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Full Length Research Paper

Antioxidant activities of solvent extracts from endemic *Cyclamen mirabile* Hildebr. tubers and leaves

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In this study, solvent extracts were prepared from different parts of *Cyclamen mirabile* (CM) and their antioxidant activities were evaluated. Other antioxidant properties of all extracts of CM tubers and leaves, including free radical scavenging activity, reducing power and total phenolic compound content, were also determined. Leaves extracts of CM exhibited higher antioxidant activity than tuber extracts with all the types of solvent used. All concentrations of petroleum ether, acetone, methanol and water extracts of CM leaves showed higher antioxidant activities than that of 0.5 mg of α-tocopherol (42%) and close to BHT (99.30%) and had 96.60, 96.00, 96.10 and 97.40% inhibition of lipid peroxidation of linoleic acid at same doses, respectively. All extracts of CM tubers and leaves had effective free radical scavenging and reducing power. In addition, total phenolic compounds in all the extracts of CM tubers and leaves were determined as pyrocatechol equivalents.

Key words: *Cyclamen mirabile*, antioxidant activity, reducing power, scavenging activity, total phenolic compound.

INTRODUCTION

One of the principal causes of food quality deterioration is the oxidation of unsaturated lipids initiated by free radicals. When lipids are exposed to environmental factors such as air, light and temperature, oxidation reactions start to produce undesirable flavours, rancid odours, discoloration and other forms of spoilage. The primary autoxidation products are hydroperoxides, that have no taste and flavour, but their degradation products (aldehydes, ketone, etc.) have very potent taste and flavour modifiers (Gordon, 1991).

Reactive oxygen species (ROS), such as hydroxyl radical, hydrogen peroxide and superoxide anions, are produced as by products in aerobic organisms and have been implicated in the pathology of a vast variety of human diseases including cancer, atherosclerosis, diabetic mellitus, hypertension, AIDS and aging (Halliwell and Gutteridge, 1984; Wallace, 1999; Lee et al., 2000a). Therefore, antioxidant activity is an important in-view of the free radical theory of aging and associated diseases.

To retard or prevent the oxidative deterioration, the antioxidants are added in food. The added antioxidants then maintain the quality and extend the shelf-life of many food products (Andreja et al., 2000). The antioxidants can be of synthetic or natural origin. The use of synthetic antioxidants is restricted in several countries, because of their possible undesirable effects on human health (Branen, 1975; Chen et al., 1992; Kahl and Kappus, 1993). Hence, the studies on natural antioxidant have gained increasingly greater importance.

In the search for sources of natural antioxidants, in the last few years some medicinal plants have been extensively studied for their antioxidant activity and radical scavenging activity (De las Heras et al., 1998; Desmarchelier et al., 2000; Schinella et al., 2002; VanderJagt et al., 2002). A number of studies on the antioxidant activities of various aromatic plants have been reported over the last 20 years (Brraco et al., 1981; Herrmann et al., 1981; Kramer, 1985; Lagouri et al., 1993). Flavonoids and other phenolic compounds of plant origin have been reported as scavenger of ROS, thus, they are viewed as promising therapeutic drugs for free radical pathologies (Parshad et al., 1998; Lee et al., 2000b).

The antioxidant activity of plant origin is dependent on the type and polarity of the extracting solvent as well as

on the test system and the substrate to be protected by the antioxidant (Heinonen et al., 1998; Moure et al., 2000; Kang and Lee, 2001). Solvent extraction is frequently used for isolation of the antioxidants and both extraction yield and antioxidant activity of the extracts are strongly dependent on the solvent, due to the different antioxidant potentials of compounds with different polarity. For these reasons, comparative studies for selecting the optimal solvent providing maximum antioxidant activity are required for each substrate. Although, the use of different polarity substances can provide more exhaustive information on the properties of the extracts, literature contains few reports of the polarity-based solvent extraction of medicinal plants (Kang et al., 2003).

Cyclamen mirabile Hildebr, is an endemic species grown in South-west Anatolia (C2 Mugla, C3 Isparta), Turkey, where it grows in *Pinus brutia* forests and hill slopes with maquis, on limestone, metamorphic and granitic rocks, at altitudes of 400 to 1600 m and flowers from September to November (Davis, 1978).

