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Antioxidant and free radical scavenging potential of *Achillea santolina* extracts

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Abstract

The antioxidative activities of hydroalcoholic extract of *Achillea santolina* were investigated employing various established in vitro systems including total antioxidant activity in linoleic acid emulsion system, 1,1-diphenyl-2-picrylhydrazyl (DPPH), superoxide and hydroxyl radicals scavenging, reducing power, and inhibitory effect on protein oxidation as well as the inhibition of Fe^{2+}/ascorbate induced lipid peroxidation in rat liver homogenate. Total phenolic and flavonoid content of *A. santolina* extract (ASE) was also determined by a colorimetric method. The results revealed that ASE has notable inhibitory activity on peroxides formation in linoleic acid emulsion system along with concentration-dependent quenching of DPPH and superoxide radicals. Furthermore, the extract showed both nonsite-specific ($Fe^{2+} + H_2O_2 + EDTA$) and site-specific ($Fe^{2+} + H_2O_2$) hydroxyl radical scavenging suggesting potent hydroxyl radical scavenging and chelating ability for iron ions in deoxyribose degradation model. A linear correlation between ASE and the reducing power was also observed ($r^2 = 0.9981$). ASE prevents thiobarbituric acid reactive substances formation in Fe^{2+}/ascorbate induced lipid peroxidation in rat liver tissue in a dose-dependent manner. Moreover, free radical induced protein oxidation was suppressed significantly by the addition of ASE over a range of concentration. These results clearly demonstrated that *A. santolina* extract possess a marked antioxidant activity.

Keywords: *Achillea santolina*; Antioxidant; Reactive oxygen species; Lipid peroxidation

1. Introduction

Oxidative stress is defined in general as excess formation and/or incomplete removal of highly reactive molecules such as reactive oxygen species (ROS). ROS include free radicals such as superoxide ($O_2^{\cdot -}$), hydroxyl radical ($^{\cdot}OH$), peroxyl radical ($RO_2^{\cdot}$) as well as nonradical species such as hydrogen peroxide (H_2O_2) (Cerutti, 1991; Fridovich, 1978). In vivo, some of these ROS play a positive role such as energy production, phagocytosis, regulation of cell growth and intracellular signaling (Halliwell & Gutteridge, 1999). On the other hand, ROS are also capable of damaging a wide range of essential biomolecules such as proteins, DNA and lipids (Farber, 1994). ROS are not only strongly associated with lipid peroxidation resulting in deterioration of food materials, but also are involved in development of a variety of diseases including aging, carcinogenesis, coronary heart disease, diabetes and neurodegeneration (Cerutti, 1985; Harman, 1980; Moskovitz, Yim, & Choke, 2002). Cells have several antioxidant defense mechanisms that help to prevent the destructive effects of ROS. These defense mechanisms include antioxidative enzymes, such as superoxide dismutase, catalase, and glutathione peroxidase and of small molecules such as glutathione and vitamins C and E (Fridovich, 1999). The efficiency of the antioxidant defense system is altered under pathological

Abbreviations: ASE, *Achillea santolina* extract; BHT, butylated hydroxytoluene; DPPH, 1,1-diphenyl-2-picrylhydrazyl; MDA, malondialdehyde; NADH, nicotinamide adenine dinucleotide; NBT, nitroblue tetrazolium; PMS, phenazine methosulphate; TBA, thiobarbituric acid; TBARS, thiobarbituric acid reactive substances.

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conditions and, therefore, the ineffective scavenging and/or overproduction of free radicals may play a crucial role in determining tissue damages (Aruoma, 1994; Halliwell, 1994).

In aerobic organisms, one of the major targets of ROS are the cellular biomembranes, where they induce lipid peroxidation. Under this process, not only the membrane structure and its function are affected, but also some oxidation reaction products, for example, malondialdehyde (MDA), can react with biomolecules and exert cytotoxic and genotoxic effects. High levels of lipid peroxides have been found in the serum of patients suffering from liver disease, diabetes, vascular disorders, and tumors (Pezzuto & Park, 2002). Substances termed antioxidants can influence the oxidation process through simple or complex mechanisms including prevention of chain initiation, binding of transitional metal ion catalysts, decomposition of peroxides, prevention of continued hydrogen abstraction, and radical scavenging (Ames, Shigrenaga, & Hagen, 1993). Antioxidants are believed to play an important role in preventing or alleviating chronic diseases by reducing the oxidative damage to cellular components caused by ROS (Ceriello, 2003; Vinson, Dabbag, Serry, & Jang, 1995). There is growing interest in natural phenolic antioxidants, present in medicinal and dietary plants, that might help attenuate oxidative damage (Rice-Evans, Miller, & Paganga, 1997; Silva, Ferreres, Malva, & Dias, 2005). These natural antioxidants not only protect food lipids from oxidation, but may also provide health benefits associated with preventing damages due to biological degeneration (Hu & Kitts, 2005; Shahidi & Wanasundara, 1992).

