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ASSESSMENT OF AERIDES ODORATA'S ANTIMICROBIAL, CYTOTOXIC, THROMBOLYTIC, AND ANTIARTHRITIC PROPERTIES: A COMPARATIVE IN VITRO ANALYSIS OF ITS DIFFERENT PARTS

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ABSTRACT

Historically, thrombosis, arthritis, cancer, wounds, and infections have all been treated with various herbal preparations. The aim of this work was to evaluate the antibacterial, thrombolytic, cytotoxic, and protein denaturation activities of ethanolic extracts from various Aerides odorata (AO) sections in vitro. Fourteen distinct microorganisms were used in the antimicrobial study; for orchid samples, ciprofloxacin was the most effective agent. AO/Leaf was the only one to exhibit antimicrobial potential; AO/Stem and AO/Root did not. Based on their LC_{50} values, we evaluated the cytotoxicity of plant extracts. AO/Root extract had the greatest cytotoxic activity when compared to AO/Leaf and AO/Stem extracts. Two incubation times (1.5 hours and 24 hours) and two concentrations (one thousand and one hundred ppm) were used to assess the thrombolytic activity of the extracts. The results obtained with regular streptokinase were then compared with the former. Compared to AO/Leaf and AO/Stem extract, it was discovered that AO/Root extract exhibited more thrombolytic activity. Furthermore, the herbal extract's protein denaturation was investigated at four different concentrations, and the results were contrasted with those of Diclofenac sodium. In relation to other sections, the maximum protein denaturation values for AO/Stem extract were determined to be 60.07±2.33%, 47.22±1.43%, 35.05±2.07 %, and 23.74±1.66% for 500–62.5 ppm, respectively.

KEYWORDS: AERIDES ODORATA; TRADITIONAL HERBAL MEDICINE; ANTIMICROBIAL ACTIVITIES; BRINE SHRIMP LETHALITY BIOASSAY; ANTI-ARTHRITIC; THROMBOLYTIC.

INTRODUCTION

Herbal medicine has been used to treat a variety of illnesses for thousands of years (Mintah et al., 2019). In addition to utilizing centuries of medical knowledge, these remedies were developed by doctors still in practice (Lemonnier et al., 2017). Medicinal plants are nature's gift for treating and preventing diseases (Mintah et al., 2019). It is important to value orchids as ornamental plants in society. Also, orchids are known for their medicinal use (Li et al., 2021).

Aerides odorata (AO) is a plant in the Orchidaceae (Singh Jalal & Jayanthi, 2018). Plants that grow easily and freely provide pendant sprays of flowers that resemble foxtails. Numerous 1" to 1.5" cream to white flowers with a deeper purple/magenta lip are used to embellish them. The petals and sepals have splashes of different shades of purple and pink, and sometimes there are pure pink varieties. It has been used in leaf juice, often in traditional medicine for healing boils and nose and ear diseases, and the ground fruits are used to treat wounds. In vitro phytochemical investigation of the methanolic extract of leaf is attributed to Alkaloids, Glycosides, Flavonoids, Tannins, Saponins, Terpenoids, Steroids, Anthraquinone, Quinine and Coumarin (Akter, Huda, & Hoque, 2018). Ethanolic, Methanolic, Petroleum ether and Chloroform extract of the leaf revealed antibacterial activity, and leaf water extract showed antifungal activity (Hoque, Khaleda, & Al-Forkan, 2016). Different fractions (Ethyl Acetate and Methanol) of Leaf extract possessed anticancer activity due to Alkaloids, Glycosides, Flavonoids, Terpenoids, Steroids, Phenolic compounds, Quinone and Coumarin (Katta, Rampilla, & Khasim, 2019). The antimicrobial efficacy of water and acetone extract from the leaf showed a potential inhibitor of antibiotic-resistant strains of Escherichia coli (Paul, Chowdhury, Nath, & Bhattacharjee, 2013).

Overuse and misuse of antibiotics increases antimicrobial resistance worldwide (particularly in developing countries). Antibiotic resistance causes millions of deaths every year (Ayukekbong, Ntemgwa, & Atabe, 2017). It's time to address antimicrobial resistance. Many medicinal plants contain antimicrobial compounds that can effectively treat microbial infections (Stan et al., 2021). It is possible to obtain a wide variety of pharmaceuticals from medicinal plants (Salmerón-Manzano, Garrido-Cardenas, & Manzano-Agugliaro, 2020). The effectiveness of old and newly introduced antimicrobial agents is limited, for example, short life expectancy, higher side effects, etc. (Mohsen, Dickinson, & Somayaji, 2020). In light of rapidly spreading resistant clinical isolates, it is crucial to find new antimicrobial agents.

