Effect of *Hibiscus rosa sinensis* on reserpine-induced neurobehavioral and biochemical alterations in rats

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Effect of methanolic extract of *Hibiscus rosa sinensis* (100-300 mg/kg) was studied on reserpine-induced orofacial dyskinesia and neurochemical alterations. The rats were treated with intraperitoneal reserpine (1 mg/kg, ip) for 3 days every other day. On day 5, vacuous chewing movements and tongue protrusions were counted for 5 min. Reserpine treated rats significantly developed vacuous chewing movements and tongue protrusions however, coadministration of *Hibiscus rosa sinensis* roots extract (100, 200 and 300 mg/kg, per orally) attenuated the effects. Biochemical analysis of brain revealed that the reserpine treatment significantly increased lipid peroxidation and decreased levels of superoxide dismutase (SOD), catalase (CAT) and glutathione reductase (GSH), an index of oxidative stress process. Coadministration of extract significantly reduced the lipid peroxidation and reversed the decrease in brain SOD, CAT and GSH levels. The results of the present study suggested that *Hibiscus rosa sinensis* had a protective role against reserpine-induced orofacial dyskinesia and oxidative stress.

**Keywords:** *Hibiscus rosa sinensis*, Lipid peroxidation, Orofacial dyskinesia, Oxidative stress, Vacuous chewing movement

Treatment with monoamine depleting agent, reserpine, develops orofacial dyskinesia characterized by twitching of facial musculature, vacuous chewing movements and tongue protrusions in experimental animals¹ ². Neisewander et al have suggested that reserpine-induced oral dyskinesia may provide a new model of tardive dyskinesia³.

Evidence are there to indicate that striatal neurodegeneration caused by oxidative stress and excitotoxicity mechanisms may play an important role in the development of Tardive Dyskinesia (TD) disorder⁴. It has been suggested that rats with vacuous chewing movements have significantly higher thiobarbituric acid reactive substances (TBARS) in striatum, suggesting increased lipid peroxidation and free radical production in these animals. Chronic use of neuroleptic is also reported to cause decrease in the activity of the antioxidant defence enzymes, superoxide dismutase (SOD), catalase (CAT), glutathione reductase. All these accumulating data strongly support of free radical hypothesis of TD⁵.

*Hibiscus rosa sinensis* L. (Malvaceae), is a popular herb in traditional system of medicine. Ethnomedical information states that the plant possesses powerful antioxidant⁶, anti-inflammatory⁷, antidiabetic⁸, cardioprotective⁹, hepatoprotective¹⁰, and anticancer¹¹ properties in various models. *H. rosa sinensis* contains numerous compounds, including quercetin, glycosides, riboflavin, niacin, carotene, anthocyanins, anthocyanidine, malvalic acid, gentisic acid, marginic acid and lauric acid⁷. Many of these compounds have proven their potential as natural antioxidants in various oxidative stress models and also attributed many pharmacological properties. Gentisic acid has been shown to possess antioxidant properties in various studies as it scavenges free radicals¹¹. Plant derived flavonoids exhibit variety of biological properties. Certain phenolic compounds attenuate neuronal death induced by oxidative stress.

In view of the reported antioxidant properties of *H. rosa sinensis* in a variety of animal models, there is no major investigative reports available pertaining to its neuroprotective effect. Therefore, the present study was undertaken to investigate the neuroprotective potential and antioxidant status of *H. rosa sinensis* in reserpine-induced orofacial dyskinesia in rats.

**Materials and Methods**

**Animals**—Male Wistar rats (200-230 g) were used for the study. Animals were housed in colony cages
and maintained at 25°C ± 2°C, 12 : 12 h L/D cycle and 50 ± 5 % RH with free access to food and water ad libitum. Animals were acclimatized to laboratory conditions before the test. All the experiments were carried out during the light period (0.800-16.00 hour). The studies were carried out in accordance with the guidelines given by 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).

