Research Article

In vitro Studies on the Neuroprotective Potential of *Rhododendron arboreum* Flower

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ABSTRACT

Rhododendron arboreum (Buransh) is the national flower of Nepal and is predominantly found in the Himalayan belt. Since ancient times, the bright red flowers have been utilised in various medicinal practices. Floral extracts have been used in ethnomedicine to treat a wide spectrum of health conditions like gout, rheumatism, diarrhoea, blood dysentery, and high-altitude sickness. Studies have elucidated that the floral parts of the plant are rich in phenols, alkaloids, and other bioactives, making it an elixir of life. Although the plant has established medicinal value among the locals, due to the ephemeral blooming phase of the flower and meagre availability, the neuroprotective potential of *Rhododendron arboreum* flowers is yet to be confirmed by scientific studies. In this study, we aim to establish the therapeutic role of the flower extracts in oxidative stress-based neurodegeneration. There have been predictions about a colossal surge in the prevalence of neurodegenerative diseases in the upcoming decades. Furthermore, we are yet to develop a potential cure. Here, we aim to impart preliminary information about *Rhododendron arboreum* flower (RAF) potency, including antioxidant potential as well as its impact on oxidative stress markers in a neurodegenerative disease model, essential for further clinical development of novel therapeutic drugs.

Keywords: Neurodegeneration, Oxidative stress, *Rhododendron arboreum*, Phytomedicine, Flavonoids.

INTRODUCTION

Health organizations are predicting a surge in the prevalence of neurodegenerative diseases in the coming decades[1]. Degenerative diseases, defined as progressive atrophy of neurons, can be categorized based on the type of neurons affected. Alzheimer's disease (AD), the most prevalent neurodegenerative disorder, is described by the progressive loss of cholinergic neurons of the entorhinal cortex[2, 3]. Parkinson's disease (PD) is the second-most prevalent ND. In this case, the dopaminergic neurons are affected, consequently hampering the signal transmission from the substantia nigra pars compacta to the basal ganglia[4]. Previously mentioned neuronal atrophies are a consequence of a wide spectrum of factors like oxidative stress, genetic mutation, specific toxicity, etc. Most of the research samples belong to Caucasian populations, which do not correlate to global populations. However, nitrosative stress has been distinguished as a common factor across the global population. Recent research has also revealed the importance of oxidative stress in the manifestation of neurodegenerative disease[5-7]. Consequently, researchers are focusing on developing therapies targeting nitroxidative stress as either adjunct therapy or to delay the onset of disease[8-10].

In a cellular environment, oxidative stress is mainly characterized by elevated levels of reactive oxidative species (ROS), a by-product of mitochondrial respiration, lipid peroxidation, ion imbalance, and metal toxicity. To combat such conditions, the cells amplify the synthesis of antioxidants and antioxidant enzymes like glutathione, thioredoxin, superoxide dismutase, catalase, glutamate peroxidase, etc. In circumstances where these ROS are not neutralized, they end up hampering the normal functioning of the cells, leading to mitochondrial dysfunction, inflammation, apoptosis, and DNA damage[11]. Neurons have a higher ATP requirement and a higher rate of oxidative metabolism, making them more susceptible to ROS[12]. Besides neuronal cells, high-altitude plants are also extremely susceptible to high oxidative stress due to UV exposure. Hence, they have evolved mechanisms to synthesize significant amounts of antioxidants. These plants are a good source of exogenous antioxidants, making them plausible candidates for phytomedicine. In this study, we aim to elucidate the protective potential of one such high-altitude plant, i.e., *Rhododendron arboreum* flower's (RAF) bioactives against oxidative stress markers in a toxin-based neurodegeneration model.

Rhododendron arboreum is an evergreen flowering plant belonging to the same family as that of blueberries and cranberries, which are well-established sources of antioxidants[13,14]. The blooming flowers of this plant have been part of the ayurvedpractises practices of the locals to treat conditions like menstrual problems, high altitude sickness, and rheumatism[15]. Although leaves, bark, and

roots of other species of the *Rhododendron* genus have scientific data proving their neuroprotective potential, not much research has focused on the potential of RAF as a lead for neurodegenerative disease. Other *Rhododendron* species like *R. simsii, R. yedoense,* and *R. fortune* have shown significant potency against neurodegenerative models.[16-18]

MATERIALS AND METHODS

Reagents and Chemicals

2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2'-azino-bis-(-3-ethylbenzothiozoline-6-sulfonic acid) disodium salt (ABTS), hydrogen peroxide (H_2O_2), Folin Ciocalteu reagent, mercuric chloride ($HgCl_2$), potassium persulphate, quercetin, trichloroacetic acid (TCA), thiobarbituric acid (TBA) was obtained from Sigma Chemicals Co., Sisco Research Laboratories Pvt. Ltd., and Himedia Laboratories Pvt. Ltd. Rest of the solvents and chemicals used were obtained of analytical grade.

