

**ANTIOXIDANT AND ANTICHOLINERGIC ACTIVITY OF *RUBIA CORDIFOLIA***

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**Summary**

*Rubia cordifolia* Linn (Rubiaceae) is an important medicinal plant commonly used in the traditional and Ayurvedic system of medicine for treatment of different ailments. In this study, the *in vitro* antioxidant status of methanolic extract of roots and rhizomes of *R. cordifolia* (M-RC) was determined. The effect of M-RC on tacrine-induced tremulous jaw movements in rats and sodium nitrite-induced hypoxia in mice was studied. IC<sub>50</sub> value for lipid peroxidation of a linoleic acid emulsion was found to be 120 µg/ml. IC<sub>50</sub> value for free radical and hydroxyl radical scavenging activity were found to be 130 ppm and 135 ppm, respectively. In M-RC, 1.8679 ± 0.29 µg/g gallic acid phenol equivalents were detected. M-RC significantly inhibited tacrine-induced vacuous chewing movements (VCM), tongue protrusions (TP) and orofacial bursts (OB). M-RC also significantly potentiates sodium nitrite-induced hypoxia and decreased the latency for death after sodium nitrite administration. The study concludes that *R. cordifolia* has an anticholinergic activity which may be attributed to antioxidant activity and presence of phenolic compounds.

**Keywords:** Phenol content, hypoxia, tacrine, vacuous chewing movements, *Rubia cordifolia*

**Introduction**

Free radicals are generated in the body due to the metabolism and disease.<sup>[1]</sup> In order to protect themselves against free radicals, organisms are having endogenous (catalase, superoxide dismutase, glutathione peroxidase/reductase) and exogenous (vitamin C and E) defenses. But these defense systems are not sufficient in critical situations like oxidative stress, UV exposure etc., when the production of free radicals significantly increases.<sup>[2]</sup> An excess generation of free radicals and a deficient cellular antioxidant defense system may lead to a state of oxidative stress. Oxidative stress and products of lipid peroxidation are involved in the physiology of various neurological disorders such as stroke, Parkinson's disease, Alzheimer's disease,<sup>[3]</sup> carcinogenesis.<sup>[4]</sup> Idiopathic and neuroleptic-induced parkinsonism are often treated with muscarinic receptor antagonists.<sup>[5,6,7]</sup>

The anticholinesterase inhibitor tacrine is used therapeutically to improve memory function in patients with early and late onset Alzheimer's disease. But it can lead to the production of parkinsonian side-effects such as bradykinesia, rigidity, tremor.<sup>[8,9]</sup> Ott and Lannon<sup>[8]</sup> demonstrated that tacrine-induced parkinsonism could be ameliorated by L-3, 4-dihydroxyphenylalanine (L-DOPA). One of the motor effects produced by cholinomimetics is tremulous jaw movements (also known as 'VCM' or 'purposeless' chewing). These are characterised as rapid, vertical deflections of the lower jaw that resemble chewing but are not directed at any stimulus. They share some characteristics with human parkinsonian symptoms.<sup>[10,11]</sup> Tacrine-induced tremulous jaw movements can be suppressed by anti-parkinsonian agents. Apomorphine, bntropine block tacrine-induced jaw movements.<sup>[12]</sup>

*Rubia Cordifolia* Linn (Rubiaceae) also known as 'manjistha' is an important medicinal plant. It possesses anti-tumor,<sup>[13]</sup> antistress and antihyperglycemic,<sup>[14]</sup> antimicrobial,<sup>[15]</sup> hepatoprotective,<sup>[16]</sup> radio protective,<sup>[17]</sup> anticancer<sup>[18]</sup> activities. This plant has also been listed officially as herbal medicine in the Chinese Pharmacopeia for the treatment of arthritis, dysmenorrhea, hematorrhea and hemostasis, which are free radical related diseases. In the present study, we have assessed the *in vitro* antioxidant activity and anticholinergic activity. Total phenolic content was determined using Folin-Ciocalteu reagent.

## Materials and methods

### Animals

Male Wistar rats (150 ± 20 g) and Swiss albino mice (22 ± 2g) were used for the study. Animals were housed in a colony cages and maintained at 25° ± 2°C, 12:12 h light/dark cycle and 50 ± 5% RH with free access to food and water *ad libitum*. Animals were acclimatized to laboratory conditions before test. All the experiments were carried out during the light period (08.00-16.00 h). The Institutional Animal Ethical Committee of MGVS Pharmacy College, Nashik approved the protocol of the study.

### Chemicals

Folin-ciocalteu reagent (Merck, India), tacrine (Sigma, MO), Vitamin E (Merck, India) were used for the study. All other chemicals and reagents were of analytical grade.

### Plant material and Extraction

Roots and rhizomes of *Rubia cordifolia* Linn. were obtained from Ayurved Seva Sangh, Nashik, India and authenticated by Dr. P. G. Diwakar, Joint director, Botanical Survey of India, Pune (Voucher specimen number: RAP 1) The collected material was extracted with acetone. The marc was dried and extracted with methanol by Soxhlet extractor. The extract was filtered and dried. The yield of methanolic extract (M-RC) was found to be 5.2 % w/w.

