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Research Article

PHYTOCHEMICAL ANALYSIS AND EVALUATION OF *IN VITRO* IMMUNOMODULATORY ACTIVITY OF *RHODODENDRON ARBOREUM* LEAVES

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ABSTRACT

Objectives: The objectives of this study are to analyze phytoconstituents in *Rhododendron arboreum* leaves (qualitatively and quantitatively) and to estimate the immunomodulatory effects of extracts using different *in vitro* methods for intracellular and phagocytic killing potency of neutrophils, which are subsequent, involved in the process of phagocytosis by neutrophils.

Methods: *R. arboreum* leaves extracts were separately extracted in 100% methanol, 50% methanol, and aqueous by a hot percolation technique using Soxhlet apparatus and concentrated with a vacuum rotary evaporator apparatus. The percentage yield of the extracts wares calculated. The extract was used to analyze phytoconstituents qualitatively, and total phenolic and flavonoid content were estimated. In another study, already purified methanolic extract of *R. arboreum* leaves, namely cytoplasmic male sterility (CMS-3) (kaempferol), RAM fr2, and transcranial magnetic stimulation (TMS-10) (ursolic acid) was used to evaluate immunomodulatory activities by nitroblue tetrazolium test, phagocytosis of killed *Candida albicans*.

Results: Phytochemical screening of the extracts showed the presence of major classes of phytochemicals, i.e., phytosterols, flavonoids, phenols, alkaloids, carbohydrate, glycoside, sterols and steroids, terpenoids, and tannin while saponin was absent in all extract. The percent yield of leaves extract was 12.97%. Total phenolic content (TPC) expressed as gallic acid equivalents (GAE) ranged 1327.5±0.009 (in 100% methanolic extract), 1309.5±0.011 (in 50% methanolic), and 1468.5±0.006 mg GAE g^{-1} dry weight (in aqueous extract). Total flavonoid content expressed as rutin equivalents (RE) ranged 219±0.025 (in 100% methanolic extract), 184±0.02 (in 50% methanolic), and 262±0.58 mg (in aqueous extract) mg RE g^{-1} dry weight. *R. arboreum* purified extract CMS-3 (kaempferol), RAM fr2, and TMS-10 (ursolic acid) stimulated the neutrophils to phagocytic activity to the extent of 67.67±2.08%, 40.3±1.5%, and 79.67±0.57%, respectively. The above-said extracts stimulated the phagocytosis of killed *C. albicans*. The mean particle numbers for CMS- 3, RAM fr2, and TMS-10 were found to be 6.7, 6.3, and 7, respectively, at concentration 1 mg/ml.

Conclusion: The phytochemical screening of all the extracts showed the presence of various phytochemicals that are biologically important. The total phenolic and flavonoid content of the plant are comparable to other medicinal plants. Even though the enormous progress on the phytochemistry and pharmacology of *R. arboreum* have been made, there still require more conclusive studies on the safety, efficacy, and *in vivo* toxicity of extracts and pure compounds to gain a better understanding.

Keywords: *Rhododendron arboreum*, Phytochemical, Ursolic acid, Kaempferol, Immunomodulatory activity.

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INTRODUCTION

Well-functioning and a strong immune system is the foundation of an excellent physical condition. The immunity is the balanced state of getting sufficient natural resistance to fight infection, the other redundant biological invasions although has tolerance to avoid autoimmune diseases and allergic reaction. Immune responses are a result of effective interaction between innate and acquired mechanism of an immune system. For the last three decades, researchers have shown a remarkable interest in the immune system as a potential target of toxicity due to exposure to environmental pollutants, chemicals and drugs. Immunodeficiencies arise as one or more components of the immune system are inactive. Several factors play an important role in altering the immune competence such as stress, lifestyle, sex, age, environmental contamination, malnutrition, alcohol/drug abuse, and genetic variability [1]. Immunomodulation is a wide term which refers to any changes or may involve amplification, expression, induction or inhibition of any part of the immune response. The immunomodulation concept has been gaining importance globally while researchers are realizing the immune system play a critical role in maintaining a disease-free condition. The incidence of life-threatening infections has increased significantly among AIDS patients, cancer patients, and transplant recipients, those receiving wide range cytotoxic drugs, corticosteroids, and antibiotics [1]. It seems that antibiotics have disappeared with their magic touch due to decades of improper use; reckless medication and unavoidable extent of bacterial genes give medicine resistance [2].

