Protective effect of *Embelia ribes* Burm on methionine-induced hyperhomocysteinemia and oxidative stress in rat brain

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The present study was aimed to find out the protective effect of ethanolic extract of *E. ribes* fruits on homocysteine, lactate dehydrogenase (LDH) and lipid profile in serum, lipid peroxidation (LPO) and non-enzymatic antioxidant glutathione (GSH) levels in brain homogenates and histopathological examination of brain tissue in methionine (1 g/kg body weight, orally for 30 days) induced hyperhomocysteinemic rats. A significant increase in homocysteine, LDH, total cholesterol, triglycerides, low density lipoprotein (LDL-C) and very low density lipoprotein (VLDL-C) levels was observed in serum. Increased LPO levels in brain homogenates with reduced serum high density lipoprotein (HDL-C) levels and decreased GSH content were other salient features observed in methionine treated pathogenic control rats. Administration of ethanolic *E. ribes* extract (100 mg/kg body weight, orally) for 30 days to methionine-induced hyperhomocysteinemic rats produced a significant decrease in the levels of homocysteine, LDH, total cholesterol, triglycerides, LDL-C, VLDL-C in serum and LPO levels in brain homogenates with significant increase in serum HDL-C levels and GSH content in brain homogenates, when compared with pathogenic control rats. Biochemical observations were further substantiated with histological examination of brain. Degenerative changes of neuronal cells in methionine treated rats were minimized to near normal morphology by ethanolic *E. ribes* extract administration as evident by histopathological examination. The results provide clear evidence for the first time, that ethanolic *E. ribes* extract treatment enhances the antioxidant defense against methionine-induced hyperhomocysteinemia and oxidative stress in brain.

**Keywords:** *Embelia ribes*, Homocysteine, Lipid peroxidation, Methionine, Oxidative stress

Homocysteine is toxic to neuronal cells and animals exposed to homocysteine accumulate this compound in the brain. Hyperhomocysteinemia has been implicated as a risk factor for vascular disease as well as brain atrophy and therefore, may be related to the development of dementia and possibly Alzheimer’s disease. About 5 to 7% of the population has mildly elevated levels of homocysteine. Concentrations of homocysteine in the brain and cerebrospinal fluid (CSF) are elevated in several neurological disorders.

Although its pathophysiological mechanisms are complex and not fully understood, much evidence suggests that hyperhomocysteinemia induces vascular and brain damage because of the highly reactive thiol group in homocysteine that is readily oxidized leading to the formation of homocysteine, homocysteine mixed disulfides and homocysteine thiolactone. During these oxidative processes, several reactive species are generated. The methionine cycle is responsible for the creation for all homocysteine in the body.

Homocysteine remethylation is an important source of methyl groups in the brain. The remethylation of homocysteine into methionine is mediated by methionine synthase and cofactor, vitamin B12; 5-methyltetrahydrofolate donates a methyl group in this reaction. Numerous methylation reactions take place in the brain including synthesis and degradation of neurotransmitters, membrane phospholipids and controlled DNA-methylation. The alternative remethylation pathway of homocysteine via betaine-homocysteine methyl transferase seems to be absent in the brain.

The recent interest in homocysteine metabolism in neuropsychiatry stems from an increasing appreciation of homocysteine as a neurotoxin and a risk factor of cerebrovascular disease. Homocysteine has now been implicated in increased oxidative stress, DNA damage, apoptosis induction and excitotoxicity in neurodegeneration. Homocysteine is rapidly taken up by neurons through a specific membrane transporter, leading to high intracellular levels of...
homocysteine. The brain may be particularly vulnerable to high levels of homocysteine in the blood because it lacks two major metabolic pathways for its elimination; betain remethylation and transsulfuration. The association between plasma homocysteine and the severity of cerebral atherosclerosis was explored by Yoo et al. The oxidation of homocysteine promotes the oxidation of low density lipoprotein cholesterol, which causes injury to vascular endothelial cells and leads to endothelial dysfunction. The cerebral infarction is the consequence of cellular necrosis due to the perfusion defect of brain. Neurotoxicity of homocysteine may play a role in the pathogenesis of cerebral infarction. Recently, neurotoxicity of homocysteine through over stimulation of N-methyl-D-aspartate receptors was observed.

