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Original article

IN VITRO EVALUATION OF THE ANTIMICROBIAL, TOXIC, ANTI-ARTHRITIC AND THROMBOLYTIC PROPERTIES OF DIFFERENT PARTS OF PLANT ORCHID *RHYNCHOSTYLIS RETUSA* (L.) BLUME.

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Abstract

Background: Infection due to microbes, trauma, cancer, arthritis and thrombosis are the most common problems worldwide. Traditionally, a large number of herbal extracts are used for the treatment of those disorders.
Aim: Ethanolic extracts from leaves, roots and stems of *Rhynchostylis retusa* (L.) Blume were phytochemically characterised and then tested for their antibacterial, toxicity, anti-arthritic, and thrombolytic properties in-vitro.
Methods and Results: Disk diffusion inhibition tests with a panel of fourteen different microorganisms was used as the antimicrobial assays. Antimicrobial activity of the stem extract found to be higher (10-11 mm, 400 µg/disk) than that of root extract whereas leaf showed no activity with ciprofloxacin (43-46 mm, 5 µg/disc) serving as reference standard. Toxicity of the plant extracts in terms of their LC50 values against the brine shrimp (*A. salina*) was assessed. The toxic potential of leaf extract was 6.7 µg/ml, which is higher than that of stem and root extract (*c.a.* 8.5 µg/ml). Thrombolytic properties were tested in vitro using streptokinase as reference (%Clot lysis after 1.5h & 24h was 76.15±1.94 & 92.59±2.35, 30000iu). The leaf extract exhibited higher activity. Leaf extract (1000 ppm) lysed of 33.08±1.24% of the clot and 60.47±2.33 after 1.5 h and 24 h of incubation, respectively. Anti-arthritic effects measured as albumin denaturation using diclofenac sodium as reference (IC50=60.25 ppm) revealed the stem extract as more active.
Conclusion: our research will help to provide evidence for some of the traditional uses of this medicinal plant.

INTRODUCTION

Herbal medicine is still recognized as the preferred primary health care system and have been used to treat a wide range of illnesses (Mintah et al, 2019). These remedies date back centuries and were developed using the medicinal knowledge of generations of doctors in active practice (Lemonnier et al, 2017). Nature has given us a gift in the form of medicinal plants that can be used to treat and prevent diseases (Mintah et al, 2019). Orchids are attractive flowers that have high value in society as ornamental plants. In addition, orchids are also known for their usage, especially in traditional medicine (Li et al, 2021).

Rhynchostylis retusa (L.) Blume is known as the 'Foxtail Orchid', and an exotic blooming orchid belonging to the Vanda alliance has been used in leaves, pseudo bulbs and whole plants for the treatment of paralysis, rheumatism, rheumatic pain, piles, menstrual disorders, fractures, cough and cold, fever, allergy, asthma, earache, wound, bone fracture and inflammation (Akter et al, 2018; Akhter et al, 2017; Dash et al, 2008; Sivaraj et al,2017; Bhatti et al, 2017; Das et al, 2012; Mehmud et al, 2017). The root of this orchid and fresh leaf buds of *Pisum sativum* is taken and made into a paste with water to cure blood dysentery, and the whole plant is also used as an emollient (Akhter et al, 2017; Kumar, 2015). Phytochemical test of the methanolic extract of the leaf found alkaloids, glycosides, flavonoids, coumarin, tannins, saponins, terpenoids, steroids and quinine (Kumar & Shiddamallayya, 2016). Fresh leaf has been reported as a potential treatment for paralysis. Fresh leaves combined with clarified 50-70 years old butter to create a paste would be applied daily to the paralyzed region. If no recovery was seen after a few days, fresh *R. retusa* leaves were combined with *Datura metel* L. (Solanaceae) roots and 12-13 fruits of *Piper nigrum* L. (Piperaceae). The combination had to be applied to the paralyzed region 1-2 times daily for 5-7days.The FMP reported significant improvements and maintained massage till full cure. (Rohani et al, 2018). The methanolic leaf extract of this plant showed significant analgesic activity in the writhing test and a beneficial anti-inflammatory effect in carrageenan and formaldehyde-induced paw oedema (Al-Amin et al, 2011). Hexane, chloroform, and methanol extracts of this orchid exhibited potential antifungal activity against six phytopathogens and considerable toxicity to the brine shrimp (Radhika, et al, 2013). Methanol, ethanol, and chloroform extracts of this plant showed different antimicrobial activity that potential against tested bacteria, while hexane extract did not show any antimicrobial activity on studied bacteria (Bhattacharjee & Islam, 2015). Various fractions of this medicinal orchid possessed significant antimycobacterial, leishmanicidal and antibacterial activity (Bhatnagar et al, 2017). Fresh leaf extract showed better antimicrobial activity to *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa* and *Salmonella typhi* than dried water extract of this orchid (Joyti et al, 2013).

Antimicrobial resistance is increasing globally (particularly in developing countries) because of the overuse and misuse of antibiotics. As a result, antibiotic resistance causes several million deaths per year. The threat of antimicrobial resistance needs to be fixed as soon as possible (Ayukekbong et al, 2017). Natural antimicrobial compounds that can treat microbial infections well have been found in a lot of medicinal plants (Stan et al, 2021). Medicinal plants provide the optimal means by which to get a wide variety of pharmaceuticals (Salmerón-Manzano et al, 2020). However, there are some limitations to old and newly introduced antimicrobial agents, e.g., short life expectancy, higher side effects, etc. (Mohsen et al, 2020). Since resistant clinical isolates are quickly spreading around the world, it is most important to find new antimicrobial agents.