Phytochemicals give flavor, color, fragrance, and create a natural defense system for host plants. Till date, more than 4000 of these compounds have been discovered. The phytochemicals give protection to plants and also possess curative potential such as immunomodulatory activity, anti-diabetic, anticancer, anti-oxidant, adaptogenic property, enhancing memory, and cholesterol reducing effects. Discovery of thousands of phytochemicals was grouped based on source and function. Potential of natural compounds with immunostimulating activity will be classified as low molecular immunomodulatory compounds such as alkaloids, phenolic, and terpenoids whereas high molecular weight compounds are polysaccharides [3].

Most of the chemotherapeutic agents usually have an immunosuppressive activity which exerts a variety of side effects and cytotoxic. Due to which researchers motivated for investigating natural resources showing immunomodulatory activity. Several medicinal plants are recognized to have immunomodulatory properties and maintain natural resistance against disease through re-establishing the body's immune system such as *Azadirachta indica* [4], *Terminalia chebula* [5], and *Lawsonia alba* [6]. The phytochemical constituents such as tannins, proteins, steroids, and terpenoids [7], Flavonoids from *Plantago* species [8] and *Syzygium samarangense* [9] have also shown immunomodulatory activity. *In vitro*

study of Vacha leaves alcohol, petroleum ether, and volatile oil extracts stimulate the phagocytosis in human neutrophils [10].

Various *in vivo* and *in vitro* test systems are used for assessing immunomodulatory activity. Phagocytosis is one such commonly used technique for screening the immune response [11]. Phagocytosis is the primary defense mechanism against any foreign bodies entering the body which is offered by macrophages and neutrophils. The process of phagocytosis consists of chronological stages such as motility, adhesion to microorganisms, ingestion of microorganisms, degranulation, and intracellular killing of microorganisms [12].

Rhododendron arboreum is one of the stateliest and impressive *Rhododendron* species where *arboreum* means tree-like [13]. *Rhododendron* is the state flower of Nepal and the state tree of Uttarakhand. It is enormously variable in stature, leaf characteristics, hardness, and flower color. It grows at elevations of 4500–10,500 ft. The tree attains a height of 40–50 ft tall occasionally attaining above 100 ft [14]. This is an evergreen much-branched tree up to 2.4 m in girth and 14 m in height [15]. Flowering period is from March to April/June to September bearing crimson to pale pink or deep red flowers. *R. arboreum* methanolic extract of different parts (roots, stem, leaves, bark, and flowers) shows good antimicrobial and antifungal activity [16-18]. Chauhan *et al*. [19] discovered that the antibacterial activity of *R. arboreum* leaf extract was found effective as compared to flower extract against *Staphylococcus aureus*. Methanolic extract of *R. arboreum* leaves through gas chromatography-mass spectrometry (GC-MS) analysis confirms the presence of main phytoconstituents, i.e., 22-stigmasten-3-one (14.59%) with highest area percentage, followed by 1, 1, 6-trimethyl-3-methylene-2-(3, 6, 10, 13,14-pentamethyl-3-ethenyl-pentadec-4-enye) cyclohexane (12.26%), beta-amyrin (7.62%), and linoleyl alcohol (6.50%) having antimicrobial, anticancer, antiarthritic, anti-inflammatory, and antiviral properties [20]. This plant is reported to possess several medicinal and pharmacological properties such as hepatoprotective, antioxidant, immunomodulatory, anti-inflammatory, anti-diabetic, anticancer, antinociceptive, adaptogenic, and antidiarrheal, oxytocic, estrogenic, prostragl, synthetase inhibitory activity, and central nervous system depressant activity [17,21-31]. In the present study, ursolic acid and kaempferol were quantitatively estimated from the *R. arboreum* leaves collected from Himachal Pradesh and its immunomodulatory properties were determined.