Achillea santolina L. (compositae) is a traditional plant used as an herbal remedy for anti-diabetic (unpublished data) and anti-inflammatory (Al-Hindawi, Al-Deen, Nabi, & Ismail, 1989) purposes in many parts of Iraq. To clarify the mechanism of action of this medicinal plant, particularly with respect to its anti-diabetic effects, we evaluated the antioxidative and free radical scavenging capabilities of the extract. Based on the results obtained in this study, *Achillea santolina* extract possess a high antioxidative, free radical scavenging and reducing potential activity in all in vitro systems used to investigate the subject matter.

2. Materials and methods

2.1. Materials

2-Deoxy-2-ribose, NBT, NADH, sulfanilamide, PMS, BHT, TBA, and hydrogen peroxide (H_2O_2) were obtained from Merck (Germany). DPPH was obtained from Fluka (Buchs, Switzerland). Linoleic acid, catechin, ascorbic acid, vitamin E, TCA (Trichloroacetic acid), and ferric chloride were obtained from Sigma (St. Louis, MO, USA). Pottassium ferric cyanide, and ethylenediamine tetraacetic acid (EDTA), were obtained from Aldrich Chemical Co. Ltd. (England). All other reagents were of analytical reagent (AR) grade.

2.2. Plant material

Aerial parts of the plant were collected from Tikrit in May 2005. The plant was identified and authenticated as: *Achillea santolina* L. by Dr. khalil I. Al-Shemmary (Biology Dep., Faculty of Sciences, Tikrit University, Iraq) and a voucher specimen (No. 5625) was deposited at the herbarium of the Faculty of sciences, Tikrit University.

2.3. Extraction

The powdered plant material (100 g) was extracted three times with ethanol–water (7:3, v/v), at room temperature. The combined extracts were filtered and concentrated under reduced pressure, and lyophilized. The freeze-dried extract was dissolved in water to a 10 mg/ml concentration and aliquots were kept at −20 °C for investigation.

2.4. Total antioxidant activity

The total antioxidant activity of ASE was measured by use of a linoleic acid system (Mitsuda, Yasumodo, & Iwami, 1996). The linoleic acid emulsion was prepared by mixing 0.2804 g of linoleic acid, 0.2804 g of Tween 20 emulsifier and 50 ml of phosphate buffer (0.2 M, pH 7.0). The mixture was then homogenized. A 0.5 ml of different concentration of the extract and standard sample (in ethanol) was mixed with linoleic acid emulsion (2.5 ml, 0.2 M, pH 7.0) and phosphate buffer (2 ml, 0.2 M, pH 7.0). The reaction mixture was incubated at 37 °C in the dark to accelerate the peroxidation process. The levels of peroxidation were determined according to the thiocyanate method by sequentially adding ethanol (5 ml, 75%), ammonium thiocyanate (0.1 ml, 30%), sample solution (0.1 ml), and ferrous chloride (0.1 ml, 20 mM in 3.5% HCl). Butylated hydroxytoluene (BHT) was used as positive control. After mixing for 3 min, the peroxide values were determined by reading the absorbance at 500 nm.

2.5. DPPH radical scavenging activity

Radical scavenging activity of *A. santolina* extract was measured according to the method of Blois (1958). Briefly, 1 ml of the crude extract at variable concentrations (25–400 μg/ml in ethanol) was added to 1 ml of a DPPH (1,1-diphenyl 2-picrylhydrazyl) solution (0.2 mM in ethanol) as the free radical source and kept for 30 min at room temperature. The decrease in the solution absorbance, due to proton donating activity by ASE component(s), was measured at 517 nm. L-Ascorbic acid was used as the positive control. The DPPH radical scavenging activity was calculated using the following formula:

$$\text{DPPH radical scavenging activity } (\%) = [(A_0 - A_1/A_0) \times 100],$$

where A_0 is the absorbance of the control, and A_1 is the absorbance of ASE or the standard sample.

2.6. Superoxide radical scavenging activity

Superoxide anion scavenging activity was measured based on the described method by Robak and Gryglewski (1988). Superoxide radicals were generated in nicotineamide adenine dinucleotide, phenazine methosulphate (PMS–NADH) system by oxidation of NADH and assayed by reduction of nitroblue tetrazolium (NBT). In this experiment, the superoxide radical was generated in 3 ml of sodium phosphate buffer (100 mM, pH 7.4) containing 1 ml of NBT (150 μM) solution, 1 ml of NADH (468 μM) solution and different concentrations of the extract (25–400 μg/ml) in water. The reaction started by adding 1 ml of PMS solution (60 μM) to the mixture. The reaction mixture was incubated at 25 °C for 5 min, and the absorbance was measured against the corresponding blank solution. L-Ascorbic acid was used as the positive control. The decrease of NBT reduction, measured by the absorbance of the reaction mixture, correlates with the superoxide radical scavenging activity of the ASE. The superoxide radical scavenging activity was calculated using the following formula:

$$\text{Superoxide radical scavenging activity } (\%) = [(A_0 - A_1/A_0) \times 100],$$

where A_0 is the absorbance of the control, and A_1 is the absorbance of ASE or the standard sample.

