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In vitro antioxidant activity of two edible Timbur fruits of Darjeeling Himalaya

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Abstract
Free radicals are implicated for many chronic, painful and near-fatal diseases including Diabetes mellitus, arthritis, cancer, apoptosis, neurodegenerative disorders etc. In treatment of these diseases, antioxidant therapy has gained an utmost importance and current research is now directed towards finding naturally occurring antioxidants of plant origin. Edible Timburs namely *Zanthoxylum acanthopodium* DC. and *Litsea cubeba* (Loureiro) Persoon are traditionally used as spices in different Nepali recipes. In the present study, the fruits of these plants were extracted with aqueous methanol (1:4) to examine the *in vitro* antioxidant property, phenol content and phytochemical constituents. The scavenging activities on DPPH free radicals, superoxide anions and per-oxidized lipid molecules were determined as well as the flavonoid and phenolic constituents of the extracts. The extracts exhibited significant scavenging activity towards DPPH free radicals and high anti-lipid peroxidation values due to the presence of relatively high total phenol contents. Also, these spices contain a vast array of different phytochemicals in their dry form. These results suggest that both *Zanthoxylum acanthopodium* and *Litsea cubeba* fruits are endowed with antioxidant phytochemicals and could serve as basal ingredients for nutraceutical formulations.

Keywords: Antioxidant, DPPH, *Zanthoxylum acanthopodium*, *Litsea cubeba*

An extensive diversity of medicinal plants and edible fruits is observed in Darjeeling hills. Two plants of these hills viz. *Zanthoxylum acanthopodium* DC. and *Litsea cubeba* (Loureiro) Persoon are locally known as 'Boke Timbur' and 'Sil Timbur' respectively. These plants are wild, tree like in habit under the families of Rutaceae and Lauraceae and are widely spread in the forest of Darjeeling hills. Fruits of these plants ('Timburs') have been used traditionally for healing diarrhea, vomiting, and gastric ulcer and as warm killer. These fruits are also used as spices in different Nepali recipes. Several studies have been conducted to determine the antioxidant properties of many plants, especially those used in traditional medicine (Jang *et al.* 2007, Surveswaran *et al.* 2007). Currently, there is a great interest in the field of antioxidant substances mainly due to the findings concerned with the effects of free radicals in the organism. Free radicals have significant role in creation of several metabolic, mutagenic and age-related disorders like diabetes, cirrhosis, cancer and cardiovascular diseases (Hertong and Feskns 1993). Reactive oxygen species (ROS), which include free radicals such as superoxide anion (SO^{2-}), hydroxyl radicals (OH) and non-free-radical species like H_2O_2 and singlet oxygen ($^1O_2$) are various forms of activated oxygen (Gulcin *et al.* 2002, Halliwell and Gutteridge 1999, Yildirim *et al.* 2000). It is commonly recognized that antioxidants can neutralize potentially harmful reactive free radicals in body cells before they cause lipid and protein oxidation and may reduce potential mutation risks and therefore, help to prevent cancer or heart diseases. Recently, there is a growing attention on the discovery of natural antioxidants because epidemiological and clinical evidences suggest that consumption of vegetables and fruits reduce the risk of developing chronic diseases like cancer and in this respect phytochemicals are generally safer than synthetic chemicals (Dastmalchi *et al.*, 2007). Therefore, the search for natural antioxidants as alternatives to synthetic ones is of great interest among researchers. Plants contain a wide variety of free radical scavenging molecules like phenols, flavonoids, vitamins, terpenoids etc. that are rich in antioxidant activity (Cai *et al.*, 2003). However, the use of natural antioxidants are limited due to lack of knowledge about their molecular composition and dynamics, amount of active ingredients in the source material and the availability of relevant toxicity data (Shahidi *et al.* 1994). Natural antioxidants tend to be safer and they also posses antiviral, anti-inflammatory, anti-tumour and hepatoprotective properties (Lim and Murtijaya, 2007). Information related to antioxidant activity and phenolic compounds on traditional Darjeeling medicinal and underexplored edible plants is scarce. Literature survey revealed no relevant phyto-pharmacological records on *Zanthoxylum acanthopodium* and *Litsea cubeba*, and the fruits of these plants have not yet been screened for their antioxidant activity. This present study, therefore investigated the phytochemical compositions and polyphenol content, the *in vitro* antioxidant, lipid peroxidation and superoxide scavenging potential of this plant.

