A comparative study of tocopherols composition and antioxidant properties of *in vivo* and *in vitro* ectomycorrhizal fungi

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ABSTRACT

In aerobic organisms, the free radicals are constantly being produced during the normal cellular metabolism. The antioxidant properties of many organisms and particularly of wild mushrooms with their content in antioxidant compounds such as tocopherols, can detoxify potentially damaging forms of activated oxygen. Herein, a comparative study of tocopherols composition and antioxidant properties of *in vivo* (fruiting bodies) and *in vitro* (mycelia) ectomycorrhizal fungi: *Paxillus involutus* and *Pisolithus arhizus*. Tocopherols were determined by high performance liquid chromatography (HPLC) coupled to a fluorescence detector. The antioxidant properties were studied in terms of DPPH radical-scavenging activity, reducing power and inhibition of β-carotene bleaching. Fruiting bodies revealed the highest antioxidant properties, including scavenging effects on free radicals (EC_{50} = 0.61 and 0.56 mg/ml) and inhibition of lipid peroxidation capacity (EC_{50} = 0.40 and 0.24 mg/ml for *Paxillus involutus* and *Pisolithus arhizus*, respectively), than mycelia produced *in vitro* cultures. Nevertheless, mycelia revealed higher levels of total tocopherols than fruiting bodies, and particularly *Pisolithus arhizus* mycelium proved to be a powerful source of γ-tocopherol (154.39 μg/g dry weight).

Keywords: Paxillus involutus; *Pisolithus arhizus*; Fruiting bodies/mycelia; Tocopherols; HPLC-fluorescence; Antioxidant activity.

1. Introduction

Maintenance of equilibrium between free radical production and antioxidant defences (enzymatic and non enzymatic) is an essential condition for normal organism functioning. When this equilibrium has a tendency to the production of free radicals we say that the organism is in oxidative stress. In this situation, excess free radicals may damage cellular lipids, proteins and DNA, affecting his normal function and leading to various diseases. In aerobic organisms, the free radicals are constantly being produced during the normal cellular metabolism, mainly in the form of Reactive Oxygen Species (ROS) and Reactive Nitrogen Species (RNS). The organism exposure to free radicals has led to the development of endogenous defence mechanisms to eliminate them (Ferreira, Barros, & Abreu, 2009; Goetz & Luch 2008; Valko et al., 2007). The antioxidants present on the diet may help the endogenous defence system to reduce oxidative damage (Fang, Yang, & Wu, 2002; Liu 2003; Temple, 2000). Our research group has been interested in the antioxidant properties of wild mushrooms and their content in antioxidant compounds such as tocopherols (Ferreira et al., 2009; Heleno, Barros, Sousa, Martins, & Ferreira, 2010).

Tocopherols act as free radical scavengers (i.e., chain-breaking antioxidants). Only a small number of published studies have compared α-tocopherol and γ-tocopherol with respect to their ability to specifically inhibit nitration stress *in vitro*. Among four of the most cited studies, three found that γ-tocopherol was a superior protectant against RNS while one study found no such benefit (Hensley et al., 2004).

Paxillus involutus (Batsch) Fr. and *Pisolithus arhizus* (Scop.) Rauschert are two widely used mycorrhizal mushroom species with a broad range of host plants and vast application in forestry programmes of mycorrhization. Mycorrhizal fungi are symbiotic organisms that establish associations with the root system of plants. This special form of

living implicates a recognizing process where chemical signals are involved and oxidative stress mechanisms are most probably activated and overcome. Maintenance and stress resistance of tree plantlets is considerably improved when seedlings become inoculated by ectomycorrhizal fungi in the nursery (Le Tacon, 1992; Marx, Ruehle, & Cordell, 1991; Rudawska & Kieliszewska-Rokicka, 1997). The establishment of ectomycorrhizal symbiosis is triggered by signals produced by both partners. These signals lead to morphological changes and a complex development of specific structures in both the plant and the fungus (Ditengou & Lapeyrie, 2000; Heller et al., 2008; Kawano, Kawano, Hosoya, & Lapeyrie, 2001; Lagrange, Jay-Allgmand, & Lapeyrie, 2001; Martin et al., 2001). Up and down regulation of several genes belonging to stress or defense responses underlines the complex nature of the ectomycorrhizal interaction (Heller et al., 2008). With these features of the mycorrhization process in mind we took two mycorrhizal mushrooms to investigate their differential capacity to produce the antioxidant compounds tocopherols *in vivo* (fruiting bodies) and after isolation *in vitro* (mycelia). *In vitro* assays intend to bypass the genetic and environmentally induced chemical variability of mushrooms thus enabling a continuous and homogeneous production of these compounds under standard conditions. Furthermore, the *in vitro* produced mycelia could be important sources of bioactive compounds to be used in pharmaceutical or food industries.