2.7. Hydroxyl radical scavenging activity

The hydroxyl radical scavenging activity was determined according to the method of Halliwell, Gutteridge, and Aruoma (1987). The hydroxyl radical scavenging activity of ASE was measured by the competition between deoxyribose and ASE for the hydroxyl radicals generated from the Fe^{3+}/ascorbate/EDTA/H_2O_2 system (nonsite-specific assay) or Fe^{3+}/ascorbate/H_2O_2 (site-specific assay). Briefly, for the nonsite-specific hydroxyl radical system, the reaction mixture, contained different concentrations of the extract (250–2500 μg/ml), 2.8 mM deoxyribose, 0.1 mM $FeCl_3$, 0.1 mM ascorbic acid, 0.1 mM EDTA and 1 mM H_2O_2 in KH_2PO_4–KOH buffer (20 mM pH 7.4), was incubated in a water bath at 37 °C for 1 h. For the site-specific hydroxyl radical system, EDTA was replaced by phosphate buffer. The extent of deoxyribose degradation was measured by thiobarbituric acid (TBA) method. TBA (1 ml, 1% w/v) and trichloroacetic acid (TCA) (1 ml, 2% w/v) were added to the mixture and heated at 100 °C for 20 min. After cooling to room temperature, the absorbance was measured at 532 nm. Mannitol, a classical hydroxyl radical scavenger was used as positive control. The hydroxyl radical scavenging activity was calculated using the following formula:

$$\text{Hydroxyl radical scavenging activity } (\%) = [(A_0 - A_1/A_0) \times 100],$$

where A_0 is the absorbance of the control, and A_1 is the absorbance of ASE or the standard sample.

2.8. Non-enzymatic lipid peroxidation induced by Fe^{2+}/ascorbate

Male wistar albino rats weighing 200–250 g (purchased from Pasteur Institute, Tehran, Iran) were housed under conventional conditions and were allowed free access to food and water, ad libitum. All experiments were carried out according to the guidelines for the care and use of experimental animals and approved by state veterinary administration of the University of Tehran. The rats were anesthetized using diethyl ether and abdomen was opened and their liver was quickly removed. The livers were then cut into small pieces and homogenized in phosphate buffer (50 mM, pH 7.4) with a homogenizer to give a 10% (w/v) liver homogenate. The liver homogenate was further centrifuge at 5000*g* for 10 min. Supernatant of the liver homogenate was collected and the amount of protein was determined by the method of Lowry, Rosebrough, Farr, and Randall (1951). The extent of lipid peroxidation was evaluated by measuring the product of thiobarbituric acid reactive substances (TBARS) in the rat liver homogenate using the modified method previously described (Mahakunakorn, Tohda, Murakami, Matsumoto, & Watanabe, 2004). The reaction mixture was composed of tissue homogenate 0.5 ml, phosphate buffer (50 mM, pH 7.4) 0.9 ml, $FeSO_4$ (0.01 mM) 0.25 ml, ascorbic acid (0.1 mM) 0.25 ml, and 0.1 ml of different concentration of the extract and the standard sample. The reaction mixture was incubated at 37 °C for 30 min and the reaction was then terminated by adding BHT (2% w/v in 95% v/v ethanol), followed by addition of 1 ml of TCA (20% w/v) to the mixture. After centrifugation at 3000*g* for 15 min, the supernatant was incubated with 1 ml of TBA (0.67%) at 100 °C for 15 min. Tocopherol was used as the positive control. The colour of the complex of TBARS with TBA was detected at 532 nm. The amount TBARS formed was calculated using the absorption coefficient of $1.56 \times 10^5\ cm^{-1}\ M^{-1}$ (Silva, Ferreres, Malva, & Dias, 2005).

2.9. Assay of protein oxidation

The effects of ASE on protein oxidation were carried out according to the slightly modified method of Wang et al. (2006). Bovine serum albumin (BSA) was oxidized by a Fenton-type reaction. The reaction mixture (1.2 ml), containing sample extract (100–1000 μg/ml), potassium phosphate buffer (20 mM, pH 7.4), BSA (4 mg/ml), $FeCl_3$ (50 μM), H_2O_2 (1 mM) and ascorbic acid (100 μM) were incubated for 30 min at 37 °C. For determination of protein carbonyl content in the samples, 1 ml of 10 mM 2,4-dinitrophenylhydrazine (DNPH) in 2 M HCl was added to the reaction mixture. Samples were incubated for 30 min at room temperature. Then, 1 ml of cold TCA (10%, w/v) was added to the mixture and centrifuged at

3000*g* for 10 min. The protein pellet was washed three times with 2 ml of ethanol/ethyl acetate (1:1, v/v) and dissolved in 1 ml of guanidine hydrocholoride (6 M, pH 2.3). The absorbance of the sample was read at 370 nm. The data were expressed in terms of percentage inhibition, calculated from a control measurement of the reaction mixture without the test sample.

2.10. Reducing power assay

The reducing power of the prepared *A. santolina* extract was determined according to method of Oyaizu (1986). Briefly, variable concentrations of the extract and the standard compound (BHT) in 1 ml of distilled water were mixed with 2.5 ml of phosphate buffer (0.2 M, pH 6.6) and 2.5 ml of a potassium ferricyanide solution (1%, w/v). The mixture was incubated in a water bath at 50 °C for 20 min. Then, 2.5 ml of a TCA solution (10%, w/v) was added, and the mixture was then centrifuged at 3000*g* for 10 min. A 2.5 ml aliquot of the upper layer was combined with 2.5 ml of distilled water and 0.5 ml of a ferric chloride solution (0.1%, w/v), and the absorbance was measured at 700 nm. Increased absorbance of the reaction mixture correlates with greater reducing power.