The tubers of *C. mirabile* contain glycosides such as starch, glue, organic acids and saponins. In addition, it has emetic purgative and stimulant effects. The infusions that are made by fresh tubers cause medium intensity diarrhea. The overdoses that are the medical dosage cause dangerous toxication which appears with vomit and strong diarrhea. The tubers are eaten usually by pigs (Baytop, 1984). Calis et al. (1997) had previously reported the study of the triterpenoid saponins of *C. mirabile* Hildebr. This resulted in the isolation of six saponins and their biological activities (antibacterial and antifungal). There are no sufficient studies on *C. mirabile*. Additionally, systemic and multi - method evaluations on antioxidant activities of solvent extracts of this species has not previously been reported. Therefore, the data presented here will be the first record on *C. mirabile.*

The purpose of this study was to evaluate the antioxidant activity of various solvent extracts from different parts (tuber and leaves) of *C. mirabile* using petroleum ether, acetone, methanol and water. Other antioxidant properties of all solvent extracts, including scavenging activity on 1,1- diphenyl - 2 - picrylhydrazyl, reducing power and total phenolic content were also determined.

MATERIALS AND METHODS

Plant materials

Different parts (leaves and tubers) of *C. mirabile* were collected from the natural environment of Mugla city in Turkey in October 2002 and cleaned to remove any residual compost. The air-dried leaves and tubers were ground to fine powder and then, stored in an air-tight container until further use.

Chemicals

Ammonium thiocyanate, ascorbic acid, potassium ferricyanide, ferrous chloride, ferric chloride, Folin-Ciocalteu's reagent (FCR), polyoxyethylenesorbitan monolaurate (Tween-20), methanol, ethyl acetate and trichloracetic acid (TCA) were obtained from E. Merck (Darmstadt, Germany). 1,1-diphenyl-2-picrylhydrazyl (DPPH), butylated hydroxytoluene (BHT) and α-tocopherol were obtained from Sigma Chemical Co. (St. Lois, MO). All other chemicals and solvents were of analytical grade.

Extraction

10 g of air-dried parts of *C. mirabile* were extracted with four different solvents (petroleum ether, acetone, methanol and water). 10 g of powdered of *C. mirabile* leaves and tubers were extracted in soxhlet apparatus with 100 ml of petroleum ether until the extraction solvent became colourless. 10 g of powdered *C. mirabile* leaves and tubers were extracted with 200 ml of acetone and methanol solvents in a shaking incubator at 25 ± 1°C for 24 h. The extraction was repeated twice at same condition. These extracts were filtered and the organic solvents were removed in vacuum by a rotary evaporator (Heidolph Laborota 4010, Germany). For water extraction, 10 g of powdered of *C. mirabile* leaves and tubers was mixed with boiling water (500 ml) for 15 min. The extract was filtered and evaporated in vacuum below 70°C on a rotary evaporator (Duh and Yen, 1997).

Determination of antioxidant activity

The antioxidant activities of extracts from different parts (tubers and leaves) of *C. mirabile* were determined by the thiocyanate method (Mitsuda et al., 1996). 1 ml of extracts at different concentrations were mixed with 2.5 ml of linoleic acid emulsion, including 155 µl linoleic acid, 175 µg Tween-20 and potassium phosphate buffer (0.04 M, pH 7.0) in 50 ml of its solution and 1.5 ml of phosphate buffer (0.04 M, pH 7.0). The mixed solution (5 ml) was incubated at 37°C for 110 h in the dark. 0.1 ml of the sample solution was added to ethanol (4.7 ml, 75%), ferrous chloride (0.1 ml, 20 mM in 3.5% HCl) and ammonium thiocyanate (0.1 ml, 30%). After the mixture was stirred for 3 min, the peroxide value was determined by measuring the absorbance at 500 nm. The percent inhibition of lipid peroxidation was calculated using the following equation:

$$\text{Inhibition \%} = 100 - ((A_1/A_0) \times 100)$$

Where, A_0 is the absorbance of control and A_1 is the absorbance of the sample of *C. mirabile*.

Determination of total phenolic compounds

The concentrations of phenolic compounds in all extracts of *C. mirabile*, expressed as microgram of pyrocatechol equivalents (PEs), were determined with Folin-Ciocalteu reagent (FCR) according to the method of Slinkard and Singleton (1977). 1 ml of the solution extracts (1 mg) was added to 46 ml of distilled water and 1 ml of FCR and was mixed thoroughly. After 3 min, the mixture was added to 3 ml of sodium carbonate (2%) and shaken intermittently for 2 h. The absorbance was read at 760 nm. The concentrations of phenolic compounds were calculated to follow the equation that was obtained from standard pyrocatechol graph:

$$\text{Absorbance} = 0.002248 \text{ pyrocatechol } (\mu g) + 0.0024 \ (R^2: 0.999)$$

Free radical scavenging activity

Radical scavenging activity of all extracts of *C. mirabile* was

Table 1. Yield of extracts from *C. mirabile* leaves and tubers using various solvents.