*Source of drugs and chemicals*—Reserpine (Research Lab, Mumbai, India), thiobarbituric acid (TBA; Research-Lab Fine Chem Industries, Mumbai, India), nitroblue tetrazolium chloride (NBT; Himedia Laboratories Pvt. Ltd. Mumbai, India), 5, 5’- dithiobis (2-nitro benzoic acid) (DTNB; Alfa Aesar, A Johnson Mathey Company). Bovine serum albumin (Spectrochem Pvt. Ltd., Mumbai, India), carboxy methyl cellulose (Research Lab, Mumbai, India). All the chemicals used were of analytical grade and purchased from standard manufacturers.

*Plant material*—Roots of *Hibiscus rosa 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 was deposited at Botanical survey of India, Pune (Voucher Specimen No. NVHR3). The plant material was shade dried and coarsely powdered. The powdered plant material (1 kg) was defatted with petroleum ether (60°C–80°C) by Soxhlet extractor. The extract was filtered and concentrated under reduced pressure. The yield of methanolic extract of *H. rosa sinensis* roots (HRS) was found to be 6.0 % w/w. The dried extract was suspended in 0.5 % carboxy methyl cellulose (CMC) in distilled water (0.5 ml/100g of body weight) and administered per orally (po).

*Phytochemical screening of *H. rosa sinensis* roots*—For phytochemical screening, methanolic extract of *H. rosa sinensis* roots was subjected to analysis using methods as described earlier 12,13.

*Treatment*—Animals were divided into 5 groups having 5 animals in each group. Group I - vehicle (0.5 % CMC in distilled water po, vehicle for HRS + 0.1 % acetic acid solution sc, vehicle for reserpine in a dose of 5 ml/kg); Group II - reserpine (1 mg/kg, sc + vehicle for HRS); Group III - HRS (100 mg/kg, po) + reserpine (1 mg/kg, sc); Group IV - HRS (200 mg/kg, po) + reserpine (1 mg/kg, sc); Group V-HRS (300 mg/kg, po) + reserpine (1 mg/kg, sc).

*Induction of orofacial dyskinesia*—Reserpine (1 mg/kg, sc) was administered to rats for 3 days every other day to induce orofacial dyskinesia. Vehicle treated group was injected with 0.5 % CMC in distilled water orally for 5 days and with 0.1 % acetic acid solution (vehicle for reserpine) subcutaneously (sc) for 3 days every other day. The first injection of acetic acid was given 24 h after the administration of 0.5 % CMC in distilled water. Reserpine group received 1 mg/kg of reserpine for 3 days every other day and vehicle of HRS for 5 days. HRS plus reserpine group was injected with 100, 200 and 300 mg/kg of HRS per orally for 5 days and with 1 mg/kg reserpine sc for 3 days every other day. The first dose of HRS was administered 24 h before reserpine. HRS was administered 30 min before administration of reserpine. Behavioural assessments were carried out on day 5 after 24 h administration of the last dose of reserpine.

*Behavioural testing*—To quantify the occurrence of oral dyskinesia on the test day, rats were placed individually into a small Plexiglass observation cage (30 × 20 × 20 cm) to score vacuous chewing movements (VCMs) and tongue protrusion frequencies. Animals were allowed for 10 min to acclimatize the observation cage before behavioral assessments. Mirrors were placed under the floor and behind the back wall of the cage to permit observation of oral dyskinesia when the animal was faced away from the observer. VCMs and tongue protrusions were defined as a single mouth openings in the vertical plane not directed towards physical material and visible extension of the tongue outside of the mouth, respectively. If VCMs or tongue protrusions occurred during a period of grooming, they were not taken into account. The behavioral parameters of oral dyskinesia were measured continuously for a period of 10 min. In all the experiments, the observer was blind to the identity of the animals5,14.

*Biochemical analysis*

*Dissection and Homogenization*—On the 5th day immediately after behavioral assessments the animals were killed by decapitation. The brain was removed, rinsed with isotonic saline and weighed. 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 done at 12000 × g for 60 min at 4°C. A Shimadzu-160A spectrophotometer was used for subsequent assays.