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MATERIALS AND METHODS

Plant materials

Two fruit samples of the selected species *viz.*, *Zanthoxylum acanthopodium* (Figure 1) and *Litsea cubeba* (Figure 2) were collected from the Chwak Bazar market of Darjeeling Town, Darjeeling , West Bengal and species authentication were done by the Taxonomy and Environmental Biology Laboratory, Department of Botany, University of North Bengal.

Animal material

Goat liver, used for anti-lipid peroxidation assay, were collected from slaughter house immediately after slay and the experiment was conducted within one hour after collection.

Chemicals

Methanol; 2.2-diphenyl-1-picryl hydrazyl (DPPH); nitro blue tetrazolium (NBT); reduced nicotinamide adenine dinucleotide phosphate sodium salt, monohydrate (NADPH); phenazine methosulphate (PMS); trichloroacetic acid (TCA); thiobarbituric acid (TBA); $FeSO_4$. $7H_2O$; acetic acid; Folin-Ciocalteu reagent; NaOH; Arnow's reagent; quercetin; $NaNO_2$; $AlCl_3$; sodium carbonate (Na_2CO_3); ferric chloride solution; Fehling's solution, cupper acetate solution; petroleum ether; ninhydrin reagent; ammonia; lead acetate; acetic anhydride; olive oil; ferric chloride; hydrochloric acid; Dragendroff's reagent; pyridine; sodium nitroprusside; chloroform; Conc. H_2SO_4 were either purchased from Sigma Chemicals (USA) or Merck (Germany). All the chemicals and reagents, used for experimental purposes were of analytical grade.

Extraction procedure

Under Soxhlet extractor powdered drug of fruits were separately extracted with methanol water in ratio 4:1 for eight hours. The refluxed samples were separated from the residues by filtering through Whatman No. 1 filter paper. The filtrates were dried under reduced pressure and their total extractive values were calculated on dry weight basis by the formula:

$$\% \text{ extractive value (yield \%)} = \frac{\text{Weight of dry extract}}{\text{Weight taken for extraction}} \text{X100}$$

The samples were then kept in freeze for further use.

DPPH based free radical scavenging activity

The scavenging reaction between (DPPH•) and an antioxidant (H-A) can be written as:

(DPPH•) + (H-A) → DPPH-H + (A•)

Purple Yellow

Antioxidants react with DPPH•, which is a stable free radical and is reduced to the DPPH-H and as consequence, the absorbance is decreased from the DPPH• radical to the DPPH-H form. The degree of discoloration indicates the scavenging potential of the antioxidant compounds or extracts in terms of hydrogen donating ability (Benabadji *et al.* 2004).The free radical scavenging activities of each fraction were assayed using a stable DPPH, following standard method of Blois (1958). The reaction mixture contained 1.8 ml of 0.1mM DPPH and 0.2 ml of each serial dilution (200 mg/ml, 40 mg/ml, 20 mg/ml, 8 mg/ml FW) of methanolic fruit extracts. Simultaneously, a control was prepared by replacing extracts with methanol. The reaction mixture was shaken and allowed to incubate for 30 min at room temperature (25° C) in the dark and OD values were measured at 517 nm with a spectrophotometer. Radical scavenging activity was expressed as percent inhibition from the given formula:

$$\text{Percent inhibition of DPPH radical} = \frac{\text{Abs. of control} - \text{Abs. of sample}}{\text{Abs. of control}} \text{X100}$$

Superoxide radical scavenging assay

Measurement of superoxide radical scavenging activity of *Zanthoxylum acanthopodium* and *Litsea cubeba* were done by using standard method followed by Nishikimi *et al.*, 1972 with minor modifications. The reaction mixture contained 1 ml of NBT solution (312 µM prepared in phosphate buffer, pH-7.4), 1ml of NADH solution (936 µM prepared in phosphate buffer, pH-7.4) and differentially diluted sample extracts. Finally, reaction were accelerated by adding 100 µL PMS solution (120 µM prepared in phosphate buffer, pH -7.4) to the mixture. The reaction mixtures were incubated at 25° C for 5 min and absorbance was measured at 560 nm against methanol as control. Percentage inhibition was calculated using the same formula mentioned above.