Solvent	Yield (%)	
	Leaf	Tuber
Petroleum ether	2.34	0.20
Acetone	5.88	0.64
Methanol	30.29	28.78
Water	12.59	7.79

determined using DPPH as a reagent (Cuendet et al., 1997; Kirby and Scmidt, 1997) with slight modification. Different amounts (0.2 to 1 mg) of extracts from CM in 1 ml of solution were added to 4 ml of a 0.004% methanol solution of DPPH. The samples were incubated for 30 min in the dark at room temperature. Scavenging capacity was read spectrophotometrically by monitoring the decrease in absorbance at 517 nm using a spectrophotometer (Shimadzu UV-1601, Japan). The capability to scavenge $DPPH^{\cdot}$ radical was calculated using the following equation:

$DPPH^{\cdot}$ scavenging effect (%) = $100 - [(A_1 / A_o) \times 100]$

Where, A_0 is the absorbance of control and A_1 is the absorbance of the sample of *C. mirabile* (Duh and Yen, 1997).

Reducing power

The reducing power of extracts from *C. mirabile* was determined according to the method of Oyaizu (1986). Extracts solution in methanol and water at different amounts (0.2 to 1 mg) were mixed with 2.5 ml of 0.2 M phosphate buffer (pH 6.6) and 2.5 ml of potassium ferricyanide (1%). The mixture was incubated at 50°C for 20 min. After 2.5 ml of TCA (10%) was added, the mixture was centrifuged at 3000 rpm for 10 min. Supernatant (2.5 ml) was mixed with distilled water (2.5 ml) and 0.5 ml of ferric chloride (0.1%) and the absorbance was measured at 700 nm. Higher absorbance of the reaction mixture indicates greater reducing power.

RESULTS AND DISCUSSION

Yield of extracts

The yields of different solvent extracts from *C. mirabile* tubers and leaves are shown in Table 1. For tubers and leaves, the highest yield were obtained with methanol (28.78 to 30.29%), followed by water (7.79 to 12.59%). This observation is in agreement with that reported by some researchers, that solvents with high polarity are effective for extraction of natural antioxidants (Chang et al., 1977; Duh et al., 1992; Economou et al., 1991; Tian and White, 1994). Results showed that the leaves gave the highest yield.

Determination of antioxidant activity

The antioxidant activity of different parts of *C. mirabile* was determined by the thiocyanate method. In this method, peroxides which oxidized ferrous ions to ferric ions are formed during the linoleic acid oxidation. Ferric ions form a complex with SCN^{-} and this complex has a maximum absorbance at 500 nm. Therefore, lower absorbance indicates high antioxidant activity.

The activities of different amounts (0.2 to 1 mg) of the extracts of CM tubers and leaves on peroxidation of linoleic acid were measured spectrophotometrically by monitoring absorbance at 500 nm. For the sake of simplification, only the results of extracts of CM tubers and leaves containing 0.5 mg amounts were given in Figures 1 and 2, respectively.

α-Tocopherol and BHT were used as comparison and positive control. All the extracts from CM tubers and leaves, except for water extract (34.60%) of CM tubers showed stronger antioxidant activities (>71%) than that of α-tocopherol (42.0%) and lower antioxidant activities than that of BHT (99.3%).

For the extracts of petroleum ether, methanol and water, the antioxidant activity of the extracts was increased with increasing amounts of extracts, but the antioxidant activity of acetone extracts of CM tubers and leaves was decreased with increasing amounts of extracts due to prooxidant activity. This might be explained by the fact that, at higher amount, the extract served as an oxygen-carrying agent (Holloway and Gainer, 1988) and as a prooxidant in the co-oxidation of linoleic acid.

Determination of total phenolic compounds

Some investigations have reported that phenolic compounds are very important plant materials because of their inhibitory effect on autoxidation of oils (Ramarathnam et al., 1986) and their radical scavenging ability (Hatano et al., 1989). Therefore, it is important to determine the effect of the total phenolic compound on the antioxidant activity of extracts of the different parts of CM. The concentration of phenolics in all the extracts of CM tubers and leaves was expressed as mg pyrocatechol equivalent g of extracts as shown in Table 2.

