

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

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1 **ABSTRACT**

2 In aerobic organisms, the free radicals are constantly being produced during the normal
3 cellular metabolism. The antioxidant properties of many organisms and particularly of
4 wild mushrooms with their content in antioxidant compounds such as tocopherols, can
5 detoxify potentially damaging forms of activated oxygen. Herein, a comparative study
6 of tocopherols composition and antioxidant properties of *in vivo* (fruiting bodies) and *in*
7 *vitro* (mycelia) ectomycorrhizal fungi: *Paxillus involutus* and *Pisolithus arhizus*.
8 Tocopherols were determined by high performance liquid chromatography (HPLC)
9 coupled to a fluorescence detector. The antioxidant properties were studied in terms of
10 DPPH radical-scavenging activity, reducing power and inhibition of β -carotene
11 bleaching. Fruiting bodies revealed the highest antioxidant properties, including
12 scavenging effects on free radicals ($EC_{50} = 0.61$ and 0.56 mg/ml) and inhibition of lipid
13 peroxidation capacity ($EC_{50} = 0.40$ and 0.24 mg/ml for *Paxillus involutus* and *Pisolithus*
14 *arhizus*, respectively), than mycelia produced *in vitro* cultures. Nevertheless, mycelia
15 revealed higher levels of total tocopherols than fruiting bodies, and particularly
16 *Pisolithus arhizus* mycelium proved to be a powerful source of γ -tocopherol (154.39
17 $\mu\text{g/g}$ dry weight).

18

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

1 **1. Introduction**

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

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

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

1 living implicates a recognizing process where chemical signals are involved and
2 oxidative stress mechanisms are most probably activated and overcome. Maintenance
3 and stress resistance of tree plantlets is considerably improved when seedlings become
4 inoculated by ectomycorrhizal fungi in the nursery (Le Tacon, 1992; Marx, Ruehle, &
5 Cordell, 1991; Rudawska & Kieliszewska-Rokicka, 1997). The establishment of
6 ectomycorrhizal symbiosis is triggered by signals produced by both partners. These
7 signals lead to morphological changes and a complex development of specific structures
8 in both the plant and the fungus (Ditengou & Lapeyrie, 2000; Heller et al., 2008;
9 Kawano, Kawano, Hosoya, & Lapeyrie, 2001; Lagrange, Jay-Allgmand, & Lapeyrie,
10 2001; Martin et al., 2001). Up and down regulation of several genes belonging to stress
11 or defense responses underlines the complex nature of the ectomycorrhizal interaction
12 (Heller et al., 2008). With these features of the mycorrhization process in mind we took
13 two mycorrhizal mushrooms to investigate their differential capacity to produce the
14 antioxidant compounds tocopherols *in vivo* (fruiting bodies) and after isolation *in vitro*
15 (mycelia). *In vitro* assays intend to bypass the genetic and environmentally induced
16 chemical variability of mushrooms thus enabling a continuous and homogeneous
17 production of these compounds under standard conditions. Furthermore, the *in vitro*
18 produced mycelia could be important sources of bioactive compounds to be used in
19 pharmaceutical or food industries.

20

21 **2. Materials and methods**

22

23 *2.1. Mushroom species*

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

1 2008. Taxonomic identification of sporocarps was made according to [Benguria \(1985\)](#)
2 and representative voucher specimens were deposited at the herbarium of Escola
3 Superior Agrária of Instituto Politécnico de Bragança. Macrofungi samples were
4 lyophilised (Ly-8-FM-ULE, Snijders) and reduced to a fine dried powder (20 mesh).

5

6 *2.2. In vitro production of mushrooms mycelia*

7 Mycelia of each one of the mushrooms were isolated from sporocarps on solid modified
8 Melin-Norkans medium (MMNm) pH 6.6 (NaCl 0.025 g/l; (NH₄)₂HPO₄ 0.25 g/l;
9 KH₂PO₄ 0.50 g/l; FeCl₃ 0.0050 g/l; CaCl₂ 0.050 g/l; MgSO₄.7H₂O 0.15 g/l; thiamine
10 0.10 g/l; glucose 10 g/l; agar 20 g/l in tap water) ([Marx, 1969](#)). The strains were
11 maintained in Petri dishes (9 cm diameter) containing the same medium at 25 °C in the
12 dark and subcultured every 4 to 6 weeks.