Lipid peroxidation assay (LPO)—Quantitative measurement of lipid peroxidation in brain was done by the method of Wills. The amount of malondialdehyde (MDA) formed was measured by reaction with thiobarbituric acid at 532 nm. The results were expressed as nmole of MDA/mg protein using the molar extension coefficient of chromophore (1.56 ×10^5 M⁻¹ cm⁻¹).

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

Catalase activity (CAT) - Catalase activity was assessed by the method of Luck, 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 and the change in the absorbance was measured at 240 nm. The enzyme activity was calculated using the millimolar extension coefficient of H₂O₂ (0.07). The results were expressed as micromole of H₂O₂ decomposed per min per milligram of protein.

Estimation of reduced glutathione—Reduced glutathione (GSH) in the brain was assayed according to the method of Ellman. Sample (0.75 ml) of homogenate was precipitated with 0.75 ml of 4 % sulphosalicylic acid and centrifuged at 1,200 g for 15 min at 4 °C. The assay mixture contained 0.5 ml of supernatant and 4.5 ml of 0.01 M, DTNB [5-5'-dithiobis (2-nitrobenzoic acid)] in 0.1 M, phosphate buffer (pH 8.0). The yellow colour developed was read immediately at 412 nm. The results were expressed as micromole of GSH per milligram of proteins.

Protein estimation—The protein content was measured according to the method of Lowery, using bovine serum albumin as standard and expressed as µg protein / mg of tissue.

Statistical analysis—For statistical analysis, data was subjected to one-way analysis of variance (ANOVA) followed by Dunnett’s test. Probability level less of 0.05 was considered statistically significant.

Results

Phytochemical screening—The results of the phytochemical screening of methanolic extract of HRS revealed the presence of alkaloids, flavonoids, sterols, saponins, phenolic compounds and tannins.

Assessment of orofacial dyskinesia—Effect of reserpine treatment on VCMs and tongue protrusions in rats are depicted in Fig. 1. The frequency of VCMs and tongue protrusions in rats were significantly increased after acute treatment with reserpine (1 mg/kg), compared to vehicle treated group. Administration of HRS (100 - 300 mg/kg) for a period

![Fig. 1](image-url)—Effect of *H. sinensis* on reserpine - induced (A) orofacial dyskinesia (B) tongue protrusions. [Statistical analysis was done using one-way ANOVA followed by Dunnett’s test. Each column represents mean ± SEM (n = 5). *Compared with vehicle treated group; **Compared with reserpine treated group. *P < 0.05; **P < 0.01]
of 5 days significantly attenuated the reserpine-induced VCMs and tongue protrusions dose dependently \((P < 0.01)\). HRS alone did not induce any VCMs or tongue protrusions (Fig. 1a, b).

**Biochemical effects**

*Lipid peroxidation assay (LPO)—*Level of MDA was increased significantly in reserpine treated group, as compared with vehicle treated group, while administration of HRS (100–300 mg/kg) significantly brought down the levels of MDA as compared with rats treated with reserpine (Table 1).

**Effect on brain SOD and CAT levels—**Levels of the defensive antioxidant enzymes SOD and CAT were decreased after reserpine administration in rats as compared with vehicle treated group. Pretreatment with HRS (100–300 mg/kg) resulted in significant elevation of SOD and CAT, as compared with rats treated with reserpine (Table 1).

**Effect on brain GSH level—**Content of GSH was depleted significantly in reserpine treated group, as compared with vehicle treated group, indicating the neurotoxicity induced by reserpine in rats. On the other hand, GSH levels elevated significantly after HRS (100–300 mg/kg) treatment as compared with reserpine (Table 1).

**Discussion**

The results of the present study demonstrated that acute administration of reserpine induced an increase in frequency of VCMs and tongue protrusions. These effects were significantly attenuated by administration of HRS in rats.

Concerning critical issues and debatable results of different studies for evaluating animal models of tardive dyskinesia, reserpine-induced VCMs and tongue protrusions seems to be a better model of TD. The characteristics of reserpine-induced oral dyskinesia resemble the symptoms of TD, and historically reserpine has been associated with development of TD in humans. Reserpine produces spontaneous oral dyskinesia that develops as a result of persistent neuropathological changes in brain⁴.