In petroleum ether, acetone, methanol and water extracts of CM leaves (1 mg), 37.36, 21.06, 17.30 and 30.14 µg pyrocatechol equivalent of phenols was detected. In addition, the amount of total phenolic compounds of acetone extract of CM tubers was 10 times more than that of methanol extract.

Free radical scavenging activity

When lipids containing polyunsaturated fatty acids are readily oxidised by molecular oxygen, reactive oxygen species such as $^{\cdot}OH$, $O_2^{\cdot -}$, $LOO^{\cdot}$, $LO^{\cdot}$ and $L^{\cdot}$ are formed (Aruoma, 1998). These species can rapidly react with susceptible food and biological substrates, such as

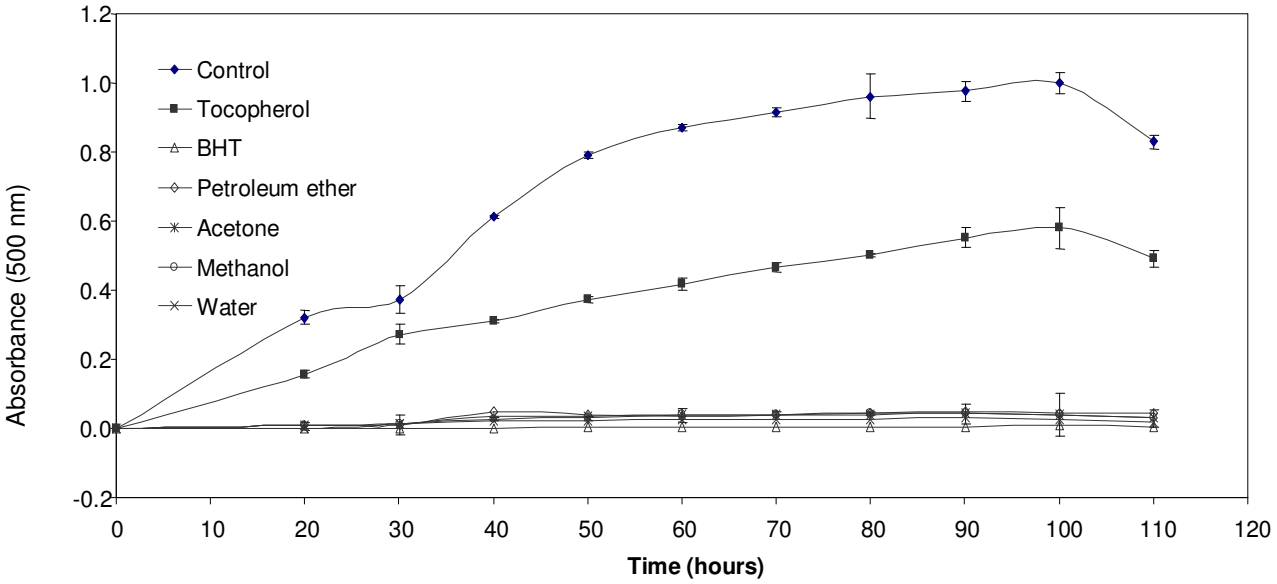

Figure 1. Antioxidant activity of solvent extracts (0.5 mg) of *C. mirabile* tubers using the thiocyanate method. BHT; Butylated hydroxytoluene (values are means ± standard deviation of three replicate analyses).