Anti-lipid peroxidation (ALP) assay

The anti-lipid peroxidation activity of the extracts of two timbur fruits was determined by the standard method (Bauchet *et al.*, 1998) followed by slight modification with the goat liver homogenate. 2.8 ml of 10% goat liver homogenate, 0.1ml of 50mM $FeSO_4$ and 0.1 ml extract was mixed. The reaction mixture was incubated for 30 minutes at 37°C. 1 ml of reaction mixture was taken with 2ml 10%TCA-0.67%TBA in acetic acid (50%) for blocking the reaction. Then the mixture was boiled for 1hour at 100°C and centrifuged at 10,000 rpm for 5 minutes. Supernatant was taken for absorbance at 535nm. Vitamin E was used for standard. ALP % was calculated using the following formula:

$$ALP\ percent = \frac{Abs\ of\ Fe^{2+}\ induced\ peroxidation - abs\ of\ sample}{Abs\ of\ Fe^{2+}\ induced\ peroxidation - abs\ of\ control} \text{x100}$$

Total phenol Estimation

Total phenolic compounds of fruit extracts were determined by Folin-Ciocalteu method (Folin and Ciocalteu, 1927). For the preparation of the calibration curve, 1 ml aliquot of 0.025, 0.05, 0.075, 0.1, 0.2 and 0.3 mg/ml methanolic gallic acid solution was mixed with 5 ml of Folin-Ciocaltue reagent (10 times diluted) and 4 ml sodium carbonate (75 g/L). The absorbance at 765 nm was measured after 1 hr. at 20° C and the calibration curve was drawn. To the same reagent, 1 ml methanolic fruit extracts (10 mg/ml) was mixed as described above and after 1 hr. the absorbance was measured. Total phenolic content in fruit methanolic extracts in Gallic Acid Equivalents (GAE) was

measured by the formula:

$C = c.V/m$

Where, C - total content of phenolic compounds, mg/g of plant extract, in GAE; c - the concentration of gallic acid deduced from the calibration curve (mg/ml); V - the volume of extracts (ml); m - the dry weight of the plant material.

Total flavonoids determination

Aluminum chloride spectrophotometric method was used for flavonoids determination (Sultana *et al*., 2009). . Each fruit methanol extracts (0.5 ml of 200mg/ml FW) were separately diluted with 4 ml double distilled water. Then the diluted extracts of fruits were mixed with 5% (0.3 ml) $NaNO_2$ 10% aluminum chloride was then added with reaction mixture. After 6 minute 2ml (1.0 M) NaOH and 2.4 ml D.D. water was added and mixed well. Thereafter, absorbance was measured at 510 nm in spectrophotometer. Standard solutions quercetin (0-500 mg L^{-1}) was used as calibration curve.

Phytochemicals evaluation of the crude extracts

The methanolic crude extract (200 mg/ml) of the fruits of the plant was subjected to various chemical tests in order to determine the secondary metabolites present by employing the use of various methods as follows:

Test for Reducing Sugars

To 0.5ml of the extract, 2ml of a mixture (1:1) of Fehling's solution I (A) and Fehling's solution II (B) was added and the mixture was boiled in a water bath for five minutes. A brick-red precipitate indicated the presence of free reducing sugars (Brain and Turner, 1975).

Test for Flavonoids

To 1ml of methanolic extract, a few drops of 10 % ferric chloride solution were added. A green or blue colour indicated the presence of phenolic nucleus (Brain and Turner, 1975).

Test for resins

0.5ml of extracts were evaporated and dissolved in 2ml of petroleum ether, 2ml of 2% cupper acetate solution was then added and the mixture was shaken vigorously and allowed to separate, a green colour indicated the presence of resin (Trease and Evans, 1983).

Test for amino acid

0.5 ml methanolic fruit extracts were treated with few drops of ninhydrin reagent, heated in water bath, a purple colour indicated the presence of amino acids (Kumar *et al*., 2009).

Test for anthraquinones

1ml methanolic fruit extracts were evaporated and dissolved in 2ml chloroform. 2ml of ammonia was added. Occurrence of Red/orange colour suggested the presence of anthraquinones (Kumar *et al*., 2009).

