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The efficacy of cashew nut (*Anacardium occidentale* L.) skin extract as a free radical scavenger

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Abstract

The free radical scavenging activity of ethanolic extracts of cashew nut (*Anacardium occidentale*, L.) skin powder (CSP) was evaluated by employing various *in vitro* antioxidant assay systems. The yield of the extract as well as the total phenolic content was also determined. The yield of ethanolic extract of the skin powder was quite high (0.45 g/g powder) with a total phenolic content of 243 mg/g extract. The cashew nut skin extract (CSE) demonstrated promising antioxidant activity with EC_{50} of 1.30 ± 0.02 μg/ml in 2,2′-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) radical scavenging assay, 10.69 ± 1.13 μg/ml in superoxide scavenging assay, 17.70 ± 0.05 μg/ml in deoxyribose oxidation assay, 24.66 ± 0.32 μg/ml in lipid peroxidation (LPO) assay and 6.00 mg/ml in iron chelation assay. To identify the compounds in the CSE responsible for the antioxidant activity, thin layer chromatography (TLC) was performed with the extract. The spot showing protection towards β-carotene bleaching was extracted and analyzed by high performance liquid chromatography (HPLC); epicatechin was found to be the major polyphenol present. The results of the present study suggest that cashew nut skin, a byproduct of cashew processing industry, can be used as an economical source of natural antioxidants.

Keywords: Cashew nut skin; Ethanolic extract; Radical scavenging; Antioxidant activity; ABTS; Total polyphenolics; Brain homogenate; Epicatechin

1. Introduction

Reactive oxygen species (ROS) are implicated in numerous pathophysiological events such as aging, cancer, atherosclerosis and diabetes (Halliwell, Guteridge, & Cross, 1992). Natural antioxidants from fruits and vegetables are reported to provide substantial protection that slows down the process of oxidative damage caused by ROS (Jacob & Burri, 1996). Hence there has been growing interest in natural antioxidants of plant origin since they also find use as nutracueticals due to their impact on the status of human health and disease prevention (Nagochi & Nikki, 2000). Several fruits, nuts, seeds, leaves, roots and barks have been exploited as potential sources of natural antioxidants (Schuler, 1990). There are considerably higher ratios of byproducts arising from seed/ nut processing plants, and hence it would be beneficial if they could be exploited as a source of natural antioxidants. Certain byproducts of agro-industries such as seed testa (Huang, Yen, Chang, Yen, & Duh, 2003), hulls (Yen & Duh, 1994), coats (Chang, Yen, Huang, & Duh, 2002) and peels (Larrauri, Ruperez, & Saura-Calixto, 1998; Singh & Rajini, 2004) have been reported to possess significant antioxidant activity.

The seed coat/testa which forms a protective barrier for the cotyledon in seeds, is reported to have the highest concentration of phenolic compounds (Duenas, Hernandez, & Estrella, 2004, 2006; Duenas, Sun, Hernandez, Estrella, & Spranger, 2003; Shahidi, Chavan, Naczk, & Amarowicz, 2001). Several recent studies have shown that extracts of almond skins (Sang et al., 2002; Siriwardhana & Shahidi, 2002; Chen, Milbury, Lapsley, & Blumberg, 2005), peanut skins (Lou et al., 2004; Lou et al., 1999), hazelnut testa and green leafy cover (Alasalwar, Karamac, Amarowicz, & Shahidi, 2006; Senter, Horvat, & Forbus, 1983), canola/ rapeseed hulls (Amarowicz, Naczk, Zadernowski, & Shah-

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idi, 2000), tamarind pericarp (Sudjaroen et al., 2005) and seed coats of lentils (Duenas et al., 2006; Duenas et al., 2003; Troszynska and Kubicka, 2001) possess strong antioxidant activities. Other authors have observed high antioxidant activity in lentils, faba beans and peas, mainly in the seed coat, due to the presence of large amounts of phenolic compounds in this part of the seed (Amarowicz et al., 2000; Nilsson, Stegmark, & Akesson, 2004; Shahidi et al., 2001; Takahata, Ohnishi-Kameyan, Furuta, Takahasi, & Suda, 2001; Troszynska & Ciska, 2002).