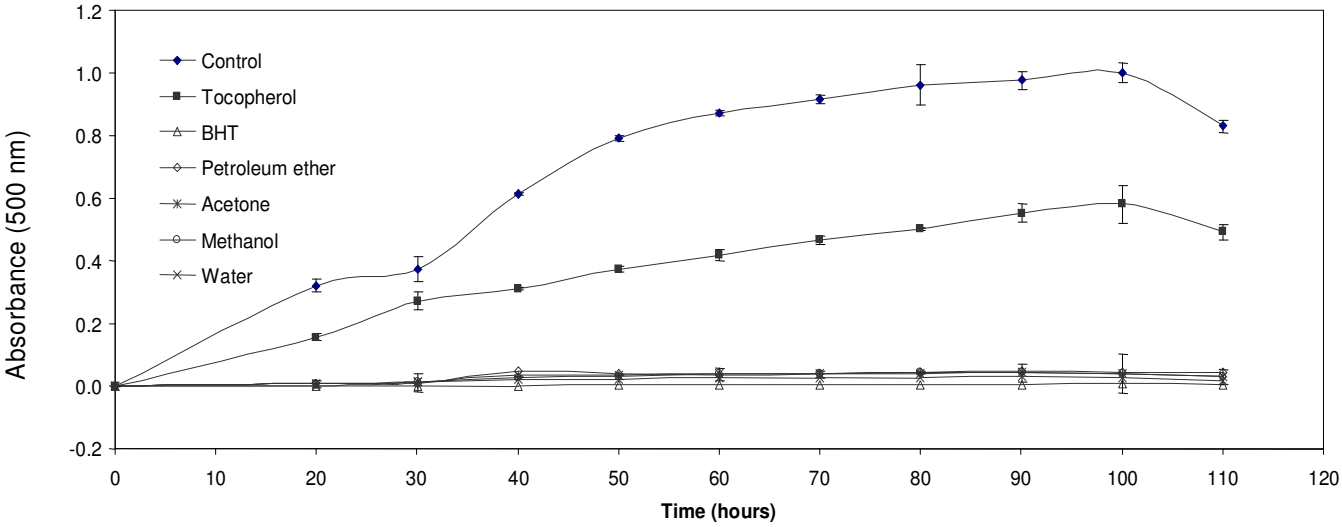

Figure 2. Antioxidant activity of solvent extracts (0.5 mg) of *C. mirabile* leaves using the thiocyanate method. BHT; Butylated hydroxytoluene (values are means ± standard deviation of three replicate analyses).

Table 2. Total phenolic content (µg of PEs[a] / mg of extract) of extracts from different parts of *C. mirabile* using various solvents[b].

Solvent	Tuber	Leaf
Petroleum ether	17.42 ± 0.35	37.36 ± 0.87
Acetone	51.76 ± 0.16	21.06 ± 0.46
Methanol	5.18 ± 0.03	17.30 ± 0.82
Water	6.36 ± 0.19	30.14 ± 0.09

[a] PEs, pyrocatechol equivalents; [b] values are means ± standard deviation of three replicate analyses.

polyunsaturated fatty acids, proteins and sugars (Halliwell, 1994).

Relatively stable radical, DPPH, has been widely used in the assessment of radical scavenging activity of some natural sources (Imai et al., 1994; Duh and Yen, 1997) of foods (Yamaguchi et al., 1998) and pure compounds (Sanchez-Moreno et al., 1998). In this method, the stable radical DPPH in alcohol is reduced to non-radicalic DPPH-H in the presence of a hydrogen-donating anti-oxidant.

Figures 3 and 4 show a significant decrease in the

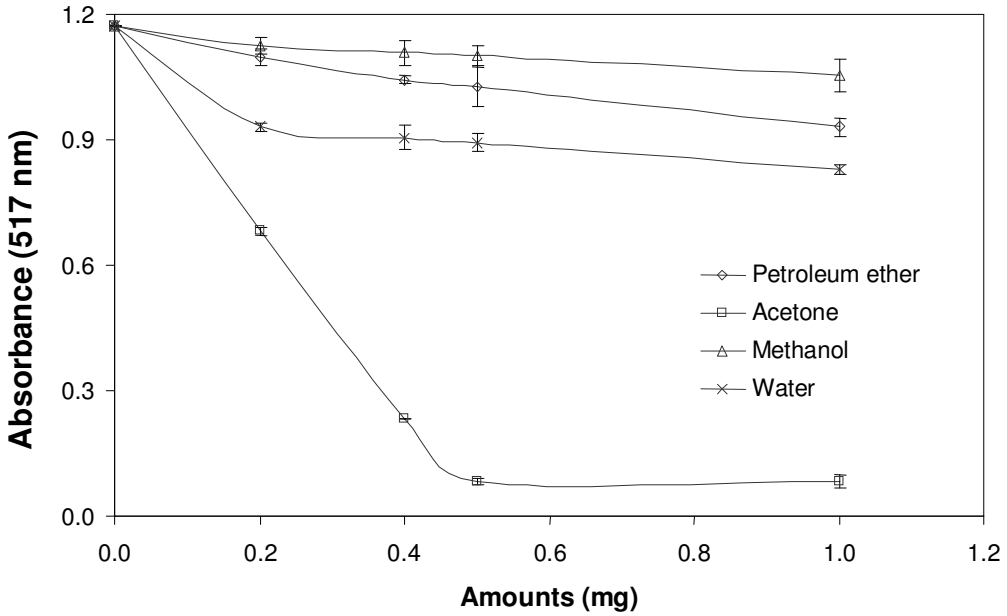

Figure 3. DPPH scavenging activity of tuber extracts of *C. mirabile* (values are means ± standard deviation of three replicate analyses).