METHODS

Materials

All the chemicals used in the present study including standards were procured from Sigma-Aldrich, India; HiMedia.

Collection of the samples

R. arboreum leaves were collected from Ani-Jalori bypass district from Himachal Pradesh (31°28′28.9524″N, 77°25′21.3852″E). The collected leaves were identified by Dr. S. K. Srivastava, Scientist "D" Botanical survey of India, Dehradun (Accession No: 115589). The collected leaves were washed thoroughly beneath fresh running water and were left for drying under a shed for 2 weeks. The dried leaves were then crushed in a blender and kept in sealed small bag in a refrigerator at 4°C until further use.

Preparation of extract

Initially, *R. arboreum* air-dried leaves (10 g) were crushed. The crushed *R. arboreum* leaves extracts were separately extracted in 100% methanol, 50% methanol (methanol: water: 1:1) and aqueous by hot percolation method using Soxhlet apparatus continuously for 8 h. All the extracts were filtered, collected and concentrated using vacuum rotary evaporator dried and stored in the refrigerator at 4°C separately [32]. This extract was used to analyze phytoconstituents (qualitatively and quantitatively). *R. arboreum* air-dried leaves (130 g) were separately crushed and were extracted in different solvents with increasing polarity, namely petroleum ether <diethyl ether < methanol by hot percolation method using Soxhlet apparatus continuously for 16 h. The extract was filtered and concentrated using vacuum rotary evaporator, dried and stored in the refrigerator at 4°C. The percentage yield of the extract was calculated after drying. Percentage yield of the plant extract was calculated using a formula:

$$\% \text{ Yield of the extract} = \frac{C_x}{C_y} \times 100$$

Where C_x= plant material weight after extraction process; C_Y= plant material weight taken for extraction.

In our previous studies, three constituents, namely cytoplasmic male sterility (CMS-3) (kaempferol), RAM fr2, and transcranial magnetic stimulation (TMS-10) (ursolic acid) were isolated/purified from them an ethanolic extract of *R. arboreum* leaves through thin-layer chromatography, column chromatography, and confirmed by HPLC. These purified extracts were used to evaluate *in vitro* immunomodulatory activities.

Qualitative phytochemical screening

The standard procedures were pursued to trace out the presence of phytochemicals in *R. arboreum* leaves, i.e., glycosides, flavonoids, anthocyanin, phenols, alkaloids, tannins, and saponins [33-35].

Phytosterol identification

Salkowski reaction

Half (0.5) mg of the extract was treated with 3 ml concentrated sulfuric acid followed by 2 ml of chloroform. Allow the mixture to stand for a minute. Formation of reddish-brown color in chloroform layer confirmed the presence of phytosterol [36,37].

Liebermann Burchard's test

Four mg of extract was treated with 0.5 ml of chloroform and 0.5 ml of acetic anhydride and then filtered. To the filtrate, few drops of concentrated sulfuric acid were added carefully along the side's wall of the test tubes. Formation of greenish-blue color indicates the existence of steroids [38].

Test for phenols

Two ml of distilled water was added to 1 ml of extracts (1 mg/ml) followed by addition of some drops of 10% $FeCl_3$ (ferric chloride) and waited for the development of blue or green color.

Alkaloids

The extract was treated with 2% diluted HCL in boiling water bath for 2 min. Allow to cool the mixture was filtered and treated with some drops of 5% NaOH solution. The samples were observed for the presence of yellow precipitate or turbidity [39].