Cashew nut (*Anacardium occidentale* L.) is a major cash crop in the world. India is the largest producer and exporter of cashew kernel, accounting for almost 50% of world export (Paramashivappa, Kumar, Vithayathil, & Rao, 2001). Cashew nut shell liquid, a byproduct obtained during the processing of cashew nuts is reported to possess antioxidant activity (Singh, Kale, & Rao, 2004). The kernel of cashew nut valued in trade is covered with a thin reddish-brown skin or testa. The testa has been reported to be a good source of hydrolysable tannins (Pillai, Kedlaya, & Selvarangan, 1963) with catechin and epicatechin as the major polyphenols (Mathew & Parpia, 1970). However, there are no reports describing the radical scavenging activities of extracts of the skin of cashew nut.

The purpose of this study was to determine whether the ethanolic extracts of cashew nut skin exhibit radical scavenging activity in a set of antioxidant assay systems. Attempt was also made to identify the major active fraction in the extract responsible for the antioxidant activity.

2. Materials and methods

2.1. Materials

Butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), nitro blue terazolium (NBT), phenazine methosulphate (PMS), hydrogen peroxide (H_2O_2), 2-thiobarbituric acid (TBA), 2,2′-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) and ethylenediaminetetraacetic acid (EDTA) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Ferrozine, nicotinamide adenine dinucleotide-reduced (NADH), trichloroacetic acid (TCA) and deoxyribose (DR) were purchased from M/s Sisco Research Laboratories, Mumbai, India. All other reagents used were of analytical grade.

2.2. Preparation of ethanolic extract of cashew skin powder

Dried cashew nut skins were a gift from a cashew processing unit (M/s Mangala Cashews, Mangalore, India). The skins were sun dried and pulverized in a multi-mill and passed through a 0.5 mm sieve to obtain a fine powder. The skin powder (CSP) was mixed with five parts of ethanol and kept in a rotatory shaker at 37 °C for 3 h. The extract was separated by centrifugation, and the resultant cashew nut skin extract (CSE) was stored in dark at 4 °C until further use within a week.

2.3. Determination of total polyphenolic compounds

The total phenolic content in the CSE was quantified using the Folin-Ciocalteu reagent according to Singleton and Rossi (1965). Aliquots of the sample with volumes adjusted to 3.0 ml with distilled water were incubated with 0.5 ml 95% ethanol and 0.25 ml of Folins reagent (1:1 diluted with distilled water) for 5 min at room temperature. Na_2CO_3 (5%) solution (0.5 ml) was added, mixed and the mixture held for 60 min at room temperature. The absorbance of the solution was then measured at 720 nm against reagent blank using a spectrophotometer. Gallic acid (0.1 mg/ml) was used as the standard and the phenolic content in the extract was expressed as milligram equivalents of gallic acid (GAE) per gram CSP.

2.4. ABTS radical scavenging activity

The radical scavenging activity of the CSE was determined by employing the $ABTS^{\bullet+}$ decolorization assay (Re et al., 1999). $ABTS^{\bullet+}$ was produced by reacting ABTS (7 mM) and ammonium persulphate (2.45 mM) in 10 ml water and keeping the mixture in the dark at room temperature for 12–16 h before use. The aqueous $ABTS^{\bullet+}$ solution was diluted with ethanol (1:100 v/v) to an absorbance of 0.7 (±0.02) at 734 nm. Aliquots of extract (20 μl) were added to $ABTS^{\bullet+}$ solution (2 ml), mixed and the absorbance was read at 734 nm after 5 min. The percentage inhibition of radical scavenging was calculated.

