Evaluation of in vitro antioxidant activity of Indian bay leaf, Cinnamomum tamala (Buch.-Ham.) T. Nees & Eberm using rat brain synaptosomes as model system

S Lakshmi Devi, S Kannappan & C V Anuradha*
Department of Biochemistry and Biotechnology, Annamalai University, Annamalai Nagar-608 002, India

Received 23 January 2007; revised 22 June 2007

The study investigated the perturbation of oxidant-antioxidant balance in brain synaptosomes of diabetic rats and determined the antioxidant and free radical-scavenging property of the Indian bay leaf. Brain synaptosomes were isolated from control and streptozotocin-induced diabetic animals and oxidative stress parameters were assayed. A methanolic extract of bay leaf (BLE) was tested for the polyphenolic content and antioxidant activity by in vitro assays. A significant increase in the levels of lipids and lipid peroxidation products and a decline in antioxidant potential were observed in diabetic rat brain synaptosomes. The total polyphenolic content of BLE was found to be 6.7mg gallic acid equivalents (GAE)/100g. BLE displayed scavenging activity against superoxide and hydroxyl radicals in a concentration-dependent manner. Further, BLE showed inhibition of Fe²⁺-ascorbate induced lipid peroxidation in both control and diabetic rat brain synaptosomes. Maximum inhibition of lipid peroxidation, radical scavenging action and reducing power of BLE were observed at a concentration of 220 μg GAE. These effects of BLE in vitro were comparable with that of butylated hydroxy toluene (BHT), a synthetic antioxidant. It can be concluded that synaptosomes from diabetic rats are susceptible to oxidative damage and the positive effects of bay leaf in vitro, could be attributed to the presence of antioxidant phytochemicals.

Keywords: Antiradical property, Diabetes, Indian bay leaf, Lipid peroxidation, Oxidative stress, Synaptosomes.

Spices are dried parts of herbs used as flavouring agents in cooking in oriental countries owing to their taste and aroma. Indian bay leaf (Cinnamomum tamala; Tejpatta in Hindi) is one among them. The dried leaf of this plant is a spice commonly used in Indian homes for seasoning. It belongs to the family Lauraceae and is indigenous to the Asia minor and southern Europe. It contains active volatile compounds such as mono- and sesqui-terpenes. Some of them are pinene, phellandrene, linalool and geraniol. Linalool is a monoterpene present in high proportion in the bay leaf and has some effects on the central nervous system. It has dose-dependent, marked sedative effects including hypnotic, anticonvulsant and hypothermic properties.

Synaptosomes, the isolated terminal portions of the axons contain neurotransmitters that diffuse across the synaptic cleft, relaying neuronal messages to the next cell. They behave as metabolically autonomous mini-cells and provide a good experimental model to evaluate the neurodegenerative processes and peroxidative events in the cerebral cells. Deleterious effects of diabetes on the central nervous system (brain) are related to the oxidative imbalance set forth by hyperglycemia. The end products of lipid peroxidation can cause changes in the physical properties i.e., permeability and fluidity of the nerve terminals and can inactivate the receptors.

Scientific data on the benefits of Indian bay leaf are scanty. Studies have shown that bay leaf has hypoglycemic activity in alloxan-induced diabetic animals and anticarcinogenic activity in dimethyl benzanthracene-induced colon carcinogenesis in the rat. A study by Celik and Ozkaya has shown that intraperitoneal administration of linalool prevents H₂O₂-induced oxidative stress in guinea pig brain when used in high concentrations. However, data regarding the antioxidant and radical scavenging activity of Indian bay leaf are not available. Further, oxidative stress is particularly active in brain whose membranes are rich in polyunsaturated, highly peroxidable fatty acids. This prompted the present study on the antiradical efficiency of Indian bay leaf and the extent of oxidative damage in diabetic rat brain synaptosomes. The diabetic rat brain is defective in neurotransmission that is attributable to oxidative damage. Hence, the protective role of...
Indian bay leaf extract on Fe$^{2+}$-ascorbate induced lipid peroxidation in diabetic brain synaptosomes as a model system has been investigated.

**Materials and Methods**

**Animals**—Adult male albino rats of Wistar strain weighing 150-170g were maintained in the Central Animal House, Rajah Muthiah Medical College and Hospital, Annamalai Nagar. The animals were individually housed under controlled temperature and hygienic conditions in polypropylene cages under 12hr-light and dark cycle. They all received a standard pellet diet (Karnataka State Agro Corporation Ltd, Agro Feeds Division, Bangalore, India) and water *ad libitum*. The procedures used in the study were approved by the Institutional Animal Ethics Committee.