Thrombosis, or blood clot is a serious is a leading cause of cardiovascular disease. Damage from this can include permanent impairment or even death if not treated (Naringrekar et al, 2019; Seifi et al. 2018; Parker & Thachil, 2018). Tissue plasminogen activator, urokinase, anti-streptokinase, and streptokinase, etc. are all examples of thrombolytic drugs. The primary purpose of these components is clot dissolution. (Ali et al, 2014). To be most effective, thrombolytic medicines need to be potent, administered in large doses, and directed just at fibrin (Verstraete, 2000). The extensive research and development of thrombolytic, antithrombotic, antiplatelet, and anticoagulant agents derived from natural constituents, such as those found in a wide variety of animal and plant species, has opened up promising new avenues for the development of effective alternative thrombolytic therapy (Chen et al. 2015).

Arthritis, one of the autoimmune diseases, can be diagnosed when the three symptoms of pain, stiffness, and swelling appear together. Inflammation of the synovial joint, brought on by an immunological response, is the cause. One out of every five persons over the age of 60 suffers from arthritis (Murugananthan & Mohan, 2013). NSAIDs, sulfasalazine, D-penicillamine, cyclophosphamide, cyclosporine, methotrexate,

azathioprine, glucocorticoids, anakinra, etanercept, abatacept, and infliximab, among others, have been used to treat arthritis (Mazumder et al, 2012; Rader & Kathiresan, 2018; Rajkapoor et al. 2007). But there are some risks, such as gastrointestinal ulcers, stomatitis, problems with the heart and blood vessels, pulmonary toxicity, myelosuppression, hematologic nephrotoxicity, hepatic fibrosis, cirrhosis, diarrhea, and local reactions at the injection site (Chitme & Patel, 2009). Because of this, it is very important to make new anti-arthritis medicines from medicinal plants that are cheap and have few side effects.

Secondary metabolites like alkaloids, saponins, terpenoids, tannins, flavonoids, glycosides, inulin, steroids, terpenoids, phlorotannin, phenols, essential oils, resins, naphthoquinone, etc. give plants their strong pharmacological properties. As treatments for diseases, these substances work by changing the way the body works Hamidi et al, 2014; Saadabi, 2006). Because people don't know enough about safe dosages and because some plants have toxic byproducts, using local plants as a source of medicine may have some bad effects (Olowa & Nuñeza, 2013).

In the current study, the in-vitro antimicrobial, thrombolytic, protein denaturation, and cytotoxic effects of these plant orchid extracts were evaluated because there is no scientific evidence of the antimicrobial, thrombolytic, protein denaturation, and cytotoxic potential of ethanolic extract of *R. retusa.*

MATERIALS AND METHODS

Materials

The chemicals used in this investigation were all purchased from Sigma-Aldrich and all of them were of analytical reagent quality. Moreover, the chemicals were used without further purification.

Plant extract preparation

Dr. Anjuman Ara Begum, Principal Scientific Officer, Bangladesh Agriculture Research Institute, Gazipur, Bangladesh, identified *R. retusa (L.) Blume,* which was grown at the Department of Agriculture's Floriculture Division, Horticulture Research Centre at the Bangladesh Agriculture Research Institute (Gazipur, Bangladesh). Following an ethanol wash and 15 days of drying at room temperature (22 ± 0.5 °C), the plants were powdered. Then 250 g of ground plant parts were soaked in 1.0 L ethanol for seven days using screw capped reagent bottles. After collecting filtrates with a Buchner funnel and Whatman filter paper (no. 11), we concentrated them using a rotary evaporator (working at temperatures and pressures below 40 °C). Then, a vortex mixer was used to vigorously mix a suspension of several plant extracts in ethanol until the appropriate concentration was reached.

Phytochemical screening

The presence of *p*rimary (carbohydrates and proteins) and secondary (alkaloids, flavonoids, steroids, terpenoids, total phenols, glycosides, and tannins) metabolites were tested for in the extracts in accordance with the protocols described in Egbuna et al., 2018, Tona (1998) and Harbone (1984).,

Test for Alkaloids (Mayer's Test)

0.2 gm of each extract was taken in test tubes and boiled using a steam bath after adding 5 ml of 2 % HCl. The portions were then sorted by filtering out unnecessary ones. The next step involved placing filtrates (1 ml each) in separate test tubes and Mayer's reagent (2 drops in each one) adding. Alkaloids can be seen in the form of precipitation if it has a milky white hue.

Test for Flavonoids

0.2 gm of each extract and 10 ml ethyl acetate were taken in test tubes. A water bath was then used to heat the test tubes to 100 °C for 3 minutes. After that, filters were used to separate the filtrates from each extract, and these samples were analyzed further.

(1) Ammonium test

The filtrates (4 ml) and diluted ammonia (1 ml, 1 %) were vigorously shaken. The layers were eventually permitted to separate. As flavonoids are responsible for giving an ammonia layer its characteristic yellow hue, their presence can be definitively established.

(2) Aluminum chloride test

$AlCl_3$ (1 ml of 1% solution) was added to 4 ml of each filtrate and allowed to settle for precipitation after vigorous shaking. The presence of flavonoids is supported by the production of yellowish-colored precipitation.

Test for Glycosides (Keller-Kiliani Test)

The extracts (0.5 g each) were dissolved in chloroform (2 ml). Then those were filtered, and each filtrate was assigned to separate test tubes. These filtrates were then dried up using evaporation. After the filtrates were dried, glacial acetic acid (1 cc) and $FeCl_3$ (3 drops of 5 % w/v) were added to each of the tubes. After that, 1 cc of fuming sulfuric acid was added to the test tubes carefully. To verify the existence of glycosides, a bluish-green hue must occur in the upper layer.