Test for tannin

0.5 ml methanolic extract of each fruit was added with 0.5 ml 1% lead acetate; a yellow colour precipitation indicated the presence of tannin (Kumar *et al*., 2009).

Test for triterpenoids

0.5ml of methanolic fruit extracts were evaporated and dissolved in 1ml chloroform.1ml acetic anhydride was then added and chilled. After cooling, conc.H_2SO_4 was added. If reddish violate colour appeared, the existence of triterpenoids was confirmed (Kumar *et al*., 2009).

Test for alkaloids

0.5 ml of each fruit extract was added with 0.2ml of 36.5% hydrochloric acid and 0.2 ml Dragendroff's reagent. Production of orange or red precipitation denoted the presence of alkaloids (Kumar *et al*., 2009).

Test for glycosides

0.5 ml methanolic extract of fruits were added with 2ml of 50% hydrochloric acid. The mixtures were hydrolyzed for 2 hrs on a water bath. After that 1ml pyridine, few drops of 1% sodium nitroprusside solution, and 5% sodium hydroxide solution were added. Pink to red colour designated the presence of glycosides (Kumar *et al*., 2009).

Test for steroid

0.5ml methanolic fruit extracts were evaporated and dissolved in 2ml chloroform. 2ml of conc. H_2SO_4 was introduced carefully by the side wall of the test tube. Formation of red colour ring confirmed the presence of steroid (Kumar *et al*., 2009).

Test for Saponins

2ml of double distilled water was added with 1ml of each methanolic extract. Few drops of olive oil were added and agitated. Formation of soluble emulsion indicated the presence of saponin (Ngbede *et al*., 2008).

Test for cardiac glycoside

0.5ml of methanolic fruit extracts were evaporated and dissolved in 1ml glacial acetic acid. One drop of 10% ferric chloride was then added. 1ml of conc.H_2SO_4 was added by the side of the test tube. Appearance of brown colour ring at the interface indicated of presence of cardiac glycosides (Ngbede *et al*., 2008).

RESULTS AND DISCUSSION

Free radicals are involved in many disorders like neurodegenerative diseases, cancer and AIDS. Antioxidants through their scavenging power are useful for the management of those diseases. DPPH stable free radical method is an easy, rapid and sensitive way to survey the antioxidant activity of a specific compound or plant extracts (Koleva *et al*., 2002). Unlike other free radicals like hydroxyl radicals and superoxide anions, DPPH has the advantage of being unaffected by certain side reactions, such as metal ion chelating and enzyme inhibition (Jayasri *et al*., 2009). Figure 3 shows that the methanol extracts of *Zanthoxylum acanthopodium* and *Litsea cubeba* have antiradical activity by inhibiting DPPH radical with the maximum inhibition value of about 98.32% and 85.12% in their plateau phase at a concentration range of 200 mg FW/ml. The inhibition percentage of DPPH radical at variable concentration of fruit extracts obey logarithmic equation at correlation co -efficient (R^2) of about 0.984 and 0.987 for *Zanthoxylum*

Table 1: Phytochemical profile of two different timbur fruits (semi-quantitative screening)

Plant samples	1	2	3	4	5	6	7	8	9	10	11	12
Sil Timbur	+++	+	++	+++	++	+++	+++	+++	+++	+++	++++	+++
Boke Timbur	++	++	+	++	+++	+++	++	++	++	++	++	++

1. Alkaloid 2. amino acid 3. anthraquinones 4. steroids 5. glycosides 6. flavonoids 7. saponins 8. tannins 9. reducing sugar 10. triterpenes 11. cardiac glycosides 12. resin

Fig 1: Fruits of Boke Timbur [*Zanthoxylum acanthopodium* DC]

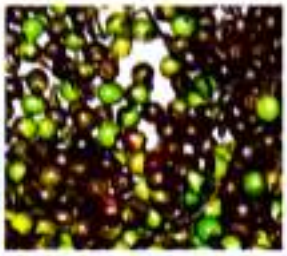

Fig 2: Fruits of Sil Timbur [*Litsea cubeba* (Loureiro) Persoon]