Though homocysteine is proposed to cause oxidative stress related neurotoxicity and natural antioxidants play a protective role in hyperhomocysteinemia, there is a need to undertake experimental studies eliciting the protective role of medicinal plants in hyperhomocysteinemia. Embelia ribes Burm is commonly known as Vidangam, Babrang, (Hindi), Babding (Gujrati), Vaayu vilanga, Hulimeese (Kannada), Vivilanganam, Vaivelangum (Tamil), Vaividungalu (Telugu) False Black Pepper (English), Krimighna, Tandula (Sanskrit). It is a large woody climbing shrub belongs to the family, Myrsinaceae, which is widely distributed in India, Sri Lanka, Malaysia and South China. It is esteemed in Ayurveda as a powerful anthelmintic. The plant is used as anti-inflammatory drug to relieve rheumatism and fever. In a preliminary study, Bhandari et al. have reported the dyslipidemic and antioxidant activity of ethanolic E. ribes extract in streptozotocin-induced diabetes in rats using gliclazide as a standard antidiabetic drug.

In the present communication, the effect of ethanolic extract of Embelia ribes fruits on serum homocysteine and lipid levels as well as parameters involved in oxidative stress levels in brain homogenates in methionine induced hyperhomocysteinemic albino rats have been reported.

Materials and Methods

Extraction of Embelia ribes—The dried fruits of Embelia ribes Burm were purchased locally, and identified by the Department of Botany, Faculty of Science, Hamdard University, New Delhi, India (voucher specimen no. UB 2). The dried and coarsely powdered fruits (100 g) were packed in a soxhlet apparatus and were subjected to extraction with ethanol (150 ml) for 72 hr. The filtrate was evaporated under vacuum drier (Narang Scientific Works Pvt. Ltd., New Delhi, India) and brown mass residue obtained was stored at 4°C for further use. The average yield of the ethanolic ER extract was approximately 7.9%. For experimental study, the weighed amount of ethanolic ER extract (100 mg/kg body weight) was dissolved in Tween 80 (1%) in distilled water.

Chemicals and reagents—Methionine was obtained from Central Dug House (CDH), Bombay. All other chemicals used were of analytical grade and purchased locally. Double distilled water was used for all biochemical assays. Methionine was administered in a dose of 1 g/kg body weight in distilled water.

Experimental animals—The experimental protocol was approved by the Institutional Animal Ethics Committee (IAEC) of Hamdard University, New Delhi, which was registered with Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India, India (Registration no. 173/CPCSEA, dated 28 January, 2000). Male adult Wistar albino rats, weighing between 200-250 g, procured from the Central Animal House Facility, Hamdard University, New Delhi, were acclimatized under standard laboratory conditions at 25±2°C, 50±15 % RH and 12:12 hr light / dark cycle for 7 days. Commercial pellet diet (Nav Maharashtra Chakan Oil Mills Ltd, Delhi, India) and water were provided ad libitum. After acclimatization, 40 rats were randomly divided into four groups of 10 animals each and subjected to following treatments: Group I (vehicle control) received only Tween 80 (1%) in distilled water; Group II (pathogenic control) rats were administered with methionine (1 g/kg body weight orally) dissolved in distilled water for 30 days; Group III (ER-100 mg/kg treated) rats received ethanolic ER extract (100 mg/kg body weight) orally for 30 days co-administered with methionine; Group IV (ER-100 mg/kg per se) rats received only ethanolic ER extract (100 mg/kg body weight) orally for 30 days.

After 30 days of treatment schedule, blood samples were collected from the retro-orbital plexus using micro-capillary technique from all the groups of overnight fasted rats and serum was separated for biochemical estimation. After blood collection, all animals were sacrificed by cervical dislocation and...
whole brains were dissected out for biochemical estimation and histopathology.