Glycosides

The extracts were neutralized by NaOH and hydrolyzed by HCl solution. Little drops of Fehling solution A and B were added and observed for red precipitate [33].

Terpenoids

Half ml of acetic anhydride was added to 4 g of extract. Then add 0.5 ml of chloroform and concentrated H_2SO_4 to the mixture and observed for red violet color [39].

Flavonoids

Lead acetate test

The extract was treated with some drops of lead acetate solution. Development of yellow color precipitate indicates the presence of flavonoids [40].

Alkaline reagent test

Some drops of NaOH solution were added to the extract. Development of deep yellow color which disappears after adding diluted HCL shows the presence of flavonoids [41].

Tannins test
Mixed little amount of extract with water and heated on the water bath and filtered. Add few drops of ferric chloride to the filtrate. A dark green solution shows the existence of tannins [33].

Saponins test
Half ml of extract was dissolved in 5 ml of distilled water and shaken for 15 min. Persistence of frothing indicates that leaves extract contains Saponins [39].

Anthocyanin and Betacyanin test
One ml of leaves extract was dissolved in 1 ml of 2N NaOH and was heated at 100°C in a water bath for 5 min. The appearance of bluish-green color shows the presence of anthocyanin while the development of yellow color indicated the presence of Betacyanin [42].

Carbohydrate test
Molisch test: Add 2 ml of leaves extract to 1 ml of Molisch reagent, and little drops of concentrated H_2SO_4 were added. Development of purple or reddish color shows the presence of carbohydrates [37-42].

Fehling's test
Add 2 ml of leaves extract, 5 ml of Fehling's solution A and B was kept in hot water bath for 5 min. The development of yellow or red color shows the presence of carbohydrates [43].

Quantitative phytochemical screening

Determination of total phenolic content (TPC)

Folin–Ciocalteu reagent (FCR) assay was performed for the evaluation of TPC in the isolated plant extract [37,44-46]. Take 150 µl of the extract and mixed in 240 µl of water and 150 µl of 0.25 N FCR. It was incubated in the dark at room temperature for 3 min. After incubation, add 300 µl of 1N Na_2CO_3, then the mixture was incubated further for 2 h inside dark at room temperature. Different dilutions of gallic acid (0.01 mg/ml, 0.02 mg/ml, 0.04 mg/ml, 0.08 mg/ml, and 0.1 mg/ml) were used as a standard for drawing the calibration curve. The absorbance of each sample was measured at 765 nm, and results were expressed in terms of mg of gallic acid equivalent g^{-1} of extract* and were calculated using a formula:

GAE* = X×V÷M

Where X: Concentration of extract/standard (mg/ml), M: Weight of extract (g), V: Volume of extract (ml).

Determination of total flavonoid content

Total flavonoid content (TFC) assay was performed as suggested by Dae *et al*. [47], Dae through minor modifications. To 10 ml extract, add 2 ml of water and 150 µl of 5% $NaNO_2$ solution. Allow the mixture to react for 6 min and then add 150 µl of 10%, $AlCl_3$ solution and absorbance were measured after 15 min at the wavelength of 510 nm. Rutin at different concentrations (0.01 mg/ml, 0.02 mg/ml, 0.04 mg/ml, 0.08 mg/ml, and 0.1 mg/ml) were used as a standard for the quantification of total flavonoid. Triplicate measurements were carried out, and total flavonoid content was expressed in milligram of rutin equivalents (RE) mg/g of extract (mg RE g^{-1})* and was calculated using formula.

RE* = X×V÷M

Where X: Concentration of extract/standard (mg/ml), M: Weight of extract (g), V: Volume of extract (ml).

Study of immunomodulatory activity

Nitroblue tetrazolium test (NBT) [48,49].