2.5. Superoxide-radical scavenging assay

The superoxide scavenging ability of the extract was assessed by the method of Nishikimi, Rao, and Yagi (1972). The reaction mixture, containing CSE (5.5–22 μg), NADH (100 μM) and NBT (100 μM) in tris–HCl (0.02 M pH 8.3), was added to a spectrophotometric cuvette and the reaction was started by adding PMS (1 μM). The change in absorbance (ΔA) was monitored for one min and the capability of the extract in scavenging superoxide radical was calculated using the following equation:

$$\text{Scavenging effect}(\%) = [1 - \Delta A_{\text{Sample 560 nm}}/\Delta A_{\text{Control 560 nm}}] \times 100$$

2.6. Deoxyribose oxidation assay

The reaction mixture, containing CSE (5–20 μg), was incubated with deoxyribose (3.75 mM), H_2O_2 (1 mM), $FeCl_3$ (100 μM), EDTA (100 μM) and ascorbic acid

(100 μM) in potassium phosphate buffer (20 mM, pH 7.4) for 60 min at 37 °C (Halliwell, Gutteridge, & Aruoma, 1987). The reaction was terminated by adding 1 ml TBA (1% w/v) and 1 ml TCA (2% w/v) and then heating tubes in a boiling water bath for 15 min. The contents were cooled and the absorbance of the mixture containing the thiobarbituric acid reactive substances (TBARS) was measured at 535 nm against reagent blank. Decreased absorbance of the reaction mixture indicated decreased oxidation of deoxyribose.

2.7. Metal ion chelating assay

The Fe^{2+}-chelating ability of the extract was monitored by measuring the ferrous iron–ferrozine complex at 562 nm according to the method of Decker and Welch (1990). Briefly, the reaction mixture, containing CSE (1–5 mg), $FeCl_2$ (2 mM), and ferrozine (5 mM), was adjusted to total volume of 0.8 ml with methanol, shaken well and incubated for 10 min at room temperature. The absorbance of the mixture was measured at 562 nm against the blank. EDTA (5–25 μg) was used as positive control. The ability of the extract to chelate ferrous ion was calculated using the following equation:

$$\text{Chelating effect}(\%) = [1 - A_{\text{Sample 560 nm}}/A_{\text{Control 560 nm}}] \times 100$$

2.8. Antioxidant assay using β-carotene linoleate model system

The antioxidant activity was evaluated in a β-carotene-linoleate model system based on the procedure of Taga, Miller, and Pratt (1984). CSE (20 μg) was added to 3 ml of β-carotene emulsion and the mixture was shaken well and incubated at 50 °C in a water bath for various periods. The absorbance of the mixture was read at 470 nm at 10, 20, 30, 40 and 60-min intervals. Each sample was read against an emulsion prepared as described earlier but without β-carotene (blank). BHA and BHT (20 μg) were employed as model antioxidants for the purpose of comparison.

2.9. Inhibition of induced lipid peroxidation in rat brain homogenate

Rat brain excised from male Wistar rats (120–125 g) was homogenized (10% w/v) in potassium chloride solution (0.154 M). The homogenate was centrifuged at 800*g* at 4 °C for 10 min and the supernatant used for the assay. Peroxidation was induced in the brain homogenate by $FeCl_2$–H_2O_2 (Yen & Hsieh, 1998). Briefly, brain homogenate (1%) was incubated with 0.5 mM each of $FeCl_2$ and H_2O_2 with or without CSE (8.4–33.6μg). After incubation at 37 °C for 60 min, the TBARS formed in the incubation mixture was measured at 535 nm (Buege & Aust, 1978).

2.10. High performance liquid chromatography (HPLC) characterization of the active molecule from CSE

The antioxidant rich ethanolic extract (CSE) was applied on silica gel thin layer chromatography plate (TLC pre-coated plates, M/s Merck KGaA, Darmstadt, Germany) and resolved using chloroform: methanol (8:2, v/v). The antioxidant-active spot was visualized after spraying with β-carotene linoleate solution (9 mg of β-carotene dissolved in 30 ml of chloroform to which 2 drops of linoleic acid were added and then added to 60 ml of ethanol, Pratt & Miller, 1984). The β-carotene positive spot was scraped, extracted in acetonitrile and subjected to HPLC.

The HPLC (Hewlett Packard 1100 Series, Palo Alto, CA) was equipped with a quaternary pump fitted with a Zorbax C18 analytical column (25 cm × 4.6 cm I.D 5 μ particle size) (M/s S. V. Scientific, Bangalore, India). Detection was done by an HP 1250 m series variable wavelength detector at wavelength of 280 nm. The gradient mobile phase consisted of acetonitrile (A) and 1% trifluoroacetic acid (TFA) (B) with flow rate of 0.1 ml/min. The elution program involved a linear gradient from 100 - 0% for 15 min followed by 5 min equilibrium in a total program time of 25 min. The sample and the standard were dissolved in mobile phase and 10 μl of each was injected.