Sample collection and preparation

The dried petals of *Rhododendron arboreum* were sourced from Devbhoomi Naturals, an Ayurveda company based in Uttarakhand, India in the month of January 2022. A 70% w/v methanolic extraction was prepared of the dried and powdered RAF in a magnetic stirrer for 3 h at 130 rpm at room temperature. Subsequently, the homogenate was subjected to centrifugation at 10,000 rpm for 15 min at 4 °C. The RAF centrifugate was stored at 4 °C until use.

Phytochemical screening

The RAF extracts were screened for carbohydrates, proteins, alkaloids, glycosides, phytosterols, terpenoids, phenols, and flavonoids using standardized protocols[19, 20].

Total flavonoid content

The quantitative analysis of flavonoids in RAF extract was performed with slight modifications[21]. To 0.2 ml of the RAF extract, 4 ml of distilled water and 0.3 ml of sodium nitrite (5% v/v) were added and incubated for 5 min at room temperature. This was followed by 0.3 ml of aluminium chloride (10% v/v) and incubated for 1 min. Lastly, 2 ml of sodium hydroxide (1 M) was added, and the volume was made up to 10 ml with distilled water. The solutions were briefly vortexed prior to the recording of absorbance against a suitable blank at 510 nm. The total flavonoid content was determined from the Quercetin calibration curve and expressed as mg QE equivalents/g of dry weight.

In vitro antioxidant potential

DPPH• scavenging assay: 1 ml of 0.06 M DPPH solution was mixed with 0.1 ml of serial dilutions of RAF extract. The aliquots were wrapped in foil and incubated for 30 min at room temperature in the dark. The absorbance was recorded at the end of the incubation period, against a suitable blank at 517 nm[22]. The scavenging potential of the extracts was calculated using the formula mentioned below and the regression graph was plotted. Ascorbic acid

(1 mg/ml) was used as a standard for the calibration curve. The IC_{50} values were obtained using an online tool provided by AAT Bioquest, Inc.

$$\text{Scavenging (\%)} = \frac{(\text{Acontrol} - \text{Atest})}{\text{Acontrol}} \times 100$$

ABTS•+ scavenging assay: The ABTS solution (equivolume 2.45 mM $K_2S_2O_8$ and 7 mM ABTS) was incubated at 4 ºC for 72 h. It was freshly diluted to obtain an OD of 0.700±0.02 at 734 nm. The RAF extract was serially diluted in aliquots of 0.1 ml and mixed with a previously prepared 2 ml ABTS solution. The absorbance was recorded after a 6 min incubation at room temperature in the dark at 734 nm against a suitable blank[23]. The scavenging potential of the tests was calculated using the formula mentioned below and the regression plot was prepared. Ascorbic acid (1 mg/ml) was used as a standard for the calibration curve. The IC50 values were obtained using an online tool provided by AAT Bioquest, Inc.

$$\text{Scavenging (\%)} = \frac{(\text{Acontrol} - \text{Atest})}{\text{Acontrol}} \times 100$$

Ex vivo Analysis

Preparation of culture: A local abattoir was selected for sourcing goat brains. The cerebellum and brain stem were discarded while the cerebrum was dissected along the longitudinal fissure. Further thin sections were obtained along the sagittal plane. The petri plate was sterilized and supplied with phosphate buffer saline, supplemented with 124 µg/ml streptomycin. Corresponding sections were submerged in the supplemented petri plates. The experiment design for the toxin models was prepared as mentioned in Table 1. All the cultures were prepared in duplicates and incubated overnight. At the end of incubation, the media was drained from the culture and the plates were stored at a temperature of -20 ℃ until further use.

Table 1: Experimental design for the culture

S. no.	Test Group	H$_2$O$_2$ Induced model		HgCl$_2$ Induced model	
		H$_2$O$_2$ (µM)	RAF (%)	HgCl$_2$ (ppm)	RAF (%)
A	Control	-	-	-	-
B	ND induced	20	-	50	-
C		120	-	150	-
D	ND induced + RAF treatment	20	1	50	1
E		20	0.25	50	0.25
F		120	1	150	1
G		120	0.25	150	0.25
H	RAF treatment	-	1	-	1
I		-	0.25	-	0.25

CAT assay: The catalase activity of treated, untreated, and control was estimated following the method of Aebi[24] with slight modifications. The enzyme extraction was

performed on all the cultures in 0.05 M phosphate buffer, pH 7. Further enzyme activity was estimated by mixing the reaction mixture (1.2 ml of 0.05M phosphate buffer, 0.2 ml of 10 mM H_2O_2) and 0.05 ml of enzyme extract to initiate the reaction. A time scan of H_2O_2 consumption over 3 min at an interval of 10 s was recorded at 234 nm. A mixture of 1.45 ml of 0.05 M phosphate buffer along with 0.05 ml of the respective enzyme extract was used as a blank. The catalase activity was determined using the molar extinction coefficient of H_2O_2 ($\varepsilon_{H_2O_2}$ = 0.039 mM^{-1}cm^{-1}).