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

16

17 *2.3. Standards and reagents*

18 The eluents *n*-hexane 95% and ethyl acetate 99.98% were of HPLC grade from Lab-
19 Scan (Lisbon, Portugal). Methanol was of analytical grade purity and supplied by
20 Pronalab (Lisbon, Portugal). Tocopherol standards (α , β , γ and δ), trolox (6-hydroxy-
21 2,5,7,8-tetramethylchroman-2-carboxylic acid) and gallic acid were purchased from
22 Sigma (St. Louis, MO, USA). Racemic tocol, 50 mg/ml, was purchased from Matreya
23 (PA, USA). 2,2-Diphenyl-1-picrylhydrazyl (DPPH) was obtained from Alfa Aesar
24 (Ward Hill, MA, USA). All other chemicals were obtained from Sigma Chemical Co.

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

3 4 *2.4. Determination of tocopherols*

5 Tocopherols content was determined following a procedure previously optimized and
6 described by [Barros, Correia, Ferreira, Baptista, & Santos-Buelga \(2008\)](#), using tocol as
7 internal standard. The equipment consisted of an integrated system with a Smartline
8 1000 pump (Knauer, Berlin, Germany), a Smartline manager 5000 degasser, an AS-
9 2057 auto-sampler (Jasco, Easton, MD) and a FP-2020 fluorescence detector (Jasco,
10 Easton, MD) programmed for excitation at 290 nm and emission at 330 nm. Data were
11 analysed using Clarity DataApex 2.4 Software. The column used was a normal-phase
12 250 mm × 4.6 mm i.d., 5 µm, Polyamide II, with a 10 mm × 4 mm i.d. guard column of
13 the same material (YMC Waters, Dinslaken, Germany), operating at 30 °C. The mobile
14 phase used was a mixture of *n*-hexane and ethyl acetate (70:30, v/v) at a flow rate of 1
15 ml/min. The compounds were identified by chromatographic comparisons with
16 authentic standards. Quantification was based on the fluorescence signal response, using
17 the internal standard method. Tocopherols content in the samples was expressed in µg
18 per g of dry weight.

19 20 *2.5. Antioxidant activity.*

21 *2.5.1. General*

22 The samples were extracted by stirring with methanol (1: 30 w/v; 25 °C at 150 rpm) for
23 1 h and subsequently filtered through Whatman No. 4 paper. The residue was then re-
24 extracted for 1 h. The combined methanolic extracts were evaporated at 40 °C to

1 dryness and redissolved in methanol at a concentration of 50 mg/ml, and stored at 4 °C
2 until analysis.

3 For total phenolics estimation, it was followed a spectrophotometric assay previously
4 described by the authors (Heleno et al., 2010), with measurement of absorbance at
5 765 nm. Gallic acid was used to calculate the standard curve (0.05-0.8 mM), and the
6 results were expressed as mg of gallic acid equivalents (GAE) per g of extract.

7 Chemical assays already described by the authors in previous studies (Heleno et al.,
8 2010), were applied to evaluate the antioxidant activity of all samples. The extract
9 concentrations providing 50% of antioxidant activity or 0.5 of absorbance (EC₅₀) were
10 calculated from the graphs of antioxidant activity percentages (DPPH and β-carotene
11 bleaching assays) or absorbance at 690 nm (reducing power assay) against extract
12 concentrations. Trolox was used as standard.

13

14 2.5.2. DPPH radical-scavenging activity

15 This methodology was performed using an ELX800 Microplate Reader (BioTek
16 Instruments, Inc., Winooski, VT). The reaction mixture in each one of the 96 wells
17 consisted of one of the different concentrations of the extracts (30 µl) and methanolic
18 solution (270 µl) containing DPPH radicals (6×10⁻⁵ mol/l). The mixture was kept for 60
19 min in the dark. The reduction of the DPPH radical was determined by measuring the
20 absorption at 515 nm. The radical scavenging activity (RSA) was calculated as a
21 percentage of DPPH discolouration using the equation: % RSA = [(A_{DPPH}-A_S)/A_{DPPH}] ×
22 100, where A_S is the absorbance of the solution when the sample extract has been added
23 at a particular level, and A_{DPPH} is the absorbance of the DPPH solution.