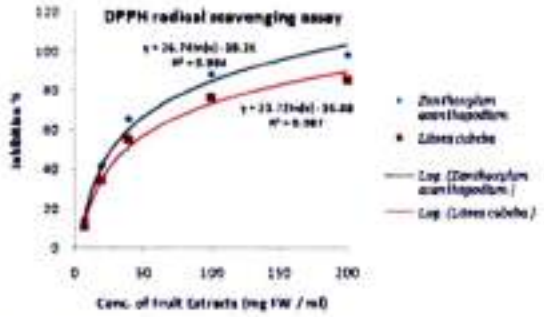

Fig.1: DPPH radical scavenging assay

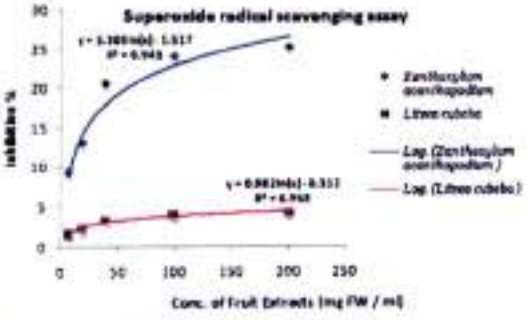

Fig.2: Superoxide radical scavenging assay

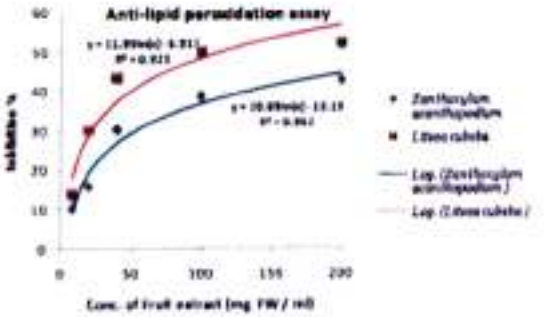

Fig. 3: Anti-Lipid Peroxidation Assay

and *Litsea* respectively. DPPH is a stable free radical at room temperature and accepts an electron or hydrogen radical to become stable diamagnetic molecule. Antioxidant molecules can quench DPPH free radicals (i.e., by providing hydrogen atoms or by electron donation, conceivably via a free-radical attack on the DPPH molecule) and convert them to a colourless molecule (i.e., hydrazine or a substituted analogous of hydrazine), resulting in a decrease in absorbance at 517 nm. It appears that extracts of two timbur fruits possess hydrogen donating abilities to act as an antioxidant.

Superoxide anion radical is one of the strongest reactive oxygen species among the free radicals that could be generated; it also has the ability to change to other harmful reactive oxygen species like hydrogen peroxide, hydroxyl radical, and singlet oxygen, which induce oxidative damage in lipids, proteins and DNA (Yen and Chen, 1995; Pietta, 2000). In the PMS / NADH-NBT system, superoxide anion derived from dissolved oxygen by PMS / NADH coupling reaction reduces NBT. The decrease of absorbance at 560 nm with antioxidants thus indicates the consumption of superoxide anion in the reaction mixture. Addition of the extracts of two fruits in above coupling reaction showed minimum decrease in absorbance (Figure 4). The extracts of *Zanthoxylum acanthopodium* demonstrated a logarithmic (R^2=0.943) dose-response inhibition of superoxide anion radicals with maximum value of 25.1%; whereas in case of *Litsea cubeba*, inhibition was almost insignificant (Figure 4).

Free radicals react with serum lipoprotein (LDL) and cause the formation of atheromatous plagues or react with the cell membranes' lipid and cause the peroxidation of polyunsaturated fatty acids. TBA method was used for evaluating the extent of lipid peroxidation. The extracts with liver homogenate undergo rapid peroxidation when incubated with $FeSO_4$ and produce peroxide (Aruma, 1996), which attack the biological material. This leads to the formation of MDA