Prepare the suspension of leukocytes (5×10^6/ml) with 0.5 ml of phosphate buffer saline (PBS) solution in 5 test tubes. A volume of 0.1 ml PBS control and 0.1 ml of endotoxin-activated plasma (LPS standard) was added to the 1st and 2nd tube, respectively. 100 µl of test samples CMS-3, RAM fr2, and TMS-10 in 1000 µg/ml concentration were added in test tube no. 3, 4, and 5, respectively. Prepare fresh 0.15% NBT solution, add 0.2 ml in each test tube and incubate at 37°C for 20 min then centrifuged at 1410 rpm for 3–4 min to throw away the supernatant. These cells were re-suspended in the little quantity of PBS solution. A thin film was prepared on a glass slide, by air drying and followed by heat fixation and then counterstained by dilute carbol-fuchsin dye for 15 s. The slides were rinsed under tap water, dried and observe under (100X) oil immersion microscope. The percent of NBT-positive cells those hold blue granules/lumps were calculated by counting 200 neutrophils.

Phagocytosis of killed *Candida albicans* [48-51]

Preparation of C. albicans suspension

The *C. albicans* (MTCC -3017) was cultured in Sabouraud broth at 35°C for 24 h and then centrifuged at 4000 rpm to pellet the cells at the bottom then the supernatant was discarded. The pellet cells were rinsed with sterile Hank's Balanced Salt Solution (HBSS) and centrifuged once more at 4000 rpm and repeated the step for 3 to 4 times. The final cell pellet was mixed with a mixture of sterile HBSS and human serum in 4:1 proportion. The concentration of cell suspension 1×10^8 was used for the experiment.

Slide preparation

Put 0.2 ml human blood on a sterile glass slide using finger prick method and allow to clot in an incubated at 37°C for 25 min. Then blood clot was gently detached, and the slide was washed carefully with sterile normal saline so the adhered polymorphonuclear neutrophils (PMNs) (invisible) should be not removed. The slide was flooded with test samples RAM fr2, TMS-10, and CMS-3 in 1000 µg/ml concentration and incubated at 37°C for 15 min. Then *C. albicans* suspension was added again incubated at 37°C for 1 h. The glass slide was washed, fixed with methanol and stained with Giemsa stain.

Phagocytosis evaluation

The mean number of Candida cells phagocytosed through PMNs on the slide was determined microscopically for 100 granulocytes through morphological criteria. This number was used as a phagocytic index (PI) and was compared through basal PI control. A number of 200 neutrophils were examined to count the number of ingested *Candida*-associated with each cell. The mean particle number associated with each cell was calculated.

RESULTS

Qualitative phytochemical analysis

The qualitative phytochemical analysis of *R. arboreum* leaves extract indicated the presence of phytosterols, flavonoids, phenols, alkaloids, carbohydrate, glycoside, sterols and steroids, terpenoids, and tannin while saponin was absent in all extract. The percentage yield of RAM extract was 12.97%. Nisar *et al*. [33] explored that methanolic leaves extract contain flavonoids, steroids, terpenoids, anthraquinones, and tannins. Roy *et al*. [30] also showed the presence of phenolics, flavonoids, tannin, steroids, diterpenes, and triterpenes in the methanolic extract. Revealed the presence of hydrocarbon alkane, steroids, ester, fatty acid**s,** flavonoids, terpenes, and organic compounds in the methanolic extract by GC-MS analysis and 49 phytochemicals were identified in the methanolic leaves extract of *R. arboretum* [20].

Quantitative phytochemical analyses

Analyze the antioxidant activity, total phenol content and total flavonoid content and their results were expressed as means ± standard deviation (Table 1).

TPC

Different dilutions of the standard (gallic acid) were used to plot a standard calibration curve, resulting in with the linear equation, Y = 4.0917X+0.0498, and regression coefficient, R^2=0.988. The TPC of *R. arboreum* in 100% methanolic, 50% methanolic, and 50% aqueous extract was found to be 1327.5±0.009 mg/g, 1468.5±0.006 mg/g, and 1309.5±0.011 mg gallic acid g^{-1} of extract, respectively (Fig. 1 and Table 1).