24

25 2.5.3. Reducing power

1 This methodology was performed using the Microplate Reader described above. The
2 different concentrations of the extracts (0.5 ml) were mixed with sodium phosphate
3 buffer (200 mmol/l, pH 6.6, 0.5 ml) and potassium ferricyanide (1% w/v, 0.5 ml). The
4 mixture was incubated at 50 °C for 20 min, and trichloroacetic acid (10% w/v, 0.5 ml)
5 was added. The mixture (0.8 ml) was poured in the 48-wells, as also deionised water
6 (0.8 ml) and ferric chloride (0.1% w/v, 0.16 ml), and the absorbance was measured at
7 690 nm.

8

9 *2.5.4. Inhibition of β -carotene bleaching.* A solution of β -carotene was prepared by
10 dissolving β -carotene (2 mg) in chloroform (10 ml). Two millilitres of this solution
11 were added to a 100 ml round-bottom flask. After the chloroform was removed at 40 °C
12 under vacuum, linoleic acid (40 mg), Tween® 80 emulsifier (400 mg), and distilled
13 water (100 ml) were added to the flask and vigorously shaken. Aliquots (4.8 ml) of this
14 emulsion were transferred into different test tubes containing different concentrations
15 (0.2 ml) of the extracts. The tubes were shaken and incubated at 50 °C in a water bath.
16 As soon as the emulsion was added to each tube, the zero time absorbance at 470 nm
17 was measured (AnalytikJena 200 spectrophotometer). Lipid peroxidation inhibition was
18 calculated using the following equation: $(\beta\text{-carotene content after 2 h of assay})/(\text{initial}$
19 $\beta\text{-carotene content}) \times 100$.

20

21 *2.6. Statistical analysis*

22 For each one of the species three samples were analysed and also all the assays were
23 carried out in triplicate. The results are expressed as mean values and standard deviation
24 (SD). The results were analyzed using one-way analysis of variance (ANOVA)
25 followed by Tukey's HSD Test with $\alpha = 0.05$. This treatment was carried out using

1 SPSS v.16.0 software. The ANOVA results were classified using letters (different
2 letters mean significant differences among results). The letters are alphabetically
3 ordered according to the decrease of the results values.

4

5 **3. Results and discussion**

6 *Paxillus involutus* and *Pisolithus arhizus* mycelia grown until 37 days of inoculation.

7 *Paxillus involutus* mycelium started to grow only after 8 days; nevertheless its radial
8 growth was higher than the one obtained for *Pisolithus arhizus* (Fig. 1).

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

11 *Pisolithus arhizus* samples, especially mycelium (158 µg/g dry weight; Table 1),

12 revealed higher total tocopherols content than *Paxillus involutus* due to the significant

13 contribution of γ-tocopherol (154 µg/g dry weight) (Fig. 2). For both species, mycelia

14 samples showed higher total tocopherols content than fruiting bodies and culture media.

15 The highest levels of α-tocopherol were found in *Pisolithus arhizus* fruiting body, while

16 the highest levels of β-tocopherol were found in *Paxillus involutus* mycelium, γ-

17 tocopherol in *Pisolithus arhizus* mycelium and δ-tocopherol in *Pisolithus arhizus*

18 fruiting body and mycelium, and in *Paxillus involutus* mycelium.

19 The predominant vitamers were γ-tocopherol (*Paxillus involutus* fruiting body and

20 mycelium, and *Pisolithus arhizus* mycelium and culture medium) and β-tocopherol

21 (*Paxillus involutus* culture medium and *Pisolithus arhizus* fruiting body).

22 Tocopherols have been recognized as one of the most important antioxidants. They

23 inhibit ROS-induced generation of lipid peroxy radicals, thereby protecting cells from

24 peroxidation of PUFA (polyunsaturated fatty acids) in membrane phospholipids, from

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

3 In the lipid peroxidation process, tocopherols (TOH) act as antioxidants by donating a
4 hydrogen atom to peroxy radicals (LOO[•]) formed from PUFA giving a stable lipid
5 hydroperoxide (LOOH) and a tocopheroxyl radical (TO[•]), which reacts with other
6 peroxy or tocopheroxyl radicals forming more stable adducts (Ferreira et al., 2009;
7 Kamal-Eldin & Appelqvist, 1996):



9 Tocopherols can also react with alkoxy radicals (LO[•]) formed in the propagation step
10 (LO[•] + TOH → LOH + TO[•]), or in very special cases, when oxygen is present in trace
11 amounts and hydroperoxides are present in negligible conditions, tocopherols can react
12 directly with alkyl radicals L[•] (L[•] + TOH → LH + TO[•]) (Kamal-Eldin & Appelqvist,
13 1996).