TBARS assay: The estimation of malondialdehyde content was done according to the method of Heath and Packer[25] with slight modifications. 0.5 ml of 10 % of the brain extract was mixed with 2.5 ml of TCA-TBA reagent and incubated in a boiling water bath for 30 min. The mixture was instantaneously cooled on an ice bath followed by centrifugation at 7000 rpm for 5 min at 4 ℃. The supernatants were collected for recording absorbance against suitable blanks at 532 nm and 600 nm. The MDA content was calculated using the formula:

$$MDA\ (mM\ cm) = ((A_{532} - A_{600}))/155$$

RESULTS AND DISCUSSION

The extraction solvent plays a crucial role in determining the potency of the medicinal value of the plant. In this study, we focused on a specific polyphenol class, i.e., the flavonoids. Research has elucidated 70% methanol as an efficient solvent for phenol extraction with the highest yield[26]. In this study, the same was used for the extraction of bioactives that were further used for analytical assays.

Phytochemical screening

Rhododendron arboreum flower has been part of ethnomedicine since ancient times as it is a source of numerous bioactives with medicinal properties. As indicated in Table 2, the RAF extract shows the presence of various phytochemicals confirmed via multiple standard phytochemical screening protocols. It can be elucidated that RAF extracts are rich in polyphenols and alkaloids.

Total flavonoid content

Flavonoids are naturally occurring polyphenols known for their various medicinal properties. As drug discovery and development is shifting more towards the plant-based approach, small bioactive molecules have found their way into the pharmaceutical industry with properties like antioxidant, antimicrobial, anticancer, and antidiabetic[27]. The flavonoid content in RAF was estimated to be 1059.01 ± 38.20 mg QE/g dry weight of RAF. Recent research has also revealed their potential role in altering multiple pathways, thereby improving brain health[28].

Antioxidant potential

An imbalance in ROS is known for its significant contribution to the manifestation of various diseases. In the case of neurodegeneration, oxidative stress has been designated as one of the common causes across the whole spectrum of these multifactorial diseases. Consequently, in

order to establish phytomedicine with neuroprotective potential to delay the onset or slow the progression, determining the antioxidant potential of RAF is necessary.

Table 2: Results of phytochemical screening performed on methanolic extract of RAF

| Sl. no	Phytochemical Screening	Results
Alkaloid		
1	Mayer's test	+
2	Wagner's test	++
3	Dragendroff's test	+
Carbohydrates		
1	Molisch's test	++
2	Benedict's test	++
Glycosides		
1	Borntragner's test	+
2	Keller Killiani's test	++
Phytosterols		
1	Salkowski test	++
2	Lieberman's test	+
Terpenoids		
1	Salkowski test	++
Phenols		
1	Ferric chloride test	++
2	Lead acetate Test	++
Flavonoids		
1	Lead acetate Test	++
2	dil. Ammonia Test	++
Protein & Amino Acids		
1	Xanthoprotic test	++

In this study, the RAF extract exhibited a dose-dependent regression in DPPH as well as ABTS content, thereby confirming the antioxidant nature of the sample. In the present investigation, RAF extract showed the highest % inhibition of 82.4 ± 0.1046 % at a 10 mg/ml concentration, whereas a 50 % inhibition of the DPPH radical was obtained at 1.35 mg/ml of RAF methanolic extract. The value obtained is noteworthy when compared to that of standard ascorbate (0.0432 mg/ml). Furthermore, the sample showed better inhibition against the ABTS assay, with the highest % inhibition of 93.62 ± 0.077 % at a 4 mg/ml concentration. Based on the regression plot, a 50 % inhibition of the ABTS radical was obtained at 0.629 mg/ml of RAF methanolic extract and 0.0503 mg/ml of ascorbate. Several studies have also reported that rutin, coumaric acid, and quercetin are the flavonoids predominantly present in R. arboreum[29]. These findings, as well as the results of our study, are in accordance with previous studies, consequently confirming the antioxidant potency

of RAF. Another review has illuminated the molecular mechanism of rutin to attenuate crucial pathological mechanisms associated with neuro-inflammation, and oxidative/nitrosative stress[30].