Existing evidences strongly indicated that reserpine-induced oral dyskinesia was closely associated with oxidative stress process. Free radicals are effectively involved in the development of orofacial dyskinesia in rats. In support of this, melatonin attenuated both reserpine-induced orofacial movements and enhanced lipid peroxidation¹⁴. Antioxidant agent such as monosialogangliosides attenuated reserpine-induced tongue protrusions in rats, which also scavenged oxygen free radicals both *in vitro* and *in vivo*⁵.

Acute administration of reserpine causes an increase in dopamine turnover, which may reflect oxidative metabolism of dopamine. Increased oxidative metabolism of dopamine resulted into production of dopamine quinones and hydrogen peroxide. These reactive species would subsequently promote lipoperoxidation.

In the present study, reserpine treated animals showed increase in levels of lipid peroxidation and also exhibited low levels of detoxifying enzymes such as SOD, CAT and GSH suggesting possible induction of free radicals generation. However, HRS roots extract attenuated these increase levels of lipid peroxidation and also increased the levels of antioxidant defensive enzymes suggesting its possible antioxidant action.

These findings supported the earlier observations about cardioprotective effect of flowers of *H. rosa sinensis* in an oxidative stress model of myocardial ischemic reperfusion injury. This cardioprotective effect has been explained on the basis of antioxidant

<table>
<thead>
<tr>
<th>Treatment</th>
<th>LPO</th>
<th>SOD</th>
<th>CAT</th>
<th>GSH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>5.50 ±1.25</td>
<td>10.23 ± 4.72</td>
<td>9.99 ± 0.43</td>
<td>7.48 ± 0.11</td>
</tr>
<tr>
<td>Reserpine (1 mg/kg)</td>
<td>20.89 ± 1.49 a**</td>
<td>1.00 ± 0.39</td>
<td>7.74 ± 0.71 a**</td>
<td>1.76 ± 0.11 a**</td>
</tr>
<tr>
<td>Reserpine (1 mg/kg) + HRS (100 mg/kg)</td>
<td>15.64 ± 2.73</td>
<td>2.95 ± 1.12</td>
<td>8.95 ± 0.22</td>
<td>4.6 ± 0.39 a**</td>
</tr>
<tr>
<td>Reserpine (1 mg/kg) + HRS (200 mg/kg)</td>
<td>12.43 ± 2.35 b*</td>
<td>4.33 ± 0.77</td>
<td>9.71 ± 0.39 b*</td>
<td>5.22 ± 0.23 b**</td>
</tr>
<tr>
<td>Reserpine (1 mg/kg) + HRS (300 mg/kg)</td>
<td>12.56 ± 1.00 b*</td>
<td>6.5 ± 3.31</td>
<td>10.82 ± 0.40 b**</td>
<td>6.10 ± 0.69 b**</td>
</tr>
</tbody>
</table>

aCompared with vehicle treated group; bCompared with reserpine treated group.

\(*P < 0.05; ** P < 0.01. \) (One-way ANOVA followed by Dunnett’s test).

LPO = nmole of MDA / mg protein; SOD = U / mg protein; CAT = μmole of H₂O₂ decomposed/ min /mg protein; GSH = μmole / mg protein.
action of the plant constituents, in which anthocyanins, anthocyanidine may be responsible for its antioxidant effect.

The observed beneficial effects of HRS in reserpine-induced changes in biochemical parameters may, thus, be attributed to its diversified chemical components viz. quercetin, glycosides, riboflavin, niacin, carotene, anthocyanins, anthocyanidine, malvalic acid, gentisic acid, margaric acid and lauric acid. Further investigations of the mechanism(s) of action of the plant extract, and the active substance(s) responsible for its biological actions, are necessary.

Thus, *H. sinensis* treatment enabled to attenuate reserpine-induced increase in VCMs and tongue protrusions as well as helps in the restoration of antioxidant enzymes, together with inhibition of lipid peroxidation in a dose-dependent manner. These results supported the oxidative stress hypothesis of tardive dyskinesia and suggested a beneficial role of *H. sinensis* in the treatment of this motor disorder.

**Acknowledgement**

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**References**


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