There are several types of blood clots that develop in veins of the arms, pelvis, thighs, lower legs, etc. Cardiovascular disease is caused by it. Without treatment, this can result in permanent disabilities or even death (Naringrekar, Sun, Ko, & Rodgers, 2019; Seifi, Dengler, Martinez, & Godoy, 2018). Heart disease was the leading cause of death worldwide in 2015, with over 17.9 million deaths; this number is projected to reach nearly 23.6 million by 2030 (Benjamin et al., 2018). There are many examples of thrombolytic drugs, such as tissue plasminogen activator, urokinase, anti-streptokinase, and streptokinase. Their primary purpose is to dissolve clots (Ali et al., 2014). It is important that thrombolytic drugs be potent, administered in large doses, and targeted at fibrin only in order to be most effective (Verstraete, 2000). It has been demonstrated that extensive research and development has been carried out on thrombolytic, antithrombotic, antiplatelet, and anticoagulant agents derived from natural constituents, such as those found in a wide range of animals and plants, in order to identify new avenues for developing alternative thrombolytic treatments (Chen et al., 2015).

One of the autoimmune diseases, arthritis, can be diagnosed when the three symptoms of pain, stiffness, and swelling occur at the same time. An immune response causes inflammation of the synovial joint. Arthritis affects one out of every five adults over 60 (Murugananthan & Mohan, 2013). A number of medications have been used to treat arthritis, including NSAIDs, sulfasalazine, D-penicillamine, cyclophosphamide, cyclosporine, methotrexate, azathioprine, glucocorticoids, etanercept, abatacept, and infliximab (Jameson et al., 2018; Mazumder, Mondal, Sasmal, Arulmozhi, & Rathinavelusamy, 2012; Rajkapoor et al., 2007).

Although there are some risks, including gastrointestinal ulcers, stomatitis, breathing issues, myelosuppression, hematologic nephrotoxicity, hepatic fibrosis, cirrhosis, diarrhea, and local reactions at injection sites (Chitme & Patel, 2009). As a consequence, it is extremely important to develop cheap and effective anti-arthritis medicines from medicinal plants.

In addition to alkaloids, saponins, terpenoids, tannins, flavonoids, glycosides, inulin, steroids, terpenoids, phlorotannins, phenols, essential oils, resins, naphthoquinone, etc., plants contain a wide range of secondary metabolites. When these substances are used as treatments for diseases, they alter the way the body functions (Hamidi, Jovanova, & Panovska, 2014; Saadabi, 2006). Using local plants as a source of medicine may have some negative effects because of a lack of knowledge about safe dosages and toxic byproducts in some plants (Olowa & Nuñeza, 2013).

As there is no scientific evidence to support the antimicrobial, thrombolytic, antiarthritic, and cytotoxic effects of ethanolic extract of Aerides Odorata, the present study evaluated the in-vitro antimicrobial, thrombolytic, antiarthritic, and cytotoxic effects of this plant orchid extracts.

MATERIALS AND METHODS

Materials

Sigma-Aldrich provided all of the chemicals used in this investigation, and they were of analytical reagent quality. Also, no further purification of the chemicals was conducted.

Plant extract preparation

The collection of AO, was made at the Horticulture Division, Bangladesh Agriculture Research Institute, Gazipur, Bangladesh. Plants were powdered after being washed with ethanol and dried at (22 ± 0.5 °C), for 15 days. After grinding the plant parts, 250 g were soaked in 1.0 L ethanol for seven days in screw-capped reagent bottles. Using a Buchner funnel and Whatman filter paper (no. 11), we collected filtrates and concentrated them in a rotary evaporator (at temperatures and pressures below 40 °C). An ethanol suspension of several plant extracts was vigorously mixed with a vortex mixer until the appropriate concentration was obtained. Then, the extracts were stored at 4℃ for further experiment.

Phytochemical screening

To analyze AO, we followed protocols outlined in Trease & Evans (2009), Tona (1998), and Harbone (1998). It is noteworthy that extracts were tested for macronutrients and secondary metabolites (Evans, 2009; Harborne, 1998; Tona, Kambu, Ngimbi, Cimanga, & Vlietinck, 1998).

Test for Alkaloids (Mayer's Test)

Each extract was boiled in 5ml of 2 % HCl after adding 0.2 g in test tubes. Afterwards, unnecessary portions were filtered out. To proceed, separate test tubes were filled with filtrates (1 ml each) and Mayer's reagent (2 drops per tube) was added. Milky white hue precipitation indicates alkaloids.

Test for Flavonoids

Test tubes were filled with 0.2 g of each extract and 10 ml of ethyl acetate. For 3 minutes, the test tubes were heated in a water bath to 100 °C. Each extract was then filtered to separate the filtrates, and these samples were then analyzed.