2. Materials and methods

2.1. Mushroom species

Samples of *Paxillus involutus* (Batsch) Fr. and *Pisolithus arhizus* (Scop.) Rauschert were collected under *Castanea sativa* Mill. in Bragança (Northeast Portugal), in autumn

2008. Taxonomic identification of sporocarps was made according to Benguria (1985) and representative voucher specimens were deposited at the herbarium of Escola Superior Agrária of Instituto Politécnico de Bragança. Macrofungi samples were lyophilised (Ly-8-FM-ULE, Snijders) and reduced to a fine dried powder (20 mesh).

2.2. In vitro production of mushrooms mycelia

Mycelia of each one of the mushrooms were isolated from sporocarps on solid modified Melin-Norkans medium (MMNm) pH 6.6 (NaCl 0.025 g/l; $(NH_4)_2HPO_4$ 0.25 g/l; KH_2PO_4 0.50 g/l; $FeCl_3$ 0.0050 g/l; $CaCl_2$ 0.050 g/l; $MgSO_4.7H_2O$ 0.15 g/l; thiamine 0.10 g/l; glucose 10 g/l; agar 20 g/l in tap water) (Marx, 1969). The strains were maintained in Petri dishes (9 cm diameter) containing the same medium at 25 ºC in the dark and subcultured every 4 to 6 weeks.

After 45 days of growth the mycelium was recovered from the medium. Both mycelium and culture medium were dried separately at 30 ºC, during 24 h, and weighted to obtain the dry biomass (dw).

2.3. Standards and reagents

The eluents *n*-hexane 95% and ethyl acetate 99.98% were of HPLC grade from Lab-Scan (Lisbon, Portugal). Methanol was of analytical grade purity and supplied by Pronalab (Lisbon, Portugal). Tocopherol standards (α, β, γ and δ), trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) and gallic acid were purchased from Sigma (St. Louis, MO, USA). Racemic tocol, 50 mg/ml, was purchased from Matreya (PA, USA). 2,2-Diphenyl-1-picrylhydrazyl (DPPH) was obtained from Alfa Aesar (Ward Hill, MA, USA). All other chemicals were obtained from Sigma Chemical Co.

(St. Louis, MO, USA). Water was treated in a Milli-Q water purification system (TGI Pure Water Systems, USA).

2.4. Determination of tocopherols

Tocopherols content was determined following a procedure previously optimized and described by Barros, Correia, Ferreira, Baptista, & Santos-Buelga (2008), using tocol as internal standard. The equipment consisted of an integrated system with a Smartline 1000 pump (Knauer, Berlin, Germany), a Smartline manager 5000 degasser, an AS-2057 auto-sampler (Jasco, Easton, MD) and a FP-2020 fluorescence detector (Jasco, Easton, MD) programmed for excitation at 290 nm and emission at 330 nm. Data were analysed using Clarity DataApex 2.4 Software. The column used was a normal-phase 250 mm × 4.6 mm i.d., 5 µm, Polyamide II, with a 10 mm × 4 mm i.d. guard column of the same material (YMC Waters, Dinslaken, Germany), operating at 30 ºC. The mobile phase used was a mixture of *n*-hexane and ethyl acetate (70:30, v/v) at a flow rate of 1 ml/min. The compounds were identified by chromatographic comparisons with authentic standards. Quantification was based on the fluorescence signal response, using the internal standard method. Tocopherols content in the samples was expressed in µg per g of dry weight.

2.5. Antioxidant activity.

2.5.1. General

The samples were extracted by stirring with methanol (1: 30 w/v; 25 ºC at 150 rpm) for 1 h and subsequently filtered through Whatman No. 4 paper. The residue was then re-extracted for 1 h. The combined methanolic extracts were evaporated at 40 ºC to

dryness and redissolved in methanol at a concentration of 50 mg/ml, and stored at 4 ºC until analysis.

