Journal of Food Technology 1 (1): 9-16, 2003

Antioxidant and Antimicrobial Activities of *Teucrium polium* L.

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Abstract: The present work examines the potential *Teucrium polium* L. extracts as a source of natural antioxidant and antimicrobial compounds. Total antioxidant activity, DPPH radical scavenging, superoxide anion radical scavenging, hydrogen peroxide scavenging, metal chelating activities, reducing power, antimicrobial activity and total contents of phenolic compounds of dried *T. polium* samples, which extracted with chloroform and acetone were studied. The total antioxidant and the others antioxidant activities of *T. polium* are increased with increasing amount of sample (50-250 μg). However, the chloroform extract of *T. polium* exhibited stronger antioxidant activity than the acetone extract. In the both extracts, the highest total antioxidant activity, DPPH· radical scavenging activity, metal chelating activity and total phenolic compounds were found in chloroform extract of *T. Polium*. In addition, the both extracts showed strong antioxidant activity comparable with α-tocopherol. Antimicrobial activity of the both *T. polium* extracts were examined by means of disc-diffusion methods with eleven microbial species (*Bacillus megaterium, Proteus vulgaris, Listeria monocytogenes, Bacillus cereus, Staphylococcus aureus, Bacillus brevis, Klebsiella pneumoniae, Micrococcus luteus, Pseudomonas aeruginosa, Escherichia coli* and *Mycobacterium smegmatis* and four fungal species (*Penicillum frequentans, Fusarium equiseti, Aspergillus candidus*, and *Byssochlamys fulves*). The both extracts were effective in inhibiting the growth of the organisms except for *Escherichia coli*. Antifungal activity of each of above extracts is lower than antimicrobial activity relatively.

Key words: Antioxidant activity, antimicrobial activity, *Teucrium polium* L.

Introduction

Reactive oxygen species (ROS) such as superoxide anion radicals (O2.-), hydroxyl radical (OH.), non free radical species such as hydrogen peroxide (H2O2) and singled oxygen (1O2) are formed *in vivo* and play a positive role such as energy production, phagocytosis, regulation of cell growth intercellular signalling, and synthesis of biologically important compounds. However, ROS may also be very damaging; they can attack to lipids of cell membranes and DNA. As a result, they can easy initiate the peroxidation of the membrane lipids. ROS are continuously produced during normal physiologic event and are removed by antioxidant defence mechanism (Büyükokuroğlu *et al.* 2001; Chang *et al.*, 2001; Gülçin *et al.*, 2002a; Gülçin *et al.*, 2002b).

The major pathway of lipid peroxidation contains a self-catalytic free radical chain reaction. However, lipid peroxidation can be catalysed environmental factor, such as light, oxygen, free radicals and metal ions (Frankel, 1991). The discovery of the inhibition of lipid peroxidation by some phenolic compounds during the late 1940s has contributed to the application of synthetic antioxidants in food industry (Sherwin, 1990). Lipid peroxidation is a major cause of food deterioration, leading to loss of functional properties and nutritional value (Yen *et al.* 1999). Synthetic antioxidants such as propyl gallate (PG), butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), and tert-butylhydroquinone (TBHQ) have been dominant since their introduction. Because of this effect, they have been used as antioxidant in foods for years. However, some physical properties of BHA and BHT, such as their high volatility and instability at elevated temperatures, strict legislation on the use of synthetic food additives and consumer preferences have shifted the attention manufacturers from synthetic to natural antioxidants (Porter, 1980; Porkorny, 1991). Consumers are increasingly avoiding foods prepared with preservatives of chemical origin, and natural alternatives are therefore needed to achieve sufficiently long shelf life of foods and a high degree of safety with respect to food borne pathogenic microorganisms (Rauha *et al.* 2000). Especially, in the past few years, there has been increasing interest in finding natural antioxidants because they can protect the human body from free radicals, ROS related effects and retard the progress of many chronic diseases as well as retard lipid oxidative rancidity in foods (Pryor, 1991; Kinsella *et al.* 1993).

Recent studies showed that a number of plants products were including polyphenolic substances such as flavonoids and tannins. Those natural antioxidant substances usually have a phenolic moiety in their molecular structure. They have been found among flavonoids, tocopherols and catechines. Organic acids,

carotenoids, protein hydrolysates and tannins can act as antioxidants or have synergistic effects when used together with phenolic antioxidants. Currently materials, which inhibit lipid oxidation, can be obtained from plant materials, food waste, microorganisms, and animal cells (Dugan, 1980; Langseth, 1995).

