Bangladesh J. Bot. **46**(3): 1015-1020, 2017 (September)

ANTIOXIDANT EFFICACY, LIPID PEROXIDATION INHIBITION AND PHENOLIC CONTENT OF ESSENTIAL OIL OF FRUITS OF *CUDRANIA TRICUSPIDATA*

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Keywords: Cudrania tricuspidata, Essential oil, Phenolics, Antioxidant, Lipid peroxidation

Abstract

This study was aimed to confirm the potential of *Cudrania tricuspidata*(Carrière.) Bur. ex Lav. fruit essential oil (CTEO) in terms of its antioxidant efficacy in a panel of free radical scavenging models. CTEO was also assessed for its lipid peroxidation inhibitory activity and reducing power ability along with total phenolic content. The CTEO was obtained by hydrodistillation of *C. tricuspidata* fruits using a microwave-assisted extraction technique. The oil showed antioxidant capacity through the inhibition of DPPH, nitric oxide, superoxide and hydroxyl radicals by 74.91, 74.95, 72.97 and 73.75%, respectively. Moreover, it displayed concentration-dependent reducing power and remarkable ferric ion-induced lipid peroxidation inhibitory activity. In addition, the CTEO yielded 8.61 ± 0.02 mg/g gallic acid equivalent of phenolic content. These findings confirm significantefficacy of *C. tricuspidata* as a potential source of natural antioxidants.

Introduction

In recent years, there is a dramatic upsurge in the areas related to newer developments in prophylaxis and treatment of diseases, particularly the role of free radicals and antioxidants. Free radicals are primarily derived from oxygen (reactive oxygen species; ROS) and are generated in the body *milieu* by a number of endogenous physiological and pathological processes (Halliwell and Gutteridge 2006). They can adversely alter macromolecules such as lipids, proteins and DNA and have been implicated in cancer, atherosclerosis, cardiovascular diseases, ageing and inflammatory diseases (Braca *et al.* 2002). Lipids are highly prone to free radical damage resulting in lipid peroxidation that can lead to adverse alterations in cell membrane leading to cell damage (Devasagayam and Kesavan 1996). There are epidemiological evidences correlating higher intake of components/foods with antioxidant ability to lower the incidences of various human morbidities or mortalities (Devasagayam *et al.* 2004).

Recently much attention has been directed towards the development of less toxic ethnomedicines and plant-based volatile essential oils have wide applications in dietary supplements, food flavoring and preservation, folk medicine and fragrance industry (Huang *et al.* 2005). Several reports have confirmed the *in vitro* and *in vivo* antioxidant efficacy of plant-based essential oils (Bajpai *et al.* 2013a). Nowadays, essential oils and their components are gaining increasing attention because of their relatively safe status, wide acceptance by consumers, and the possibility of their exploitation for potential multi-purpose functional uses.

Cudrania tricuspidata(Carrière.) Bur. ex Lav. is a deciduous tree found in the eastern area of Russia, and northern regions of Korea, Japan and China, and known as one of the most ubiquitous traditional herbal remedies in East Asia (Park *et al.* 2006). The beneficial effects of this plant have been traditionally associated with anti-inflammatory (Park *et al.* 2006), anti-tumor (Zou *et al.* 2004), α-glucosidase inhibitiory (Seo *et al.* 2007) and cytotoxic (Park 2005) activities.

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Previously present authors reported the chemical composition of essential oil of *C. tricuspidata* fruit (CTEO) and the mechanism of its antibacterial activity against food-borne pathogenic bacteria (Bajpai *et al.* 2013b). However, the antioxidant activity of essential oil of*C. tricuspidata* fruit (CTEO) has not been reported so far. Hence, the objective of the present study was to evaluate the antioxidant capacity and free radical scavenging efficacy of CTEO using various *in vitro* modelsas well as ferric ion-induced lipid peroxidation inhibitory activity, reducing power ability and total phenolic contents.