For total phenolics estimation, it was followed a spectrophotometric assay previously described by the authors (Heleno et al., 2010), with measurement of absorbance at 765 nm. Gallic acid was used to calculate the standard curve (0.05-0.8 mM), and the results were expressed as mg of gallic acid equivalents (GAE) per g of extract.

Chemical assays already described by the authors in previous studies (Heleno et al., 2010), were applied to evaluate the antioxidant activity of all samples. 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 and β-carotene bleaching assays) or absorbance at 690 nm (reducing power assay) against extract concentrations. Trolox was used as standard.

2.5.2. DPPH radical-scavenging activity

This methodology was performed using an ELX800 Microplate Reader (BioTek Instruments, Inc., Winooski, VT). The reaction mixture in each one of the 96 wells consisted of one of the different concentrations of the extracts (30 µl) and methanolic solution (270 µl) containing DPPH radicals (6×10^{-5} mol/l). The mixture was kept for 60 min in the dark. The reduction of the DPPH radical was determined by measuring the absorption at 515 nm. The radical scavenging activity (RSA) was calculated as a percentage of DPPH discolouration using the equation: % RSA = $[(A_{DPPH}-A_S)/A_{DPPH}] \times 100$, where A_S is the absorbance of the solution when the sample extract has been added at a particular level, and A_{DPPH} is the absorbance of the DPPH solution.

2.5.3. Reducing power

This methodology was performed using the Microplate Reader described above. The different concentrations of the extracts (0.5 ml) were mixed with sodium phosphate buffer (200 mmol/l, pH 6.6, 0.5 ml) and potassium ferricyanide (1% w/v, 0.5 ml). The mixture was incubated at 50 ºC for 20 min, and trichloroacetic acid (10% w/v, 0.5 ml) was added. The mixture (0.8 ml) was poured in the 48-wells, as also deionised water (0.8 ml) and ferric chloride (0.1% w/v, 0.16 ml), and the absorbance was measured at 690 nm.

2.5.4. Inhibition of β-carotene bleaching. A solution of β-carotene was prepared by dissolving β-carotene (2 mg) in chloroform (10 ml). Two millilitres of this solution were added to a 100 ml round-bottom flask. After the chloroform was removed at 40 ºC under vacuum, linoleic acid (40 mg), Tween® 80 emulsifier (400 mg), and distilled water (100 ml) were added to the flask and vigorously shacked. Aliquots (4.8 ml) of this emulsion were transferred into different test tubes containing different concentrations (0.2 ml) of the extracts. The tubes were shaken and incubated at 50 ºC in a water bath. As soon as the emulsion was added to each tube, the zero time absorbance at 470 nm was measured (AnalytikJena 200 spectrophotometer). Lipid peroxidation inhibition was calculated using the following equation: (β-carotene content after 2 h of assay)/(initial β-carotene content) × 100.

2.6. Statistical analysis

For each one of the species three samples were analysed and also all the assays were carried out in triplicate. 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. The ANOVA results were classified using letters (different letters mean significant differences among results). The letters are alphabetically ordered according to the decrease of the results values.

3. Results and discussion

Paxillus involutus and *Pisolithus arhizus* mycelia grown until 37 days of inoculation. *Paxillus involutus* mycelium started to grow only after 8 days; nevertheless its radial growth was higher than the one obtained for *Pisolithus arhizus* (Fig. 1).

Fruiting bodies, mycelia and culture media of *Paxillus involutus* and *Pisolithus arhizus* were analysed for their content in tocopherols and antioxidant properties.

Pisolithus arhizus samples, especially mycelium (158 μg/g dry weight; Table 1), revealed higher total tocopherols content than *Paxillus involutus* due to the significant contribution of γ-tocopherol (154 μg/g dry weight) (Fig. 2). For both species, mycelia samples showed higher total tocopherols content than fruiting bodies and culture media. The highest levels of α-tocopherol were found in *Pisolithus arhizus* fruiting body, while the highest levels of β-tocopherol were found in *Paxillus involutus* mycelium, γ-tocopherol in *Pisolithus arhizus* mycelium and δ-tocopherol in *Pisolithus arhizus* fruiting body and mycelium, and in *Paxillus involutus* mycelium.

The predominant vitamers were γ-tocopherol (*Paxillus involutus* fruiting body and mycelium, and *Pisolithus arhizus* mycelium and culture medium) and β-tocopherol (*Paxillus involutus* culture medium and *Pisolithus arhizus* fruiting body).

