Protective role of *Hibiscus rosa sinensis* L. in aluminium-induced neurotoxicity and oxidative stress

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Abstract

The present study was designed to investigate the neuroprotective effect of *Hibiscus rosa sinensis* L. in aluminium-induced neurotoxicity in rats. The animals were treated with aluminium chloride (4.2 mg/kg, p.o) for 21 days to induce oxidative stress. The methanolic extract of *Hibiscus rosa sinensis* (HRS - 100, 200 and 300 mg/kg/day, p.o, for 21 days) was administered 30 min before aluminum. The elevated plus-maze paradigm was used to assess the effect of HRS on aluminium-induced memory impairment. Animals were sacrificed on 21st day, brains were isolated and biochemical estimations were performed. The aluminium treated group showed significant increase in transfer latency (TL) on day 20th and 21st indicating impairment of memory. Administration of HRS (100 - 300 mg /kg, p.o) significantly (p < 0.01) reversed the memory impairment. Biochemical analysis of brain revealed that the chronic administration of aluminium significantly increased lipid peroxidation and decreased levels of catalase (CAT) and glutathione reductase (GSH), an index of oxidative stress process. Administration of extract significantly (p < 0.01) attenuated the lipid peroxidation; and reversed the decrease in brain CAT and GSH levels (p < 0.01). The superoxide dismutase (SOD) levels were increased after aluminium treatment which was normalized by HRS (p < 0.01). The results strengthen the oxidative stress hypothesis of aluminium- induced neurotoxicity and suggest the beneficial role of HRS in the management of Alzheimer's disease and oxidative stress. Cognitive-enhancing activity of HRS may be exerted through antioxidant mechanism.

Keywords: *Hibiscus rosa sinensis*, aluminium, elevated plus-maze, lipid peroxidation, oxidative stress.

1. Introduction

Aluminum is the most widespread metal in the earth's crust. In addition to its presence and increasing bioavailability in ground water, because of acid rain, it is also found in significant amounts in industrialized countries. Some foods with substantial amounts of aluminum-containing additives include processed cheeses, cakes, and juices. There are also aluminum-containing drugs, including antacids. The role of aluminum in the etiology of human neurodegenerative
diseases is highly controversial [1]. A number of observations indicate that high concentration of aluminium may be toxic to the nervous system. The role of heavy metals in the pathogenesis of neurodegenerative disease, and the possible link between aluminum and Alzheimer's disease (AD), is currently receiving considerable attention. The implication of aluminum in Alzheimer's disease was suggested by elevated levels of metal present in the brains of these patients [2]. Possible mechanisms of aluminum induced neurotoxicity have been related to cell damage via free radical production resulting oxidative stress. Increased lipid peroxidation (LPO) is the major consequence associated with oxidative stress. Aluminum has been shown to be associated with both plaques and tangles in the Alzheimer disease. Various investigations have suggested that Alzheimer's disease is more common in areas where the aluminum content in water supplies is the highest [3]. Several antioxidants have been studied for the reduction of oxidative stress occurring during memory impairment. Elucidation of the role of oxidative stress is important because therapy with agents that scavenge reactive oxygen species and augment endogenous antioxidant capacity may prove useful in therapeutic modulation of these devastating neurological conditions [4].

Hibiscus rosa sinensis L. (Malvaceae), also known as china rose, is a popular herb in traditional system of medicine. Ethnomedical information states that this herb is used for the treatment of cough, fever, dysentery, venereal disease, and also applied topically to cancerous swelling [5]. The experimental and clinical studies have shown that the dried flower powder of H. sinensis has significant protective effects in ischemic heart disease [6]. It has hepatoprotective action through antioxidant effect [7]. Moreover it has also known to have radical scavenging effect [8]. Experimental reports indicated that H. sinensis possess a protective effect against the tumor promotion stage of cancer development [9]. The flowers and leaves of the plant found to exhibit significant hypoglycemic and lipid lowering activity [10]. The chemical constituents of H. sinensis include quercetin, carotene, niacin, riboflavin, malvalic acid, gentisic acid, margaric acid, lauric acid, anthocyanin and anthocyanidine [6, 11]. H. sinensis has been shown to possess antioxidant property in a variety of models. Hence, this study was undertaken to assess the possible beneficial effects of H. sinensis in aluminium-induced neurotoxicity.