**Chemicals**—Streptozotocin (STZ) was obtained from Sigma-Aldrich Pvt Ltd, MO, USA. All other chemicals and solvents used for the study were of analytical grade and were obtained from Sisco Research Laboratories, Mumbai, India.

**In vivo studies**

**Experimental design**—After acclimatization, the rats were divided at random into two groups consisting of 6 rats each. One set of rats was maintained as control and fed commercial diet and tap water *ad libitum*. These rats received 0.5 ml 0.6M citrate buffer (vehicle) intraperitoneally. The second set of rats was injected with STZ (45 mg/kg in 0.6M citrate buffer, pH 6.5) intraperitoneally.

The body weights of the animals were recorded every day. Blood glucose was determined to confirm diabetes. Diabetic animals with blood glucose values above 180mg/dl were included in the study. At the end of 15 days, the animals were anesthetized by administering ketamine hydrochloride (35mg/kg) and sacrificed by cervical decapitation. Blood was collected from the jugular vein with heparin as anticoagulant and glucose level was determined.

**Preparation of synaptosomes**—Synaptosomal fraction was isolated from the brain homogenate following the method of Hajos.

After sacrifice, the forebrain was dissected out on ice. The tissue was minced and homogenized gently in 10 volumes w/v of ice-cold 0.32M sucrose with a Teflon homogenizer. After centrifugation of the homogenate at 1000g for 10 min at 4°C, the supernatant was again centrifuged at 12,500g for 20 min. The final pellet was gently resuspended in 10 volumes of 0.32M sucrose and used as the crude synaptosomal fraction.

**Determination of lipids**—Contents of cholesterol, phospholipids, triglycerides (TG) and free fatty acids (FFA) were measured in synaptosomal fraction after lipid extraction by the method of Folch et al.

**Assay of oxidative stress parameters**—The levels of thiobarbituric acid reactive substances (TBARS) and lipid hydroperoxides (LHP), levels of non-enzymatic antioxidants vitamin C, vitamin E and GSH and the activities of enzymatic antioxidants such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione reductase (GR) and glutathione S-transferase (GST) were assayed in the synaptosomes.

**In vitro studies**

**Preparation of bay leaf extract**—Bay leaves were purchased locally and authenticated by Dr S.Venkatesalu, Reader in Botany, Annamalai University. A voucher specimen of the plant (No: 318-N) has been deposited at the herbarium of the Department of Botany, Annamalai University. The leaves were dried and finely powdered in a mechanical mixer. The extract was prepared as follows: 20g of finely powdered bay leaf was weighed and mixed with 100ml of 80% methanol and kept for 5 days at room temperature. After 5 days, it was filtered and evaporated under the hood. The residue was dissolved in 5ml of water, washed with petroleum ether several times, till a clear upper layer of petroleum ether was obtained. The lower aqueous layer was measured and diluted in water (1:3). This solution was called BLE and was used for various *in vitro* studies.

**Estimation of total phenolic content in BLE**—Total phenolic content in BLE was determined using 2,2′-diphenyl-1-picrylhydrazyl reagent by the method of Singleton and Rossi. The values were expressed as mg gallic acid equivalents (GAE)/100g.

**Inhibition of Fe$^{2+}$-ascorbate-induced lipid peroxidation by BLE**—The effect of varying volumes of the aqueous extract of BLE on Fe$^{2+}$/ascorbate-induced lipid peroxidation in control and diabetic rat brain synaptosomes was assessed by the method of Hogberg et al.

The incubation mixture in a total volume of 2 ml contained 0.2ml of synaptosomal fraction, 50 μM FeSO$_4$, 1 mM KH$_2$PO$_4$ and 0.2 mM ascorbic acid in 0.15M Tris-HCl buffer, pH 7.4 and
varying volumes of BLE (containing 44, 88, 132, 176, 220 µg GAE). Incubation was carried out in a shaking water bath at 37°C for 20 min. The reaction was stopped by the addition of 1ml of 10% trichloro acetic acid (TCA) after the incubation period. The tubes were shaken well and 1.5ml of thiobarbituric acid (TBA) (1% in 0.05N sodium hydroxide) reagent was added and was heated in a boiling water bath at 90°C for 20 min. The tubes were centrifuged and the colour developed in the supernatant was read at 532nm.