Test for Steroids

Extracts (0.2 g each) were dissolved in 2 ml of $CHCl_3$. Next, the tests detailed below were conducted.

(1) Salkowski test

Fuming H_2SO_4 (2 cc) was added to each test tube and shaken for several minutes. Steroid presence is unmistakably indicated by reddening of the chloroform layer, which happens when they are present.

(2) Liebermann-Burchard test

The test tubes were prepared by adding 10 drops of acetic anhydride and mixing them thoroughly. Then H_2SO_4 (2 ml, concentrated) was added to them. The presence of steroids is confirmed by the transient greenish coloration.

Test for Terpenoids

0.2 g of the extracts were taken in test tubes containing chloroform (2 ml) for dissolving followed by drying by evaporation. After that, the test tubes received H_2SO_4 (2 ml, concentrated), which was heated for about 2 minutes. Terpenoids are present when a bluish-gray color develops.

Test for Saponins (Froth Test)

The test tubes containing the extracts (0.1 g each) were vigorously shaken for 15 minutes after being diluted with 15 cc of distilled water. Saponins may be present, as indicated by the formation of a 1 cm layer.

Test for Phenols ($FeCl_3$ Test)

Extracts were heated for 5 minutes in 45% ethanol after 0.2 g was added to test tubes. These were then chilled and filtered. The filtrate (1 ml) was then combined with distilled water (5 ml), and 5 % $FeCl_3$ (3 drops). The presence of phenols is indicated by a momentary greenish-to-black hue.

Test for Tannins (Gelatin Test)

Extracts (0.2 g each), 1 % gelatin (0.3 ml) and 10 % NaCl (a few drops) and 0.2 g of extracts were placed in test tubes. The presence of tannins is indicated by white precipitate.

Test for Carbohydrates (Molisch's Test)

Extracts (0.1 g each) were dissolved in distilled water (5 ml) in separated test tubes with vigorous shaking followed by filtration. To the filtrates, Molisch reagent (5 drops) was added with vigorous shaking again.

The appearance of a ring, brownish in hue, by the addition of conc. H_2SO_4 (1 cc), at the interface indicates carbohydrates exist.

Test for Proteins

(1) Biuret's Test

A total of 3 cc of the extracts, 1 ml $CuSO_4$ (1% w/v), and 1 ml NaOH (4% w/v) were placed in test tubes. An indication of protein content is a shift in color from blue to purple.

(2) Xanthoproteic Test

Extracts (3 cc each) and concentrated sulfuric acid (1 ml each) were placed in test tubes. Firstly, white precipitate forms, which then turns yellow on boiling; orange precipitation forms after the addition of 1 ml ammonium hydroxide, confirming the presence of proteins, e.g., tyrosine and tryptophan.

Ultraviolet and infrared analyses

The UV-visible analysis was utilized to find out the bond characteristics of the plants extracts and was performed at a range of 200-800 nm wavelength at the same condition by using UV- 2401PC, Shimadzu UV spectrophotometer. Fourier transform infrared spectroscopy – attenuated total reflectance (FTIR–ATR) analyses of the plant extract samples were availed for investigating the functional groups present in the plant extracts and was accomplished by using the IRAffinity-1S (Shimadzu, Japan) spectrometer.

Antimicrobial Screening

Plant extracts were tested for their antibacterial and antifungal effects using the disc diffusion method (CLSI guideline), as detailed by Daoud et al., 2015; Bauer et al., 1966. 5 gram-positive bacterial strains (*Staphylococcus aureus* ATCC-25923 , *Sarcina lutea* ATCC-9341, *Bacillus subtilis* ATCC-6633, *Bacillus cereus* ATCC-11778, and *Bacillus megaterium* ATCC-14581), 8 gram-negative bacterial strains (*Escherichia coli* ATCC-25922, *Pseudomonas aeruginosa* ATCC-49189, *Vibrio parahaemolyticus* ATCC-17802, *Salmonella typhi* ATCC-14028, *Shigella boydii* ATCC-9207, *Vibrio mimicus* ATCC-33653, *Salmonella paratyphi* ATCC-9150 and *Shigella dysenteriae* ATCC-13313), and 3 fungi stains (*Aspergillus niger* ATCC-16404, *Candida albicans* ATCC-10231, and *Saccharomyces cerevisiae* ATCC-9763) were collected from the Pharmacy Department, University of Dhaka, Bangladesh. Those stains were used as pure culture and maintained on the nutrient agar medium (Oxoid, UK). The 400 µg of each extract, dissolved in methanol, was applied to dried, sterile Matricel filter paper discs (6.0 mm in diameter, BBL, USA). Then those were dried to evaporate the residual methanol. The positive control was ciprofloxacin (5 µg/disc), and the negative control was a blank disc. The test bacteria and fungus were evenly seeded in nutrient agar medium in petri dishes before sample discs, dried blank discs, and standard antibiotic discs were added on top. These were then stored in a refrigerator at 4°C for roughly 24 hours. Petri dishes were inverted so that the bottom faced up substances could easily diffuse from the discs into the agar substrate. After being turned over, the petri dishes were incubated for 24 hours at 37°C in an incubator. The diameter of the inhibition zone was used as a measure of their efficacy, and the value was reported in millimetres (Raju et al. 2013; Wilkinson, 2006). This showed how well they stopped microorganisms from growing.