The spoilage and poisoning of foods by microorganisms are problems that have not yet been brought under adequate control despite the range of robust preservation techniques available. In nature, there are a large number of different types of antimicrobial compounds that play an important role in the natural defence of all kinds of living organism.

The genus *Teucrium* (Lamiaceae) is represented by 27 species in the flora of Turkey (Davis 1982). *T. polium* is used in the treatment of diabetes, hepatitis, haemorrhoid and stomach pain in Turkish folk medicine (Baytop, 1984; Bedir *et al.* 1999). In addition, this plant was commonly used against headache, cold and pain (Tuzlaci and Erol, 1999). Also, anti-inflammatory, antibacterial, antihypertensive, hypoglycaemic, hypolipidemic, analgesic effects and anorectic properties of *T. polium* have been reported. In addition, active compounds such as diterpenoids, flavonoids, iridoids, sterols, teulolin, and terpenoids have been isolated from *T. polium* (Tuzlaci and Erol, 1999, Bedir *et al.* 1999, Rasekh *et al.* 2001). Recent biological screenings of *T. polium* have shown that a number of them have anticandidal and antidermatophytic activities (Ali-Shtayeh *et al.* 1997; Ali-Shtayeh and Abu Ghdeib, 1999).

The aim of present study was to investigate the antioxidant and antimicrobial properties of *T. polium* in order to evaluate its medicinal value and to point to an easily accessible source of natural antioxidants that could be used as a possible food supplement.

Materials and Methods

Chemicals: Ammonium thiocyanate was purchased from E. Merck (Darmstadt, Germany). Ferrous chloride, polyoxyethylenesorbitan monolaurate (Tween-20), β-tocopherol, 1,1-diphenyl-2-picryl-hydrazil (DPPH.), 3-(2-Pyridyl)-5,6-bis (4-phenyl-sulfonic acid)-1,2,4-triazine (Ferrozine), linoleic acid, nicotinamide adenine dinucleotide (NADH), pyrocatechol, Folin-Ciocalteu reagent and trichloraceticacid (TCA) were purchased from Sigma Chemical Co. (St. Louis, MO). All other chemicals were of analytical grade.

Plant Materials and Preparation of Extracts: *Teucrium polium* L. plant were collected from Kahramanmara° in May and authenticated by Dr. Ahmet Ilçim, Department of Biology, Faculty of Science and Arts, Sütçü Imam University, Kahramanmara°, Turkey. The plant materials were left on a bench to dry. The dried sample was chopped into small parts with a blender. For chloroform and acetone extracts, 20 g dried powder of each *T. polium* was extracted with 400 mL of solvent until extraction solvents become colourless, then extracts of each solvent were evaporated under reduced pressure and the final residues were used for the bioassays.

Microorganisms: Bacteria and fungi were obtained from the stock cultures of Microbiology Laboratory, Department of Biology, Faculty of Science and Arts, Sütçü İmam University, Kahramanmaraþ. The bacterial and fungal stock cultures were maintained on Muller Hinton Agar (Oxoid) slants, respectively, which were stored at 4 oC. For the purpose of antimicrobial evaluation eleven microorganisms, i.e. *Staphylococcus aureus* (gram-positive), methicillin-resistant cultured in the appropriated broths at 37°C overnight.

Antimicrobial Activity Determination: Agar cultures of the test microorganisms were prepared as described by Mackeen at al (Mackeen *et al.*, 1997). For screening 30 mg of extract was loaded onto each discs (Schleicher & Schül, Germany) and placed on the previously inoculated agar. The plates were incubated for 18 h at 30°C for bacterial species and 48 h at 30°C for fungal species. Clear inhibition zones around the discs indicated the presence of antimicrobial activity.