2. Materials and methods

2.1 Plant material

The roots of H. sinensis were collected in the month of November from local area of Nashik (India) and authenticated by P. S. N. Rao (Director, Botanical survey of India, Pune). A voucher specimen of the plant has been deposited at Botanical survey of India, Pune (Voucher Specimen No. NVHR3). The plant material was shade dried and coarsely powdered. The powdered plant material was defatted with petroleum ether (60 - 80°C) by Soxhlet extractor. The defatted marc was further extracted with methanol for 72 h. Extract was filtered and concentrated under reduced pressure. The yield of methanolic extract of H. sinensis roots (HRS) was found to be 6.2 % w/ w. The dried extract was suspended in 0.5 % carboxy methyl cellulose (CMC) in distilled water and administered orally (p.o).

2.2 Experimental animals

Male Wistar strain rats (120 - 180 g) were used for the study. Animals were housed in colony cages and maintained under the standard laboratory environmental conditions; temperature 25 ± 2°C, 12 h light: 12 h dark cycle and 50 ± 5 % relative humidity with free access to food and water ad libitum. Animals
were acclimatized to laboratory conditions before the test. Each group consisted of six (n = 6) animals. All the experiments were carried out during the light period (08:00 - 16:00 h). The studies were carried out in accordance with the guidelines given by Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), New Delhi (India). The Institutional Animal Ethical Committee of M.V.P.S College of Pharmacy, Nashik approved the protocol of the study (IAEC/2008/02).

### 2.3 Drugs and chemicals

Aluminium chloride anhydrous LR (S.D Fine Chemical Ltd. Mumbai, India), Thioborbituric acid (TBA) (Research Lab Fine Chem Industries, Mumbai, India.), Nitroblue tetrazolium (NBT) (Himedia Laboratories Pvt. Ltd. Mumbai, India), 5, 5'- Dithiobis (2-nitro benzoic acid) (DTNB) (Alfa Aesar, A Johnson Mathey Company). All the chemicals used were of analytical grade and purchased from standard manufacturers.

### 2.4 Phytochemical screening of the H. sinensis roots

Phytochemical screening of the methanolic extract of H. rosa sinensis roots for the presence of flavonoids, glycosides, saponins, alkaloids and sterols were carried out in accordance with procedures previously described [12, 13].

### 2.5 Acute toxicity Test

The HRS was administered orally in doses of 100, 200, 400, 800, 1000 and 2000 mg/kg to group of mice (n = 3) and percentage mortality was recorded for 24 h period. During the first 1 h after the drug administration, the mice were observed for gross behavioral changes as described by Irwin et al. (1968). The parameters observed were hyperactivity, grooming, convulsions, sedation, loss of righting reflex, respiration, salivation, urination and defecation [14].

### 2.6 Treatment schedule

Animals were divided into five groups (n = 6 for each group). Group I – Control (vehicle for HRS, 0.5% CMC in distilled water). Group II – Aluminium (4.2 mg/kg, p.o). Group III - Aluminium (4.2 mg/kg, p.o) + HRS (100 mg/kg, p.o). Group IV - Aluminium (4.2 mg/kg, p.o) + HRS (200 mg/kg, p.o). Group V - Aluminium (4.2 mg/kg, p.o) + HRS (300 mg/kg, p.o). HRS was administered 30 min before aluminium.