Tocopherols have been recognized as one of the most important antioxidants. They inhibit ROS-induced generation of lipid peroxyl radicals, thereby protecting cells from peroxidation of PUFA (polyunsaturated fatty acids) in membrane phospholipids, from

oxidative damage of plasma very low-density lipoprotein, cellular proteins, DNA, and from membrane degeneration (Fang et al., 2002).

In the lipid peroxidation process, tocopherols (TOH) act as antioxidants by donating a hydrogen atom to peroxyl radicals ($LOO^{\bullet}$) formed from PUFA giving a stable lipid hydroperoxide (LOOH) and a tocopheroxyl radical ($TO^{\bullet}$), which reacts with other peroxyl or tocopheroxyl radicals forming more stable adducts (Ferreira et al., 2009; Kamal-Eldin & Appelqvist, 1996):

$$LOO^{\bullet} + TOH \rightarrow LOOH + TO^{\bullet}$$

Tocopherols can also react with alkoxy radicals ($LO^{\bullet}$) formed in the propagation step ($LO^{\bullet} + TOH \rightarrow LOH + TO^{\bullet}$), or in very special cases, when oxygen is present in trace amounts and hydroperoxides are present in negligible conditions, tocopherols can react directlly with alkyl radicals $L^{\bullet}$ ($L^{\bullet} + TOH \rightarrow LH + TO^{\bullet}$) (Kamal-Eldin & Appelqvist, 1996).

In the past α-tocopherol was considered the most active form of vitamin E in humans and it was reported as having the highest biological activity. Recent work has begun to focus on so-called desmethyl tocopherols such as γ-tocopherol. Under specific conditions, γ-tocopherol might scavenge RNS more efficiently than α-tocopherol and thereby provide special benefits. This vitamer also reflects anti-inflammatory, antineoplastic, and natriuretic functions possibly mediated through specific binding interactions. Moreover, a nascent body of epidemiological data suggests that γ-tocopherol is a better negative risk factor for certain types of cancer and myocardial infarction than is α-tocopherol (Hensley et al., 2004). Therefore, the results herein obtained are very relevant once *Pisolithus arhizus* mycelium proved to be a powerful source of γ-tocopherol.

The antioxidant properties of all the samples were evaluated through scavenging

activity on DPPH radicals (examined by the capacity to decrease the absorbance at 517 nm of DPPH solution), reducing power (evaluated measuring the conversion of a Fe^{3+}/ferricyanide complex to the ferrous form by the samples) and lipid peroxidation inhibition by β-carotene-linoleate system (measured by the inhibition of β-carotene bleaching, by neutralizing the linoleate-free radical and other free radicals formed in the system which attack the highly unsaturated β-carotene models). The results showed an increase in antioxidant properties with the increase of extract concentration (Fig. 3).

All the samples revealed antioxidant properties but in the order fruiting bodies > mycelia > culture mediua (Table 2). *Paxillus involutus* fruting body revealed the highest radical scavenging effects (82% at 20 mg/ml; Fig. 3) but *Pisolithus arhizus* fruiting body gave the highest phenolics content (298 mg GAE/g extract) and the lowest EC_{50} value (0.56 mg/ml, Table 2). Concerning reducing power, *Paxillus involutus* mycelium gave the highest absorbance at 1.25 mg/ml (1.7; Fig. 3). Both fruiting bodies gave the lowest reducing power EC_{50} values without significant statistical differences (Table 2). *Pisolithus arhizus* fruiting body showed the highest percentage of lipid peroxidation inhibition (96% at 20 mg/ml; Fig. 3) with the lowest EC_{50} values without significant statistical differences with *Paxillus involutus* fruting body sample (Table 2).

In fruiting bodies and culture media samples, *Pisolithus arhizus* revealed the highest antioxidant properties, while in mycelia samples *Paxillus involutus* gave the highest antioxidant potential.

Overall, fruiting bodies seem to have highest antioxidant properties, including scavenging effects on free radicals and inhibition of lipid peroxidation capacity, than mycelia produced *in vitro* cultures. Nevertheless, mycelia produced higher levels of total tocopherols than fruiting bodies, and particularly *Pisolithus arhizus* mycelium proved to be a powerful source of γ-tocopherol.

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Figure 1. Mean radial growth of *Paxillus involutus* (■) and *Pisolithus arhizus* (■) in MMNm (modified Melin-Norkans medium) along time of inoculation.