Antioxidant Activity Determination: The antioxidant activity of each solvent extract of *T. polium* was determined according to the thiocyanate method (Mitsuda *et al.* 1996). Each samples (1.0 mg) in 1 mL of ethanol was added to a solution of linoleic acid (2.5 mL, 0.02 M) in potassium phosphate buffer (2mL, 0.04 M, pH 7.0). The mixed solution was incubated at 37 oC in a glass flask. The peroxide value was determined by reading the absorbance at 500 nm in a spectrophotometer (8500 II, Bio-Crom Gmb, Zurich, Switzerland), after reaction with FeCl2 and thiocyanate at intervals during incubation. The solutions without added solvent extracts were used as blank samples. All test data were average of duplicate analyses. The inhibition of lipid peroxidation in percent was calculated by following equation:

% Inhibition = 100-[(A1 / Ao) x100]

where Ao was the absorbance of the control reaction and A1 was the absorbance in the presence of the *T. polium* extracts sample (Duh *et al.*, 1999)

Reducing Power: The reducing power of *T. polium* extracts was determined according to the method of Oyaizu (Oyaizu, 1986). Different amount of *T. polium* extracts (from 50 to 250 mg) in 1mL of distilled water were mixed with phosphate buffer (2.5 mL, 0.2 M, pH 6.6) and 2.5 mL of potassium ferricyanide [K3Fe(CN)6]

(1%). Then the mixture was incubated at 50 oC for 20 min. A portion (2.5 mL) of TCA (10%) was added to the mixture, which was then centrifuged at then centrifugation for 10 min at 1000μg (MSE Mistral 2000, U.K, Serial No: S693/02/444) for 10 min. The upper layer of solution (2.5 mL) was mixed with distilled water (2.5 mL) and FeCl3 (0.5 mL, 0.1%), and the absorbance was measured at 700 nm in a spectrophotometer (8500 II, Bio-Crom Gmb, Zurich, Switzerland). Increased absorbance of the reaction mixture indicated increased reducing power. a-Tocopherol, a commercial antioxidant, was used as reference standard.

Superoxide Anion Scavenging Activity: Measurement of superoxide anion scavenging activity of *T. polium* extracts was done based on the method described by Nishimiki (Nishimiki *et al.* 1972) and slightly modified. One mL of nitroblue tetrazolium (NBT) solution (156 μM NBT in 100 mM phosphate buffer, pH 7.4), 1 mL NADH solution (468 μM in 100 mM phosphate buffer, pH 7.4) and 1 mL of sample solution of *T. polium* extracts (contains 100 μg dried sample) in methanol were mixed. The reaction started by adding 1 mL of phenazine methosulphate (PMS) solution (60 μM PMS in 100 μM phosphate buffer, pH 7.4) to the mixture. The reaction mixture was incubated at 25 oC for 5 min, and the absorbance at 560 nm was measured against blank samples.

Free Radical Scavenging Activity: The free radical scavenging activity of *T. polium* extracts was measured using the method of Blois (Blois, 1958) with a slight modification. A 0.1 mM solution of DPPH in ethanol was prepared. Then 1 mL of this solution was added 3 mL of extracts solution in ethanol, which containing 100 μg of extract samples. After 30 minutes at room temperature, the decrease in absorbance was measured at 517 nm in a spectrophotometer (8500 II, Bio-Crom Gmb, Zurich, Switzerland).

Metal Chelating Activity: The chelating of ferrous ions by the extracts of *T. polium* was estimated by the method of Dinis (Dinis, 1994). Briefly the samples of *T. polium* extracts (50-250 μg) were added to a solution of 2 mM FeCl2 (0.05 mL). The reaction was initiated by the addition of 5 mM ferrozine (0.2 mL) and the mixture was shaken vigorously and left standing at room temperature for ten minute. Absorbance of the solution was then measured spectrophotometrically at 562 nm in a spectrophotometer (8500 II, Bio-Crom Gmb, Zurich, Switzerland). All test and analyses were run in triplicate and averaged.

Hydrogen Peroxide Scavenging Activity: The ability of both extracts of *T. polium* to scavenge hydrogen peroxide was determined according to the method of Ruch (Ruch, 1989). A solution of hydrogen peroxide (2 mM) was prepared in phosphate buffer (pH 7.4). Hydrogen peroxide concentration was determined spectrophotometrically from absorption at 230 nm with molar absorbtivity 81 (mol/L)-1cm1. The samples of *T. polium* extracts (50-250 μg) in distilled water were added to a hydrogen peroxide solution (0.6 mL). Absorbance of hydrogen peroxide at 230 nm in a spectrophotometer (8500 II, Bio-Crom Gmb, Zurich, Switzerland) was determined after ten minute against a blank solution containing in phosphate buffer without hydrogen peroxide.