### 2.7 Behavioral testing

#### 2.7.1 Elevated plus maze test (EPM)

The nootropic activity was evaluated by using elevated plus maze (EPM). Aluminium (4.2 mg/kg, p.o) was administered chronically to rats for a period of 21 days to induce oxidative stress. Behavioral assessments were carried on 20th and 21st day. The EPM consisted of two open arms (35 × 5 cm) crossed with two closed arms (35 × 5 × 20 cm). The arms were connected together with a central square of 5 × 5 cm. The apparatus was elevated to the height of 50 cm in a dimly illuminated room. Animals were placed individually at the end of either of the open arms facing away from the central platform. The time taken by each animal to move from open arm to either of the closed arms was recorded. This duration of time was called transfer latency (TL). If the animal does not enter into any of the enclosed arms within 120 s, it was gently pushed into any of the enclosed arms and TL was considered as 120 s. Later the animal was allowed to explore the plus maze for 5 min and send back to home cage. TL was noted on day 20th and 21st day. TL measured on day 20th serves as a parameter for acquisition (learning) while TL on day 21st indicates retention (memory) [15].

#### 2.8 Biochemical analysis: Dissection and Homogenization

On the 21st day of aluminium treatment, immediately after behavioral assessments the
### Table 1. Effects of *H. rosa sinensis* root extract (HRS) in aluminium-induced alterations in rat brain CAT, SOD, LPO and GSH.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>CAT (µmol of H₂O₂ decomposed / min / mg protein)</th>
<th>SOD (U / mg protein)</th>
<th>LPO (nmol of MDA / mg protein)</th>
<th>GSH (µmol of GSH / mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>14.0 ± 2.0</td>
<td>3.3 ± 0.8</td>
<td>4.5 ± 2.3</td>
<td>6.1 ± 0.7</td>
</tr>
<tr>
<td>Aluminium</td>
<td>1.6 ± 0.3 a**</td>
<td>6.5 ± 1.7 a**</td>
<td>30.2 ± 3.5 a**</td>
<td>1.5 ± 0.1 a**</td>
</tr>
<tr>
<td>HRS – 100 + Al</td>
<td>8.3 ± 1.7 b***</td>
<td>5.1 ± 1.7</td>
<td>22.5 ± 5.0</td>
<td>1.8 ± 0.4</td>
</tr>
<tr>
<td>HRS – 200 + Al</td>
<td>12.1 ± 0.6 b***</td>
<td>3.8 ± 1.3 b**</td>
<td>16.9 ± 5.5 b**</td>
<td>4.3 ± 0.8 b**</td>
</tr>
<tr>
<td>HRS – 300 + Al</td>
<td>13.3 ± 0.5 b**</td>
<td>2.9 ± 0.9 b**</td>
<td>14.3 ± 2.2 b**</td>
<td>5.0 ± 0.5 b**</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± SEM (n = 6). a Compared with control treated group; b Compared with vehicle treated group. **p < 0.01. (One-way ANOVA followed by Dunnett’s test).

**Fig 1.** Effect of *H. sinensis* roots extract (HRS) on transfer latency (s) in aluminium-induced neurotoxicity on (a) day 20 (b) day 21. Each column represents mean ± SD (n = 6).

* a Compared with control treated group; b Compared with vehicle treated group.

**p < 0.01. (One-way ANOVA followed by Dunnett’s test).**
animals were killed by decapitation. The brain was removed, rinsed with isotonic saline and weighted. A 10% (w/v) tissue homogenate was prepared in 0.1 M phosphate buffer (pH 7.4). The post nuclear fraction for catalase assay was obtained by centrifugation (Remi - C - 30, Remi Industries Ltd. Mumbai, India) of the homogenate at 1000 g for 20 min at 4°C; for other enzyme assays, centrifugation was at 12000 g for 60 min at 4°C. A Shimadzu -160A spectrophotometer was used for subsequent assays [16].

2.8.1 Catalase activity (CAT)
Catalase activity was assessed by the method of Luck (1971), where the breakdown of H₂O₂ was measured at 240 nm. Briefly, the assay mixture consisted of 3 ml of H₂O₂ phosphate buffer (0.0125 M H₂O₂) and 0.05 ml of supernatant of brain homogenate (10%) and the change in the absorbance were measured at 240 nm. The enzyme activity was calculated using the millimolar extension coefficient of H₂O₂ (0.07). The results were expressed as micromoles of H₂O₂ decomposed per minute per milligram of protein [17].

