Evaluation of phytoconstituents and assessment of adaptogenic activity in vivo in various extracts of *Rhododendron arboreum* (leaves)

JD Roy¹, AK Handique¹, CC Barua², A Talukdar², FA Ahmed³, IC Barua⁴

¹Department of Biotechnology, Gauhati University, Jalukbari, Guwahati, India  
²Department of Pharmacology and Toxicology, Khanapara, Guwahati, India  
³National Research Centre on Yak, Dirang, Arunachal Pradesh, India  
⁴Department of Agronomy, Assam Agriculture University, Jorhat, India

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**ABSTRACT**

Natural products particularly of plant origin with antistress property and nutraceutical value have become the focus of scores of scientific investigation in recent times. Against this background the present study was carried out to analyse the phytochemical components and adaptogenic activity of *Rhododendron arboreum* which grow in high altitudes. Adaptogenic activity of the plant extract was evaluated in vivo in rat and mice animal models. Methanol, hydroethanol and aqueous extract of the leaves were prepared and preliminary screening of the phytochemical constituents was carried out. The important phytoconstituents viz. flavonoids and phenolics were estimated and amount of gallic acid and quercetin were evaluated by HPTLC. Adaptogenic activity of the extract was studied in vivo using rat and mice as test animals. The criteria taken were forced swimming test in rat and mice and tail suspension test in mice. Methanol extract showed the presence of diterpenes, triterpenes, flavonoids, steroids, tannin, phenolics. Hydroethanol extract showed the presence of diterpenes, triterpenes, saponin, glycosides, alkaloids, tannin and aqueous extract showed the presence of tannin, glycosides, triterpenes, flavonoids, diterpenes. Quantitative analysis exhibited that the methanol extract have the highest amount of flavonoid and phenolics. The HPTLC profile of methanol extract showed the presence of highest amount of quercetin. Gallic acid was detected only in methanol extract. In vivo study in test animals revealed that the methanol extract of *R. arboreum* exhibited potent anti stress activity. Hence, presence of these compounds which are known to have strong anti-oxidant activity in high quantity might be responsible for adaptogenic activity.

**Introduction**

Plants have been used to treat or prevent illness since before recorded history. The sacred Vedas dating back between 3500 B.C and 800 B.C give many references of medicinal plants. One of the remotest works in traditional herbal medicine is “Virikshayurveda”, compiled even before the beginning of Christian era [1]. Hence, natural products and plant based products have played a vital role in prevention and mitigation of human diseases for centuries [2]. Due to their large structural diversity, the natural products have emerged as appealing sources of useful leads for the discovery of new drugs; their potential is further enhanced with the present sophisticated techniques for isolation, identification, structure elucidation and combinatorial synthesis [3]. The World Health Organisation (WHO) has described guidelines for the standardisation of medicinal plants with regard to their macroscopic and microscopic description [4]. Several studies have suggested that the pharmacognostic details of a plant may provide useful criteria in identification and authentication.

*Corresponding Author: JD Roy, PhD Scholar, C/O R. K. Pegu, Division of Animal Health, ICAR Research Complex for NEH region, Umroi Road, Umium, India. E-Mail: jayntidr@gmail.com*
of plant drugs [5]. According to World Health Organization (WHO), more than 80% of the world’s population relies on traditional medicines for their primary health care needs [6]. The medicinal value of plants lies in some chemical substances that produce a definite physiologic action on the human body. The most important of these bioactive compounds of plants are alkaloids, flavonoids, tannins and phenolic compounds [7]. *Rhododendron arboreum*, belonging to the family Ericaceae, is one of the most stately and impressive rhododendron species and one of the well known plants of North East India. Besides its ornamental value, it is also known for its wide variety of medicinal property. In Homeopathic Materia Medica, the tincture of dried leaves of *R. arboreum* has been used in gout rhematism. Ayurvedic preparation “Asoka Arishta,” containing *R. arboreum* possesses oxytocic, estrogenic, and prostaglandin synthetase-inhibiting activity [8]. Dried flowers of *R. arboreum* are highly efficacious in checking diarrhoea and blood dysentery [9] in hilly areas, the flowers of *R. arboreum* with sweet and sour taste are used in local brew which is known to be a refreshing appetizer and used to prevent high altitude sickness, young leaves are used for its’ medicinal property and applied on the forehead to alleviate headache [10], fresh and dried corolla is given when fish bones get struck in the gullet [11]. Scientific validation of the medicinal property of the plant includes potent antioxidant property of flavonoids isolated from the leaves of *R. arboreum* [12], anti-inflammatory [13], hepatoprotective activity of leaves of *R. arboreum* in CCl4 induced hepatotoxicity in rats [14], flower contains antidiabetic potential [15], ethyl acetate fraction of flowers of *R. arboreum* showed potent anti-diarrhoeal activity [16]. The present study focus on the comparative evaluation of phytoconstituents present in various extracts of *Rhododendron arboreum* (leaves) in reference with the adaptogenic activity.