Materials and Methods

The chemicals and standard reagents used in this study were of analytical grade and were purchased from Sigma-Aldrich (St. Louis, USA). Spectrophotometric measurements were done using a 96-well-microplate ELISA reader (Tecan, Infinite M200).Dried fruits of *C. tricuspidata* (200 g) were subjected to hydrodistillation using a microwave extraction apparatus, and oil was prepared as reported previously (Bajpai *et al.* 2013b).

The scavenging activity of stable DPPH free radical was determined by the previously described method (Bajpai*et al.* 2013a). Different concentrations of CTEO (100-500 µg/ml) were added to 50 µl of the methanolic solution of DPPH (0.004%) at 1:1 ratio in a 96-well-microplate. The mixture was incubated at 37^{o}C in thedark for 30 min with shaking (150 rpm). Absorbance was recorded at 517 nm against a blank. Ascorbic acid and α-tocopherol were also used as reference compounds. The percentinhibitory activity was calculated using the following formula : Inhibition % = (Absorbance of control - absorbance of test)/(Absorbance of control) × 100.

Nitric oxide radical scavenging activity of CTEO was determined as reported previously (Bajpai *et al.* 2013a). In this assay, the solution of sodium nitroprusside (SNP) (10 mM) in phosphate buffer saline (PBS pH 7.4) was mixed with different concentrations of CTEO (100-500 µg/ml). The mixture was incubated at 37ºC for 60 min in light. The half quantity of aliquots was taken and mixed with equal quantity of the Griess reagent, and the mixture was incubated at 25^{o}C for 30 min in the dark. The absorbance of pink chromophore generated was read at 546 nm against a blank. Ascorbic acid and α-tocopherol were used as reference compounds. The percent inhibition activity was calculated by the formula : Inhibition % = (absorbance of control - Absorbance of test) / (Absorbance of control) × 100.

Superoxide radical scavenging activity of CTEO was measured by the reduction of nitroblue tetrazolium (NBT) according to a previously reported method (Bajpai *et al.* 2013a). In this assay, the reaction mixture (150 µl) contained phosphate buffer (0.2 M, pH 7.4), NADH (73 µM), NBT (50 µM), PMS (15 µM) and various concentrations (50-250 µg/ml) of the CTEO. After incubation for 60 min at room temperature, the absorbance of the reaction mixture was measured at 560 nm against an appropriate blank. Ascorbic acid and α-tocopherol were used as positive controls. The per cent inhibitory activity was calculated by the previously used formula.

A previously described method was adopted for determining the hydroxyl radical scavenging activity of CTEO (Bajpai *et al.* 2013a). The reaction mixture in a final volume of 240 µl contained 2-deoxy-2-ribose (3 mM), KH_2PO_4-KOH buffer (20 mM, pH 7.4), $FeCl_3$ (0.1 mM), EDTA (0.1 mM), H_2O_2 (2 mM), ascorbic acid (0.1 mM) and various concentrations (100-500 µg/ml) of CTEO or standard compounds. After incubation for 45 min at 37^{o}C, 40 µl of 2.8% trichloroacetic acid (TCA), and 40 µl of TBA (0.5% in 0.025M NaOH solution containing 0.02% BHA) were added in the reaction mixture, and the mixture was incubated at 95^{o}C for 15 min to develop the pink color, and the absorbance was measured at 532 nm against an appropriate blank solution. Ascorbic acid and BHA were used as positive controls.

The percent inhibition activity was calculated by the formula : Inhibition % = (Absorbance of control – absorbance of test)/(Absorbance of control) × 100.

The Fe^{3+}/ascorbic acid dependent non-enzymatic lipid peroxidation in bovine brain extract was performed as reported previously (Bajpai *et al.* 2013a). The reaction mixture, in the absence and presence of CTEO or reference compounds (50-250 µg/ml), containing 50 µl of bovine brain phospholipids (5 mg/ml), 1 mM $FeCl_3$ and 1 mM ascorbic acid in 20 mM phosphate buffer with a final volume of 330 µl, was incubated at 37°C for 1 hr. The hydroxyl radicals generated in the reaction initiated the lipid peroxidation, resulting in malondialdehyde (MDA) production that was measured by TBA reaction. BHA and α-tocopherol were used as positive controls. The percent inhibition was calculated as before.