2.11. Determination of total phenolic content

Total phenolic content in the lyophilized extract was determined with the Folin–Ciocalteu's reagent (FCR) according to a published method (Slinkard & Singleton, 1977). Each sample (0.5 ml) was mixed with 2.5 ml FCR (diluted 1:10, v/v) followed by 2 ml of Na_2CO_3 (7.5%, v/v) solution. The absorbance was then measured at 765 nm after incubation at 30 °C for 90 min. Results were expressed as gallic acid equivalents (mg gallic acid/g dried extract).

2.12. Determination of total flavonoid content

The total flavonoid content of ASE was determined by a colorimetric method as described in the literature (Zhishen, Mengcheng, & Jianming, 1999). Each sample (0.5 ml) was mixed with 2 ml of distilled water and subsequently with 0.15 ml of a $NaNO_2$ solution (15%). After 6 min, 0.15 ml of an $AlCl_3$ solution (10%) was added and allowed to stand for 6 min, then 2 ml of NaOH solution (4%) was added to the mixture. Immediately, water was added to bring the final volume to 5 ml and the mixture was thoroughly mixed and allowed to stand for another 15 min. Absorbance of the mixture was then determined at 510 nm versus prepared water blank. Results were expressed as catechin equivalents (mg catechin/g dried extract).

2.13. Statistical analyses

All data are presented as means ± SD. The mean values were calculated based on the data taken from at least three independent experiments conducted on separate days using freshly prepared reagents. Statistical analyses were performed using student's *t*-test. The statistical significances were achieved when $P < 0.05$.

3. Results and discussion

3.1. Total antioxidant activity

The total antioxidant activity of ASE was measured using ferric thiocyanate test which determines the amount of peroxide produced at the initial stage of lipid peroxidation. Lower absorbance indicates a higher level of antioxidant activity. Fig. 1 shows the changes in the absorbance under the influence of different concentrations of the extract (100, 200 and 400 μg/ml) at 37 °C, compared to BHT as a positive control during 96 h. According to this figure the extent of inhibition of lipid oxidation is moderate at low (100 μg/ml) doses of ASE. However, at higher concentrations (200 and 400 μg/ml), ASE suppressed lipid oxidation by extending the lag phase and reducing the propagation rate, thus reflecting typical characteristic of a chain-breaking antioxidant, similar to that of known antioxidant, BHT. Lipid oxidation is one of the major factors causing deterioration of foods during the storage and processing. Oxidized polyunsaturated fatty acids may induce aging and carcinogenesis. Although there are some synthetic antioxidant compounds such as BHT and butylated hydroxyanisole (BHA) which are commonly used in foods processing, it has been reported that these synthetic antioxidants are not devoid of biological side effects and their consumption may lead to carcinogenecity and causes liver damages (Branien, 1975; Linderschmidt, Trylka, Good, & Witschi, 1986). Therefore, the development of alternative antioxidants mainly from natural sources has attracted considerable attention.

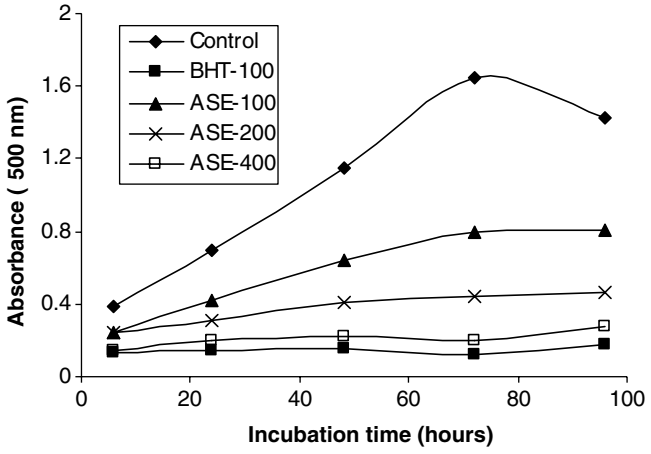

Fig. 1. Total antioxidant activity of different concentrations (100, 200, and 400 μg/ml) of *Achillea santolina* extract (ASE) and BHT (100 μg/ml) in linoleic acid emulsion determined by the thiocyanate method. For experimental details see Section 2. All values statistically different ($P < 0.05$).