Ex vivo Analysis against oxidative stress markers

It has been established that ROS hinders the normal functioning of the cell by directly or indirectly hampering the molecular mechanisms. Consequently, cells enhance the production of antioxidants and specific enzymes in order to combat this imbalance as one of the evolved defence mechanisms[31,32]. Catalase is one such crucial antioxidant enzyme that helps combat oxidative stress, and its deficiency or malfunction has been associated with several neurological disorders. Catalase itself is one of the

oxidative stress biomarkers, and it is being developed as a therapeutic agent for several diseased conditions[33]. In this study, we focus more on the therapeutic potency against sporadic neurodegenerative diseases, where oxidative stress plays a major role. In order to confirm the therapeutic potency of RAF, we estimated the catalase activity in control, treated, and untreated cultures. Upon further analysis, we observed a significant decrease in the catalase activity of the ones treated with RAF after inducing neurodegeneration using different concentrations of $HgCl_2$. As depicted in Figure 1A, treated ex vivo cultures showed the highest reduction of 64.73% in the catalase activity after treatment of 50 ppm $HgCl_2$ induced ND model with 1 % RAF extract.
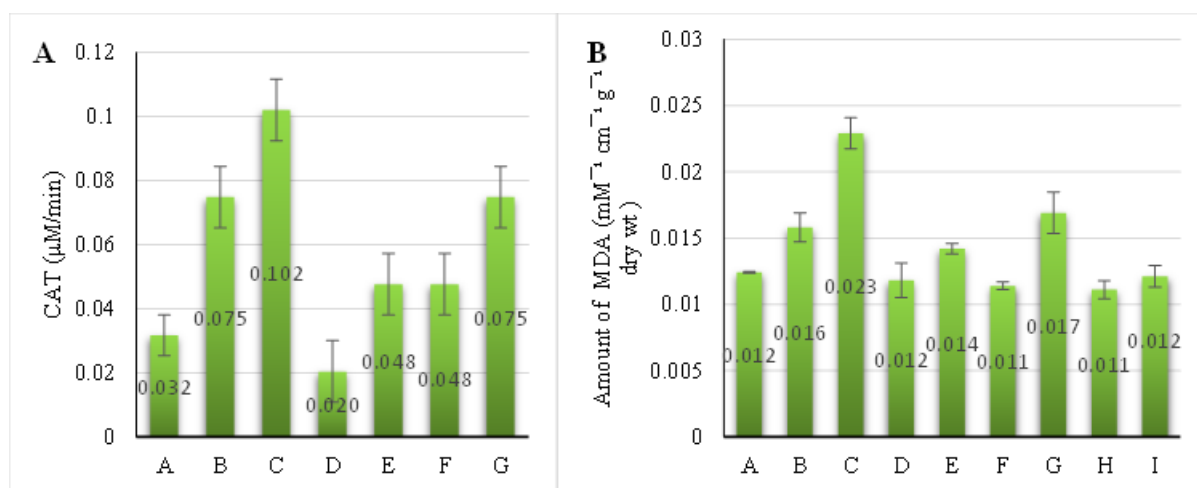
Figure 1: Effect of RAF on catalase activity in $HgCl_2$ induced model of neurodegeneration (A); and MDA content in H_2O_2 induced model of neurodegeneration (B).

Another well-established biomarker for oxidative stress is lipid peroxidation. This includes thiobarbituric acid-reactive substances (TBARS) and oxidized–LDL (ox–LDL). Further studies have elucidated its association with neurodegenerative conditions where oxidative stress triggers lipid peroxidation and the brain being saturated with polyunsaturated fatty acids makes it highly vulnerable[35,36]. In this study, we estimated the TBARS level by assaying for MDA content, an end product of the decomposition of lipid peroxidation products. Comparative analysis of MDA content in control, treated, and untreated samples revealed a noteworthy decrease in MDA content of treated cultures. As depicted in Fig 1B, treated cultures showed the highest reduction of 50.43 % in the MDA content when treated with 1 % RAF extract to the 120 µM H_2O_2 induced ND model with 1 % RAF extract. Hence, the results elucidate that RAF bioactives have the ability to mitigate oxidative stress, consequently lowering MDA production.

CONCLUSION

Current study revealed that the RAF extract is not only a potent antioxidant, but it also has potency against oxidative stress-based neurodegeneration in the corresponding ex vivo brain model. It has been established that the RAF

extract is rich in flavonoids, and the results confirm its high efficacy against both the antioxidant assays performed. As a result, its potency as a natural antioxidant source has been confirmed. Furthermore, the analysis of oxidative stress biomarkers in H_2O_2 as well as $HgCl_2$ models of neurodegeneration showed a significant decrease in biomarkers upon treatment with RAF extract. Consequently, the study confirms that RAF extract exhibits significant attenuation of neurodegeneration caused by the selected neurotoxin. However, further screening ex vivo and in vivo is required to further validate and develop RAF extract into a potent phytomedicine for neurodegenerative conditions.

ACKNOWLEDGEMENT

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