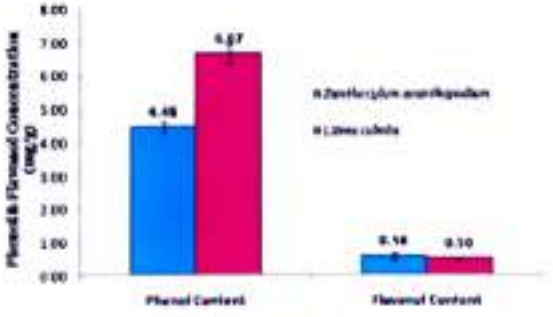

Fig 4: Phenol and flavonol content

(malonaldehyde) and other aldehydes, which form a pink chromogen with TBA, absorbing at 535nm (Kosugi *et al*., 1987). It was observed (Figure 5) that methanol extract of two timbur fruits have high anti-lipid peroxidation effect against goat liver. Addition of Fe^{2+} to the liver, cause increased rate of lipid peroxidation. The peroxides of lipids react with ferrous chloride to form a reddish ferric chloride pigment. In this method the concentration of peroxide decreases as the antioxidant activity increases. The dose dependent logarithmic inhibition curve of lipid peroxidation was observed in Figure 5 and both *Zanthoxylum* and *Litsea* showed potential bioactivity with optimized inhibition of 42.64 and 52.1% at their saturated point.

Phenolic compounds such as flavonoids, phenolic acids and tannins are widely researched, naturally occurring antioxidant components of plants, and their effects on human nutrition and healthcare are considerable. These phenolic compounds, found in medicinal plants as well as fruits and vegetables, play important roles in preventing degenerative diseases, including inflammation, cancer and arteriosclerosis, when they are consumed as a part of daily diet (Sato *et al*., 1996, Li *et al*, 2008). The mechanism of action of flavonoids is through scavenging or chelating the harmful radicals (Cook and Samman, 1996, Kessler *et al*., 2003). Basically phenolics are a class of antioxidant agents which act as free radical terminators (Shahidi and Wanasundara, 1992). The flavonoid contents of the *Zanthoxylum* and *Litsea* fruit extracts in terms of quercetin equivalent (the standard curve equation: $y = 0.0067x + 0.0132$, $r^2 = 0.999$) were 0.58 and 0.50 mg/g respectively (Figure 6). Figure 6 also demonstrated the contents of total phenols that were measured by Folin Ciocalteu reagent in terms of gallic acid equivalent (by standard curve equation: $y = 0.05x + 0.0545$, $r^2 = 0.9873$). The total phenol content of *Zanthoxylum* and *Litsea* were about 4.48 and 6.67 mg/g respectively on dry weight basis.

It is reported that the phenolic compounds constitute a major group of compounds that acts as primary antioxidants (Hatano *et al*., 1989). Though *Zanthoxylum* contains lower amount of total phenolics, as compared with *Litsea*; both Methanol Extractive Yield and radical scavenging activity is higher in *Zanthoxylum* (Methanol extractive value=105 [mg /g FW], % Yield in methanol=10.35) than in *Litsea* (Methanol extractive value=85 [mg /g FW], % Yield in methanol=8.40). So the positive correlation between antioxidant activity and phenolic compounds, as stated by several authors (Surveswaran *et al*., 2007), is not the universal rule for all plant extracts. It also indicates that the metabolites other than the phenolics are equally responsible for antioxidant activity in case of *Zanthoxylum*. The result of phytochemical screening of methanol extract of two timbur fruits showed the presence of alkaloid, amino acid, anthraquinones, steroids, glycosides, flavonoids, saponins, tannins, reducing sugar, triterpenes, cardiac glycosides and resin (Table 1), most of which are higher in *Litsea cubeba* (Sil Timbur).

CONCLUSION

The present study confirmed that the methanolic extracts of fruits of *Zanthoxylum acanthopodium* and *Litsea cubeba* contain high levels of total phenolic and flavonoid compounds and have high DPPH radical scavenging ability along with potential anti-lipid peroxidation properties. The study also indicated that these fruits contain versatile group of secondary metabolites, even when their moisture levels were sufficiently reduced. So these underexplored spices are promising for more detailed investigation of their antioxidant properties, development of therapeutic products to protect against certain diseases and show potential for use in food supplements, subject to evaluation of toxicity and immunogenicity. Additional studies are required to analyze in detail the individual compounds related to antioxidant activity of these fruits, and further scientific investigation must proceed to ensure that the medicinal properties of these spices *in vivo* correlate with its antioxidant activity.

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