Toxicity Test by Brine Shrimp Lethality Bioassay

Ethanolic extracts of *R. retusa* were evaluated for their cytotoxic effects using a brine shrimp lethality test (Meyer et al., 1982; McLaughlin et al., 1998). The preparation of the experiment solutions involved dissolving 0.8 mg of the extracts in DMSO, followed by the serial dilution method with simulated seawater to produce solutions with different concentrations (400, 200, 100, 50, 25, 12, 5, 6, 2.5, 3.13, 1.56, 0.78 and 0.39 mg/ml). the standard Vincristine sulfate was prepared by dissolving 0.08mg in DMSO, followed by the serial dilution method with simulated seawater to produce solutions with different concentrations (40, 20, 10, 5, 2.5, 1.25, 0.63, 0.31, 0.16 and 0.08 mg/ml). The solutions were put into the designated test tubes after ten live nauplii were placed in 5 cc of saltwater. A 3x magnification was used to examine the test tubes, and the quantity of brine shrimp nauplii that persisted after 24 hours was recorded. The absence of controlled forward motion during 30 seconds of observation was defined as mortality (Middleton et al., 2005).

The percentage of lethality was calculated for each extract and control from this data. The positive and negative controls were vincristine sulphate and DMSO, respectively. Logarithmic concentration versus fatality rate curves were used to calculate LC50 values in Microsoft Excel Plus 2016 (Ogbole et al., 2020). After 24 hours, the number of brine shrimp nauplii that were still alive was counted, and the following equation 1 was used to calculate the percentage of deaths: (Meyer et al., 1982; Apu et al., 2010)

Equation 1:

Percent of mortality = (no. of dead nauplii after 24h/initial no. of live nauplii) × 100

In-vitro Thrombolytic Activity

Thrombolytic properties were investigated according to the method disclosed in Prasad et al., 2007 and Prasad et al., 2006. Lyophilized streptokinase (SK) in a commercially available 1.5 million international units (IU) vial was combined with 5 ml of sterile distilled water to create a stock solution. The chemicals used in this investigation were all of the analytical reagent quality. Twenty healthy volunteers gave 60 ml of blood; none of them smoked, used oral contraceptives, or were on anticoagulant therapy. For the coagulation of collected blood in the falcon, tubes were given approximately 15 minutes. After that, a centrifuge (Remi- R-83 A model) was used to spin the blood at a velocity of 2000 rpm for 1 minute, separating the serum from the rest of the blood. After carefully removing the serum without disturbing the clot, the weight of the clot in each falcon tube was recalculated. Following proper labeling, 100 µl of various plant extracts, 100 ml of streptokinase (for the positive control), or 100 µl of distilled water (for the negative control) were placed in 100 µl falcon tubes. Once the clot lysis was being monitored, the falcon tubes were incubated for 90 minutes in an incubator operated at 37 °C and 24 hours. After carefully removing the dissolved clot, the gravimetric method was used to calculate the percentage of clot lysis after 90 minutes and 24 hours of incubation. This test was done in triplicate, and the following equation 2 was used to calculate the percentage of clot lysis.

Equation 2:

% of Clot Lysis = (Weight of Released Clot/ Weight of Clot) × 100

In-vitro Method of Egg Albumin Denaturation

The method described in Ajithkumar et al., 2006 was used to keep track of arthritic activities. In a 1000 ml volumetric flask, 8 g of NaCl, 0.2 g of KCl, 1.44 g of Na_2HPO_4, 0.24 g of KH_2PO_4, and 800 ml distilled water were mixed correctly. Then the solution's pH was adjusted to 6.4 with 1N HCl and filled with the needed amount of distilled water, making up the mark of 1000 ml. An anti-arthritic activity test used a total of 5 ml of the reaction mixture containing egg albumin (0.2 ml), PBS of pH 6.4 (2.8 ml) and different concentration of 2 ml of plant extracts (66.5, 125, 250, 500 ppm). A control sample of 2 ml of distilled water was also given. Diclofenac sodium (66.5, 125, 250, 500 ppm) in 2 ml dilutions was used as the gold standard. Then, for 15 minutes, the mixes were placed in a BOD incubator set to 37±2 °C. Then, the concoctions were placed in a water bath and cooked to 70 °C for five minutes. After cooling, the solutions' absorbance was assessed at 660 nm with a UV-Vis spectrophotometer. The use of phosphate-buffered saline served as a placebo. The tests were done in triplicate.

To calculate the percentage inhibition of protein denaturation, the equation 3 was used:

Equation 3

% Inhibition of Protein Denaturation = $100 \times [Vt / V_o - 1]$

Where, Vt = Absorbance of Test Sample, V_o = Absorbance of Control

Statistical Analysis

With the help of ANOVA and Dunnett's test, the information is represented as the standard deviation from three different analyses. The values of $P < 0.001$ and $P < 0.05$ were regarded as significant.

RESULTS AND DISCUSSION

Preliminary Phytochemical Screening

Prior to the qualitative evaluation of the phytochemicals present, a preliminary phytochemical screening was carried out on the ethanolic extracts of the various parts of *R. retusa using* standard techniques. The table below **(Table 1)** contains a list of the phytochemicals that were found.

Table 1. Presence of different phytochemicals in ethanolic extracts of different parts of *R. retusa.*

Sample Name	Chemical Group Test									
	Saponin	Steroid	Glycoside	Terpenoid	Carbohydrate	Phenol	Flavonoid	Tannin	Alkaloid	Protein
Leaf	-	-	+	+	+	+	+	-	+	-
Stem	-	+	-	+	+	+	+	+	+	-
Root	+	+	+	+	+	+	-	-	+	+

(+) presence, (-) absence.

Spectroscopic analysis

The identification of phytoconstituents found in the plant extracts was accomplished using UV-VIS analysis. The compounds with σ -bonds, π -bonds and lone pair of electrons, chromophores, and aromatic rings were identified using the UV-visible spectra. Table 2 shows the observed absorption bands for the extracts of the plant.