Ammonium test

Shaking vigorously was conducted on the filtrates (4 mL) and the diluted ammonia (1 mL, 1%). After a while, the layers separated. Ammonia layers are characterized by their characteristic yellow hue because of flavonoids.

Aluminum chloride test

4 ml of each filtrate were added to 1 ml of $AlCl_3$ solution and vigorously shaken for precipitation. Yellowish precipitation indicates the presence of flavonoids.

Test for Glycosides (Keller-kiliani Test)

In order to dissolve the extracts (0.5 g each), chloroform was used (2 ml). Following that, each filtrate was separated into test tubes. Evaporation was then used to dry up these filtrates. Following the drying of the filtrates, glacial acetic acid (1 ml) and $FeCl_3$ (3 drops 5%) were added to each tube. To complete the experiment, 1 ml fuming sulfuric acid was carefully added to the test tubes. Upper layers with a bluish-green hue indicate glycosides.

Test for Steroids

In 2ml of $CHCl_3$, 0.2 grams of extract were dissolved. Following that, we conducted the tests outlined below.

Salkowski test

Each test tube was filled with fuming H_2SO_4 (2 ml) and shaken for a few minutes. There is no doubt that steroids are present when the chloroform layer reddens.

Liebermann-Burchard test

To prepare the test tubes, 10 drops of acetic anhydride were added and thoroughly mixed. They were then treated with concentrated H_2SO_4 (2 ml). Greenish coloration confirms the presence of steroids.

Test for Terpenoids

To dissolve extracts, 0.2 g of them were placed in test tubes containing chloroform (2 ml), and then dried by evaporation. H_2SO_4 (2 ml, concentrated) was then added to the tubes and heated for 2 minutes. Blueish-gray colors indicate the presence of terpenoids.

Test for Saponins (Froth Test)

Immediately after the extracts (0.01 g each) were diluted in 15 cc of distilled water, the test tubes containing the extracts were vigorously shaken for 15 minutes at room temperature. A 1 cm layer of saponins may be present.

Test for Phenols ($FeCl_3$ Test)

0.2 g of each extract was added to test tubes and heated for 5 minutes in 45% ethanol. A cooling process followed, and the filtrate was filtered. In the next step, 1 ml of the filtrate was mixed with distilled water (5 ml), and 5 % $FeCl_3$ (3 drops). A momentary greenish-black hue indicates the presence of phenols.

Test for Tannins (Gelatin Test)

Test tubes were filled with extracts (0.2 g each), 1 % gelatin (0.3 ml), 10 % NaCl (a few drops). Precipitates of white color indicate the presence of tannins.

Test for Carbohydrates (Molisch's Test)

Each extract was dissolved in distilled water (5 ml) in separate test tubes with vigorous shaking and then filtered. The filtrates were again vigorously shaken with Molisch reagent (5 drops). Carbohydrates can be detected by the appearance of a brown ring at the interface when continuous H_2SO_4 (1 ml) is added.

Test for Proteins

Biuret's Test

The extracts were placed in test tubes along with 1 ml $CuSO_4$ (1% w/v) and 1 ml NaOH (4% w/v). When protein is present, a color shift from blue to purple is observed.

Xanthoproteic Test

Each test tube was filled with extracts (3 cc) and concentrated sulfuric acid (1 ml). Initially, a white precipitate forms, then yellow precipitate forms after boiling. Once 1 ml ammonium hydroxide is added, an orange precipitate forms, which confirms the presence of proteins.

Instrumental Characterization

An IR Affinity-1S (Shimadzu, Japan) spectrometer was used to perform Fourier transform infrared spectroscopy – attenuated total reflectance (FTIR–ATR) analyses of plant extract samples in order to investigate the functional groups present in them. Using UV-2401PC, Shimadzu UV spectrophotometer, the UV-visible analysis was used to determine the bond characteristics of the plants extracts at a wavelength of 200-800 nm under the same conditions.

Antimicrobial Screening

In a study conducted by Daoud et al., 2015 and Bauer (1996) (Bauer, 1996; Daoud et al., 2019), plant extracts for antibacterial and antifungal effects were tested using the disc diffusion method (CLSI guideline). This study was conducted on five gram-positive bacteria strains (Staphylococcus aureus ATCC-25923 , Sarcina lutea ATCC-9341, Bacillus subtilis ATCC-6633, Bacillus cereus ATCC-11778, and Bacillus megaterium ATCC-14581), eight gram-negative bacteria (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 three fungi (Aspergillus niger ATCC-16404, Candida albicans ATCC-10231, and Saccharomyces cerevisiae ATCC-9763) were collected from the Pharmacy Department, University of Dhaka, Bangladesh. In order to test the effects of each extract, 400 µg of the methanol-dissolved extract were applied to dried, sterile Matricel filter paper discs (6.0 mm in diameter, BBL, USA). After that, the residual methanol was evaporated. A blank disc served as a negative control, and ciprofloxacin (5g/disc) served as a positive control. A nutrient agar medium was used to seed test bacteria and fungi in petri dishes before dried blank discs, antibiotic discs, and sample discs were added on top. The samples were then stored in a refrigerator at 4 °C for about 24 hours. The bottom of the petri dish was inverted to allow easy diffusion of substances from the discs into the agar matrix. An incubator was used to incubate the petri dishes at 37 °C for 24 hours after they had been turned over. As a measure of their efficacy, the diameter of the inhibition zone was measured in millimeters (Raju, Moghal, Dewan, Amin, & Billah, 2013; Wilkinson, 2006). As a result, microorganisms were unable to grow.