### Materials and methods

#### Collection of plant materials

Leaves of *R. arboreum* were collected during the month of August-September, 2012–2013 from Dirang, Arunachal Pradesh by Dr, F. A. Ahmed and identified by taxonomist Dr. I. C. Barua. Department of Agronomy, AAU, Jorhat, Assam and voucher specimens were deposited in the herbarium.

#### Preparation of various extracts

Leaves of *R. arboreum* were shade dried and powdered. About 250 g of powdered leaves was soaked in 1000 ml of of methanol for 72 h in a beaker and the mixture was stirred every 18 h using a sterile glass rod. Filtrate was obtained after passing through Whatman filter paper no 1 for three times and concentrated in rotary evaporator (Buchi, India, Model No-R210) at 50°-60°C under reduced pressure. A dark brown of *R. arboreum* methanol extract (RAME) was obtained and stored in air tight container at 4°C for further use. Recovery was 17.90% (w/w) in terms of dry leaves. The procedure for preparation of hydro-ethanol extract was similar to that of the preparation of methanol extract; where instead of methanol, a mixture of ethanol and water (1:1) were used for soaking the powered leaves. The recovery of hydroethanol extract (RAHE) was 18.52 % (w/w) in terms of dry leaves. For aqueous extract, one part of the powdered plant material was boiled with sixteen parts of water for a period of 15 mins. It was filtered hot through muslin cloth. The filtrate was transferred to a round bottom flask and freezeed up overnight which was then lyophilized in a lyophilizer (HETO Power Dry LL 3000) to obtain the aqueous extract. The recovery of aqueous extract was 16.23 % (w/w) in terms of dry leaves.

#### Phytochemical screening

The plant extract was subjected to phytochemical screening as per the standard method for presence of different phytoconstituents [17].

#### Determination of total phenolics

Using modified Folin Ciocalteu method, total phenol content in the extracts was determined. An aliquot of the extract was mixed with 5 ml Folin Ciocalteu reagent (previously diluted with water 1: 10 v/v) and 4 ml (75g/l) of sodium carbonate. The tubes were vortexed for 15 secs and allowed to stand for 30 mins at 40 °C for colour development. Absorbance was then measured at 765 nm using spectrophotometer (Chemito, UV 2100). Samples of extracts were evaluated at a final concentration of 0.1 mg/ml. Total phenolics were expressed as mg/g tannic acid equivalent using the following equation based on the calibration curve: y=0.1216x where, x is the absorbance, y is the tannic acid equivalent (mg/g) [18].

#### Determination of total flavonoids

Estimation of the total flavonoids in the plant extracts was carried out using the method of Ordon Ez et al., 2006. 0.5 ml of sample, 0.5 ml of 2% AlCl3 solution in ethanol was added. After 1 hour at room temperature, the absorbance was measured at 420 nm. A yellow colour indicated the presence of flavonoids. Extracts samples were evaluated at a final concentration of 0.1 mg/ml. Total flavonoids were expressed as quercetin (mg/g) equivalent using the following equation based on the calibration curve: y=0.0255 x where, x is the absorbance, y is the quercetin equivalent (mg/g) [19].