2.11. Statistical analysis

All the data are expressed as mean ± standard error (SE) ($n = 3$) and the results were processed by the Programme: Microsoft Office Excel 2002.

3. Results and discussion

The CSP on extraction with ethanol yielded a large amount of extract (0.45 g/g powder). The total phenolic content of CSE, expressed as gallic acid equivalents (GAE) was 243 mg GAE/g CSP. Earlier studies have suggested that polyphenolic compounds are associated with antioxidant activity and play an important role in preventing lipid peroxidation (Kirakosyan et al., 2003). However, the polyphenolic compounds in the pericarp or seed coats of nuts have been scantily reported, and no studies have explored the phenolics from cashew nut skins and their antioxidant activity. The present investigation shows that cashew nut skin contains considerable amounts of phenolics clearly indicating that CSP could be a potential source of natural antioxidants.

In this study, the potential of CSE to scavenge free radical was assessed by its ability to quench $ABTS^{\bullet+}$ synthetic free radical. The stable nitrogen-centered free radical $ABTS^{\bullet+}$ is frequently used for the estimation of free radical scavenging ability (Re et al., 1999). Fig. 1 depicts the concentration-dependent decolorization of $ABTS^{\bullet+}$ by CSE in comparison with BHA. As evident from the figure, CSE was equally potent as BHA in radical scavenging.

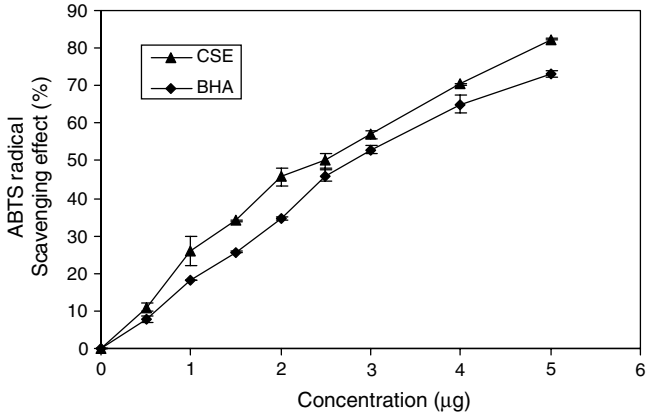

Fig. 1. ABTS radical scavenging effect of Cashew nut skin extract (CSE). Values are mean ± SE of three determinations.

Superoxide anion derived from dissolved oxygen by PMS-NADH coupling reduces NBT (Nishikimi et al., 1972). The decrease in absorbance at 560 nm with antioxidants indicates the consumption of superoxide anion in the reaction mixture. Fig. 2 shows the percentage inhibition of superoxide radical generation by various concentrations of CSE. Apparently the scavenging of the superoxide radical also increased with increasing CSE concentration, with an EC_{50} of 10.69 µg/ml (Table 1). This data shows that CSE is a potent scavenger of superoxide radicals.

Scavenging activity of CSE on hydroxyl radical is shown in Fig. 3. The scavenging activity against hydroxyl radical for CSE (0–20 µg) ranged from 0% to 50%, indicating that the scavenging effect increased with increasing amounts of CSE. The hydroxyl radical is the most reactive radical known to initiate lipid peroxidation (Halliwell, 1991). The ability of CSE to quench the hydroxyl radical seems to relate directly to the prevention of propagation of lipid peroxidation (LPO).

Plant extracts enriched in phenolic compounds are capable of complexing with and stabilize transition metal ions, rendering them unable to participate in metal-catalyzed initiation and hydroperoxide decomposition reactions (Gordon, 1990). Metal chelation is an important antioxidant property (Kehrer, 2000), and hence CSE was assessed for its ability to compete with ferozzine for iron (II) ions in the solution. As evident from Fig. 4, CSE was capable of reducing iron (III). This study revealed that CSE (EC_{50} 6 mg/ml, Table 1) had a relatively lower Fe^{2+} binding compared with that of EDTA.