Cytotoxicity Test by Brine Shrimp Lethality Bioassay

A brine shrimp lethality test was used to evaluate the cytotoxic effects of ethanolic extracts of AO (McLaughlin, Rogers, & Anderson, 1998; Meyer et al., 1982). DMSO was used to dissolve 1 mg of the extracts, followed by serial dilution with simulated seawater to produce different concentration solutions (100, 50, 25, 12,5, 6,2.5, 3.13, 1.56, 0.78, 0.39, 0.19, and 0.09 mg/ml). After placing ten live nauplii in 5 cc of saltwater, the solutions were placed into the designated test tubes. Test tubes were examined with a 3x magnification, and brine shrimp nauplii were counted after 24 hours. Mortality is defined as an absence of controlled forward movement during 30 seconds of observation (Middleton et al., 2005). This data was used to calculate the percentage of lethality for each extract and control. A positive control was vincristine sulfate, and a negative control was DMSO. Microsoft Excel Plus 2016 was used to calculate LC50 values using logarithmic concentration versus fatality rate curves (Persoone, 1980). Numbers of brine shrimp nauplii that were still alive after 24 hours were counted, and the percentage of deaths was calculated (Apu et al., 2010; Meyer et al., 1982).

Percent of mortality = (no. of dead nauplii after 24h/initial no. of live nauplii) × 100……..(1)

In-vitro Thrombolytic Activity

According to Prasad et al., 2007; Prasad et al., 2006 (Prasad et al., 2006; Prasad et al., 2007), thrombolytic properties were investigated. Stock solutions were prepared by combining lyophilized streptokinase (SK) in a 1.5 million international units (IU) vial with 5 ml of sterile distilled water. Throughout this investigation, only analytical reagent-quality chemicals were used. In the study, twenty healthy individuals donated 60 ml of blood; none smoked, used oral contraceptives, or were taking anticoagulants. Tubes were given approximately 15 minutes to coagulate the blood collected from the falcon. The serum was then separated from the remaining blood by spinning the blood at a velocity of 2000 rpm for 1 minute, using a centrifuge (Remi-83 A model). Once the serum had been carefully removed without disturbing the clots, the weight of each clot was calculated again. It was decided to place 100 µl of various plant extracts, 100 ml of streptokinase (for the positive control), or 100 µl of distilled water (for the negative control) in 100 µl falcon tubes after proper labeling. A 24-hour incubator at 37 °C was used to incubate the falcon tubes after clot

lysis was monitored for 90 minutes. A gravimetric method was used to determine the percentage of clot lysis after 90 minutes and 24 hours of incubation after removing the dissolved clot carefully. Triplicates were performed in this test.

% of Clot Lysis = (Weight of Released Clot/ Weight of Clot) × 100…….(2)

In-vitro Anti Arthritic Properties (Albumin of Egg Denaturation Method)

Pavithra et al., 2015 (Pavithra, Smitha, Kulashekar, & Kumar, 2015) described a method for tracking arthritic activities. Using 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 properly mixed. It was next necessary to adjust the pH of the solution to 6.4 using 1N HCl and then fill up the mark of 1000 ml with the needed amount of distilled water. In order to determine whether plant extracts had anti-arthritic activity, a total of five ml of a reaction mixture were used, consisting of egg albumin (0.25 ml), PBS with a pH of 6.4 (2.8 ml), and different concentrations of 2 ml of plant extracts (66.5, 125, 250, 500 ppm). In addition, 2 ml of distilled water was given as a control sample. The gold standard was diclofenac sodium (66.5, 125, 250, 500 ppm) in 2 ml dilutions. A BOD incubator was used to incubate the mixes at 37°C for 15 minutes. After that, the concoctions were placed in a water bath and cooked for five minutes at 70 °C. A UV-Vis spectrophotometer was used to measure the solutions' absorbance at 660 nm after cooling. As a placebo, phosphate-buffered saline was used. Using the formula below, the percentage inhibition of protein denaturation can be calculated:

% Inhibition of Protein Denaturation = 100 × [Vt / V_o - 1]……..(3)

Where, Vt = Absorbance of Test Sample, V_o = Absorbance of Control. The tests were done in triplicate.