3.2. DPPH radical scavenging activity

The DPPH radical is a stable organic free radical with an absorption maximum band around 515–528 nm and thus, it is a useful reagent for evaluation of antioxidant activity of compounds (Sanchez-Moreno, 2002). In the DPPH test, the antioxidants reduce the DPPH radical to a yellow-colored compound, diphenylpicrylhydrazine, and the extent of the reaction will depend on the hydrogen donating ability of the antioxidants (Bondent, Brand-Williams, & Bereset, 1997). It has been documented that cysteine, glutathione, ascorbic acid, tocopherol, polyhydroxy aromatic compounds (e.g., hydroquinone, pyrogallol, gallic acid), reduce and decolorize 1,1-diphenyl-2-picrylhydrazine by their hydrogen donating capabilities (Blois, 1958). The ASE demonstrated a concentration-dependent scavenging activity by quenching DPPH radicals (Fig. 2). The hydrogen donating activity, measured using DPPH test, showed that the ASE contained 55 mg ascorbic acid equivalents/g extract of activity (Fig. 2 and Table 2, i), with EC_{50} value (defined as the concentration of test compound required to produce 50% maximal inhibition) of 55 μg/ml (Table 1).

3.3. Superoxide radical scavenging activity

In the PMS–NADH–NBT system, superoxide anion derived from the dissolved oxygen by PMS/NADH coupling reaction reduces NBT. The decrease in the absorbance at 560 nm with antioxidants thus indicates the consumption of the generated superoxide anion in the reaction mixture. Fig. 3 shows the inhibitory effect of ASE on superoxide radical generation. The ASE demonstrated a concentration-dependent scavenging activity by neutralizing superoxide radicals with EC_{50} value of 39 μg/ml (Table 1). Moreover, using this assay, the ASE was found to contain 305 mg ascorbic acid equivalents/g extract (Fig. 3 and Table 2, ii). Superoxide, the one-electron reduced form of molecular oxygen, is a precursor of other ROS such as hydrogen peroxide, hydroxyl radical, and singlet oxygen that have the potential of reacting with biological macromolecules and thereby inducing tissue damages (Aruoma,

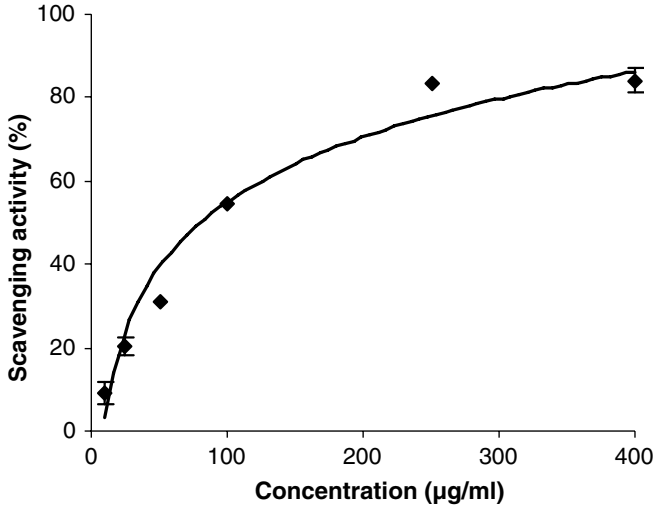

Fig. 2. DPPH radical scavenging activity of *Achillea santolina* extract (ASE). For experimental details see Section 2. Each value represents the mean ± SD ($n = 3$).

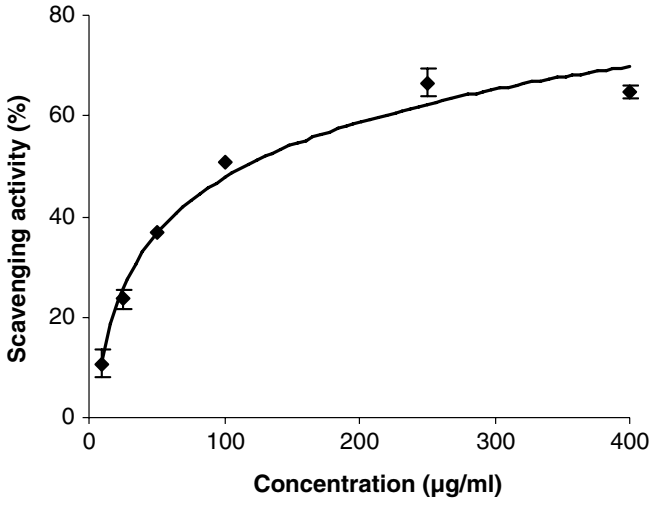

Fig. 3. Superoxide radical scavenging activity of *Achillea santolina* extract (ASE). For experimental details see Section 2. Each value represents the mean ± SD ($n = 3$).

Table 1
EC_{50} and $RP_{0.5\,AU}$ values of *Achillea santolina* extract (ASE) and the pure compounds

Antioxidant activity	EC_{50}			
	ASE	Ascorbic acid	Mannitol	BHT
(i) DPPH radical scavenging activity	55 (18)	3	–	–
(ii) Superoxide radical scavenging activity	39 (3.2)	12	–	–
(iii) Hydroxyl radical scavenging (nonsite-specific assay)	416 (1.3)	–	320	–
(iv) Hydroxyl radical scavenging (site-specific assay)	519 (1.4)	–	373	–
		$RP_{0.5\,AU}$		
(v) Reducing power	77 (1.5)	–	–	51

The EC_{50} and $RP_{0.5\,AU}$ values for ASE were calculated from data presented in Figs. 2–5 and 7. The values for the pure compounds (ascorbic acid, mannitol and BHT) were calculated from data obtained from similar experiments and scavenging effect (%) or reducing power (absorbance) versus test material plots (data not shown). Values are shown in μg extract, or μg pure compounds per ml reaction volume. Values in ( ) are relative to pure compound for the particular assay.