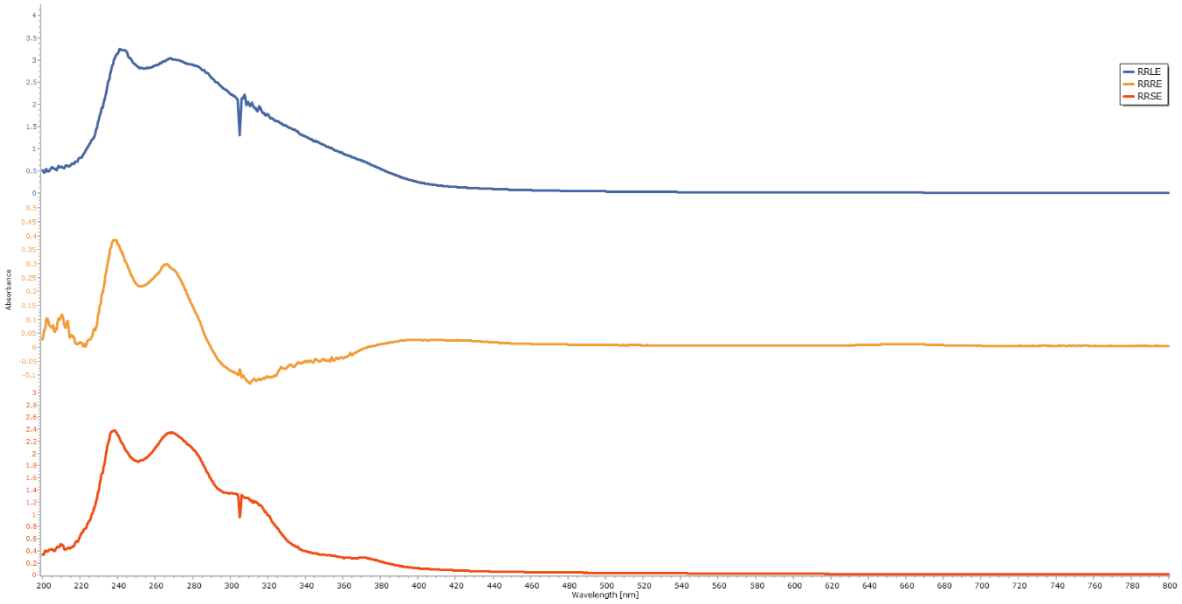

Figure 1. UV-visible spectra of *R. retusa* extracts (Leaf in Blue color; Root in Yellow color; Stem in Red color)

Table 2: UV-visible peaks of *R. retusa* extracts

Plant name, part of the plant and extracted medium	Wavelength (nm)
Leaf	241.4, 268.2, 307
Stem	238.2, 268.7
Root	210.4, 239.2, 266, 268.7

In the UV-VIS spectra the appearance of one or more peaks in the region from 200 to 400 nm is a clear indication of the presence of unsaturated groups and heteroatoms such as S, N, O (Kavitha & Nandagopalan, 2021). Uv -visible spectra of *R. retusa* / Leaf /Ethanol *and R. retusa* /Stem/Ethanol extracts revealed peaks at 268.2 nm, 268.7 nm respectively which indicated the presence of phenol and exhibited peaks at 241.4 nm and 238.2 nm individually indicated the presence of flavonoid (Tošović et al., 2015) and steroid. Peaks found at 210.4nm, 239.2 nm and 266 nm of *R. retusa* / Root /Ethanol extract indicated the existence of saponin, steroid and protein respectively.

Based on the peak value in the region of infrared radiation of the FTIR spectrum, the functional group of the active components was identified. In table 3 FTIR peaks for *R. retusa* extracts were reported.

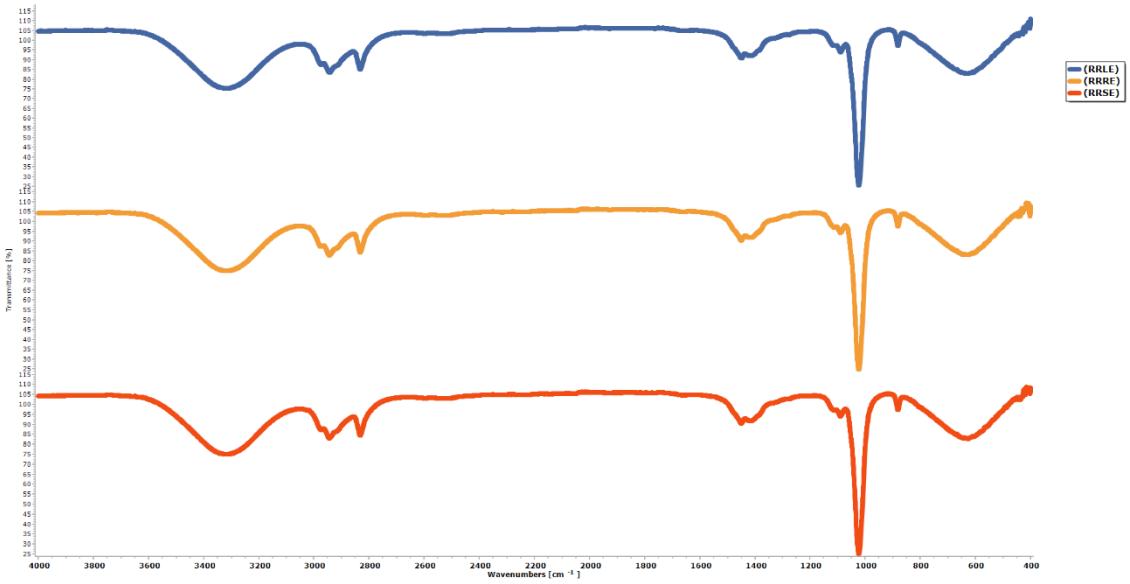

Figure 2. FTIR spectrum of *R. retusa plant* extracts (Leaf in Blue color; Root in Yellow color; Stem in Red color.