Statistical Analysis

Standard deviations from three different analyses are calculated using ANOVA and Dunnett's test. $P > 0.001$ and $P > 0.05$ were considered significant.

RESULTS AND DISCUSSION

Screening of the phytochemical composition of the sample

An initial phytochemical screening was performed using standard techniques on the ethanolic extracts of the various *AO* parts before the qualitative evaluation of the phytochemicals was performed. A list of phytochemicals that were identified can be found in the table below (**Table 1**).

Table 1. Phytochemical composition of ethanolic extracts of different parts of AO

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

NB: AO indicates *Aerides odorata*, and (+) indicate presence, (-) indicates absence.

Analysis by UV-Vis

Phytoconstituents were identified using UV-VIS analysis of plant extracts. It was possible to identify compounds that contain σ -bonds, π -bonds and a lone electron pair, chromophores, and aromatic rings by using UV-visible spectrum analysis. The absorption bands for each plant extract are shown in **Table 2**.

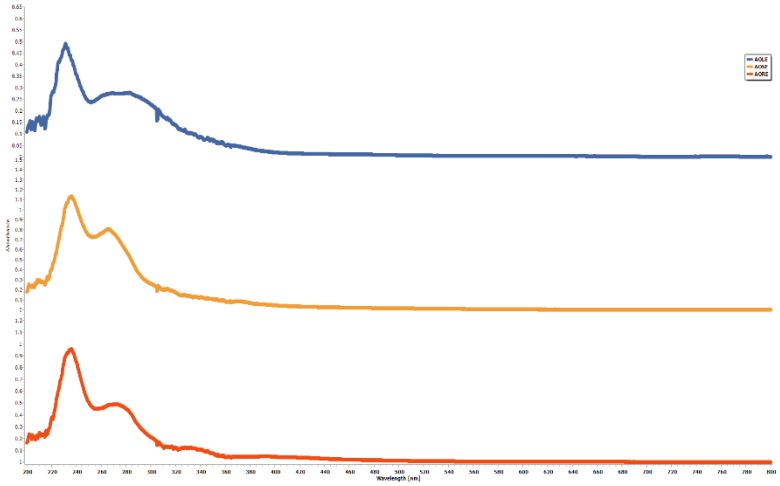

Figure 1: UV-visible spectra of *AO* plant extract

Table 2. UV-visible peaks of AO plant extract

Plant name, part of the plant and extracted medium	**Wavelength (nm)**
AO/Leaf/Ethanol (Blue Line)	210.2,230.8, 269,282.5
AO/Stem/Ethanol (Yellow Line)	208.9,236.1, 265.4, 335,371.2
AO/Root/Ethanol (Orange Line)	210,236, 271.8

Unsaturated groups and heteroatoms like S, N, and O are clearly identifiable by the emergence of one or more peaks in the area from 200 to 400 nm in UV-VIS spectra (Kavitha D, 2021)**.** Uv-visible spectra of (AO)/Leaf/Ethanol, (AO)/Stem/Ethanol, and (AO)/Root/Ethanol extracts exhibited peaks at 208.9 nm, 210 nm and 210.2 nm suggested the presence of saponin and steroid was detected individually at wavelengths of 230.8 nm and flavonoids was indicated by the peak at 236.1 nm, and 236 nm (Tošović, Milošević, & Marković). Peaks at 269 nm and 282.5 nm in the AO/Leaf/Ethanol extract also demonstrated the presence of phenols and proteins, respectively. Peaks at 265.4 nm, 335 nm, and 371.2 nm were also found in the AO/Stem/Ethanol extract, indicating the presence of proteins, alkaloids, and flavonoids individually. Root/ethanol extracts of AO also showed a peak at 271.8 nm that indicated the presence of proteins

FTIR–ATR analysis

According to the infrared peak of the FTIR spectrum, the active ingredients were identified as a functional group. The FTIR peaks for several plant extracts are presented in table 5.