Hydroxyl radical scavenging assay—The hydroxyl radical scavenging activity of BLE was determined by the method of Halliwell et al. The incubation mixture in a total volume of 1ml contained 0.2ml of 100 mM potassium dihydrogen phosphate-potassium hydroxide buffer pH 7.4, varying volumes of BLE (containing 44, 88, 132, 176 and 220 µg GAE), 0.2 ml of 500µM ferric chloride, 0.1 ml of 1mM ascorbic acid, 0.1 ml of 1mM ethylene diamine tetra acetate (EDTA), 0.1 ml of 10mM hydrogen peroxide and 0.2ml of 2-deoxyribose. The contents were mixed thoroughly and incubated at room temperature for 60min. Then 1ml of 1% TBA (1g in 100ml of 0.05N NaOH) and 1ml of 28% TCA were added. All the tubes were kept in a boiling water bath for 30 min. Butylated hydroxy toluene (BHT) was used as a positive control for comparison. The absorbance was read in a spectrophotometer at 532nm with reagent blank containing water in place of extract. Decreased absorbance of the reaction mixture indicated increased hydroxyl radical scavenging activity. The percentage scavenging was calculated as shown below.

\[
\text{Scavenging} \ (\%) = \frac{\text{control OD} - \text{test OD}}{\text{control OD}} \times 100
\]

Superoxide anion scavenging activity—Superoxide anion scavenging activity of BLE was determined by the method of Nishmiki et al with modifications. The assay was based on NADH-PMS-NBT colour reaction. The reaction mixture in a final volume of 2.5ml contained, 1ml of NBT (100µmol NBT in 100mM phosphate buffer, pH 7.4), 1ml of NADH solution (468µmol in 100mM phosphate buffer, pH 7.4) and varying volumes of BLE (containing 44, 88, 132, 176 and 220 µg GAE). The reaction was started by the addition of 100µl PMS (60µmol/100mM phosphate buffer, pH 7.4). The reaction mixture was incubated at 30°C for 15 min after which the absorbance was measured at 560 nm. Blank contained all the solutions and water in place of BLE. Butylated hydroxy toluene (BHT) was used as a positive control for comparison. Decreased absorbance of the reaction mixture indicated increased superoxide anion scavenging activity. The percentage scavenging was calculated as per the formula given above.

Reducing power—The reducing power of BLE was determined by the method of Oyaizu. Substances, which have reduction potential react with potassium ferricyanide (Fe3+) to form potassium ferrocyanide (Fe2+), which then reacts with ferric chloride to form ferric-ferrous complex that has an absorption maximum at 700nm. Varying volumes of BLE (containing 44, 88, 132, 176 and 220 µg GAE) were taken in test tubes, mixed with 2.5ml of phosphate buffer (0.2M, pH 6.6) and 2.5ml of potassium ferricyanide (1%w/v). The mixture was incubated at 50°C for 20 min. Later, 1.5ml of 10% TCA was added and centrifuged at 3000g for 10min. From all the tubes, 0.5ml of supernatant was mixed with 1ml of distilled water and 0.5 ml of FeCl3 (0.1w/v). The absorbance was measured at 700 nm in a spectrophotometer against a blank that contained water in the place of BLE. Increased absorbance of the reaction mixture indicated increasing reducing power. BHT was used as positive control for comparison.

DPPH● radical scavenging assay—The radical scavenging activity of BLE against DPPH● was determined by spectrophotometrically by the method of Brand Williams et al. The reaction mixture in a total volume of 3ml contained 1ml of 100µM DPPH● in methanol, equal volumes of BLE containing 44, 88, 132, 176 and 220 µg GAE and 1ml of phosphate buffer pH 7.4. The tubes were incubated for 10 minutes at 37°C in the dark. The absorbance was monitored at 517nm. Blank was carried out to determine the absorbance of DPPH● in the absence of extract. The percentage scavenging was calculated as per the formula given above.

Statistical analysis—The results of animal experiments are given as means ± SD of 6 rats from each group and statistically evaluated by Student’s t-test for unpaired comparisons. For in vitro assays, the results given are the average of 5 determinations and were analysed by Student’s t-test for unpaired comparisons. A value of P<0.05 was considered significant.
Results and Discussion

Hyperglycemia, the hallmark of diabetes and a reduction in body weight were observed in STZ-treated animals at the end of 15 days (Table 1). The loss of body weight has been suggested to be due to excessive breakdown of tissue proteins during diabetes.