### Phytochemical studies

M-RC was subjected to identification of phytoconstituents as suggested by Evans.<sup>[19]</sup>

### Determination of total antioxidant activity

Total antioxidant activity of M-RC was determined spectrophotometrically using the thiocyanate method.<sup>[20]</sup> The absorbance was measured at 500 nm. All data reported are the average of triplicate analyses. Percent inhibition of lipid peroxide generation was calculated using following formula.

% inhibition = [(absorbance of control - absorbance of test) / absorbance of control] x 100

**Determination of Free Radical Scavenging Activity**

DPPH scavenging activity was measured by the spectrophotometer.<sup>[21]</sup> To an ethanolic solution of DPPH (200 µM), 0.05 ml of the test compounds dissolved in ethanol were added at different concentrations. An equal amount of ethanol was added to the control. After 20 min the decrease in absorbance of the test mixtures (due to quenching of DPPH free radicals) was read at 517 nm and the percentage inhibition calculated. Each trial has been done in triplicate. IC<sub>50</sub> value was calculated by plotting graph %Scavenging verses Concentration.

% scavenging = [(absorbance of control- absorbance of test)/ absorbance of control] x 100

**Hydroxyl radical scavenging activity**

This method involves *in-vitro* generation of hydroxyl radicals using Fe<sup>3+</sup>/ascorbate/ EDTA/ H<sub>2</sub>O<sub>2</sub> system using Fenton reaction. Scavenging of this hydroxyl radical in presence of antioxidant is measured. The hydroxyl radicals formed by the oxidation is made to react with DMSO (dimethyl sulphoxide) to yield formaldehyde. The intensity of yellow color formed by reaction of formaldehyde with NASH reagent (2M ammonium acetate with 0.05M acetic acid and 0.02M acetyl acetone in distilled water) is measured at 412nm against reagent blank.<sup>[22]</sup> The activity is expressed as % hydroxyl radical scavenging.

IC<sub>50</sub> value was calculated by plotting graph %Scavenging verses Concentration.

% Hydroxyl radical scavenging activity= [(absorbance of control- absorbance of test)/ absorbance of control] x 100

**Amount of total phenolics**

Total phenolics present in the M-RC was determined spectrophotometrically with the Folin-Ciocalteu reagent, according to the method suggested by Slinkard and Singleton.<sup>[23]</sup> The absorbance was measured at 760 nm. All tests were performed in triplicate. The concentration of total phenolics in MERC was determined as µg gallic acid equivalents, using the following equation obtained from a standard gallic acid graph:

Absorbance = 0.159 x gallic acid (µg) + 0.0290

**Effect on Tacrine-induced orofacial dyskinesia**

Rats were divided in 6 groups, each containing five animals. The rats received orally, vehicle and M-RC (100, 200 and 300 mg/kg) 1 h prior to tacrine (2.5 mg/kg, i.p.). Vitamin E (10 mg/kg, p.o.) was used as a reference. Immediately after injection of tacrine, rats were placed in a Plexiglas observation box (22x22x22 cm<sup>3</sup>) for a 10 min habituation period. An observer blind to treatment recorded the number of vacuous chewing movements (VCM), tongue protrusions (TP), and number of orofacial bursts (OB) as described by Cousins.<sup>[12]</sup> All rats were observed for 1 h period.

**Effect on sodium nitrite-induced hypoxia**

Sodium nitrite (250 mg/kg, s.c.) was administered to induce hypoxia.<sup>[24,25]</sup> M-RC (100, 200 and 300 mg/kg, p.o.) was administered 1 h before treatment with sodium nitrite. Duration to induce hypoxia was recorded.

**Statistical analysis**

Data are expressed as mean ± SEM. Statistical analysis was done by using one way analysis of variance (ANOVA) followed by Dunnett's test. Values with P<0.05 were considered statistically significant.

## Results

### Phytochemical studies

M-RC revealed presence of glycosides, phenolics, flavonoids, and saponins.

### Total antioxidant activity

IC<sub>50</sub> value for M-RC was found to be 120 µg/ml.

### Free radical and hydroxyl radical Scavenging activity

The IC<sub>50</sub> values of M-RC by DPPH free radical scavenging and hydroxyl radical scavenging method was found to be 135 and 130 ppm respectively. The results are summarized in table1.

**Table 1: % Scavenging activity**

Concentration (ppm)	% Scavenging activity by	
	DPPH method	Hydroxyl radical scavenging method
10	12.88 ± 0.01	22.96 ± 0.03
25	22.88 ± 0.02	31.11 ± 0.05
50	29.26 ± 0.05	39.74 ± 0.02
100	44.75 ± 0.09	49.02 ± 0.06
250	69.02 ± 0.04	53.32 ± 0.08
IC <sub>50</sub>	135	130

Each value in the table is obtained by calculating the average of three experiments and expressed as mean ± SEM.