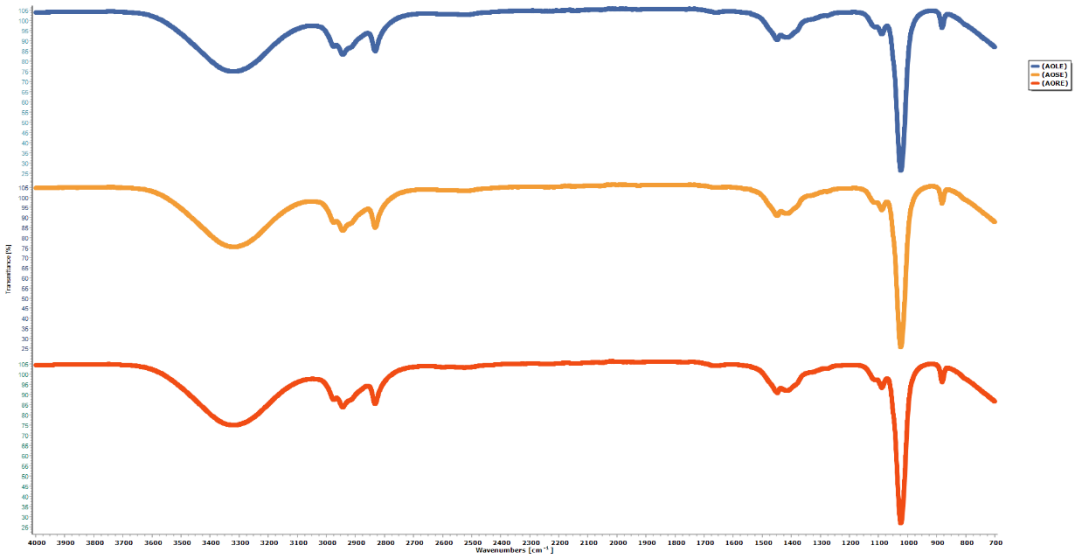

Figure 2: FTIR spectrum of AO plant extract

Table 3: FTIR peaks of AO plant extract

Plant name, part of the plant and extracted medium	Wavenumbers (cm^{-1})
AO/Leaf/Ethanol (Blue Line)	881,1023,1089,1449,1653,2832,2943,3324
AO/Stem/Ethanol (Yellow Line)	881.1,1023,1089,1449,1654,2832,2943,3320
AO/Root/Ethanol (Orange Line)	880.9,1023,1089,1449,1654,2832,2944,3324

Figure 2 demonstrates that AO plant extracts from three distinct portions, including the leaf, stem, and root, produced a peak at 880.9 cm^{-1}, 881 cm^{-1}, 881.1 cm^{-1} which are corresponds to the -CH_2-rocking group, demonstrating the presence of carbohydrates. The -C-O group, which is represented by the peak at 1023 cm-1 in three portions of this plant extract, indicated the presence of flavonoids and cardiac glycosides in the leaf, stem, root part. Three portions of the AO plant extract showed peaks at 1449 cm^{-1} and 1653 cm^{-1} to 1654 cm^{-1}, which stood for the -CH_3 group and -C=C- group, respectively, peak at 2943 cm^{-1} to 2944 cm^{-1} were responsible for the -C-H-stretching of the alkane, indicating the presence of terpenoids. The peaks at 1089 cm^{-1} and 2832 cm^{-1}, which indicated secondary -OH- and -N-H- stretching that guided the existence of alkaloids in all three portions of this plant's extracts. The peaks at 3320 cm^{-1} to 3324 cm^{-1}, which corresponded to the -OH- group, were also observed in the extracts of the three separate portions of the AO plant, indicating the presence of flavonoids in the leaf stem and root parts (Noh, Azmin, & Amid, 2017).

Activity against microorganisms

A synthetic or natural antimicrobial drug prevents or eradicates the growth of microorganisms such as bacteria, fungi, helminths, protozoa, and viruses. Antimicrobial properties depend on how they work against microbes. Agents that stop bacteria from building cell walls, depolarize cell membranes, prevent protein synthesis and nucleic acid synthesis, and stop metabolic processes are the main classes. Phytoconstituents such as alkaloids, flavonoids, glycosides, etc., have antimicrobial activity (Doughari, 2006; Kiani & Jabeen, 2019; Nugraha, Triatmoko, Wangchuk, & Keller, 2020; Yuan et al., 2021). Extracts were effective against different types of microorganisms. Against all microorganisms, extracts had a zone

of inhibition with a diameter of about 8 mm. A zone of inhibition that is active for medicinal plants is one that is ≥8 mm. 10 to 11 mm is the diameter of the zone of inhibition. There are four levels of antimicrobial activity based on the diameter of the zones of inhibition: very active (≥20 mm), less active (≥8 to ≤12 mm), moderately active (>12 to <20 mm), and inactive (<8 mm) (Raghavanpillai Sabu et al., 2022). Multiple bacterial strains were suppressed by orchid extracts, demonstrating their broad range of activity. There have been similar results with other plant extracts as well (Abate, Bachheti, Bachheti, & Husen, 2021; Sieberi, Omwenga, Wambua, Samoei, & Ngugi, 2020). Antimicrobial activity was tested using the disc diffusion method. It was found that the ethanolic extract of the leaves displayed good antimicrobial activity against the microorganisms (**Table 4**).