Heat induced oxidation of an aqueous emulsion system of β-carotene and linoleic acid is often employed as an antioxidant assay. In this particular model, β-carotene undergoes rapid discoloration in the absence of an antioxidant (Taga et al., 1984). The presence of a phenolic antioxidant

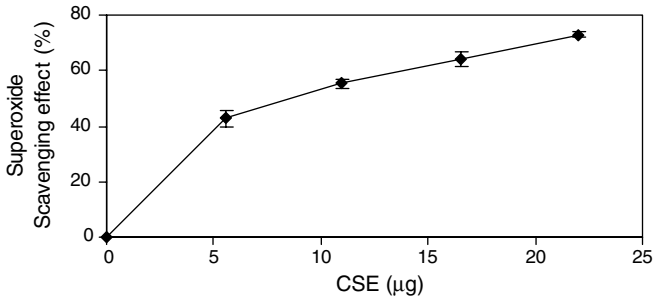

Fig. 2. Superoxide radical scavenging effect of CSE. Values are mean ± SE of three determinations.

Table 1
EC_{50} of CSE in various antioxidant assay systems

Antioxidant assay	EC_{50}[a]
ABTS[b]	1.30 ± 0.02
Superoxide scavenging[b]	10.69 ± 1.13
Deoxyribose oxidation[b]	17.70 ± 0.05
Iron chelation[c]	6.00 ± 0.24
LPO in rat brain homogenate[b]	24.66 ± 0.32

LPO: Lipid peroxidation.
Values are mean ± SE of three determinations.
[a] The EC_{50} value in each assay was determined graphically by plotting each activity as a function of CSE concentration (µg or mg).
[b] µg/ml.
[c] mg/ml.

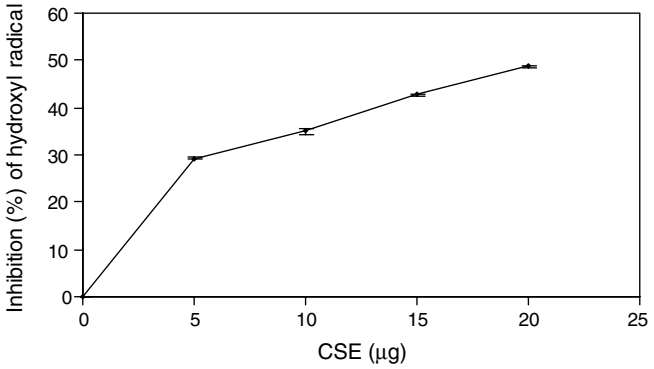

Fig. 3. Inhibitory effect of CSE on deoxyribose oxidative damage. Values are mean ± SE of three determinations.

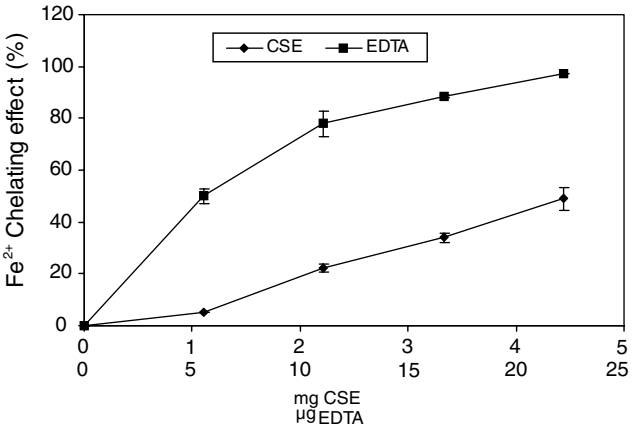

Fig. 4. Chelating effect of CSE on Fe^{2+} ion. Values are mean ± SE of three determinations.