Table 3: Main FTIR absorption peaks of *R. retusa plant* extracts.

Plant name, part of the plant and extracted medium	Wavenumbers (cm^{-1})
Leaf	881.1,1023,1449,1652,2832,2943,3321
Root	881.1,1023,1089,1653,1449,2832,2943,3319
Stem	881.1,1023,1089,1654,1449,2832,2943,3321

Figure 2 shows that, the *R. retusa* plant extracts of three different parts such as leaf, root and stem revealed peak at 881 cm^{-1} that corresponded to the $–CH_2$-rocking group which showed the existence of carbohydrate. Three parts of this plant extract showed peak at 1023 cm^{-1} which corresponded to the -C-O- group that revealed the presence of flavonoids and cardiac glycosides in leaf part, flavonoids in stem part and cardiac glycosides in root part. Three parts of *R. retusa* presented peaks at 1449 cm^{-1} and 1652 cm^{-1} to 1654 cm^{-1} which represented the $–CH_3$ group and -C=C- group, peak at 2943 cm^{-1} was responsible for -C-H- stretching of alkane indicated the presence of terpenoids. All three parts of this plant extracts exhibited peaks at 1089 cm^{-1} and 2832 cm^{-1} which denoted secondary -OH- and –N-H- stretching that directed the existence of alkaloids. *R. retusa plant* extracts of two different parts also displayed peaks at 3321 cm^{-1} that corresponded to the -OH- group which indicated the presence of flavonoids in leaf and stem part (Noh et al., 2017).

Antimicrobial Activity

Antimicrobial drugs are substances, either naturally occurring or synthesized, that prevent or eradicate the growth of microorganisms such as bacteria, fungi, helminths, protozoa, and viruses. Depending on how they work against microbes, antimicrobial. The main classes are agents that stop bacteria from building cell walls, depolarize cell membranes, stop protein synthesis, stop nucleic acid synthesis, and stop metabolic processes are the primary classes. Some basic phytoconstituents are responsible for antimicrobial activity, e.g., alkaloids, flavonoids, glycosides, etc. (Doughati et al., 2006; Kiani, 2019; Yuan et al., 2021; Nugraha et al., 2020). The effectiveness of extracts against every type of microorganism varied. The extracts' zone of inhibition values against all microorganisms had a diameter of about ≥8 mm. For medicinal plants, an active zone of inhibition value is one that is ≥8 mm. The diameter zone of inhibition varies from 8 - 11 mm. The diameter of the zones of inhibition distinguishes four levels of antimicrobial activity: very active (≥20 mm), less active (≥8 to ≤12 mm), moderately active (>12 to <20 mm), and inactive (<8 mm) (Suba et al., 2022). Orchid extracts have been shown to suppress growth of multiple bacterial strains, demonstrating their broad spectrum of activity. Similar results have been seen with other plant extracts (Abate et al., 2021; Sieberi et al., 2020). Disc diffusion method was used for antimicrobial activity testing. All the plant's extract exhibited good antimicrobial properties against the microorganisms **(Table 4)**.

Table 4. Antimicrobial properties of ethanolic extracts (400 µg/disk) of different parts of *R. retusa*.

Microbiological Test				
Name of Microorganism	**Diameter of the zones of inhibition (mm)**			
	Ciprofloxacin (5 µg/disk**)**	**Leaf extract**	**Stem extract**	**Root extract**
Gram Positive Bacteria				
Bacillus cereus	45	-	10	9
Bacillus megaterium	45	-	10	9
Bacillius subtilis	45	-	10	8
Staphylococcus aureus	46	-	11	9
Sarcina lutea	45	-	11	8
Gram Negative Bacteria				
Salmonella paratyphi	43	-	10	8
Salmonella typhi	45	-	11	8
Vibrio parahaemolyticus	45	-	10	9
Vibrio mimicus	46	-	10	9
Escherichia coli	44	-	10	10
Shigella dysenteriae	46	-	10	9
Pseudomonas aeruginosa	45	-	10	9
Shigella boydii	45	-	10	8
Fungi				
Saccharomyces cerevisiae	45	-	11	8
Candida albicans	45	-	10	9
Aspergillus niger	45	-	10	9

Though *R. retusa* /Leaf/EtOH extract contained glycosides, flavonoids and alkaloids, it still did not show any activity against any microorganism. On the other hand, our study revealed that ethanol extract of the stem and root of *R. retusa* exhibited activity against all microorganisms. The hexane, chloroform and

methanol extract of leaf of *R. retusa showed* antifungal activity against *Macrophomina phaseolina*, *Sclerotium rolfsii*, *Fusarium oxysporium*, *Altarnaria altarnata*, Rhizoctonia solani, Fusarium solani etc. assessed by Radhika et al., 2013. Bhattacharjee & Islam, 2015 reported that extract of the whole plant of *R. retusa* had antimicrobial efficiency against *Staphylococcus aureus*, *Bacillus subtilis*, *Vibrio cholerae*, *Escherichia coli*, *Klebsiella pneumonia etc.* and antifungi efficiency against *Penicillium sp., Rhizopus sp., Aspergillus niger* etc. Diethyl ether, n-butanol and water extracts of the leaf, stem and root of *R. retusa* showed activity against *Staphylococcus aureus, Enterococcus sp, Serratia sp.*, *Acinetobacter sp.* and *Escherichia coli observed by* Bhatnagar et al., 2017.
The active compounds in orchids that were the subject of this study need to be better understood and tested before they can be used in conventional and traditional medicine. Also, all the tested extracts showed different levels of antimicrobial properties that fight infections caused by microorganisms except leaf part.