Table 4. An evaluation of the antimicrobial activity of ethanolic extracts of different parts of AO

Name of Microorganism	Name of Standard/ Sample (Ethanolic Exatract)			
	Standard Ciprofloxacin	Leaf of AO (mm)	Stem of AO (mm)	Root of AO (mm)
Gram Positive Bacteria				
Bacillus cereus	45	10	-	-
Bacillus megaterium	45	10	-	-
Bacillius subtilis	45	10	-	-
Staphylococcus aureus	46	10	-	-
Sarcina lutea	45	10	-	-
Gram Negative Bacteria				
Salmonella paratyphi	43	11	-	-
Salmonella typhi	45	10	-	-
Vibrio parahaemolyticus	45	11	-	-
Vibrio mimicus	46	10	-	-
Escherichia coli	44	11	-	-
Shigella dysenteriae	46	11	-	-
Pseudomonus aeruginosa	45	11	-	-
Shigella boydii	45	11	-	-
Fungi				
Saccharomyces cerevisiae	45	10	-	-
Candida albicans	45	11	-	-
Aspergillus niger	45	11	-	-

However, stem and root of AO/EtOH showed no activity against any microorganism despite containing glycosides, flavonoids, and alkaloids. Meanwhile, ethanol extract of the leaf of AO was found to be potent against all microorganisms in our study. Paul *et al.,* 2013 observed that acetone and water extracts of leaf of AO showed activity against ampicillin and kanamycin-resistant *E. coli* (Paul et al., 2013).Haque *et al.,* 2016 reported that, Ethanol, methanol, chloroform, and petroleum ether extract of leaf of AO showed potentiality against *Bacillus subtilis, Staphylococcus aureus, Salmonella typhi, Shigella dysenteriae* and *Vibrio* and water extract of that plant showed activity against the fungi, e.g., *Alternaria alternate (Fr.) Kedissler, Botryodiplodia theobromae, Colletotrichum corchori, Curvularia lunata, Fusarium equiseti,*

Macrophomina phaseolina, (Hoque et al., 2016). Whereas our study showed especially that ethanol extract of leaf of AO was potent against all microorganisms, root and stem were not effective against any microorganisms.

In order to utilize AO orchid extracts in conventional and traditional medicine, their active compounds must be better understood and tested. Among the parts of the plant, only leaf extracts exhibited antimicrobial properties capable of fighting infections caused by microorganisms.

Bioassay of Brine Shrimp Lethality

Cancer-treating medications called cytotoxic pharmaceuticals (also called antineoplastics) contain toxic substances that prevent cells from reproducing or developing. Aside from treating rheumatoid arthritis and multiple sclerosis, they are also useful for other conditions. Traditional cancer treatments aim to kill only tumor cells or impair their growth permanently. The use of cytotoxic medicines prevents DNA from being synthesized or damages DNA chemically, which causes cancer cells to die. It is possible to measure how deadly something is by looking at the nauplii of brine shrimp (Meyer et al., 1982). An inexpensive and safe method for determining whether a chemical or plant product is harmful to cell (de Almeida, da Silva, & Echevarria, 2002). The use of this method can also be regarded as one of the primary methods for identifying anti-tumor properties. It is possible to evaluate the toxicity of plant extracts based on their LC50 values. The LC50 value should be lower than 1000 µg/ml to determine whether it is cytotoxic (Meyer et al., 1982). Leaf, stem, and root LC50 values of AO were 5.014g/ml, 2.175g/ml, and 1.124g/ml chronologically, while standard vincristine sulfate was 0.84g/ml (**Table 5**). Even though the leaf, stem, & root extracts of AO are less active than vincristine sulfate, they can be considered potent cytotoxic drugs.

Table 5. Bioassay of ethanolic extracts from different parts of *Aerides odorata* on brine shrimp lethality

Sample Name	**LC_{50}** (µg/ml)
AO/Leaf/EtOH	5.014
AO/Stem/EtOH	2.175
AO/Root/EtOH	1.124
Vincristine sulfate (Standard)	0.84

A number of studies have shown that secondary metabolites such as flavonoids, phenolics, and terpenoids are responsible for cytotoxic activity (Anderson, Goetz, McLaughlin, & Suffness, 1991; Aryal et al., 2019; Coe, Parikh, & Johnson, 2010; Rahman et al., 2021; Ren, Qiao, Wang, Zhu, & Zhang, 2003; Tungmunnithum, Thongboonyou, Pholboon, & Yangsabai, 2018; Vila et al., 2004). All extracts contain phenolics, flavonoids, terpenoids but effectiveness may vary because of the concentration of those phytochemicals.

In-Vitro Thrombolytic Effect

The purpose of thrombolysis, also known as thrombolytic therapy, breaks up large blood clots, restore blood flow, and prevent organs and tissues from damage. Blood clots are dislodged by thrombolytic medications that activate plasminogen, which splits plasmin into two. An enzyme called fibrinogen dissolves the crosslinks that make blood clots structurally stable by releasing proteolytic enzymes.