The Fe^{3+} reducing power of the CTEO was determined by the method of Bajpai *et al.* (2013a). Aliquots (50 µl) of different concentrations of CTEO (5-25µg/ml) were mixed with 50 µl phosphate buffer (0.2 M, pH 6.6) and 50 µl potassium ferricyanide (1% w/v in H_2O), followed by incubation at 50°C for 20 min in the dark. After incubation, 50 µl of TCA (10% w/v in H_2O) was added to terminate the reaction and the mixture was subjected to centrifugation at 3,000 rpm for 10 min. For final reaction mixture, the supernatant (50 µl) was mixed with 50 µl distilled water and 10 µl $FeCl_3$ solution (0.1% w/v in H_2O). The reaction mixture was incubated for 10 min at room temperature and the absorbance was measured at 700 nm against an appropriate blank. A higher absorbance of the reaction mixture indicated greater reducing power. Ascorbic acid and α-tocopherol were used as positive controls.

Total phenolic content was determined using Folin-Ciocalteu reaction according to the published method (Bajpai *et al.* 2013a). An aliquot of 50 µl CTEO (100 µg/ml) was mixed with 50 µl of 5% Folin-Ciocalteu reagent and the reaction mixture was incubated 25ºC for 5 min in the dark followed by the addition of 100 µl of 20% (w/v in H_2O)Na_2CO_3 solution. After incubation at room temperature for 20 min, the absorbance was measured at 730 nm against the appropriate methanolic blank solution. The total phenolic content was evaluated from a standard calibration curve of gallic acid using the concentration range of 5-50 µg/ml.

All data are expressed as the mean ± Sd by measuring three independent replicates. Analysis of variance using one-way ANOVA followed by DMRT was performed to test the significance of differences between the means obtained among the treatments at the 5% level of significance using a SAS software (Version: SAS 9.1, SAS Institute Inc., Cary, NC, USA).

Results and Discussion

In living systems, the free radicals are continuously generated in the body and can cause extensive damage to tissues and biomolecules resulting in various diseases and also ageing. A large number of synthetic chemical drugs protect the living cells against oxidative stress, however, they can also have severe side effects. Hence, researchers and food manufacturers are interested in using natural antioxidants from plant-derived dietary supplements and traditionally used medicinal plants (Yazdanparast *et al.* 2008). Previously we reported that the GC-MS analysis of the CTEO led to the identification of 29 different components representing 94.46% of the total oil (Bajpai *et al*. 2013b). In recent years, several researchers have reported that phenolics, hydrocarbons and oxygenated phytoconstituents of plant-derived essential oils have enormous antioxidant activity and free radical scavenging potential (Bajpai *et al*. 2013a).

DPPH radical scavenging assay is the most popular method for the determination of antioxidant capacity of compounds due to its simplicity, rapidity, sensitivity, and better reproducibility (Nakayama *et al.* 1993). Radical scavengers may protect cells, tissues from free radicals, thereby preventing diseases such as cancer and atherosclerosis (Nakayama *et al.* 1993).

The DPPH radical scavenging activities of CTEO, ascorbic acid and α-tocopherol, are presented in Fig. 1.

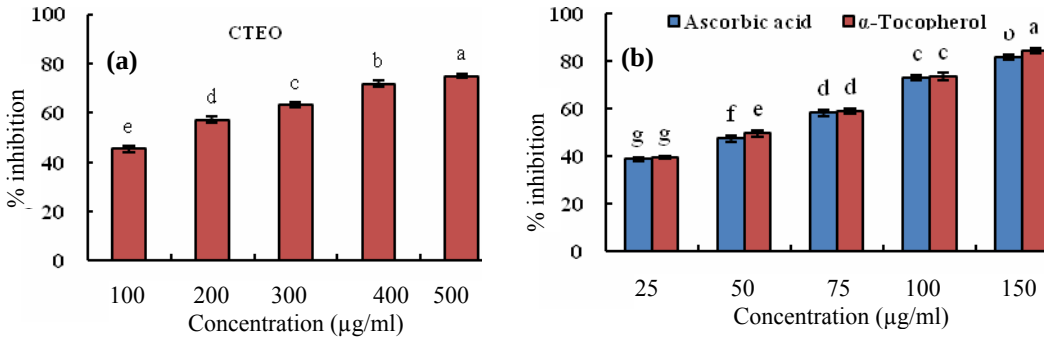

Fig. 1. DPPH radical scavenging activity of *C. tricuspidata* fruit essential oil (a) and standard compounds, ascorbic acid and α-tocopherol (b).