TFC

Different dilutions of rutin were used to plot a standard calibration curve, resulting in the linear equation, Y = 1.3225X+0.0379, and regression coefficient, R^2=0.996. TFC value for the 100% methanolic and 50% methanolic extracts of *R. arboreum* was 219±0.025 mg and 184±0.58 mg rutin g^{-1} of extract, respectively, while aqueous extract showed the highest value of 262±0.023 mg rutin g^{-1} of extract (Fig. 2 and Table 1).

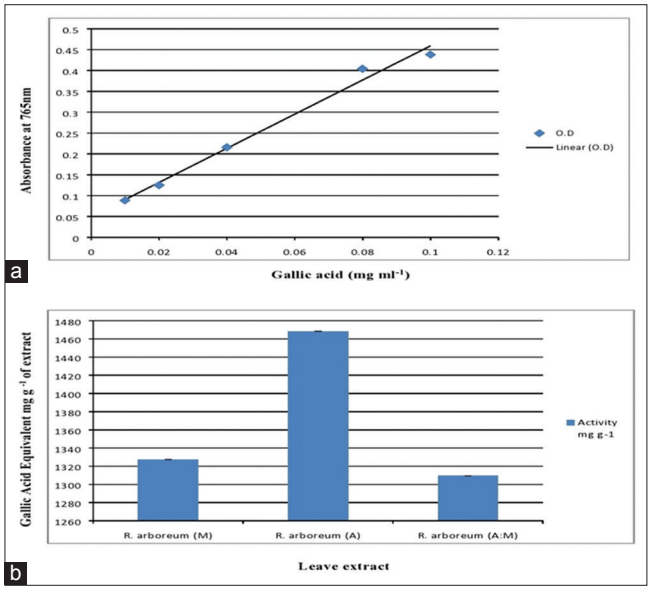

Fig. 1: Total phenolic content (TPC). (a) Calibration curve of gallic acid (b) TPC of different solvent extracts of *Rhododendron arboreum*

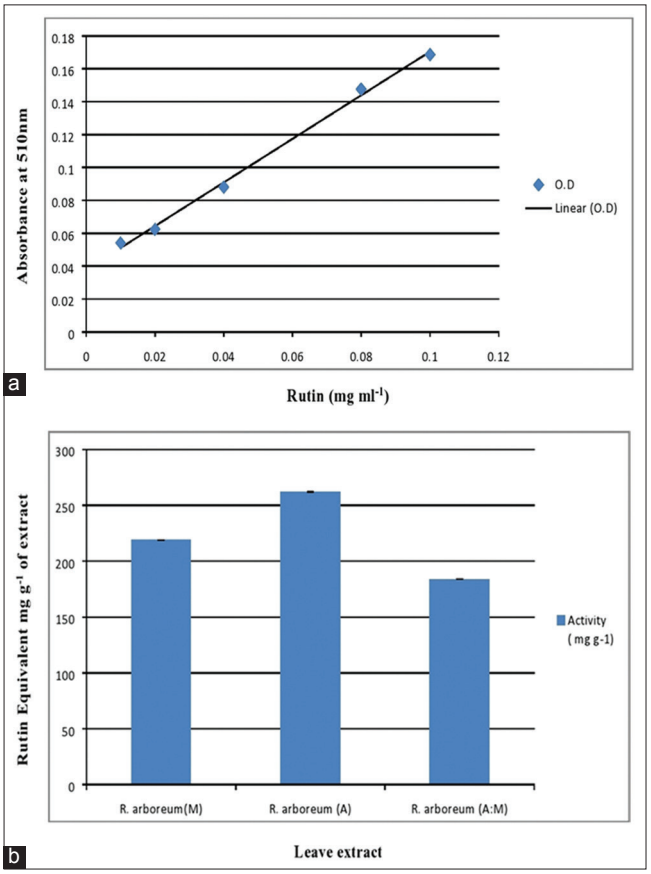

Fig. 2: Total flavonoid content (TFC). (a) Calibration curve of rutin (b) TFC of different solvent extracts of *Rhododendron arboretum*