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

25 The antioxidant properties of all the samples were evaluated through scavenging

1 activity on DPPH radicals (examined by the capacity to decrease the absorbance at 517
2 nm of DPPH solution), reducing power (evaluated measuring the conversion of a
3 Fe^{3+} /ferricyanide complex to the ferrous form by the samples) and lipid peroxidation
4 inhibition by β -carotene-linoleate system (measured by the inhibition of β -carotene
5 bleaching, by neutralizing the linoleate-free radical and other free radicals formed in the
6 system which attack the highly unsaturated β -carotene models). The results showed an
7 increase in antioxidant properties with the increase of extract concentration (Fig. 3).
8 All the samples revealed antioxidant properties but in the order fruiting bodies >
9 mycelia > culture media (Table 2). *Paxillus involutus* fruiting body revealed the highest
10 radical scavenging effects (82% at 20 mg/ml; Fig. 3) but *Pisolithus arhizus* fruiting
11 body gave the highest phenolics content (298 mg GAE/g extract) and the lowest EC_{50}
12 value (0.56 mg/ml, Table 2). Concerning reducing power, *Paxillus involutus* mycelium
13 gave the highest absorbance at 1.25 mg/ml (1.7; Fig. 3). Both fruiting bodies gave the
14 lowest reducing power EC_{50} values without significant statistical differences (Table 2).
15 *Pisolithus arhizus* fruiting body showed the highest percentage of lipid peroxidation
16 inhibition (96% at 20 mg/ml; Fig. 3) with the lowest EC_{50} values without significant
17 statistical differences with *Paxillus involutus* fruiting body sample (Table 2).
18 In fruiting bodies and culture media samples, *Pisolithus arhizus* revealed the highest
19 antioxidant properties, while in mycelia samples *Paxillus involutus* gave the highest
20 antioxidant potential.
21 Overall, fruiting bodies seem to have highest antioxidant properties, including
22 scavenging effects on free radicals and inhibition of lipid peroxidation capacity, than
23 mycelia produced *in vitro* cultures. Nevertheless, mycelia produced higher levels of
24 total tocopherols than fruiting bodies, and particularly *Pisolithus arhizus* mycelium
25 proved to be a powerful source of γ -tocopherol.

1

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

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

8
9 **Figure 3.** Radical scavenging activity on DPPH radicals, reducing power and lipid
10 peroxidation inhibition measured by the β -carotene bleaching inhibition; mean \pm SD (n
11 = 9). *Paxillus involutus* mycelium (—◆—), *Paxillus involutus* fruiting body (—◇—), *Paxillus*
12 *involutus* medium (—■—), *Pisolithus arhizus* mycelium (—▲—), *Pisolithus arhizus* fruiting
13 body (—*—), *Pisolithus arhizus* medium (—*—).

14

Table 1. Tocopherol composition ($\mu\text{g/g dw}$); mean \pm 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
<i>Paxilus involutus</i>	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
<i>Paxilus involutus</i>	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
<i>Paxilus involutus</i>	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
<i>Pisolithus arhizus</i>	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
<i>Pisolithus arhizus</i>	Mycelium	0.17 ± 0.03 c	1.83 ± 0.06 c	154.39 ± 10.82 a	1.55 ± 0.40 a	157.94 ± 10.45 a
<i>Pisolithus arhizus</i>	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 EC₅₀ 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
<i>Paxilus involutus</i>	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
<i>Paxilus involutus</i>	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
<i>Paxilus involutus</i>	Culture medium	2.27 ± 0.08	1.01 ± 0.07 e	> 20.00 a	28.56 ± 0.12 a	9.85 ± 0.93 a
<i>Pisolithus arhizus</i>	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
<i>Pisolithus arhizus</i>	Mycelium	24.94 ± 2.81	9.48 ± 0.67 d	> 20.00 a	7.29 ± 0.62 c	2.49 ± 0.18 c
<i>Pisolithus arhizus</i>	Culture medium	0.95 ± 0.05	2.36 ± 0.07 e	> 20.00 a	11.97 ± 0.01 b	4.39 ± 0.05 b