Figure 2. HPLC fluorescence chromatogram of *Pisolithus arhizus* mycelium. Peaks: 1) α-tocopherol (R^1=R^2=CH_3); 2) β-tocopherol (R^1=CH_3, R^2=H); 3) BHT (butylated hydroxytoluene); 4) γ-tocopherol (R^1=H, R^2=CH_3); 5) δ-tocopherol (R^1=R^2=H); 6) I.S.-internal standard (tocol).

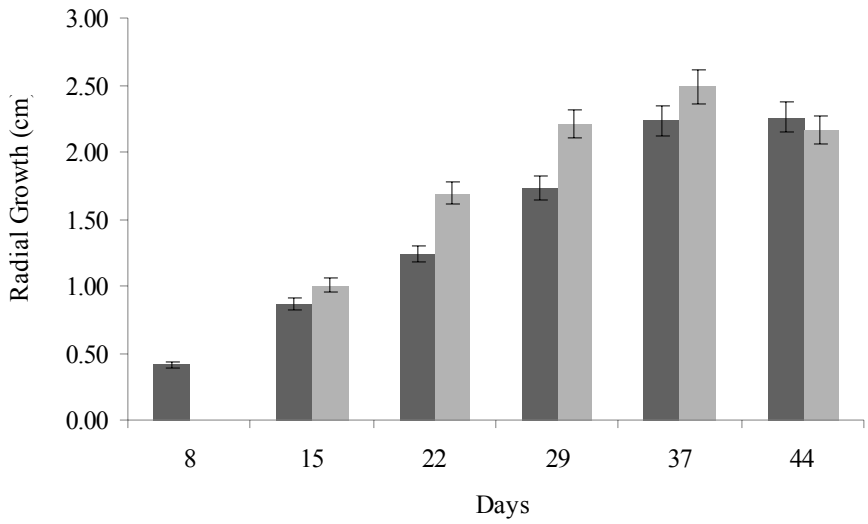
Figure 3. Radical scavenging activity on DPPH radicals, reducing power and lipid peroxidation inhibition measured by the β-carotene bleaching inhibition; mean ± SD (n = 9). *Paxillus involutus* mycelium (—◇—), *Paxillus involutus* fruiting body (—◇—), *Paxillus involutus* medium (—□—), *Pisolithus arhizus* mycelium (—△—), *Pisolithus arhizus* fruiting body (—✳—), *Pisolithus arhizus* medium (—✳—).

Table 1. Tocopherol composition (μg/g dw); mean ± SD (n = 9). In each column different letters mean significant differences between results ($p < 0.05$).

Species	Sample	α-tocopherol	β-tocopherol	γ-tocopherol	δ-tocopherol	Total tocopherols
Paxilus involutus	Fruiting body	0.15 ± 0.01 dc	0.30 ± 0.05 f	4.27 ± 0.25 cd	0.29 ± 0.02 cb	5.02 ± 0.19 c
Paxilus involutus	Mycelium	0.72 ± 0.14 b	6.18 ± 0.08 a	9.82 ± 1.40 b	1.42 ± 0.01 a	18.15 ± 1.30 b
Paxilus involutus	Culture medium	0.03 ± 0.00 d	0.62 ± 0.05 e	0.39 ± 0.04 c	0.56 ± 0.03 b	1.60 ± 0.12 c
Pisolithus arhizus	Fruiting body	2.11 ± 0.04 a	2.39 ± 0.33 b	1.67 ± 0.01 cd	1.68 ± 0.09 a	7.84 ± 0.45 c
Pisolithus arhizus	Mycelium	0.17 ± 0.03 c	1.83 ± 0.06 c	154.39 ± 10.82 a	1.55 ± 0.40 a	157.94 ± 10.45a
Pisolithus arhizus	Culture medium	0.06 ± 0.00 dc	1.28 ± 0.03 d	7.71 ± 0.26 cb	0.22 ± 0.01 c	9.28 ± 0.29 c

Table 2. Phenolic content (mg GAE/g extract), extraction yield (%) and antioxidant activity EC_{50} values (mg/ml); mean ± SD (n = 9). In each column different letters mean significant differences between results ($p < 0.05$).