Table 2
Antioxidant activities of *Achillea santolina* extract (ASE)

Antioxidant activity	ASE
(i) DPPH radical scavenging activity	55
(ii) Superoxide radical scavenging activity	305
(iii) Hydroxyl radical scavenging (nonsite-specific assay)	768
(iv) Hydroxyl radical scavenging (site-specific assay)	719
(v) Reducing power	658

The EC_{50} and $RP_{0.5\,AU}$ values for ASE from Table 1 were converted to (i and ii) mg ascorbic acid equivalents, (iii and iv) mg mannitol equivalent, and (v) mg BHT equivalents per g extract.

1998) and also it has been implicated in initiating oxidation reactions associated with aging (Wickens, 2001). These results clearly indicated that ASE is a potent scavenger of superoxide radicals in a dose-dependent manner.

3.4. Inhibition of lipid peroxidation in rat liver homogenate

Lipid peroxidation is an oxidative alteration of polyunsaturated fatty acids in the cell membranes that generates a number of degradation products. MDA, one of the products of lipid peroxidation, has been studied widely as an index of lipid peroxidation and as a marker of oxidative stress (Janero, 1990). The addition of $FeSO_4$–ascorbic acid to the liver homogenate for 30 min significantly increased the extent of TBARS formation, compared to the control sample (4.35 nmol/mg protein versus 0.28 nmol/mg protein). However, as shown in Table 3, adding 100–1000 μg/ml ASE to rat liver homogenate significantly reduced TBARS formation in the liver homogenate, indicating significant anti-lipid peroxidation activities in ASE. The extract was capable of inhibiting TBARS formation by 14.86%, 32.55%, 54.78% and 73.33% at extract concentrations of 100, 250, 500 and 1000 μg/ml, respectively. Co-incubation of Fe^{2+}-containing sample with 100 μM tocopherol resulted in 96.06% inhibition of lipid peroxidation. Initiation of lipid peroxidation by ferrous sulfate takes place either through ferryl–perferryl complex or through hydroxyl radical generation (Ko, Cheng, Lin, & Teng, 1998). Therefore, the inhibition could be caused by the absence of ferryl–perferryl complex or by scavenging hydroxyl radicals or by chelating the iron ions.

Table 3
Effect of *Achillea santolina* extract (ASE) and tocopherol on Fe^{2+}/ascorbate induced lipid peroxidation in rat liver homogenate

Concentration (μg/ml)	TBARS (nmol/mg protein)	Inhibition (%)
Fe^{2+}/ascorbate[a]	4.35 ± 0.091	–
Control[b]	0.28 ± 0.049	–
100	3.74 ± 0.063	14.86 ± 1.56*
250	3.02 ± 0.021	32.55 ± 0.52
500	2.12 ± 0.056	54.78 ± 1.39
1000	1.36 ± 0.063	73.33 ± 1.36
Tocopherol (100 μM)	0.44 ± 0.028	96.06 ± 0.70

For experimental details see Section 2.
Each value represents the mean ± SD ($n = 3$). All values statistically different ($P < 0.05$) except those marked with the *.
[a] Reaction mixture consisted of oxidant pair (Fe^{2+}/ascorbate).
[b] Reaction mixture without oxidant pair (Fe^{2+}/ascorbate).

3.5. Hydroxyl radical scavenging activity

We examined the inhibitory action of ASE on deoxyribose degradation which gives an indication of hydroxyl radical scavenging action and iron chelating activity (Lopes, Schulman, & Hermes-Lima, 1999). When hydroxyl radical, generated by the Fenton reaction, attacks deoxyribose it degrades into fragments that react with TBA on heating at low PH to form a pink color. ASE neutralized nonsite-specific (in the presence of EDTA), hydroxyl-radical induced deoxyribose cleavage in a concentration-dependent manner (Fig. 4), with EC_{50} value of 416 μg/ml (Table 1). With this assay, the ASE was found to contain 768 mg mannitol equivalent/g extract in nonsite-specific model of hydroxyl radical scavenging (Fig. 4 and Table 3, iii). Moreover, ASE exhibited a site-specific (in the absence of EDTA), hydroxyl radical scavenging activity in a dose-dependent manner (Fig. 5) with EC_{50} value of 519 μg/ml (Table 1), though such activity was relatively weaker than that of nonsite-specific hydroxyl radical at the same concentrations. Using this site-specific model, the ASE was found to contain 719 mg mannitol equivalent/g extract of activity (Fig. 5 and Table 3, iv). These results clearly demonstrated the capacity of ASE to quench hydroxyl radicals and also to chelate the iron metal ions. Hydroxyl radical is an extremely reactive oxygen species, capable of modifying almost every molecule in the living cells. This radical has the capacity to cause strand damages in DNA leading to carcinogenesis, mutagenesis, and cytotoxicity. Moreover, hydroxyl radicals are capable of the quick initiation of lipid peroxidation process as by abstracting hydrogen atoms from unsaturated fatty acids (Aruoma, 1998; Kappus, 1991). Due to this high reactivity