#### HPTLC analysis

A densitometer HPTLC of RAME was performed for the characteristic fingerprinting profile RAME was dissolved with HPLC grade methanol at the concentration of 1 mg/ml in methanol. The solution was centrifuged at 3000 rpm for 5 mins and used for HPTLC analysis. The samples (2µl) were loaded as 7 mm band length in the 10 × 10 Silica gel 60F254 TLC plate using Hamilton syringe and CAMAG LINOMAT 5 instrument. The samples loaded plates were kept in TLC twin trough developing chamber (after saturation with solvent vapour) and loaded on the TLC plate with the solvent system.
chloroform, toluene and water (3:3:1) for quercetin and methanol: acetoniitrile : water for gallic acid as mobile phase. Finally the plate was kept in scanner stage and scanning was done in 254 nm.

**Animals**

Healthy adult albino mice and Wistar rats of either sex approximately of same age, weighing between 25-30 g and 150-200 g respectively, were used for the study. The animals were group housed in polypropylene cages containing sterile paddy husk bedding under controlled conditions at 25±30°C, RH 50±5% and 10/14 h of light/dark cycles. Food and water were provided ad lib. The study was conducted after obtaining the approval of the Institutional Animal Ethics Committee (No:770/03/ac/CPCSEA/FVSc, AAU/IAEC/06/22).

**Acute toxicity studies**

The acute oral toxicity of the plant extract was estimated by following up- and down stair case method in mice as per OECD TG 425 guidelines. Based on the toxicity study, the doses of plant extract for different parameters to be studied were selected.

**Forced swimming test in mice**

Forced swimming test in mice was carried out as previously described (Porsolt et al.,1977) with slight modifications. Briefly, mice was placed individually in a clear cylinder (diameter 10 cm, height 25 cm), containing 15 cm of water at 25 ± 1°C. The water was changed between testing sessions. Treatments were given 1 h prior to test and placed in the cylinder again. Mice were forced to swim for 6 mins, and the immobility time during the last 5 mins was manually measured by a blinded observer. Mice was considered immobile when they ceased struggling, remained floating motionless, and only make those movements necessary to keep their head above the water [20].

**Forced swimming test in rat**

In the forced swimming test in rats, the animals were placed individually in glass cylinders (40 cm height × 18 cm diameter) containing 22 cm depth of water at 28°C. The procedure consisted of a pre-swimming test and swimming test separated by 24 h. During the pre-swimming, rats were placed in the cylinders for 15 mins. Rats were removed from the cylinders, dried with a cloth towel and warmed with electric heater before they were placed back to home cage. Treatments were given 1 h prior to test and placed in the cylinder again. Immobility time was recorded during the last 4 mins of the total 6 mins test period by observers blind to the treatment conditions. Rats were considered to be immobile when floating motionless or making only those movements necessary to keep their head above the water surface [21].

**Tail suspension test in mice**

The tail suspension test was conducted as previously described (Steru et al.,1985). Briefly, mice was suspended by adhesive tape that is positioned about 2.5 cm from the tail tip with the head 40 cm above the floor. Treatments were given 1 h prior to test and placed in the cylinder again. The trial was carried out for 6 mins and the duration of immobility was manually recorded by two blinded observers during the final 5 mins interval of the test. Mice was considered immobile when they hung passively and motionlessly [22].

**Statistical Analysis**

All values were expressed as mean ± SE and analysed by using one way analysis of variance (ANOVA) using Graph pad Prism Version 6. P value less than 0.0001 (P < 0.0001) was the criteria for statistical significance.

**Results and discussion**

Medicinal value of plants has assumed an important dimension in the past few decades. Plants produce a very diverse group of secondary metabolites with antioxidant potential. The present study focus on studying the diverse phyto- constituents present in various extracts by different methods and identification of phyto constituents which might be related to the adaptogenic activity. Plants used in traditional medicine contain a vast array of substances that can be used to treat chronic and infectious diseases. Secondary metabolites serve as plant defense mechanism against predation by microorganisms, insects and herbivores. Some, such as terpenoids, give plants their odours; others (quinines and tannins) are responsible for plant flavour and some of the herbs and spices used by humans to season food yield useful medicinal compounds. In the present study, methanol extract which showed a recovery of 17.9% contains diterpenes, triterpenes, flavonoids, steroids, tannin, phenolics, hydroethanol extract having a recovery of 18.52% contains alkaloids, tannins, diterpenes, triterpenes, saponin, glycosides and aqueous extract with the recovery of 16.23% showed the presence of tannin, glycosides, triterpenes, diterpenes, flavonoids. The phenolic content was found to be 0.3720±0.002 mg tannic acid/g of dry plant material in methanol extract, 0.0961±0.003 mg tannic acid/g of dry plant material in hydroethanol extract and 0.0964±0.00 mg tannic acid/g of dry plant material in aqueous extract. The flavonoid content was found to be 0.0652 ±0.004 mg quercetin /g of dry plant material in methanol extract, 0.0449 ±0.002 mg quercetin /g of dry plant material in hydroethanol extract and 0.0570 ±0.002 mg quercetin /g of dry plant material in aqueous extract. The results are shown in Table 1.