NBT

R. arboreum purified extract CMS- 3 (kaempferol), RAM fr2, and TMS-10 (ursolic acid) stimulated the neutrophils to phagocytic activity toward the extent of 67.67±2.08%, 40.3±1.5%, and 79.67±0.57%, respectively, when compared to normal control (PBS) (21.3±0.57%) and positive control, i.e., endotoxin-activated plasma (82±1%) at same concentration. Leukocytes once stimulated, a membrane permeable, yellow-colored, water-soluble, and nitroblue tetrazolium are reduced to blue NBT formazan crystals by the leukocytes (Figs. 3 and 4).

Phagocytosis of killed *C. albicans*

R. arboreum purified extract CMS- 3 (kaempferol), RAM fr2, and TMS-10 (ursolic acid) stimulated the phagocytosis of killed *C. albicans.* The mean particle numbers phagocytosis of killed *C. albicans* after treatment with CMS- 3, RAM fr2, and TMS-10 extract were found to be 6.7, 6.3, and 7, respectively, at concentration 1mg ml^{-1}, when compared to positive control [6] (Figs. 5 and 6).

CONCLUSION

The phytochemical analysis concludes that *R. arboreum* leaves extract to contain various phytochemicals that are biologically important. The total phenolic and flavonoid content of the plant are comparable to other medicinal plants. The NBT assay and phagocytosis of killed *C. albicans* study are evidence that compounds in *R. arboreum* leave extract played

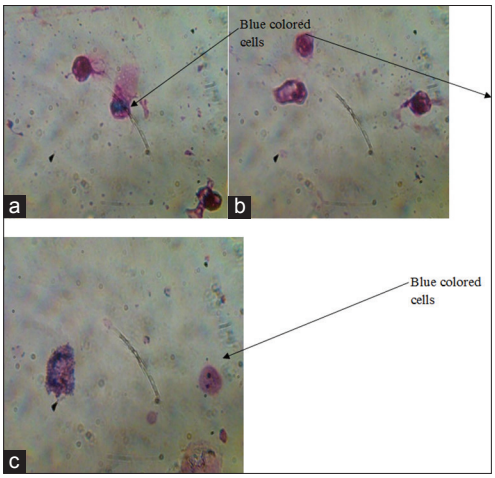

Fig. 3: Photograph showing neutrophils with reduced nitroblue tetrazolium test. (a) Neutrophil stimulation by cytoplasmic male sterility-3. (b) Neutrophil stimulation by RAM fr2. (c) Neutrophil stimulation by transcranial magnetic stimulation-10

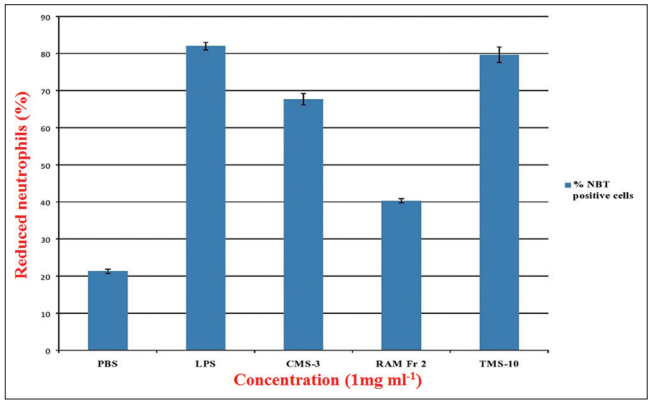

Fig. 4: Percent of reduced neutrophils treated with extracts by nitroblue tetrazolium test. Test extracts: Cytoplasmic male sterility-3, RAM fr2, and transcranial magnetic stimulation-10. Positive control: Endotoxin-activated plasma (LPS). Normal control: Phosphate buffer saline

a significant role in the modulation of the immune response and thus may have application as an immunomodulatory agent. While huge progress on the pharmacology and phytochemistry of *R. arboreum* have been made, but still require more conclusive studies on the efficacy, safety, and *in vivo* toxicity of extracts and pure active compounds to gain a better understanding.