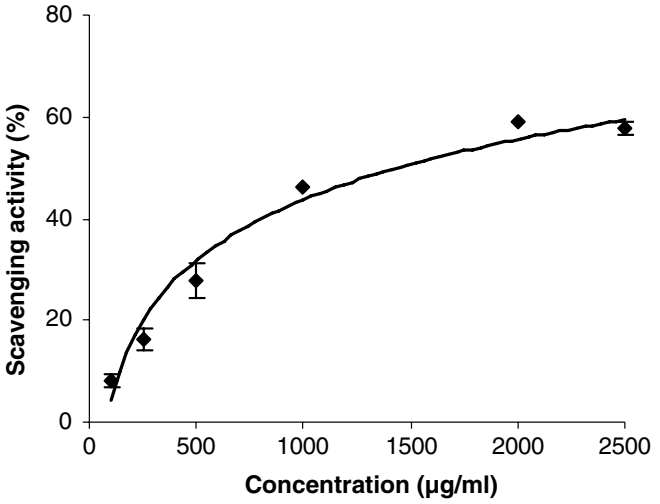

Fig. 4. Hydroxyl radical scavenging activity (nonsite-specific assay) of *Achillea santolina* extract (ASE). For experimental details see Section 2. Each value represents the mean ± SD ($n = 3$).

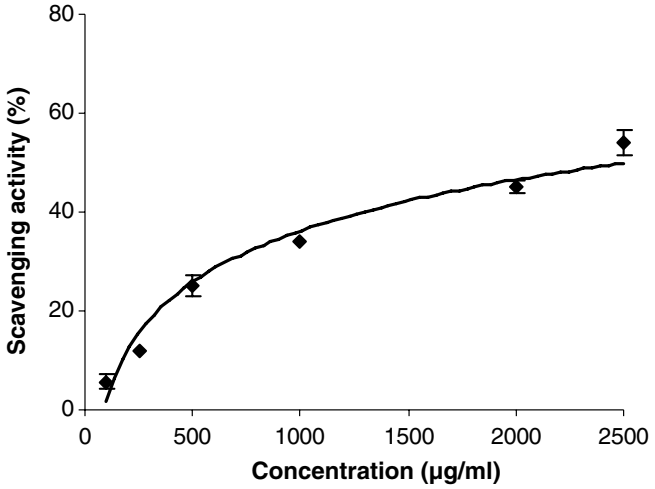

Fig. 5. Hydroxyl radical scavenging activity (site-specific assay) of *Achillea santolina* extract (ASE). For experimental details see Section 2. Each value represents the mean ± SD ($n = 3$).

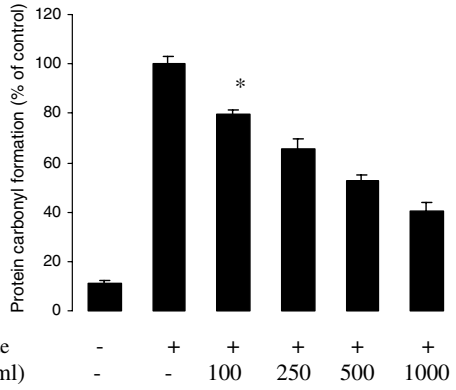

Fig. 6. Inhibitory effect of *Achillea santolina* extract (ASE) on protein (BSA) oxidation expressed as protein carbonyl formation (PCO) induced by H_2O_2/Fe^{3+}/ascorbic acid system. For experimental details see Section 2. Each value represents the mean ± SD ($n = 3$). All values statistically different ($P < 0.05$) except those marked with the *.

of hydroxyl radicals, measurements based on scavenging hydroxyl radicals, such as the nonsite-specific method, are not accurate measurement of oxidative protection of an antioxidant molecule in vivo (Halliwell & Gutteridge, 1999). This is because the radical is more likely to be scavenged by direct reaction with other surrounding molecules before it can attack its target molecule. Deoxyribose assay when performed in the absence of EDTA (site-specific model), forms hydroxyl radicals on the surface of the ribose substrate in the presence of H_2O_2 and ascorbic acid. In this model, the only substances that inhibit deoxyribose degradation are those that bind iron ions strongly enough to remove them from deoxyribose and form complexes less reactive in generating hydroxyl radicals (Aruoma, Grootveld, & Halliwell, 1987). The ability of ASE to quench hydroxyl radicals (mainly through site-specific model) seems to be directly related to the prevention of propagation of lipid peroxidation.