Original Research Article

Table 1: Consistency, recovery (%), phytoconstituents, phenolic and flavonoids content in various plant extract of leaves of *R. arboreum*

<table>
<thead>
<tr>
<th>Name of the extract</th>
<th>Consistency</th>
<th>Recovery (%)</th>
<th>Phytoconstituents Present</th>
<th>Phenolic Content (mg tannic acid/g of dry plant material)</th>
<th>Flavonoids Content (mg quercetin/g of dry plant material)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methanol extract</td>
<td>dark brown crystalline</td>
<td>17.9%</td>
<td>diterpenes, triterpenes, flavonoids, steroids, tannin, phenolics</td>
<td>0.3720*±0.002</td>
<td>0.0652±0.004</td>
</tr>
<tr>
<td>Hydroethanol Extract</td>
<td>dark brown crystalline</td>
<td>18.52%</td>
<td>Diterpenes, triterpenes, saponin, glycosides, Alkaloids, tannin</td>
<td>0.0961±0.003</td>
<td>0.0449±0.002*</td>
</tr>
<tr>
<td>Aqueous Extract</td>
<td>dark brown crystalline</td>
<td>16.23%</td>
<td>tannin, glycosides, triterpenes, Flavonoids, diterpenes</td>
<td>0.0964±0.004</td>
<td>0.0570±0.004</td>
</tr>
</tbody>
</table>

Values indicate mean ± SE. One way ANOVA show the values are highly significant *P<0.0001, Dunnett’s Test showed q value>4.320 (for phenolics) and Dunnett’s Test q value>2.660 (for flavonoids).

The HPTLC studies further confirmed the presence of quercetin in all the extracts and the highest amount of quercetin was present in methanol extract (0.83 mg/ml), followed by hydroethanol extract (0.78 mg/ml) and aqueous extract (0.82 mg/ml) (Table 2).

Table 2: Quercetin content (mg/ml) in different extracts of *R. arboreum*

<table>
<thead>
<tr>
<th>Name of the extract/fraction</th>
<th>Rf value</th>
<th>Peak area(AU)</th>
<th>Quercetin content (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Quercetin (standard)</td>
<td>0.97±0.103</td>
<td>4914.60±0.516*</td>
<td>1.00±0.051*</td>
</tr>
<tr>
<td>Methanol extract</td>
<td>0.98±0.129</td>
<td>4124.00±5.163*</td>
<td>0.83±0.007*</td>
</tr>
<tr>
<td>Hydroethanol extract</td>
<td>0.98±0.005</td>
<td>3837.50±7.745</td>
<td>0.78±0.010</td>
</tr>
<tr>
<td>Aqueous extract</td>
<td>0.98±0.010</td>
<td>4031.80±0.258*</td>
<td>0.82±0.005*</td>
</tr>
</tbody>
</table>

Values indicate mean ± SE. One way ANOVA show the values are highly significant *P<0.0001, followed by Dunnett’s Test q value>2.605.
HPTLC studies also showed the presence of gallic acid in methanol extract only whereas the hydroethanol and aqueous extract was devoid of it. The concentration of gallic acid was 0.1854 mg/ml (Table 3).