The levels of cholesterol, FFA, TG and phospholipids in brain synaptosomes of control and experimental animals are given in Table 2. The increased level of cholesterol in the diabetic rat brain synaptosomes could arise from a rise in cholesterol biosynthesis. Increased activity of hydroxy methyl glutaryl (HMG CoA) reductase, a rate-limiting enzyme in cholesterol biosynthesis has been reported in diabetic rats. The higher level of FFA in the synaptosomes may be attributed to the extraction of FFA from plasma, which in turn leads to the accumulation of toxic fatty acid metabolites. A rise in FFA contributes to elevation of TG in the synaptosomes of diabetic rats. Phospholipids are vital components of biomembranes that also play an important role in the membrane function. Oxidative deterioration of fatty acids may be responsible for decreased phospholipids in brain. Decreased phospholipids could alter membrane function in the nerve endings of diabetic rats.

Generation of reactive oxygen species (ROS) during hyperglycemia results in oxidative stress. Lipid peroxidation is one of the main consequences associated with ROS production and is particularly active in brain whose membranes are rich in polyunsaturated and highly peroxidizable fatty acids. Further, a high content of non-heme iron, relative deficiency of antioxidant enzymes, abundance of oxidizable substrates like catecholamines and remarkably high oxygen consumption make the brain tissue more vulnerable to oxidative damage. In the present study elevated levels of TBARS and LHP in brain synaptosomes of diabetic rats were observed (Table 2). These results are consistent with those of Bellush and Reid who reported increased formation of hydroperoxides in the STZ-diabetic rat brain synaptosomes. A reduction in antioxidants and an increase in TBARS and LHP provide a strong evidence of generation of the oxidative stress and an imbalance between the genesis of ROS and removal in synaptosomes.

The activities of enzymic antioxidants such as SOD, CAT, GPx, GR and GST are significantly lowered in brain synaptosomes of streptozotocin-induced diabetic rats (Table 3). The enzyme SOD removes the superoxide anion while CAT, a heme

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control</th>
<th>Diabetes-induced</th>
</tr>
</thead>
<tbody>
<tr>
<td>SOD (Units/mg protein)</td>
<td>8.40 ± 0.62</td>
<td>7.01 ± 0.54*</td>
</tr>
<tr>
<td>CAT (nmole of H$_2$O$_2$/min/mg protein)</td>
<td>12.73 ± 0.98</td>
<td>9.81 ± 0.76*</td>
</tr>
<tr>
<td>GPx (nmole/min/mg protein)</td>
<td>18.62 ± 1.45</td>
<td>15.80 ± 1.16*</td>
</tr>
<tr>
<td>GR (nmole of NADPH oxidized/hr/mg protein)</td>
<td>8.26 ± 0.62</td>
<td>6.76 ± 0.44*</td>
</tr>
<tr>
<td>GST (nmole of CDNB-GSH conjugate/ min/mg protein)</td>
<td>16.56 ± 1.24</td>
<td>13.70 ± 0.96*</td>
</tr>
<tr>
<td>GSH (nmole/mg protein)</td>
<td>8.06 ± 0.53</td>
<td>5.86 ± 0.37*</td>
</tr>
<tr>
<td>Vitamin-C (pmole/mg protein)</td>
<td>15.01 ± 1.24</td>
<td>9.04 ± 0.87*</td>
</tr>
<tr>
<td>Vitamin-E (pmole/mg protein)</td>
<td>633 ± 47.4</td>
<td>571 ± 34.8*</td>
</tr>
</tbody>
</table>

*P<0.05 as compared to control animals (Student’s t-test).

CDNB-GSH- 1-Chloro 2, 4-dinitro benzene-reduced glutathione conjugate.
protein catalyses the reduction of H₂O₂ and protects the tissues from highly reactive hydroxyl radicals that could be generated from H₂O₂. GPx, a selenium containing metalloenzyme has a well-established role in protecting cells against oxidative injury. GST catalyses the conjugation of electrophilic substances with GSH to form the corresponding GSH-S-conjugate. Antioxidant enzyme proteins are themselves susceptible to inactivation by ROS and by glycation; e.g., SOD and catalase can be inactivated by glycation while GST activity could be modulated by ROS. Insufficient availability of GSH may also reduce the activity of GPx and GST.