3.6. Inhibitory effects against protein oxidation

The oxidative protein damages, provoked by free radicals, have been demonstrated to play a significant role in aging and several pathological events (Stadtman & Levin, 2000). Radical mediated damages to proteins might be initiated by electron leakage, metal-ion dependent reactions, and autoxidation of lipids and sugars (Dean, Fu, Stocker, & Davies, 1997). Major molecular mechanisms, leading to structural changes in proteins are free-radical mediated protein oxidation characterized by carbonyl formation (PCO). In deed, measurement of PCO has been used as a sensitive assay for oxidative damages of proteins (Reznick & Packer, 1994). Protein oxidation was used as another method to measure hydroxyl radical scavenging activity of ASE beside the nonsite-specific deoxyribose assay by incubating BSA in a H_2O_2/Fe^{3+}/ascorbic acid system which generate hydroxyl radicals. The oxidation was determined in terms of PCO formation. As shown in Fig. 6, ASE dose-dependently exhibited inhibitory effects on PCO formation by 19.47%, 34.01%, 47.11% and 61.85% at the extract concentrations of 100, 250, 500 and 1000 µg/ml, respectively.

3.7. Reducing power assay

For the measurement of the reductive ability, we investigated the Fe^{3+}/Fe^{2+} transformation in the presence of ASE using the method of Oyaizu. The reducing capacity of compound may serve as a significant indicator of its potential antioxidant activity (Hsu, Coupar, & Ng, 2006). Fig. 7 shows that the reductive capability of ASE (measured at 700 nm) relative to BHT, a well known antioxidant. Similar to the antioxidant activity, the reducing potential of ASE increased in a dose-dependent manner, with a high correlation index ($r^2 = 0.9981$). The reducing power, $RP_{0.5\,AU}$ (defined as the amount of material in µg per ml reaction volume that produces 0.5 absorbance unit

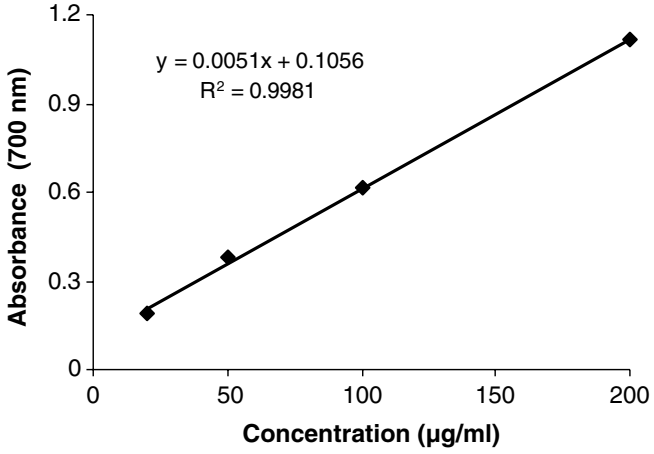

Fig. 7. Reducing power of *Achillea santolina* extract (ASE). For experimental details, see Section 2.

Table 4
Total phenolic[a] and flavonoid content[b] of *Achillea santolina* extract (ASE)

Total phenolic content (mg/g)	104.66 ± 4.39
Total flavonoid content (mg/g)	49.04 ± 1.98

For experimental details see Section 2.
Each value represents the mean ± SD ($n = 3$).
[a] Total phenolic content was expressed as mg gallic acid equivalents/g dried extract.
[b] Total flavonoid content was expressed as mg catechin equivalents/g dried extract.

at 700 nm) of ASE was 77 μg/ml (Table 1). Using this assay, the ASE was found to contain 658 mg BHT equivalents/g extract of activity (Fig. 6 and Table 2, v). Based on these results, it might be concluded that ASE is an electron donor capable of neutralizing free radicals. This would have the effect of converting free radicals to more stable products and thus terminating free radical initiated chain reactions.

3.8. Total phenolic and flavonoid contents

The antioxidant activity of AES is probably due to its phenolic contents. It is well known that phenolic compounds are constituents of many plants, and they have attracted a great deal of public and scientific interest because of their health promoting effects as antioxidants (Hollman & Katan, 1999; Rice-Evans et al., 1997).The phenolic compounds exhibit considerable free radical scavenging activities, through their reactivity as hydrogen- or electron-donating agents, and metal ion chelating properties (Rice-Evans, Miller, & Paganga, 1996). Flavonoids are a class of secondary plant phenolics with powerful antioxidant properties (Pietta, 2000) and phytochemical investigations of *A. santolina* have demonstrated the presence of some flavonoids (Khafagy, Sabri, Soliman, Abou-Donia, & Mosandl, 1976). Therefore, it would be valuable to determine the total phenolic and flavonoid content of the plant extracts. The extracts were investigated regarding their composition by different colorimetric techniques, such as the content of total phenolic compounds by the Folin–Ciocalteu assay and flavonoids by $AlCl_3$ reagent. Total phenolic and flavonoid contents of ASE were determined and expressed in terms of gallic acid and catechin equivalents. Total phenolic and flavonoid contents of each gram of dried extract were estimated to be equivalent to 104.66 mg gallic acid and 49.04 mg catechin (Table 4).

4. Conclusions

In this study, using various in vitro assay systems, the antioxidant potential of *A. santolina* extract was evaluated based on DPPH, superoxide, and hydroxyl radical scavenging activities, inhibition of lipid peroxidation in linoleic acid emulsion and rat liver homogenate. In addition, we further evaluated the inhibition of protein oxidation as well as reducing power of the extract. The results clearly confirmed the antioxidative and free radical scavenging activity of the extract. Identification of the antioxidative constituents of the plant and evaluation of their probable anti-diabetic properties is in progress.

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