**Biochemical analysis**—In serum, homocysteine levels were estimated using the Fluorescence Polarization Immunoassay (FPIA) method. Bound homocysteine (oxidized form) is reduced to free homocysteine that is enzymatically converted to S-adenosyl-L-homocysteine (SAH). UV spectrophotometric method of analysis was used for the estimation of lactate dehydrogenase (LDH), total cholesterol, triglycerides and high density lipoprotein (HDL-C). Commercial diagnostic kits from SPAN Diagnostics, Udhna, Surat, India were used for cholesterol and triglycerides estimation. HDL-C content was estimated using a commercial diagnostic kit from Reckon Diagnostics Pvt. Ltd. Baroda, India. Homogenate (10%) of whole brain tissue in ice cold KCl (0.15 M, pH 7.0) was used for the assay of the malondialdehyde according to the method of Ohkawa et al. Lipid peroxidation (LPO) was measured by estimating thiobarbituric acid reactive substances (TBARS; malondialdehyde, MDA) and glutathione assay was based on the reaction with DTNB in which DTNB (5,5'-dithiobis-(2-nitrobenzoic acid)) is reduced by -SH group to form one mole of 2-nitro-5-mercaptobenzoic acid (yellow color).

**Histopathological studies**—At the end of the experiment, whole brain tissues from all the groups were subjected to histopathological studies. The tissues were fixed in formalin (10%), routinely processed and embedded in paraffin wax. Paraffin sections (5 μm thick) were cut on glass slides and stained with hematoxylin and eosin (H and E) after dewaxing, and examined under a light microscope.

**Statistical analysis**—Statistical analysis was carried out using Graphpad Prism 3.0 (Graphpad software; San Diego, CA). All data were expressed as mean±SE. Groups of data were compared with an analysis of variance followed by Dunnett ‘t'-test. Values were considered statistically significant at P<0.01

**Results**

No mortality and morbidity were observed in any group of rats after feeding methionine for 30 days. A significant increase in serum homocysteine and lactate dehydrogenase (LDH) levels were observed in rats treated with methionine (group II) as compared with levels of vehicle control group (group I). Oral administration of ethanolic *E. ribes* (100 mg/kg body weight) in hyperhomocysteinemic rats produced a significant reduction in methionine-induced elevated levels of serum homocysteine and LDH (group III) as compared to pathogenic control rats (group II) (Table 1).

Serum total cholesterol, triglycerides, LDL-C and VLDL-C levels were significantly increased along with significant decrease in HDL-C levels in rats treated with methionine (group II) as compared with levels of vehicle control (group I) (Table 2 and 3). Ethanolic *E. ribes* extract (group III) treatment in

| Table 1—Effect of ethanolic extract of *Embelia ribes* on methionine-induced changes in the levels of serum homocysteine and LDH |
|-----------------|-----------------|-----------------|-----------------|
| Treatment       | Homocysteine (µg/ml) | LDH (IU/L) |
| Gr I            | 8.52 ± 0.19      | 28.30 ± 0.81  |
| Gr II           | 22.65 ± 0.34 a   | (+165.84)* a   | 59.36 ± 0.69 a  |
| Gr III          | 15.58 ± 0.07 b   | (-31.21) † b   | 39.03 ± 0.45 b  |
| Gr IV           | 8.92 ± 0.26      | (-34.24) †     | 30.05 ± 0.53    |
| F               | 1591.90          | 490.40         |

P values <0.01; when compared with *vehicle control group,* pathogenic control group.

| Table 2—Effect of ethanolic extract of *Embelia ribes* on methionine-induced changes in the levels of serum total cholesterol, triglycerides and HDL-C |
|-----------------|-----------------|-----------------|-----------------|
| Treatment       | Cholesterol (mg/dL) | Triglycerides (mg/dL) | HDL-C (mg/dL) |
| Gr I            | 100.59 ± 0.87  | 87.15 ± 1.31  | 39.58 ± 0.57  |
| Gr II           | 194.21 ± 1.65 a | (+93.07)* a | 179.52 ± 2.15 a |
| Gr III          | 160.85 ± 2.30 b | (-17.17) † b  | 133.91 ± 1.99 b |
| Gr IV           | 98.04 ± 1.06   | (-25.41) †   | 92.04 ± 1.10   |
| F               | 902.49         | 683.07         | 753.19         |

P values <0.01; when compared with *vehicle control group,* pathogenic control group.