2.8.2 Superoxide dismutase activity (SOD)
Superoxide dismutase activity was assayed according to the method of Kono (1978), wherein the reduction of nitroblue tetrazolium chloride (NBT) was inhibited by the superoxide dismutase was measured at 560 nm spectrophotometrically. Briefly the reaction was initiated by the addition of hydroxylamine hydrochloride to the reaction mixture containing NBT and post nuclear fraction of brain homogenate. The results were expressed as units per milligrams of protein, with one unit of enzyme defined as the amount of SOD required to inhibit the rate of reaction by 50% [18].

2.8.3 Lipid peroxidation assay (LPO)
The quantitative measurement of lipid peroxidation in brain was done by the method of Wills (1966). The amount of malondialdehyde (MDA) formed was measured by reaction with thiobarbituric acid at 532 nm. The results were expressed as nanomoles of MDA per milligram of protein, using the molar extension coefficient of chromophore (1.56 x 10⁵ M⁻¹ cm⁻¹) [19].

2.8.4 Estimation of reduced glutathione (GSH)
Reduced glutathione (GSH) in the brain was estimated according to the method of Ellman (1959). A 0.1 ml of sample of homogenate was precipitated with 0.75 ml of 4 % sulphasalicylic acid. The assay mixture contained 0.5 ml of supernatant and 4.5 ml of DTNB in 0.1 M phosphate buffer, pH 8.0. The yellow color developed was read immediately at 412 nm. The results were expressed as nanomoles of GSH per milligram of protein [20].

2.8.5 Protein estimation
The protein content was measured according to the method of Lowery et al. (1951), using bovine serum albumin as standard and expressed as µg protein/mg of tissue [21].

2.9 Statistical analysis
Results are expressed as mean ± S.E.M., and the statistical analysis of data was done using one-way analysis of variance (ANOVA) followed by Dunnett's test. Probability level less of 0.05 was considered statistically significant.

3. Results
3.1 Phytochemical screening
The phytochemical screening of the HRS has revealed the presence of flavonoids, glycosides, anthocyanins, saponins and tannins.

3.2 Acute toxicity
Oral administration of HRS up to 2 g/kg did not produce any toxic effects on normal behavior of the mice. No mortality was observed and HRS was found to be safe at given doses.

3.3 Elevated plus maze test (EPM)
The aluminium treated group showed significant increase (p < 0.01) in transfer latency on day
20th and 21st as compared to control group, indicating impairment in learning and memory. Pretreatment with HRS (100, 200 and 300 mg/kg) leads to significant (p < 0.01) decrease in transfer latency as compared to aluminium treated group, indicating improvement in retention of memory (Fig.1).

3.4 Effect on brain malondialdehyde level
Chronic administration of aluminium resulted in significant (p < 0.01) increase in the MDA levels in brain as compared to control group. Treatment of HRS (200 and 300 mg/kg) significantly (p < 0.01) reduced the MDA levels as compared to the aluminium - treated group (Table 1).

3.5 Effect on brain GSH level
Administration of aluminium for 21 days leads to decrease in GSH level significantly (p < 0.01) as compared to control group. The reduction in GSH activity by aluminium was also significantly (p < 0.01) reversed by HRS (200 and 300 mg/kg) treatment (Table 1).

3.6 Effect on brain CAT level
Aluminium administration resulted in significant (p < 0.01) depletion of antioxidant enzyme CAT as compared to control group. Treatment with HRS (100, 200, 300 mg/kg) significantly (p < 0.01) restored the level of CAT, compared to aluminium treated group (Table 1).

3.7 Effect on brain SOD level
Chronic administration of aluminium resulted in a significant increase in the SOD levels in brain as compared to control animals. Treatment of HRS (200 and 300 mg/kg) significantly (p < 0.01) normalized the SOD levels as compared to the aluminium- treated group (Table 1).