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AUTHOR'S CONTRIBUTION

Conception and design of the work were done by Dr. Nishant Rai, Dr. Navin Kumar, and Mr. Pramod Rawat. Extraction/identification of phytochemical was done by Dr. Rakesh Kumar Bachheti and Mr. Pramod Rawat. Statistical analysis, interpretation of data and drafting of the article were done by Mr. Pramod Rawat. Critical revision of the article was done by Dr. Nishant Rai, Dr. Navin Kumar Dr. Rakesh Kumar Bachheti and Mr. Pramod Rawat.

Table 1: Comparative phytochemical analyses of *R. arboretum* leaves extract in 100% methanol and 50% aqueous

Extract	Phenolic (mg/g)	Flavonoid (mg/g)
R. arboretum (M)	1327.5±0.009	219±0.025
R. arboretum (A)	1468.5±0.006	262±0.58
R. arboretum (A: M)	1309.5±0.011	184±0.023

Where M is methanol extract and A is aqueous. Each value is mean±SD value of 3 samples. SD: Standard deviation, *R. arboretum: Rhododendron arboretum*

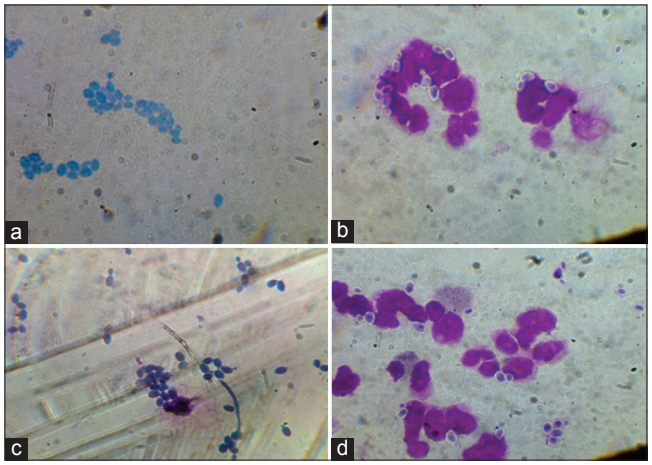

Fig. 5: Ingestion of *Candida albicans* by neutrophils. (a) *C. albicans* cells (100×). (b) Phagocytosis stimulation of *C. albicans* by cytoplasmic male sterility-3. (c) Phagocytosis stimulation of *C. albicans* by RAM fr2. (d) Phagocytosis stimulation of *C. albicans* by transcranial magnetic stimulation-10

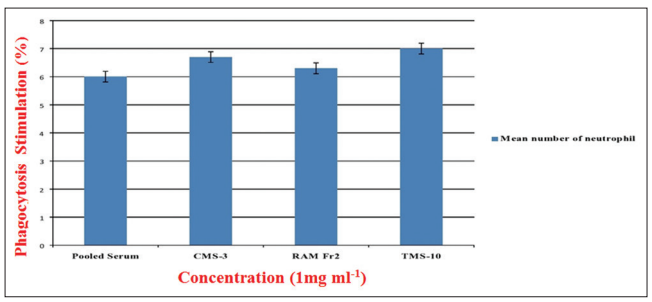

Fig. 6: Percent phagocytosis stimulation by *Rhododendron arboreum* extracts

CONFLICTS OF INTEREST

The authors confirm no conflicts of interest.

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