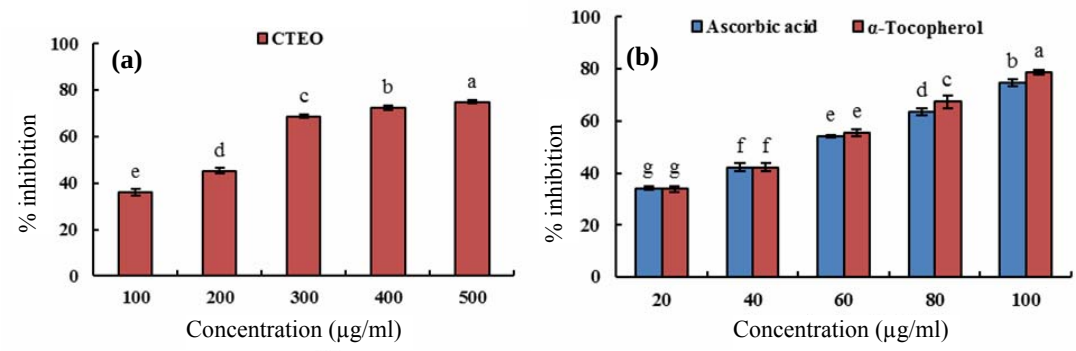

Fig. 2. Nitric oxide radical scavenging activity of *C. tricuspidata* fruit essential oil (a) and standard compounds ascorbic acid and α-tocopherol (b).

The results showed that α-tocopherol and ascorbic acid (150 µg/ml) possessed up to 73.45 and 72.91%, inhibitory effect on scavenging the DPPH radicals, respectively (Fig. 1b). Whereas the DPPH scavenging ability of CTEO (500 µg/ml) was found to be 74.91% (Fig. 1a).

Sustained levels of nitric oxideradical are cytotoxic and contribute to the vascular collapse coupled with septic shock, furthermore chronic expression of nitric oxide radical is associated with various cancers and inflammatory conditions including juvenile diabetes, multiple sclerosis, arthritis and ulcerative colitis (Tylor *et al.* 1997). The CTEO (500 µg/ml) caused significant inhibition of nitric oxide by 74.95% (Fig. 2a). Ascorbic acid and α-tocopherol caused about 74.62 and 78.61% inhibition of nitric oxide radicals at the concentration of 100 µg/ml, respectively (Fig. 2b). CTEO may inhibit nitrite formation by directly competing with oxygen in the reaction with nitric oxide.

Superoxide anion is also implicated in several pathophysiological processes, due to its transformation into more reactive species such as hydroxyl radical that initiates lipid peroxidation (Gulcin *et al.* 2006). The percentage inhibition of superoxide generation at 250 µg/ml concentration of CTEO was 72.97, which is almost equal to the inhibitory values of 73.00 and

74.45% obtained for ascorbic acid and α-tocopherol at the same concentration, respectively. CTEO had equivalent scavenging activity compared to ascorbic acid and α-tocopherol (Fig. 3a). Hydroxyl radical is the most reactive free radical. The hydroxyl radical scavenging abilities of CTEO, ascorbic acid and BHA are summarized in Fig. 4. In this assay, CTEO, ascorbic acid and BHA at the concentration of 250 µg/ml exhibited 73.75, 73.79 and 70.02% hydroxyl radical scavenging activity, respectively (Fig. 3b).