Brine Shrimp Lethality Bioassay

Their LC_{50} values can evaluate the toxicity of plant extracts. If LC_{50} values are lower than 1000 µg/ml, it is considered cytotoxic (Meyer et al., 1982). Radhika et al., 2013 reported that LC_{50} values of hexane, chloroform and methanol extract of leaf of *R. retusa showed* 86.41 2 µg /ml, 30.50 µg /ml, 18.52 µg /ml respectively. Whereas, in this study LC_{50} values of leaf, stem, & root of *R. retusa* were ten times less toxic with the standard vincristine sulfate LC50 at 0.84 µg/ml (Table 5). Despite having lower activity than vincristine sulphate, those leaf, stem, & root of *R. retusa* extract can be considered a candidate for further in vitro cytotoxicity tests.

Table 5. Brine shrimp lethality bioassay of ethanolic extracts of different parts of *R. retusa*

Sample Name	LC_{50} (µg/ml)
***R. retusa* /Leaf/EtOH**	6.711
***R. retusa* /Stem/EtOH**	8.391
***R. retusa* /Root/EtOH**	8.488
Vincristine sulphate (Standard)	0.84

Some studies confirmed that cytotoxic activity is responsible for secondary metabolites such as phenolics, flavonoids, terpenoids, etc. (Coe et al., 2010; Vila et al., 2004; Rahman et al., 2022; Ren et al., 2003; Tungmunnithum et al., 2018; Aryal et al., 2019;). The absence of flavonoids in the root extract may explain why the root of *R. retusa* is less potent than the leaf and stem part.

In-Vitro Thrombolytic Activity

Thrombolysis, also called thrombolytic therapy, is used to break up large blood clots, get blood flowing again, and protect organs and tissues from damage. Blood clots are dislodged by thrombolytic medications by activating plasminogen, which splits plasmin into two pieces. Plasmin is a proteolytic enzyme that has the potential to dissolve the cross-links that fibrin molecules form, which give blood clots their structural stability.

According to some studies, the diverse composition of plant extracts' phytoconstituents, which includes flavonoids, alkaloids, terpenoids, and tannins, is likely what causes thrombolytic activity (Dwivedi, 2007; Bhowmick et al., 2014). The presence of terpenoids, flavonoids and alkaloids may be responsible for exerting this effect by the leaf extracts of *R. retusa.* The stem extract of *R. retusa* possessed all the phytochemicals e.g., terpenoids, flavonoids, alkaloids and tannins responsible for excreting thrombolytic activity. The root extracts of *R. retusa showed* that activity may be due to the presence of terpenoids and alkaloids.

Figure 3 displays the findings of a comparison between the ethanolic extracts of the three separate portions of the *R. retusa (*leaf, stem and root), the positive (streptokinase), and the negative (water) controls in terms of the percentage of clot lysis.

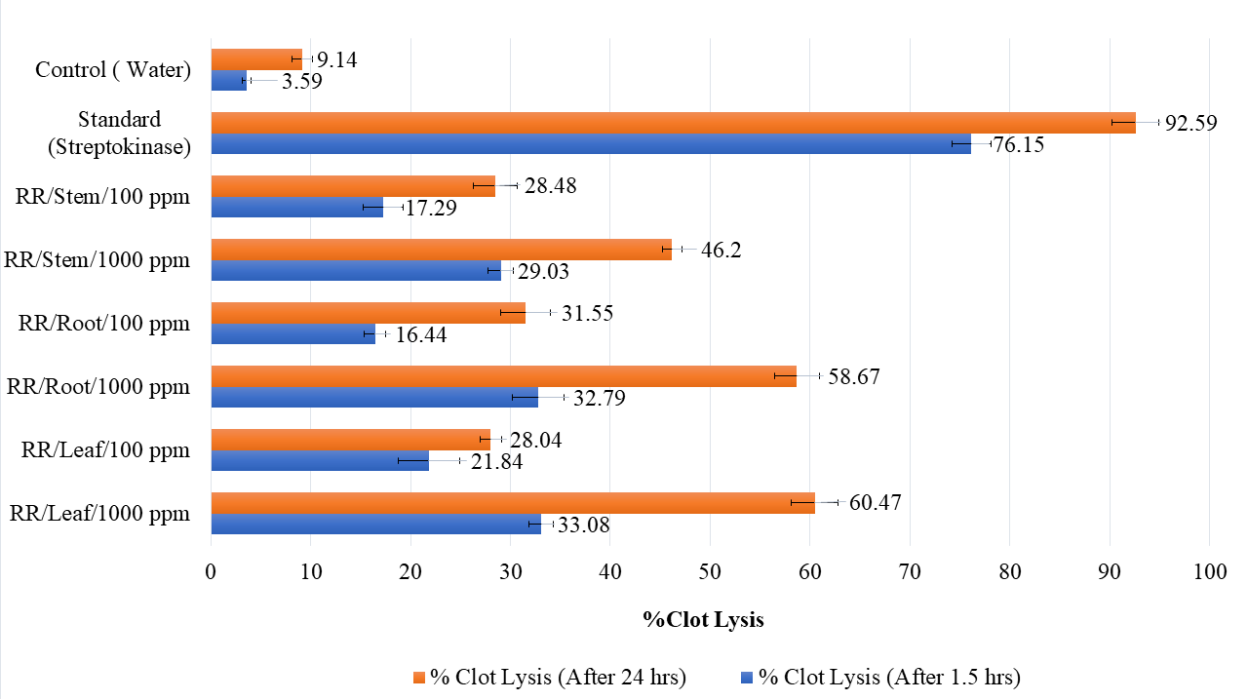

Figure 3. Thrombolytic properties of ethanolic extracts of different parts of *R. retusa* compared with streptokinase (reference).