**Table 3: Gallic acid content (mg/ml) in different extracts of *R. arboreum***

<table>
<thead>
<tr>
<th>Name</th>
<th>Rf value</th>
<th>Peak area (AU)</th>
<th>Gallic acid content (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gallic acid (standard)</td>
<td>0.40 ±0.005</td>
<td>8762.20 ±0.516</td>
<td>1.00±0.051</td>
</tr>
<tr>
<td>Methanol extract</td>
<td>0.41 ±0.002</td>
<td>4124.00±1.032</td>
<td>0.47±0.007</td>
</tr>
<tr>
<td>Hydroethanol extract</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Aqueous extract</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Values indicate mean ± SE. Paired T test show the rf values are not significant, P value = 0.1139, peak area and concentration is highly significant*P<0.0001
The animal studies showed that the methanol extract of *R. arboreum* showed the best anti-stress activity as compared to hydroethanol and aqueous extract in all the animal models. In forced swimming test in mice, the immobility time was recorded to be 206.83±1.447 secs, in forced swimming test in rat the immobility time was recorded to be 228.16 secs. and in tail suspension test in mice was recorded to be 202.32 secs (Table 4). The increase in the immobility time signifies that the animal is able to withstand the stress it is subjected to and they do not try to escape.

**Table 4: Anti-stress activity of different plant extracts in different animal models**

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Dose (mg/kg p.o.)</th>
<th>Forced Swimming in mice (Immobility time in secs)</th>
<th>Forced Swimming in rat (Immobility time in secs)</th>
<th>Tail Suspension in mice (Immobility time in secs)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>10ml/kg</td>
<td>55.08±1.290</td>
<td>55.56±2.886</td>
<td>51.50±5.170</td>
</tr>
<tr>
<td>RAME</td>
<td>50</td>
<td>115.00±2.886*</td>
<td>207.66±0.774*</td>
<td>113.20±2.695*</td>
</tr>
<tr>
<td>RAHE</td>
<td>100</td>
<td>161.33±0.258*</td>
<td>212.66±2.581*</td>
<td>151.21±0.258*</td>
</tr>
<tr>
<td>RAEE</td>
<td>200</td>
<td>206.83±1.549*</td>
<td>228.16±5.163*</td>
<td>202.32±0.522*</td>
</tr>
<tr>
<td>RAME</td>
<td>50</td>
<td>13.30±1.549*</td>
<td>109.00±0.258*</td>
<td>61.70±7.508*</td>
</tr>
<tr>
<td>RAHE</td>
<td>100</td>
<td>55.21±1.290*</td>
<td>127.33±5.221*</td>
<td>110.22±2.582*</td>
</tr>
<tr>
<td>RAEE</td>
<td>200</td>
<td>114.61±1.032*</td>
<td>197.00±0.774*</td>
<td>121.75±5.170*</td>
</tr>
<tr>
<td>Zeetress</td>
<td>50</td>
<td>100.36±5.163*</td>
<td>37.66±2.695*</td>
<td>23.22±0.776*</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>108.27±2.065*</td>
<td>60.16±0.026*</td>
<td>75.34±1.293*</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>113.50±2.695*</td>
<td>76.33±0.077*</td>
<td>88.90±2.065*</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>182.16±0.517*</td>
<td>127.33±5.221*</td>
<td>141.13±0.258*</td>
</tr>
</tbody>
</table>

Values indicate mean ± SE. One way ANOVA show the values are highly significant *P<0.0001

From the above study, it can be concluded that the methanol extract of the leaves of *R. arboreum* possess adaptogenic activity which could be due the important phyto-constituents present therein. The methanol extract showed the highest phenolic and flavonoid content among the three extracts. Phenolic compounds possess anti-inflammatory, anticarcinogenic, anti-atherosclerotic, and other properties that may be related to their antioxidant activities [23, 24]. Polyphenolic compounds have an inhibitory effect on mutagenesis and carcinogenesis in humans [25]. Flavonoids and flavonols are two polyphenolic compounds that play an important role in stabilizing lipid oxidation and are associated with antioxidant activity [26]. The adaptogenic activity of the methanol extract of the plant thus observed could be attributed to the synergistic effects of these different compounds. Further the methanol extract exhibited highest amount of quercetin and gallic acid was present in methanol extract only. Quercetin works as anti-inflammatory, antioxidant, anticancer agents [27]. Gallic acid is known to have anti-inflammatory, antimutagenic, anticancer and antioxidant activity [28-30]. Hence presence of these compounds in methanol extract further enhances the adaptogenic property of the extract. Based on the above findings, further fractionation will be
attempted for isolation and identification of compounds responsible for the adaptogenic activity of the plant extract.

Conflict of interest statement
We declare that we have no conflict of interest.

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