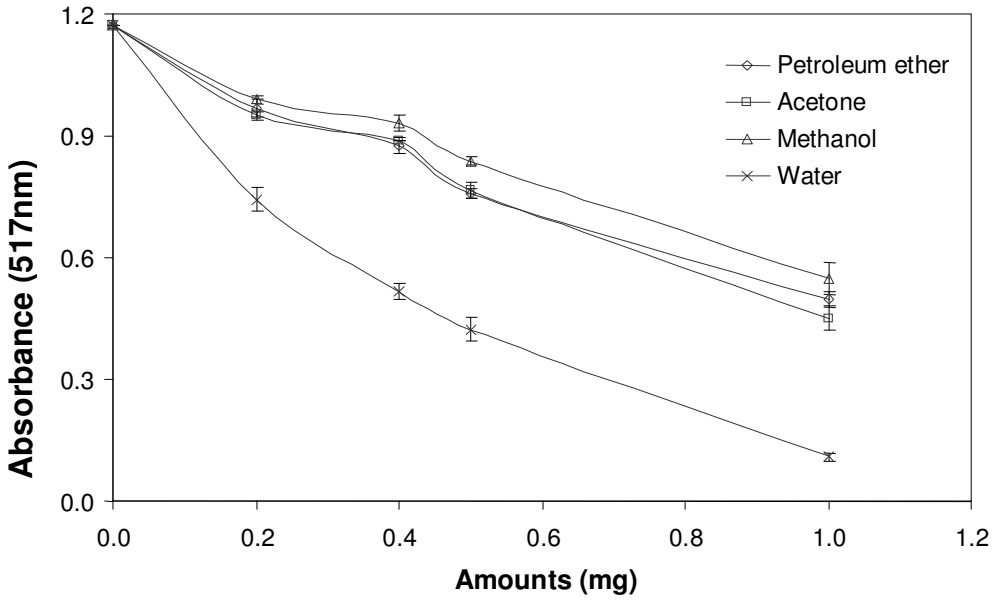

Figure 4. DPPH scavenging activity of leaf extracts of *C. mirabile* (values are means ± standard deviation of three replicate analyses).

concentration of DPPH radical due to the scavenging activity of the tuber and leaf extracts of *C. mirabile*. The scavenging activity of all extracts increased with increasing amounts of extracts. The scavenging capacity of 1 mg doses of petroleum ether, acetone, methanol and water extracts of CM leaves were found to be 57.51, 61.52, 53.07 and 88.25%, respectively and these values were greater than that of 0.1 mg dose of BHT (45.13%) and than that of 0.02 mg dose of ascorbic acid (42.44%), but lower than that of 0.1 mg of α-tocopherol (90.87%). The scavenging effect of these extracts followed the order: Acetone > petroleum ether > water > methanol for tuber extracts of CM.

The results obtained for the free radical scavenging activity suggest that, all the extracts of CM possessed the ability to quench free radical from reaching biomolecules