Details of groups are same as in Table 1.
methionine-induced hyperhomocysteinemic rats significantly decreased the total cholesterol, triglycerides, LDL-C and VLDL-C levels and increased the HDL-C levels in serum as compared to methionine treated rats (group II) (Table 2 and 3).

Methionine treatment significantly increased the LPO levels and decreased the GSH levels in brain homogenates in pathogenic control rats (group II) as compared to levels of vehicle control rats (group I). Ethanolic E. ribes extract (group III) treatment in hyperhomocysteinemic rats, significantly decreased the LPO levels and increased the GSH levels in brain, as compared to pathogenic control (group II) rats (Table 4).

There was no significant \( P>0.05 \) change observed in all the above mentioned parameters in E. ribes per se (100 mg/kg) group (i.e. group IV) as compared to the vehicle control group i.e. group I rats.

Histopathological studies—Photomicrograph of vehicle control group showed normal fibrillary background. The neuronal cell morphology and tissue architecture was maintained (Fig. 1a). Methionine-induced hyperhomocysteinemia and oxidative stress resulted in focal necrosis and vacuolar changes (Fig. 1b). Ethanolic E. ribes extract (100 mg/kg) treated groups showed normal fibrillary background and absence of degenerative changes or necrosis. The neuronal cell morphology and tissue architecture was retained (Fig. 1c). Ethanolic E. ribes extract (100 mg/kg) per se groups showed normal fibrillary background and neuronal cell morphology (Fig. 1d).

Discussion

Hyperhomocysteinemia has recently emerged as an independent risk factor for development of coronary, cerebrovascular and peripheral arterial occlusive disease. Recent epidemiological data have shown that hyperhomocysteinemia can be detected in 20 and 40% of patients with coronary artery disease and cerebrovascular disease or peripheral atherosclerosis respectively, which is in agreement with homocysteine theory of arteriosclerosis proposed by McCully and Wilson, who reported that hardening of the arteries was directly related to the amino acid, homocysteine.

Rats fed a methionine rich diet showed elevated concentrations of homocysteine in blood and a lowered activity of glutathione peroxidase. Homocysteine-induced oxidative stress may be worsened in case of a reduced glutathione production. Homocysteine has been found to induce neurological dysfunction via oxidative stress. This effect can be explained by enhancing the production of ROS, and oxidative deactivation of nitric oxide. Moreover, homocysteine causes brain lipid peroxidation by blocking NMDA receptor. Antioxidant treatment restores several toxic effects of homocysteine.

Bhandari et al. have reported the anti-diabetic and antioxidant activity of ethanolic extract of E. ribes Burm in streptozotocin-induced diabetes in rats using gliclazide as positive control drug. In the present study, we examined the homocysteine and lipid lowering potential of ethanolic extract of E. ribes in methionine-induced hyperhomocysteinemia and oxidative stress in rats.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>LDL-C (mg/dL)</th>
<th>VLDL-C (mg/dL)</th>
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<tbody>
<tr>
<td>Gr I</td>
<td>43.59 ± 0.56</td>
<td>17.43 ± 0.26</td>
</tr>
<tr>
<td>Gr II</td>
<td>144.08 ± 1.71</td>
<td>35.90 ± 0.43</td>
</tr>
<tr>
<td></td>
<td>(+230.53)*</td>
<td>(+105.97)*</td>
</tr>
<tr>
<td>Gr III</td>
<td>112.01 ± 1.40</td>
<td>26.78 ± 0.40</td>
</tr>
<tr>
<td></td>
<td>(-22.26)†</td>
<td>(-25.40)†</td>
</tr>
<tr>
<td>Gr IV</td>
<td>41.74 ± 0.53</td>
<td>17.04 ± 0.24</td>
</tr>
<tr>
<td>F</td>
<td>1894.90</td>
<td>683.19</td>
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\( P \) values <0.01; when compared with vehicle control group, pathogenic control group.