Species	Sample	Extraction yield	Phenolics	DPPH Scavenging activity	Reducing power	β-carotene bleaching inhibition
Paxilus involutus	Fruiting body	34.24 ± 1.56	78.92 ± 4.47 b	0.61 ± 0.01 c	0.39 ± 0.00 e	0.40 ± 0.00 d
Paxilus involutus	Mycelium	16.28 ± 0.71	59.47 ± 3.37 c	1.15 ± 0.07 b	0.65 ± 0.03 d	2.21 ± 0.12 c
Paxilus involutus	Culture medium	2.27 ± 0.08	1.01 ± 0.07 e	> 20.00 a	28.56 ± 0.12 a	9.85 ± 0.93 a
Pisolithus arhizus	Fruiting body	20.14 ± 1.32	297.94 ± 0.30 a	0.56 ± 0.01 d	0.37 ± 0.03 e	0.24 ± 0.03 d
Pisolithus arhizus	Mycelium	24.94 ± 2.81	9.48 ± 0.67 d	> 20.00 a	7.29 ± 0.62 c	2.49 ± 0.18 c
Pisolithus arhizus	Culture medium	0.95 ± 0.05	2.36 ± 0.07 e	> 20.00 a	11.97 ± 0.01 b	4.39 ± 0.05 b


3.00
2.50
2.00
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0.50
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Radial Growth (cm)
8
15
22
29
37
44
Days

Fig. 1.

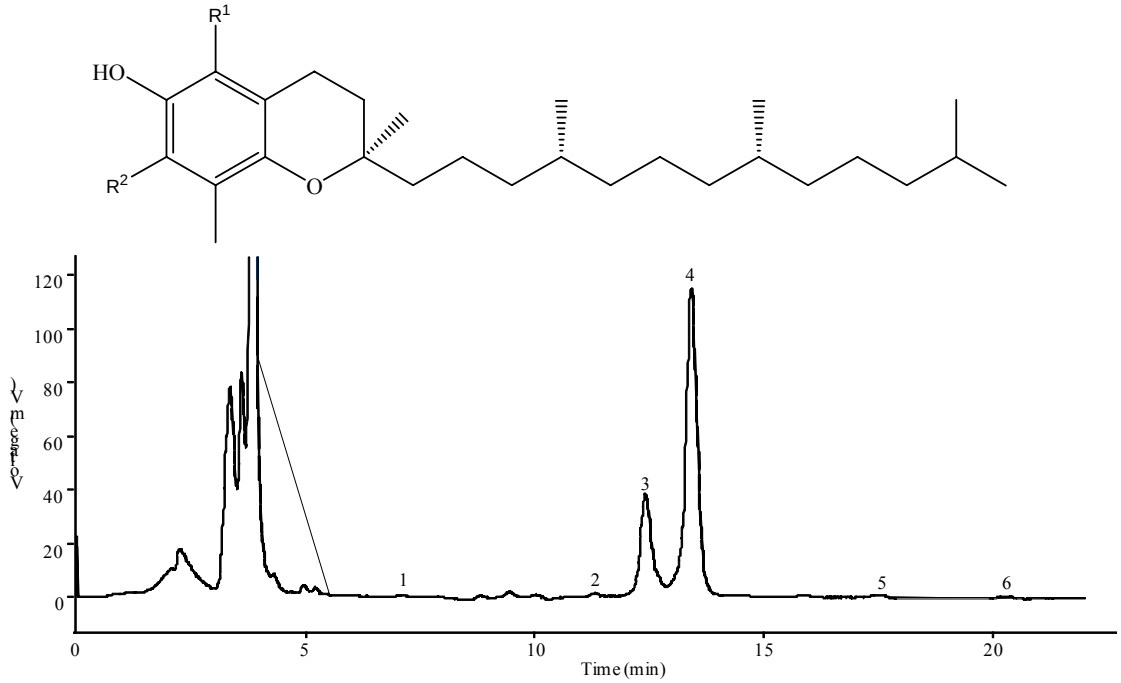
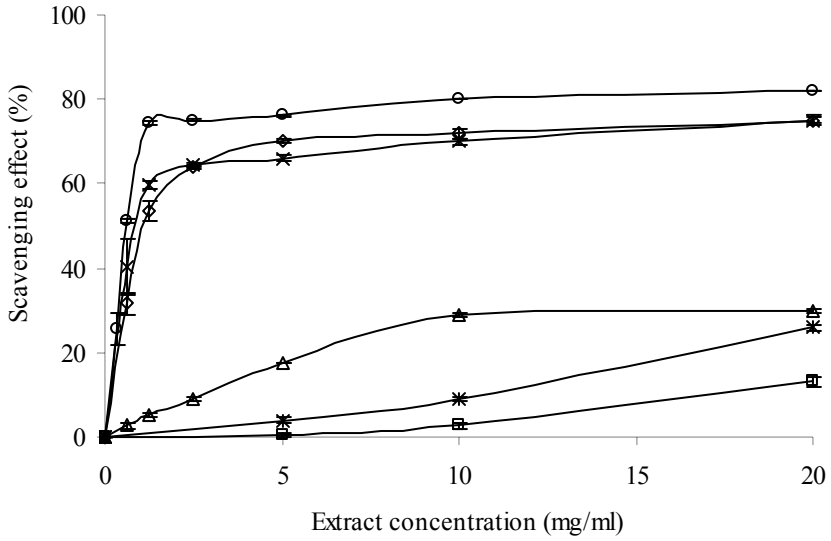
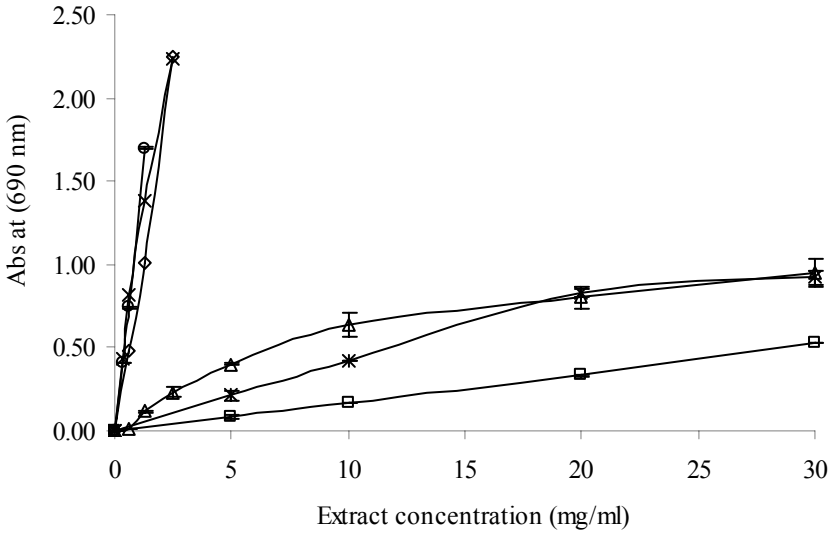