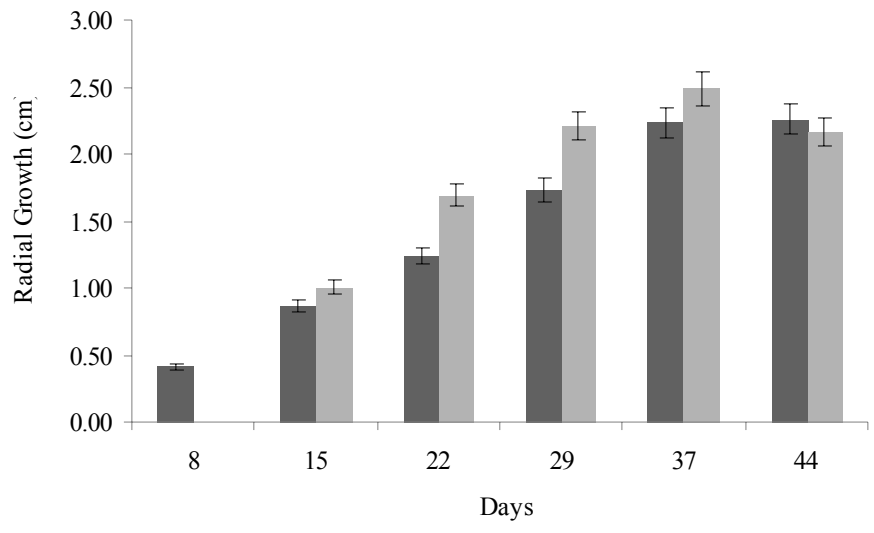


Fig. 1.

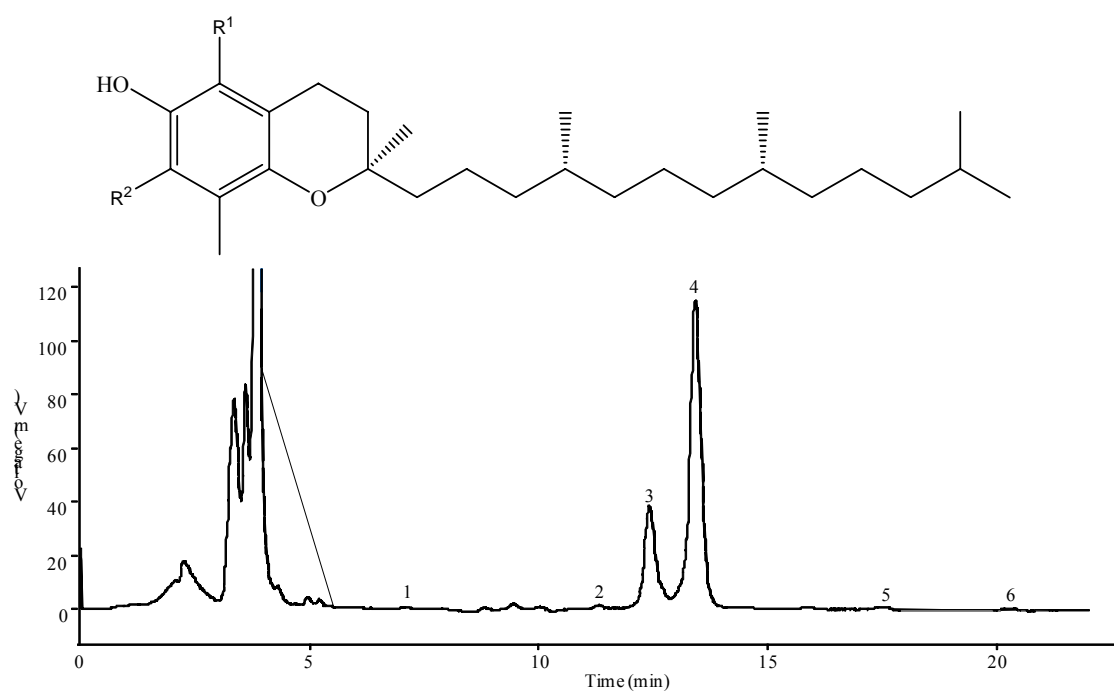


Fig. 2.

