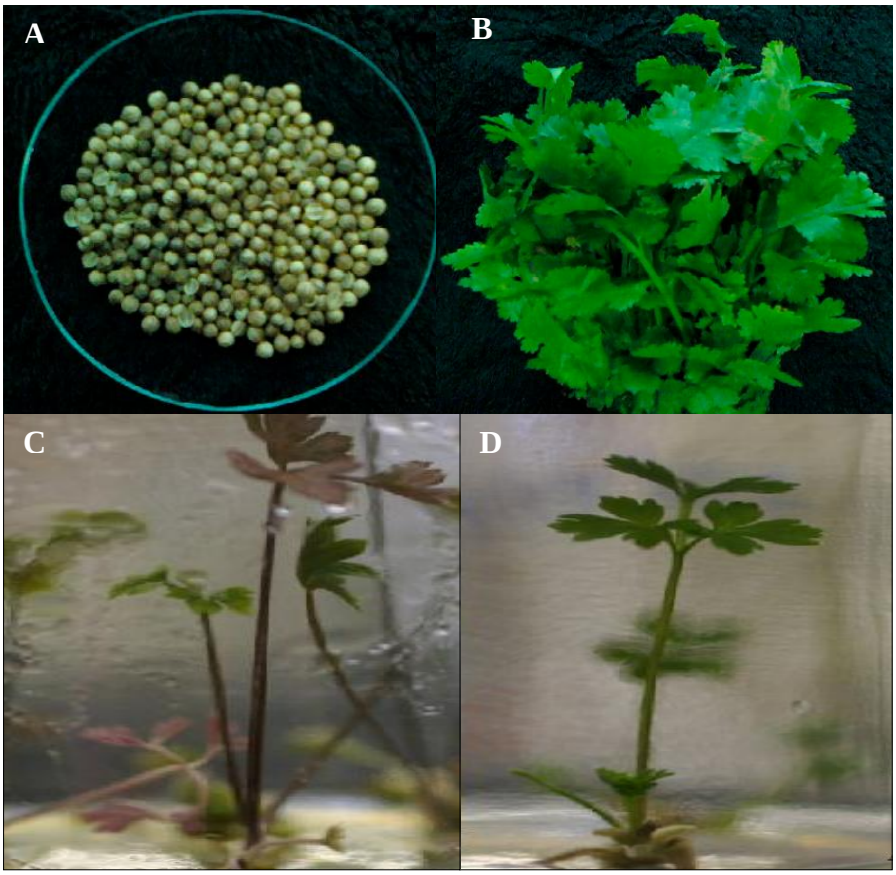


**Fig. 1** A. Seeds of coriander; B. *In vivo* grown coriander; C. Clone A- *in vitro* grown plants with purple pigmentation; D. Clone B- *in vitro* grown plants completely green.

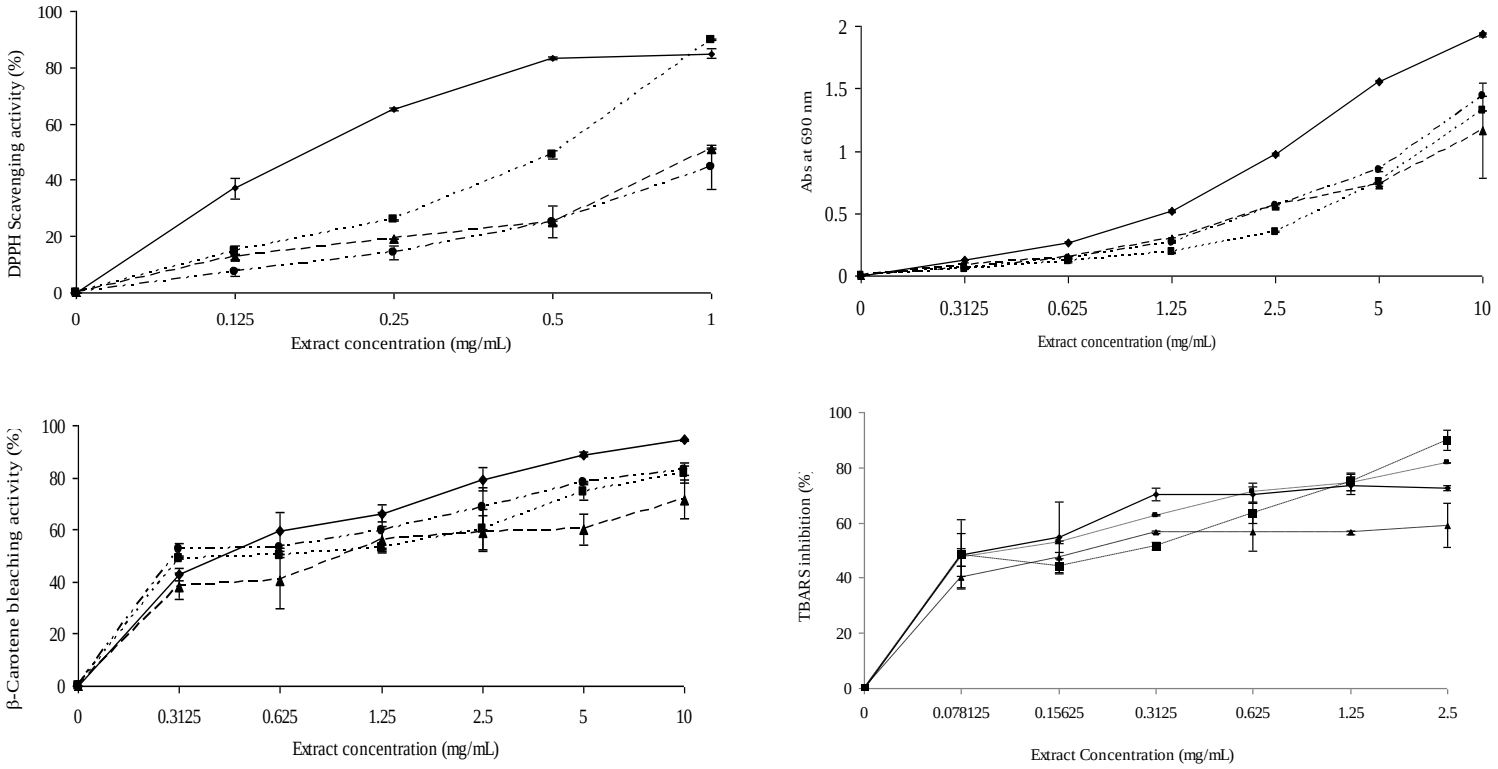


**Fig. 2** DPPH radical scavenging activity (%), Reducing power at 690 nm, β-Carotene bleaching inhibition (%) and TBARS formation inhibition (%) of *in vivo* (♦ - *in vivo* sample; ■ –seeds) and *in vitro* samples (▲ – clone A; ● – clone B). Trolox $EC_{50}$ values: 43.03 ± 1.71 μg/ml for DPPH assay; 29.62 ± 3.15 μg/ml for reducing power assay; 3.73 ± 1.90 μg/ml for TBARS assay and 2.63 ± 0.14 μg/ml for β-carotene bleaching assay.