Details of groups are same as in Table 1
Homocysteine, a thiol containing amino acid derived from demethylation of dietary methionine, may generate partially reduced ROS that are able to stimulate the lipid peroxidation involved in atherosclerotic process. Thus, an imbalance in dietary methionine may contribute to the development of atherosclerosis by increasing homocysteine levels.

The data in our present study showed that methionine (1 g/kg body weight orally) treatment for 30 days in pathogenic control group rats significantly elevated the homocysteine, LDH, total cholesterol, triglycerides, LDL-C and VLDL-C levels in serum and LPO levels in whole brain homogenates with concomitant decrease in HDL-C levels in serum and GSH content in brain homogenates.

Free radicals generated by hyperhomocysteinemia, initiate lipid peroxidation of the membrane bound polyunsaturated fatty acids, leading to impairment of the membrane structural and functional integrity. This concurs with the present findings wherein the levels of LPO were found to be significantly increased in animals subjected to methionine treatment. Due to this increased lipid peroxidation, GSH levels are lowered.

Fig. 1—Histological examination of brain in experimental animals [(a) vehicle control group (Group I) rat showing normal fibrillary background. The neuronal cell morphology and tissue’s architecture was maintained; (b) pathogenic control group (Group II) rat showing significant focal necrosis and vacuolar changes; (c) *E. ribes* (100 mg/kg) treatment group (Group III) rat showing normal fibrillary background with absence of degenerative changes or necrosis. The neuronal cell morphology and tissue’s architecture was retained; (d) *E. ribes* (100 mg/kg) per se group (Group IV) rat showing normal fibrillary background and neuronal cell morphology. Fig. 1 a-d: 400×. N-normal; FN-focal necrosis; V-vacuolar changes].
In the present study, elevated levels of homocysteine, LDH, total cholesterol, triglycerides, LDL-C and VLDL-C in serum and LPO in brain homogenates were reduced significantly by treatment with ethanolic extract of *E. ribes* (100 mg/kg body weight orally for 30 days), suggesting antioxidant and antihyperhomocysteinemic activity of *E. ribes*. The levels of HDL-C in serum were increased significantly as compared to methionine treated group i.e. group II. The levels of GSH in brain homogenates were increased significantly after the treatment of ethanolic extract of *E. ribes* as GSH scavenges free radicals and suppresses the overproduction of ROS. Therefore, ethanolic extract of *E. ribes* decreased the utilization of GSH, which, in turn, decreased oxidative stress.

Biochemical assay of various parameters in serum and brain tissues of the animals revealed that ethanolic extract of *E. ribes* favorably modified various biochemical markers in methionine-induced hyperhomocysteinemic rats significantly as compared to pathogenic hyperhomocysteinemic rats.

Chemically, *E. ribes* is reported to contain embelin, quercetin (polyphenol), tannins and alkaloids, which may contribute to its antioxidant activity. In the present study, on phytochemical analysis of ethanolic extract of *E. ribes*, it was found to contain embelin, carbohydrates, saponins and acidic compounds. It can, thus, be concluded that the antioxidant effects of ethanolic extract of *E. ribes* can be attributed to the contents of polyphenols, saponins and alkaloids.

Lastly, the light microscopic observations of the brain tissues of methionine-treated rats showed focal necrosis and vacuolar changes where as the rats treated with ethanolic extract of *E. ribes* showed normal fibrillary background and neuronal cell morphology except focal pyknosis with mild vacuolar changes, thereby, further supporting the role of *E. ribes* as a promising neuroprotective agent in methionine-induced hyperhomocysteinemia.

Over the last decade, following *in vitro* and *in vivo* observations of a homocysteine-associated vascular pathology, convincing epidemiological evidence has been gathered on the relation between moderate elevation of plasma homocysteine and vascular disease, including cerebral ischemia mainly through arterial atherothromboembolism.

Homocysteine is proposed to cause oxidative stress related neurotoxicity and natural antioxidants play a protective role in hyperhomocysteinemia. Oral administration of ethanolic extract of *E. ribes* protected the rats from methionine-induced hyperhomocysteinemia and oxidative stress. Hence, *Embelia ribes* could be useful in conditions associated with hyperhomocysteinemia including cerebral ischemia.

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