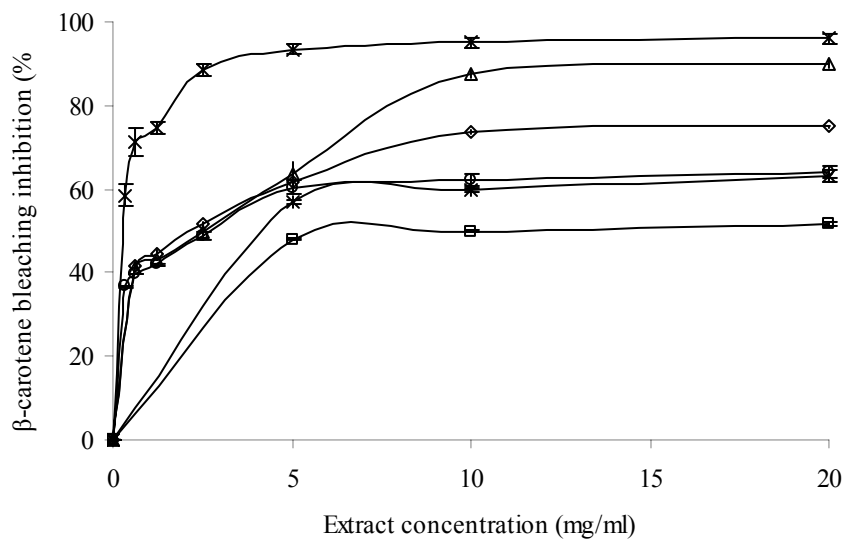
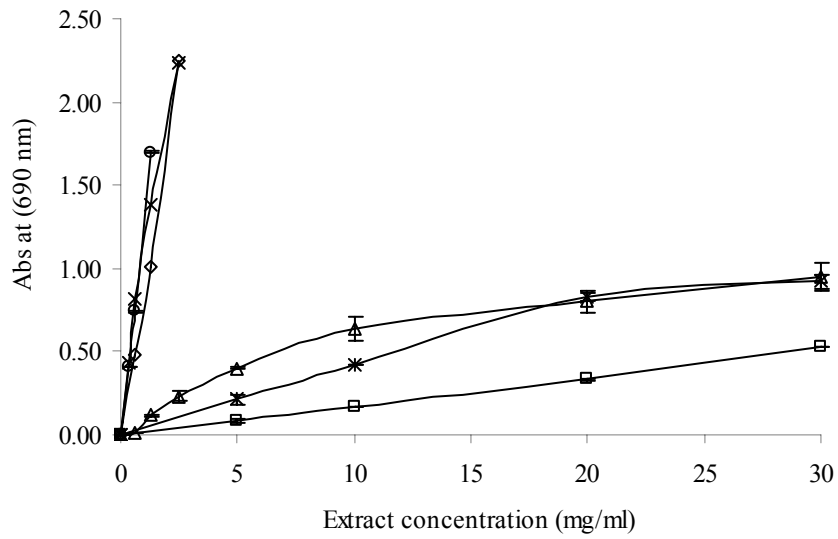
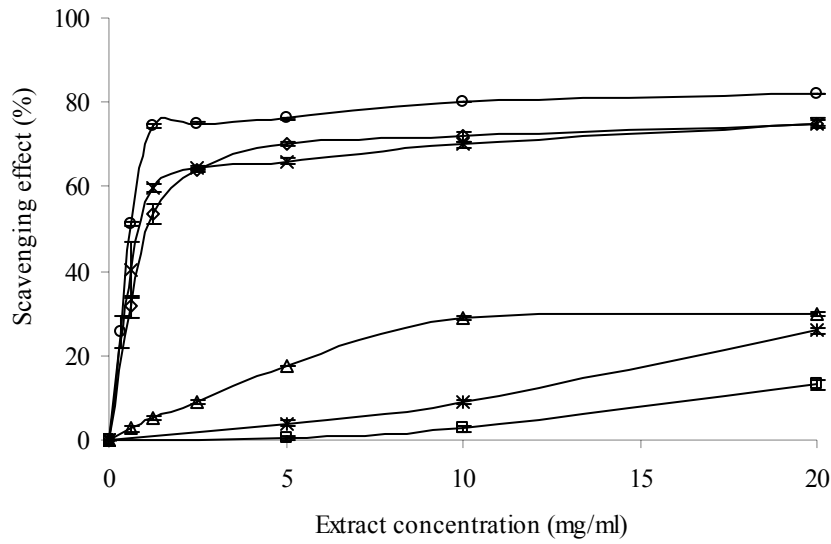


Fig. 3.