### Amount of total phenolic content

M-RC contained 1.8679 ± 0.29 µg/g Gallic acid equivalents of phenols. The phenolic compounds may contribute directly to the antioxidant action.<sup>[26]</sup>

### Tacrine-induced vacuous chewing movements

Tacrine shows significant increase in VCM, TP, OB. M-RC dose-dependently reduced tacrine-induced VCM, TP, OB. Vitamin E was used as a reference standard. It also significantly reduced tacrine-induced VCM, TP, OB (Table 2).

**Table 2: Effect of *R. cordifolia* on tacrine-induced orofacial dyskinesia**

Group	Number of		
	VCM	TP	OB
Control	119 ± 2.72	14.25 ± 0.85	55 ± 2.90
Tacrine (2.5)	1141 ± 12.93*	46 ± 0.91*	259 ± 2.58*
M-RC 100	385 ± 10.41**	21.5 ± 1.32**	137 ± 5.30**
M-RC 200	243.5 ± 3.4**	14.75 ± 1.11**	118 ± 6.31**
M-RC 300	223 ± 3.22**	14 ± 1.09**	103 ± 2.39**
Vitamin E 10	261.2 ± 9.44**	19.75 ± 0.8**	102.2 ± 6.23**

n=5. Data are mean ± SEM. \**P* < 0.05 compared to control. \*\**P* < 0.05 compared to tacrine. VCM: vacuous chewing movement, TP: tongue protrusion, OB: orofacial burst, M-RC: methanolic extract of *R. cordifolia*

**Sodium nitrite-induced hypoxia**

M-RC significantly decreased the latency for death after sodium nitrite administration (Table 3).

**Table 3: Effect of *R. cordifolia* on Sodium nitrite-induced hypoxia**

Group	Time of respiratory arrest (min)
Control	22.55 ± 2.02
M-RC 100	17.82 ± 0.63*
M-RC 200	14.79 ± 0.51*
M-RC 300	14.25 ± 1.05*

n=5. Data are mean ± SEM. \* $P < 0.05$  compared to control.

**Discussion**

Most of the mammals have an inherent mechanism to prevent and neutralize the free radical induced damage. In biochemical system, superoxide radical and  $H_2O_2$  react together to form a singlet oxygen and hydroxyl radical, which can attack and destroy almost all known biochemicals.<sup>[27]</sup> Hydroxyl radicals are the major ROS, causing lipid oxidation and enormous biological damage.<sup>[28]</sup> It is apparent from the present study that the M-RC possesses antioxidant activity. M-RC showed antioxidant activity by inhibiting hydroxyl radical and DPPH scavenging. The roots of *R. Cordifolia* could be ranked as anthraquinone rich due to the strong violet colour indicated by the Borntrreger's test. It was already reported that naturally occurring phenolic compounds have free radical scavenging properties, due to their hydroxyl groups.<sup>[29]</sup> The majority of isolated molecules from the roots of *R. Cordifolia* elucidate as anthraquinones.<sup>[30]</sup> It has been estimated that family Rubiaceae is rich in anthraquinone compounds.<sup>[31,32]</sup> M-RC showed presence of phenolic compounds. The antioxidant potential may be attributed to the presence of polyphenolic compounds and anthraquinone glycosides.

Tacrine, the acetylcholinesterase inhibitor, used in treatment of early and late onset of Alzheimer's disease. Animal studies have shown that cholinomimetic drugs produce a wide variety of motor effects.<sup>[33]</sup> Administration of tacrine is associated with a wide variety of peripheral and central side effects.<sup>[35]</sup> Some of the central side effects of tacrine are extrapyramidal motor dysfunctions; these include several Parkinsonian symptoms such as cogwheel rigidity, tremor, and bradykinesia.<sup>[8,35]</sup> There is a substantial literature showing that Parkinsonism can be exacerbated by cholinergic stimulation and alleviated by muscarinic antagonism.<sup>[5,6,36]</sup> Previous studies with animals also have shown that cholinomimetic drugs have pronounced motor effects. The induction of parkinsonian symptoms by cholinesterase inhibition is generally consistent with the substantial literature showing cholinergic involvement in idiopathic and neuroleptic-induced parkinsonism.<sup>[5,6]</sup> Cholinomimetic drugs induce perioral movements in rats.<sup>[12,37,38]</sup> In rats, drugs that stimulate muscarinic cholinergic receptors produce a number of different orofacial movements, the most common of which is known as vacuous jaw movements.<sup>[38,39]</sup> M-RC significantly decreased the latency for death after treatment with sodium nitrite. It may suggest cholinergic inhibition as observed with inhibition of tacrine-induced VCM, TP, and OB. It may be concluded that M-RC has significant antioxidant activity. The study highlights the importance of *R. Cordifolia* as an alternative system of medicine for parkinsonism.

### **Conclusion**

The study concludes that *R. cordifolia* has an anticholinergic activity which may be attributed to antioxidant activity and presence of phenolic compounds.

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