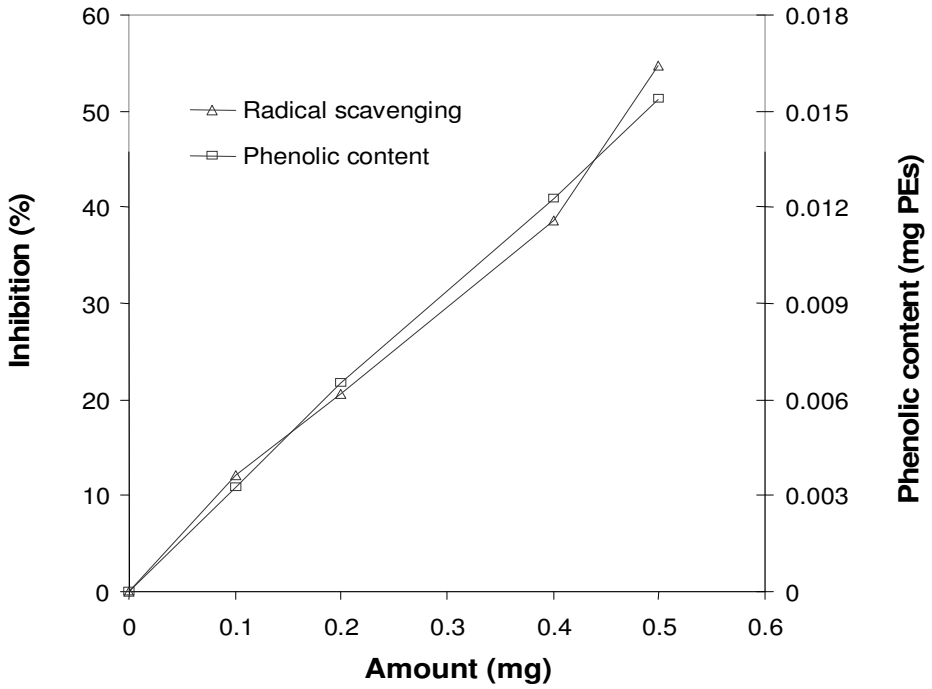

Figure 5. The scavenging activity (%) and total phenolic content (mg PEs) of different amounts of water extract of *C. mirabile* leaves.

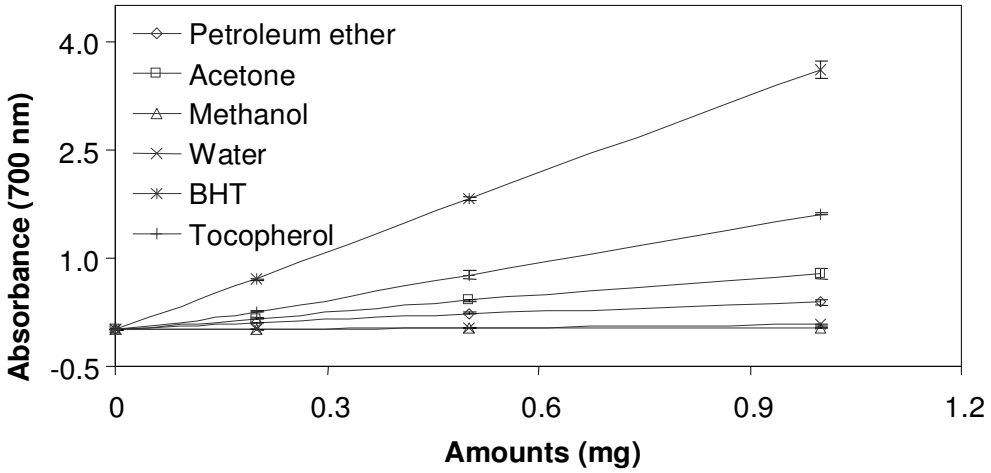

Figure 6. Reducing power of solvent extracts of *C. mirabile* tubers (values are means ± standard deviation of three replicate analyses).

(polyunsaturated fatty acids, sugars and amino acids etc.) in susceptible biological and food systems (Halliwell et al., 1995). These activities may be attributed to the hydrogen and electron donating abilities of their phenolics.

In fact, a strong and positive correlation (at 0.1 to 0.5 mg) was found between the scavenging activity of acetone and water extracts with the total phenolic content in *C. mirabile* tubers and leaves (R^2 = 0.99). Figure 5, shows the total phenolic content and the scavenging activity of water extract of CM leaves. The equation of total phenolic content (y) and scavenging activity (x) of water extract of CM leaves was y = 0.289792 x + 0.166278 (R^2 = 0.99).

Reducing power

As shown in Figures 6 and 7, all the various amount of extracts from CM tubers and leaves, BHT and α-