In-Vitro Protein Denaturation Activity

Antiarthritic medicine treats or prevents arthritic symptoms such as joint pain and stiffness. Depending on the type of anti-arthritis drug, it may help with pain, reduce inflammation, or weaken the immune system. One of the causes of rheumatoid arthritis is protein denaturation. Denaturation of proteins may contribute to the production of autoantigens in some arthritic conditions. Changes to electrostatic, hydrogen, hydrophobic, and disulphide bonds are likely part of the denaturation pathway (Arya et al., 2014). **Figure 4** represents the data on the protein denaturation of leaf, stem and root of *R. retusa* extracts.

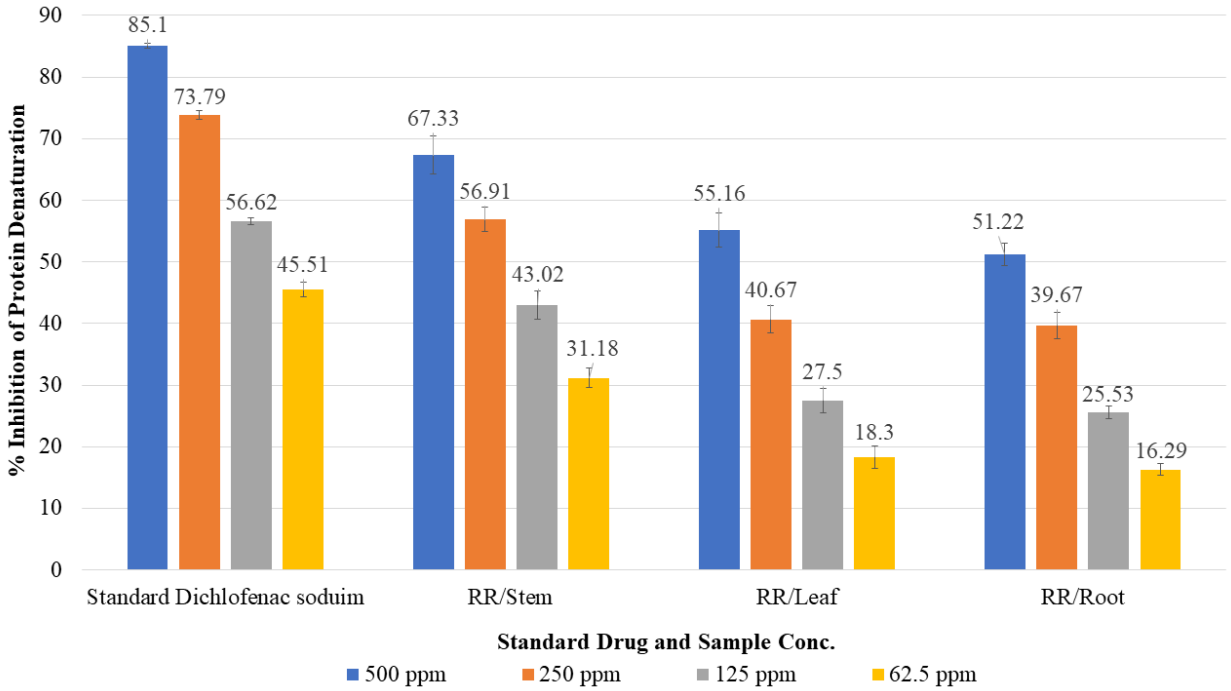

Figure 4: Protein denaturation activity of ethanolic extracts of leaf, stem and root of *R. retusa* compared to standard (Diclofenac Sodium).

Some phytochemicals -such as flavonoids- have similar anti-rheumatic, hypotensive, anti-infective, diuretic, and antiradical effects (Hossain et al., 2013; Kamtekar et al., 2014). The leaf and stem of *R. retusa* contains flavonoids, therefore being the potential active principles.

CONCLUSIONS

Stem ethanolic extracts from *R. retusa* shown the best antibacterial and protein denaturation activities, while leaf extracts were more thrombolytic. The extracts shown toxicity for *A. salina* (LC50) in the 6.7-8.5 µg/ml range. Our research will help to provide evidence for some of the traditional uses of this medicinal plant. Although our research shows that these extracts have low to moderate activities, further bio isolation efforts may yield more potent antibacterial, antifungal, anticancer, anti-trauma, thrombolytic, and antiarthritic compounds.

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Conflict of interest

The authors have no conflict of interest regarding this article.

Authors contributions

Md. Saifur Rahaman: Validation, Investigation, Writing-Original Draft, Visualization; Md. Samsur Rahaman: Conceptualization, Methodology, Resources, Data curation; Shah Md. Marzuk Hasnine: Formal Analysis, Writing-Original Draft + Review & Editing, Visualization; Md. Abdul Quaiyum Bhuiyan: Writing - Review & Editing; Visualization; Salma Sultana: Formal Analysis, Resources, Visualization; Mohammad Shahriar Kabir, Md. Abdul Bari, and Md. Ismail Hossain: Formal Analysis, Visualization; Mubarak Ahmad Khan: Specifically critical review, commentary or revision – including pre-or post-publication stages

Supplementary Materials

Datasets generated for and/or utilized in the current study will be made accessible by the relevant author upon reasonable request.

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