Determination of Total Phenolic Compounds: Total soluble phenolics in the *T. polium* extracts were determined with Folin-Ciocalteu reagent according to the method of Slinkard (Slinkard and Singleton, 1977) using pyrocatechol as a standard. Briefly, 0.1 mL of extract solution (contains 1000 μg extracts) in a volumetric flask diluted glass-distilled water (46 mL). Folin-Ciocalteu reagent (1 mL) was added and the content of the flask mixed thoroughly. After 3 minutes 3 mL of Na2CO3 (2%) was added then the mixture was allowed to stand for 2 hours with intermittent shaking at room temperature. The absorbance was measured at 760 nm in a spectrophotometer (8500 II, Bio-Crom Gmb, Zurich, Switzerland). The concentration of total phenolic compounds in the *T. polium* determined as micrograms of pyrocatechol equivalent.

Statistical Analysis: Experimental results were mean ± S. D. of five parallel measurements. Analysis of variance was performed by ANOVA procedures. Significant differences between means were determined by Duncan's Multiple Range tests. P values < 0.05 were regarded as significant and P values <0.01 very significant.

Result and Discussion

Antimicrobial Activity: Disc diffusion methods are extensively used to investigate the antibacterial activity of natural substance and plant extracts. These assay are based on the use of disc as reservoirs containing the solution of substances to be examined. In the case of solutions with a low activity, however, a large concentration or volume is needed. The limited capacity of discs means that holes or cylinders are preferably used (Bartner *et al.*, 1994).

In this study, eleven different microbial and four different fungal species were used to screen the possible antimicrobial activities of *T. polium* extracts. Ten bacterial species and four fungal species were sensitive to antimicrobial activity of extracts as a

Table 1: Anitmicrobial activities of chloroform and acetone extract of Teucrium polium L. (30μg/disc) and Streptomycin (20 μg/disc)

Microorganisms	Teucrium polium L. Extracts		
	Chloroform	Acetone	Streptomycin
Bacteria			
Bacillus megaterium	7	-	17
Proteus vulgaris	7	10	18
Listeria monocytogenes	-	10	19
Bacillus cereus	7	-	14
Staphylococceus aureus	-	13	17
Bacillus brevis	7	10	16
Klebsiella pneumoniae	7	10	15
Micrococcusluteus	-	11	16
Pseudomonas aeruginosa	7	11	14
Mycobacterium smegmatis	-	11	15
Escherchia coli	-	-	-
Fungus			
Penicillum frequentans	-	-	ND
Fusarium equiseti	-	-	ND
Aspergillus candidus	-	-	ND
Byssochlamys fulves	-	-	ND

Values are the mean of three replicate. Acetone extract (30 μg/disc), Chloroform extract (30μg/disc), Streptomycin (20μg/disc), ND: Not determined.

Table 2: The percentage of inhibition of lipid peroxidation in linoleic acid emulsion and total phenolic compounds in 1mg of chloroform and acetone extracts of *Teucrium polium* L.

	Total Phenolic Content (μg)	Inhibition of Lipid Peroxidation (%)
Acetone Extract*	87.37	84
Chloroform Extract*	49.37	89

*Microgram equivlanet of pyrocatechol.

shown Table 1. Of the species used, *S. aureus* is one of the most of gram-positive bacteria causing food poisoning. Its source is not the food itself, but the humans who contaminate foods after they have been processed (Rauha *et al.*, 2000). All of the extracts showed strong antibacterial activity against this bacterium (*S. aureus*), but the both extracts of *T. polium* didn't showed antibacterial activity against *E. coli*, in that both extracts failed to inhibit the growth of *E. coli*. Streptomycin (20 μg/disc) was used as a positive control for bacteria.