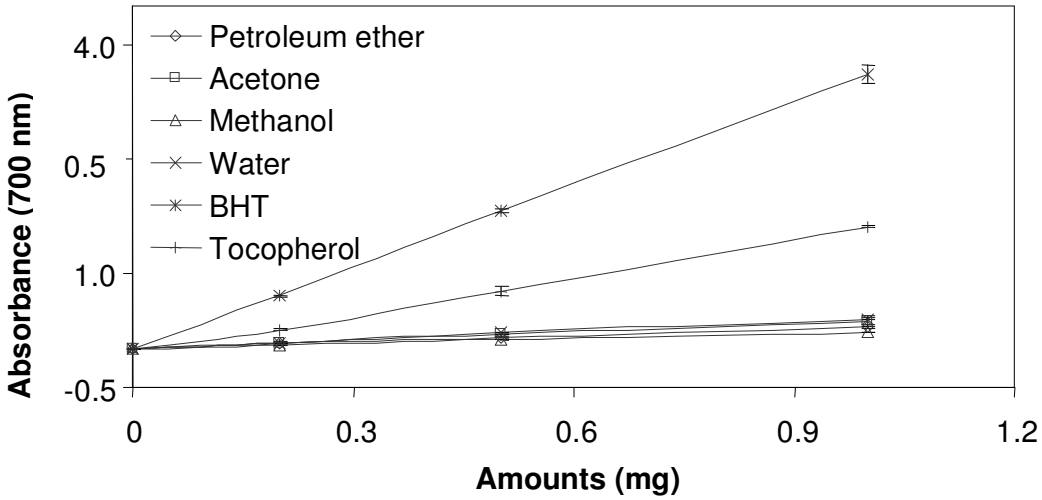

Figure 7. Reducing power of solvent extracts of *C. mirabile* leaves (values are means ± standard deviation of three replicate analyses).

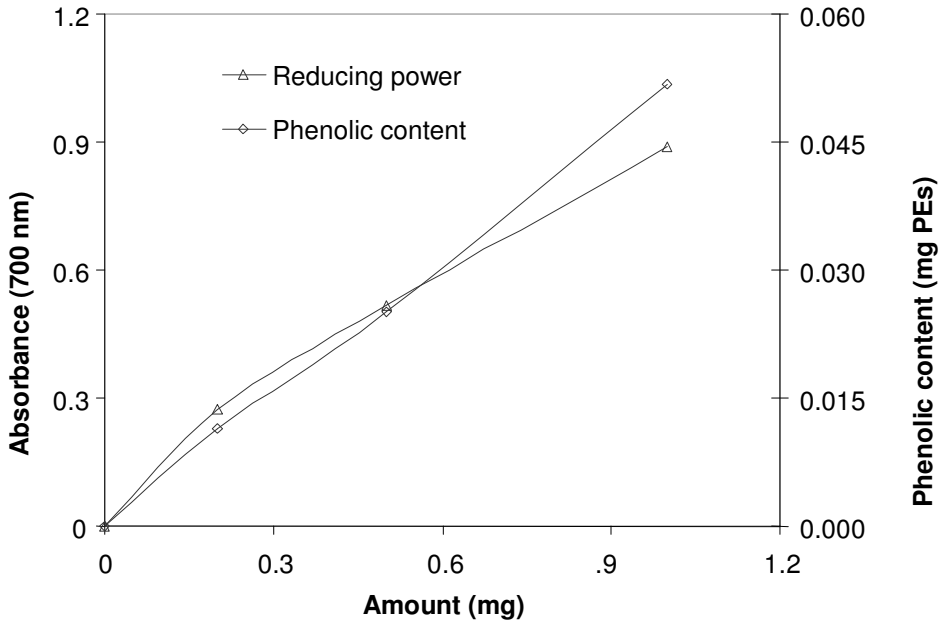

Figure 8. The reducing power (absorbance at 700 nm) and total phenolic content (mg PEs) of different amounts of acetone extract of *C. mirabile* tubers.

tocopherol showed higher activities than the control. The reducing power of solvent extracts of CM tuber and leaves increased with increasing amounts of extracts and decreased in the order acetone > petroleum ether > water > methanol, water > acetone > petroleum ether > methanol, respectively. As a whole, the reductive capacity of all extracts was less than that of BHT and α-tocopherol in the various amounts of the extracts. Like the scavenging effect, these results indicate that, there is a correlation between the reducing power and total phenolic compounds (R^2: 0.99). In addition, Figure 8 shows that the equation of the reducing power (y) and the total phenolic compounds (x) of different doses of acetone extract of CM tubers was y = 0.016729 x + 0.049617 (R^2: 0.98).

Many researchers have reported that, the reducing power is generally associated with the reductones (Duh, 1998) and might be due to hydrogen-donating ability (Shimida et al., 1992). The reducing power of the extracts of CM tubers and leaves might contain reductone, which could stabilise free radicals and terminate radical chain reactions and this might be due to the hydrogen-donating

abilities. Therefore, the antioxidant activity of the extracts may be related to their reducing power.

Conclusion

The results of this study indicate that, the extracts of CM tubers and leaves possessed high antioxidant activity *in vitro* and can be easy accessible source of natural antioxidant. However, the components responsible for antioxidant activity of the extracts of CM are unclear. Future studies will be aimed at investigating the effects of different parts of *C. mirabile* upon isolating and identifying the substances responsible for the antioxidant effects of the solvent extracts.

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