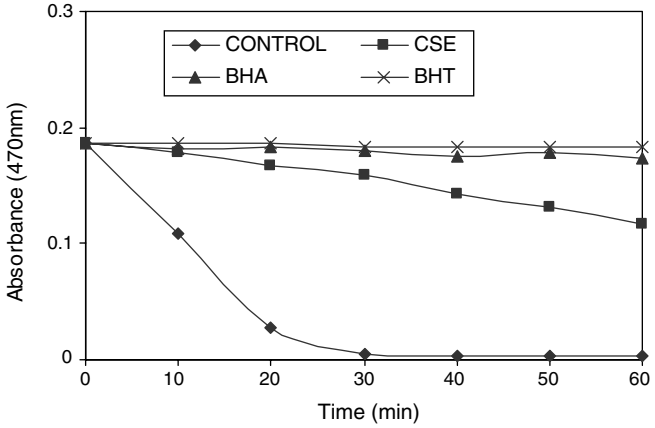

Fig. 5. Antioxidant activity of Cashew nut skin extract (CSE) in β-carotene-linoleic acid system.

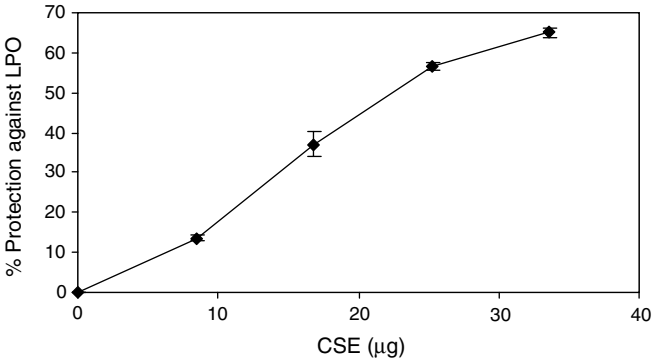

Fig. 6. Inhibition of Fe^{2+}–H_2O_2 induced lipid peroxidation by CSE in rat brain homogenate. Values are mean ± SE of three determinations.

can hinder the extent of β-carotene destruction by "neutralizing" the linoleate free radical and any other free radicals formed within the system. In the present study, CSE exhibited marked antioxidant activity, nearly equal to that of BHA as shown in Fig. 5. However, the greatest antioxidative efficacy was from the synthetic antioxidants (i.e. BHA and BHT) which practically inhibited β-carotene bleaching throughout the incubation period.

The inhibition of LPO may be considered as one of the test to determine the antioxidant activity of a compound (Cos, Calomme, Pieters, Vlietinck, & Vanden Berghe, 2000). Results have demonstrated that treatment with CSE successfully inhibited TBARS formation in the rat brain homogenate. Addition of CSE to the Fe^{2+}–H_2O_2 system resulted in a concentration-dependent decrease in the formation of tissue oxidation levels (Fig. 6) confirming that CSE was also a scavenger of $^{\cdot}OH$.

From the data obtained from the various assay systems, it is clear that the order of effectiveness of CSE in the antioxidant assays was as follows: ABTS > superoxide > deoxyribose > LPO in rat brain homogenate > iron chelation (Table 1). Hence CSE appears to be a more powerful radical scavenger than a metal chelator. The cashew nut skin antioxidant activity may principally be attributed to its phenolic composition, due to the ability of these compounds as free radical scavengers. Earlier studies have shown that cashew nut testa is a good source of hydrolysable tannins (Pillai et al., 1963). More than 40% of the total polyphenol in the testa is reported to be constituted by (+) catechin and (−) epicatechin (Mathew & Parpia, 1970). An attempt was made to elucidate the molecule responsible for the strong antioxidant characteristics of CSE observed in the present study. Ascending TLC of CSE on silica gel plates developed in chloroform–methanol (8:2, v/v) yielded a single spot which gave strong reaction with β-carotene-linoleate spray. The spot on extraction and HPLC analysis yielded a peak similar to standard epicatechin (data not shown). This finding prompted us to speculate that probably the epicatechin present in CSE may be largely responsible for the antioxidant activity of CSE. However these findings have to be confirmed by LC-MS.

It is clearly evident that ethanolic extract of cashew nut skin has significant antioxidant activity in various antioxidant assay systems. The polyphenols, in general present in the skin appears to significantly contribute to the antioxidant activity of CSE. According to the findings in our study, cashew nut skins are a natural source of phenolic compounds. Further, the presence of the potent bioactive phenolic compounds in the skins of cashew nut could be of interest to both food and pharmaceutical industries, where it could be employed as an economical source of natural antioxidants.

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