Total Antioxidant Activity: In the present study, antioxidant activities of acetone and chloroform extracts of the *T. polium* were determined by the thiocyanate methods. The amount of peroxides formed in linoleic acid emulsion during incubation is determined spectrophotometrically by measuring absorbance at 500 nm. High absorbance is the indication of high concentration of formed peroxides. Antioxidant activity was shown by both extracts of *T. polium* assayed (Fig. 1). As can be seen in Table 2, the both extract of *T. polium* showed effective levels of inhibitory activity towards lipid oxidation. The antioxidant activity of these extracts was higher than that of α-tocopherol. Chloroform extract of *T. polium* had stronger antioxidant activity than the acetone extract. This observation shows that antioxidant components present in the chloroform extract contributed towards the increased activity over the acetone extract. Although the antioxidant activity of each extract is increased with increasing amount of extract, but those values were not showed. The percentage of inhibition of 1 mg chloroform and acetone extract of *T. polium* on peroxidation in linoleic acid system was 89 and 84 %, respectively and greater than that 1 mg of α-tocopherol (46%). The percentage of inhibition on peroxidation of chloroform and acetone extracts of *T. polium* and α-tocopherol followed the order: chloroform extract of *T. polium* > acetone extract of *T. polium* > α-tocopherol.

Reducing Power: For the measurements of the reductive ability we investigated the Fe^{3+}-Fe^{2+} transformation in the presence of the samples of *T. polium* extracts using the Oyaizu method (Oyaizu,

1986). Like the antioxidant activity, the reducing power of both extracts increased with amount of extract (Fig. 2). Some literature has reported that reducing power was associated with antioxidant activity (Yen and Duh, 1993). Even in the presence of 50 μg of extract of *T. polium*, reducing power was significantly higher than that control, in which there was no extract ($p < 0.05$). The acetone extract of *T. polium* is higher than reducing power chloroform extract.

Superoxide Anion Scavenging Activity: In the PMS/NADH-NBT system, superoxide anion derived from dissolved oxygen by PMS/NADH coupling reaction reduces NBT. The decrease of absorbance at 560 nm with antioxidants thus indicates the consumption of superoxide anion in the reaction mixture. Figure 3 shows the superoxide radical scavenging activity of 100 μg of both of acetone and chloroform extracts of *T. polium*. Both of organic extracts exhibited strong superoxide radical scavenging activity. The results were found statistically significant ($P < .0.05$). Superoxide anion radical scavenging activity of those samples of *T. polium* extract followed the order: acetone extract of *T. polium* > chloroform extract of *T. polium*.

Free Radical Scavenging Activity: DPPH. is a stable free radicals in aqueous or ethanol solution and accepts an electron or hydrogen radical to become a stable diamagnetic molecule (Soares *et al.*, 1997). In order to evaluate the antioxidant potency through free radical scavenging by the test samples, the change of optical density of DPPH radicals was monitored. Hence, DPPH· is usually used as a substrate to evaluate antioxidative activity of antioxidants (Duh *et al.* 1999). Fig. 4 illustrates the decrease absorbance of DPPH radical due to the scavenging ability of soluble solids in the 100 μg of *T. polium* extracts. Chloroform extract of *T. polium* had the higher free radical scavenging activity than acetone extract. The results were found statistically significant when compared to the control values ($p < .0.05$).

Metal Chelating Activity: Ferrozine can quantitatively form complexes with Fe^{2+}. In the presence of other chelating agents, the complex formation is disrupted with the result that the red colour of the complex is decreased. Measurement of the rate of colour reduction therefore allows estimation of the chelating activity of the coexisting chelator (Yamaguchi *et al.*, 2000). In this assay the both extract of *T. polium* and standard compounds interfered with the formation of ferrous and ferrozine complex, suggesting that it has chelating activity and captures ferrous ion before ferrozine. The metal binding capacity of *T. polium* extracts and standard antioxidants determined by assessing their ability to compete with ferrozine for the ferrous ions. As shown Figure 5, he formation of ferrozine-Fe + 2 complex is not complete in presence of *T. polium* extracts. The ability of chelating ferrous ions also increased with increasing amount of *T. polium* extracts. It is indicated that *T. polium* extracts chelate the iron. The metal chelating effect of *T. polium* extracts close to α-tocopherol and BHT, but lower than that BHA, relatively and this difference was statistically significant ($p < 0.05$) when compared to control. 250 μg of chloroform and acetone extracts of *T. polium* exhibited 55.18 and 25.38, % iron binding capacity, respectively. In the other hand, 250 μg of α-tocopherol, BHA, and BHT are showed 43.04, 74.83, and 40.61% chelating activity of iron, respectively. Those values obtained from Fig. 4 demonstrated that the action of *T. polium* extracts, as peroxidation protector may be more related to its iron binding capacity.