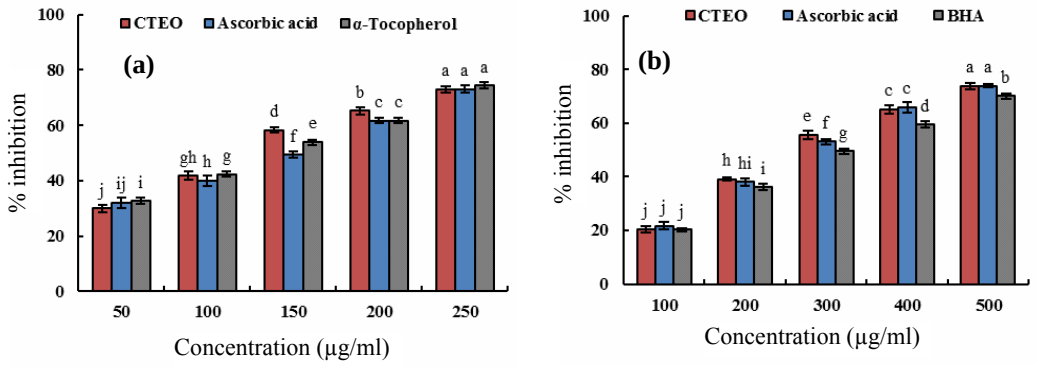

Fig. 3.Superoxide radical (3a) and hydroxyl radical (3b) scavenging activities of *C. tricuspidata* fruit essential oil (CTEO) and standard compounds.

Among all biological macromolecules, unsaturated membrane lipids are predominantly prone to oxidative damage resulting in cell injury (Niki 2008). The CTEO and reference samples at the concentration of 250 µg/ml showed significant efficacy on inhibition of lipid peroxidation activity by 72.88, 80.13 and 76.59%, respectively (Fig. 4a).

The reducing properties are generally associated with the presence of reductones (Duh *et al.* 1999), which have been shown to exert antioxidant action by breaking the free radical chain by donating a hydrogen atom (Gordon 1990). At the concentration of 20 µg/ml, the absorbance values of CTEO, ascorbic acid and α-tocopherol were found to be 0.83, 1.10, and 0.95, respectively, while the absorbance value of CTEO at 25 µg/ml was found to be 1.11, which are very close to both of reference compounds (Fig.4b).

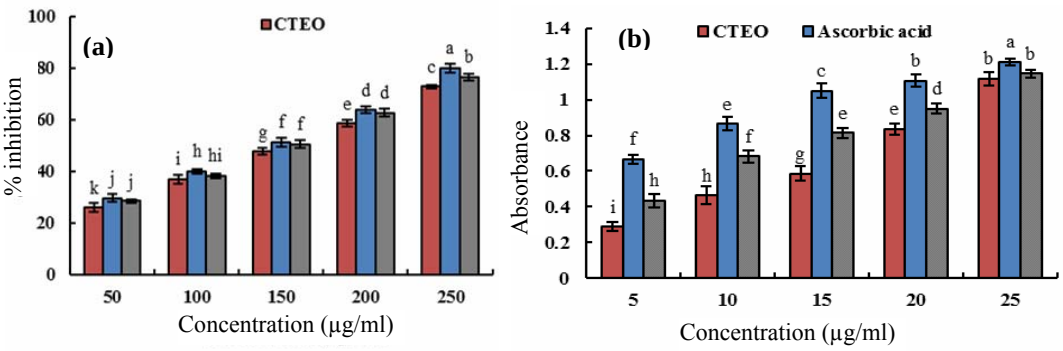

Fig. 4.Lipid peroxidation inhibition (4a) and reducing power ability (4b) of *C. tricuspidata* fruit essential oil (CTEO) and standard compounds.

The phenolic content could be used as an important indicator of the antioxidant capacity, which may be used as preliminary screening tool for utilizing the essential oils in dietary

supplements (Liu *et al.* 2008). The content of total phenolic compounds in CTEO was found to be 8.61 ± 0.02 mg/g GAE. The results of this study showed that CTEO contained phenolic compounds and exhibited high amount of antioxidant and lipid peroxidation inhibition ability. These findings indicate that CTEO can be a significant source of natural antioxidants as an alternative to chemical antioxidants for its practical application in the food industry to stabilize the food from oxidative deterioration.

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(Manuscript received on 12 June 2015; revised on 19 December 2016)