The levels of non-enzymatic antioxidants such as GSH, vitamin-C and vitamin-E in brain synaptosomes of diabetic animals are given in Table 3. The decrease may be due to an increased reaction of vitamins and the tripeptide GSH with ROS in the defense process. Vitamin E is one of the major chain breaking lipophilic antioxidants within the cell membrane where it protects membrane fatty acids from peroxidation. GSH constitutes a major reducing substance of the cytoplasm and is known to protect the cellular system against toxic effects of lipid peroxidation. GSH and vitamins E and C exist in the interconvertible reduced and oxidized forms and thus participate in neutralizing free radicals as and when they are formed. There is a well-established synergism between vitamin E, vitamin C and glutathione through the antioxidant network.

The total polyphenolic content of BLE was determined to be 6.7mg/100g in gallic acid equivalents (GAE). The aqueous solution prepared gave a concentration of 2.37mM GAE.

Figure 1 gives the dose-dependent inhibition of Fe²⁺-ascorbate induced lipid peroxidation by BLE. Owing to the susceptibility to oxidative stress, synaptosomes from control and diabetic rats were prepared and used as an *in vitro* model system for lipid peroxidation assay to determine the antilipidperoxidative activity of BLE. Incubation with Fe²⁺-ascorbate system caused an elevation in the TBARS level in both control and diabetic rat synaptosomes as compared to the respective basal levels without Fe²⁺-ascorbate (basal levels Control-0.23 ± 0.01; Diabetic-0.34 ± 0.02 nmol/mg protein). BLE inhibited Fe²⁺-induced lipid peroxidation in a dose-dependent manner in both control and diabetic rat synaptosomes suggesting the antioxidant property of bay leaf. BLE inhibited lipid peroxidation by 70% at 220µg GAE, the maximum concentration used in the study.

Figure 2 shows the percentage hydroxyl radical scavenging activity of BLE and BHT at various concentrations. Addition of BLE scavenged hydroxyl radical in a concentration dependent manner. The extract significantly inhibited (67.2±4.3%) degradation of deoxyribose mediated by the hydroxyl radicals at a dose of 220µg GAE (Fig. 1) compared to BHT (1000µg). The concentration needed for 50% inhibition was 176µg GAE. Scavenging ability of BLE may be due to the active hydrogen donating ability, which can neutralize the hydroxyl radical generated.

Figure 3 shows that scavenging of superoxide anion by BLE was proportional with increasing concentration of the extract added and was comparable to that of BHT. Superoxide is biologically important since it can form more toxic H₂O₂, by the SOD reaction and can be decomposed to form stronger oxidative species such as hydroxyl radical and singlet oxygen. The scavenging capacity of the BLE was 16.24 ± 0.91 % at 44µg GAE and 72.15 ± 3.8 % at 220µg GAE.
The reduction potential of BLE at various concentrations is presented in Fig. 4. There was a concentration-dependent increase in the reducing power of the extract as determined by the Fe$^{3+}$-Fe$^{2+}$ colour formation. However, BHT was found to be a more powerful reducing agent than BLE. The reducing power of BLE might be due to di- and mono-hydroxyl substitution in the aromatic ring, which possesses potent hydrogen donating ability.

Figure 5 shows that the inhibition of DPPH$^\ast$ radical formation was proportional to increasing concentrations of the BLE. BLE was a better scavenger compared to BHT. The inhibition capacity of BLE was 20% at 44µg GAE and 80% at 220µg GAE while BHT exhibited 29% at 200µg and 93% at 1000µg.

Bay leaf has been used as a food additive in Indian homes for its aroma. Bay leaf contains linalool as the active component, which may effectively scavenge the free radicals and terminate the radical chain reaction. Linalool has considerable protective effects against H$_2$O$_2$ induced-oxidative stress in brain tissues by decreasing oxidative reaction in unsaturated fatty acids. Other chemical entities present in the Indian bay leaf could act as electron donors and react with free radicals to form more stable products and terminate radical chain reactions. Eugenol, one such constituent, possesses free radical scavenging activity due to the presence of an electron-repelling group at O-position to phenolic group.

The results of the present study show that a perturbation of balance between the generation of ROS and scavenging capacity exists in synaptosomes of diabetic rats. Further, Indian bay leaf extract effectively scavenges ROS and suppresses Fe$^{2+}$-ascorbate-induced lipid peroxidation in brain synaptosomes. This preliminary study indicates a need for further studies on the chemical analysis of Indian bay leaf and molecular mechanism of its protective action in vivo.

References
5. Kar A, Choudhary B K & Bandyopadhyay N G, Comparative evaluation of hypoglycemic activity of some


18 Roe J H & Kuetether CA, Detection of ascorbic acid in whole blood and urine through 2, 4-DNPH derivative of dehydroascorbic acid, *J Biol Chem.*, 47 (1943) 399.