Hydrogen Peroxide Scavenging Activity: The scavenging ability of *T. polium* extracts on hydrogen peroxide is shown Figure 6 and compared with BHA, BHT and α-tocopherol as standards. The both *T. polium* extracts were capable of scavenging hydrogen peroxide in an amount-dependent manner. Acetone and chloroform extracts (500 μg) of *T. polium* exhibited 78.75 and 62.50 % scavenging activity on hydrogen peroxide, respectively. On the other hand, at the same dose; BHA, BHT, and α-tocopherol exhibited 37.25, 86.0, and 57.0% hydrogen peroxide scavenging activity. These results showed that *T. polium* extracts had stronger hydrogen peroxide scavenging activity than BHA. Those values close to α-tocopherol but lower than that BHT. There is statistically significant correlation between those values and control ($p < 0.05$). Hydrogen peroxide itself is not very reactive, but it can sometimes be toxic to cell because it may give rise to hydroxyl radical in the cells (Halliwell, 1991). Thus, the removing of H2O2 as well as O2- is very important for antioxidant defence in cell or food systems.

Total Phenolic Compounds Analysis: Phenolic antioxidants are potent free radical terminator (Shahidi and Wanasundara, 1992). The phenolic compounds, biologically active component, are the main agents that can donate hydrogen to free radicals and thus break the chain reaction of lipid oxidation at the first initiation step. This high potential of phenolic compounds to scavenge radicals may be explained by their phenolic hydroxyl groups (Sawa *et al.*, 1999). The total amount of phenolic compounds in *T. polium* extracts was determined as pyrocatechol equivalent by using an

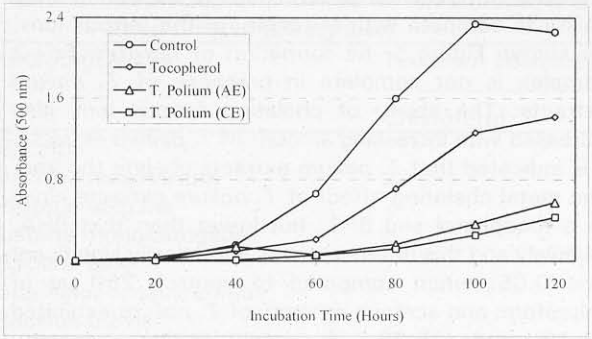

Fig. 1: Antoxidant activity of 250 μg of chloroform and acetone extracts of *Teucrium polium* L. in the linoleic acid emulsion. (CE: Chloroform extract, AE: (Acetone extract:.

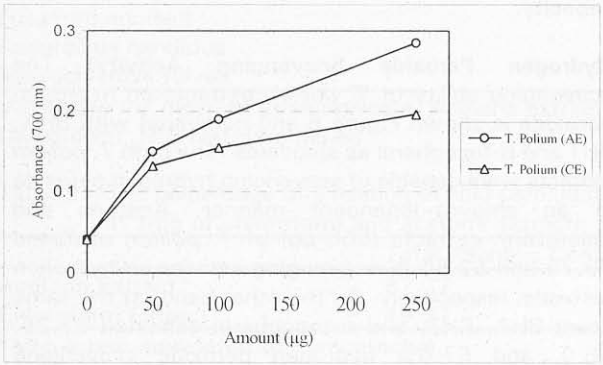

Fig. 2: Reducing power of chloroform and acetone extracts of *Teurcium polium* L. (CE: Chloroform extract, AE: Acetone extract).

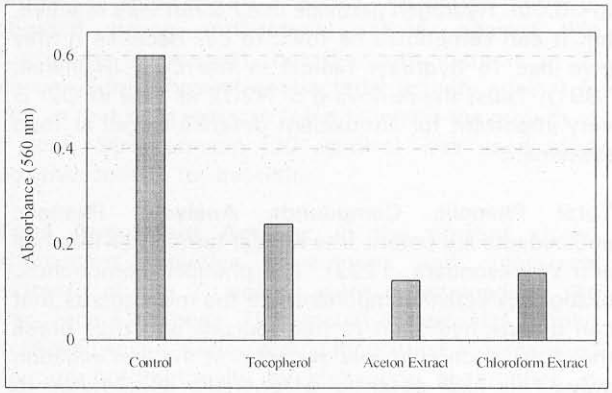

Fig. 3: Superoxide anion radical scavenging activity of 100 μg of chloroform and acetone extracts of *Teucrium polium* L.