Fig. 2.


Scavenging effect (%)
100
80
60
40
20
0
0
5
10
15
20
Extract concentration (mg/ml)


Abs at (690 nm)
2.50
2.00
1.50
1.00
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25
30
Extract concentration (mg/ml)

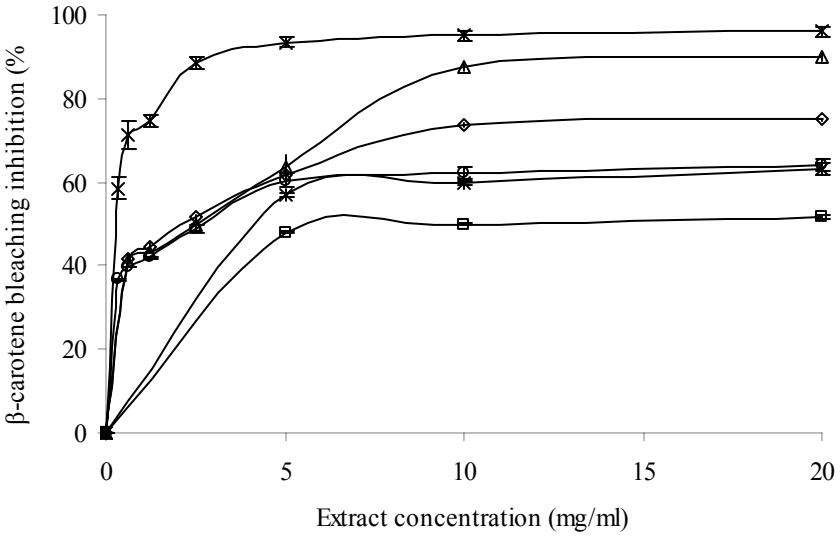
β-carotene bleaching inhibition (%
100
80
60
40
20
0
0
5
10
15
20
Extract concentration (mg/ml)

Fig. 3.