4. Discussion
Aluminium (Al) as a non-redox active metal has been shown to cause oxidative damage to neurons through Fe²⁺, which stabilizes ferrous (Fe²⁺) ion by reducing its rate of oxidation. Fe²⁺ is potent in promoting the generation of oxidative species; as it catalyzes the Fenton reaction which leads to the formation of OH, OH⁻ and Fe³⁺ from the non-enzymatic reaction of Fe²⁺ with H₂O₂. Because Al is an activator of superoxide dismutase (SOD) and an inhibitor of catalase (CAT), superoxide radical is readily converted to H₂O₂ and the breakdown of H₂O₂ to H₂O and O₂ by catalase is slowed down. Excess accumulation of H₂O₂ further leads to the production of OH radicals which in turn damage various proteins, DNA and membrane lipids. Al³⁺ ions enhance membrane oxidative damage by accelerating peroxidation of membrane lipids in the presence of Fe²⁺ [22].

The principle finding of the present study is that, the long term administration of aluminium was associated with oxidative stress, as indicated by sharp increase in the MDA levels and depletion of endogenous antioxidant enzymes (CAT and GSH), which are in agreement with the earlier reports [2, 3, 4, 22]. Lipid peroxidation is one of the main consequences associated with oxidative stress. Evidences of results indicates that aluminium, a metal without redox capacity in biological systems, in the micromolar range, has the potential to stimulate lipid peroxidation induced by Fe²⁺ in brain homogenates [23]. Increased levels of MDA reflect the stimulatory effects of aluminum on lipid peroxidation. The depletion of CAT and GSH enzymes were observed which is taken as marker of oxidative stress.

Several antioxidants have been studied for the reduction of oxidative stress occurring in neurodegenerative disorders. Co-administration of HRS (100, 200 and 300 mg/kg) to aluminium exposed groups, prevented oxidative stress revealing its protective role. In the present study, HRS has shown significant decrease in the MDA levels. Similarly, also
increased the antioxidant defensive enzymes such as CAT and GSH, indicating the antioxidant activity of the extract which also restored SOD levels. HRS contains numerous compounds, including quercetin, glycosides, riboflavin, niacin, carotene, anthocyanins, anthocyanidine, malvalic acid, gentisic acid, margaric acid and lauric acid [5, 11]. Many of these compounds have proven their potential as antioxidants in various oxidative stress models. Gentisic acid has been shown to possess antioxidant properties in various studies as it scavenges free radicals [7]. Gauthaman et al. (2006) have indicated H. sinensis as an efficient cardioprotective agent; which was explained on the basis of antioxidant action of the plant constituents, in which anthocyanins, anthocyanidine may be responsible for its antioxidant effect [6]. The observed beneficial effects of HRS in aluminium-induced changes in biochemical parameters may thus be attributed to its diversified chemical components.

Animals loaded with aluminum developed brain lesions that are similar to those found in Alzheimer's disease. The proteolytic cleavage of the amyloid precursor protein and the production of amyloidogenic beta amyloid Peptide (AB) are believed to be critical events in the etiology of Alzheimer's disease. The neurotoxicity of Aβ in whatever form may involve the formation of reactive oxygen species. Aluminum is a pro-oxidant and is known to promote the oxidation activity of Aβ in the presence of iron [24]. High levels of aluminum are reported to be a contributing factor for cognitive impairment [25]. The aluminum treated animals had deficits of learning and memory as indicated by EPM. The animals consistently showed increase in transfer latencies indicating learning and memory impairment. The HRS treatment leads to decrease transfer latencies; strengthen its memory improvement action.

5. Conclusion

Supplementation of HRS along with aluminium, a remarkable resurgence was observed in learning and memory; antioxidant defensive enzymes viz CAT, GSH and SOD together with level of lipid peroxidation. These results support the oxidative stress hypothesis of aluminium-induced neurotoxicity and suggest a beneficial role of H. rosa sinensis in the treatment of dementia.

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