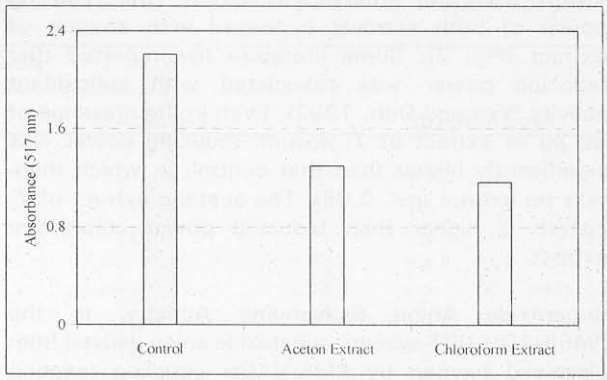

Fig. 4: Free radical scavenging activity of 100 μg of chloroform and acetone extracts of *Teucrium polium* L.

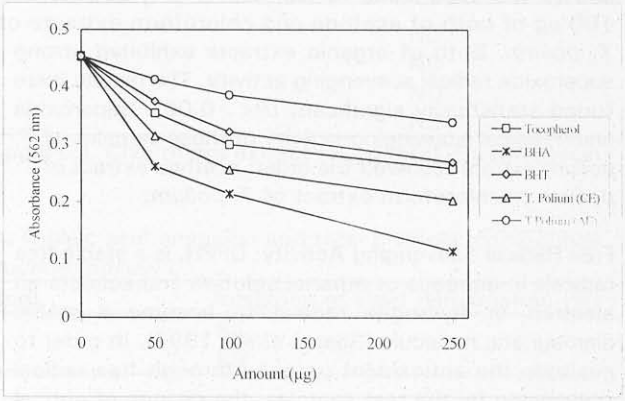

Fig. 5: Metal chelating activity of chloroform and acetone extracts *Teucrium polium* L.

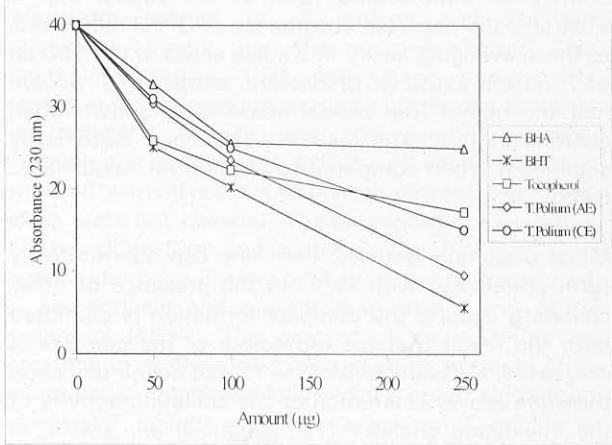

Fig. 6: Hydrogen peroxide scavenging activity of chloroform and acetone extracts of *Teucrium polium* L. (CE: Chloroform extract, AE: Acetone extract).

equation that was obtained from standard pyrocatechol graph. The equation is given below:

Absorbance = 0,001 x mg Pyrocatechol + 0,0033

Standard graph was prepared by using 0,1 ml of pyrocatechol solution contains 0-400 μg Pyrocatechol in place of *T. polium* extracts. To determine the amount of total phenolic compounds in extracts, the absorbance of sample (contains 1000 μg dried extract) was measured at 760 nm using a spectrophotometer (8500 II, Bio-Crom Gmb, Zurich, Switzerland). Absorbance value inserted in above equation and the total amount of phenolic compound was calculated. As it can be seen in Table 2, total phenolic contents acetone and chloroform extracts of *T. polium* were found as 87.37 and 49.38 μg pyrocatechol equivalent. In conclusion, *T. polium* have antioxidant and antimicrobial activities. Accordingly, this implies the inhibition of microbial pathogenesis, and cellular oxidation that is linked to pathological incidents such as heart disease, aging and cancer. However, the components responsible for the antioxidative and antimicrobial activities of *T. polium* extracts are currently unclear. Therefore, it is suggested that further work could be performed on the isolation and identification of the antioxidative and antimicrobial components from extracts of *T. polium*.

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