In **Figure 3**, clot lysis is compared between leaf, stem, and root extracts of AO ethanolic extracts, and positive (streptokinase) and negative controls (water). Following 1.5 and 24 hours of incubation, respectively, clot lysis for the streptokinase (100µL, 30,000 IU) was 76.15 ± 1.94 % and 92.59 ± 2.35 %. + On the other hand, after 1.5 and 24 hours of incubation, respectively, clot lysis was 3.59 ± 0.46 % and 9.14 ± 1.05 % in the water control group.

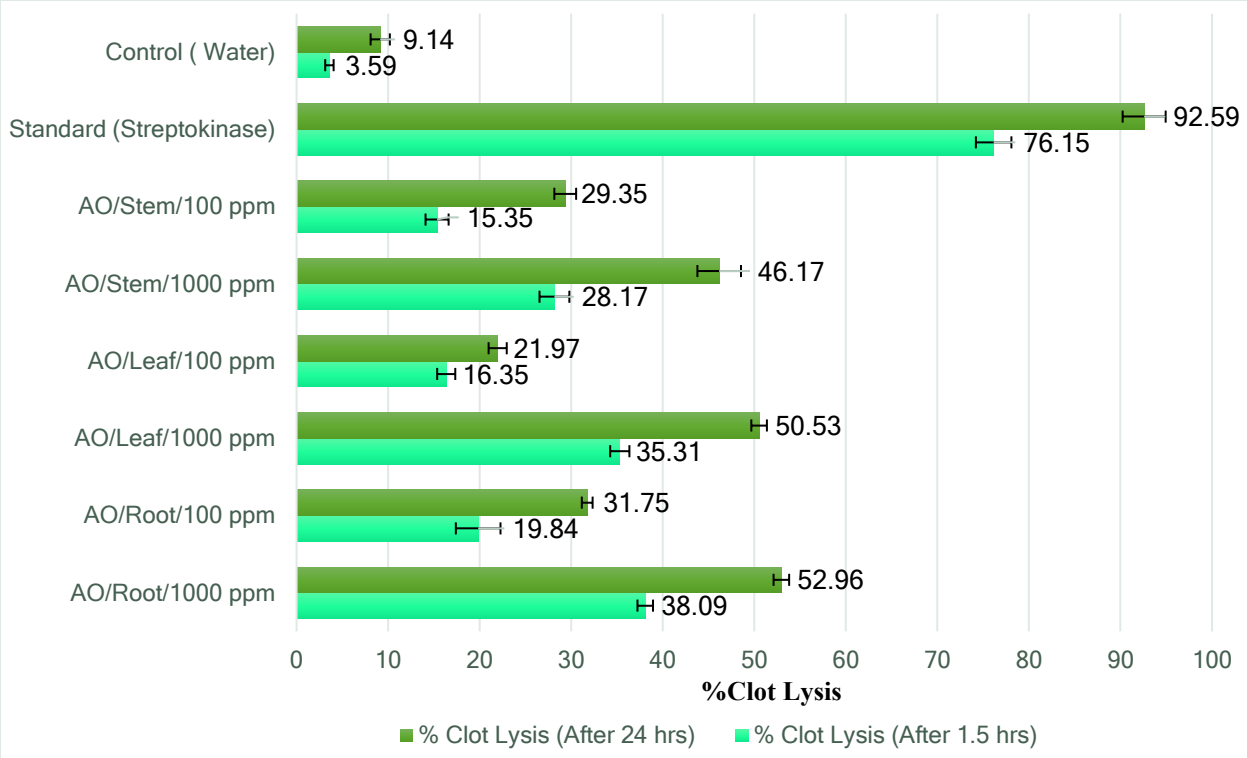

Figure 3: Comparison of the thrombolytic properties of ethanolic extracts of different parts of AO and Standard (Streptokinase)

Figure 3 illustrates that the ethanolic extract of AO leaf at 1000 ppm and 100 ppm exhibited varying percentages of clot lysis: 35.31±1.05% & 16.35±0.99% after 1.5 hours and 50.53±0.85% & 21.97±0.99% following a 24-hour incubation period. The percentage of clot lysis in the stem of AO at 1000 ppm and 100 ppm was 28.17±1.63% and 15.35±1.26% after 1.5 hours, and 46.17±2.38% and 29.35±1.19% after a 24-hour incubation period, respectively. After 1.5 hours, the clot lysis of 100 ppm AO root extract demonstrated 38.09-0.85% & 19.84-2.44%, and after 24 hours of incubation, 52.96-0.85% & 31.75-0.59%.

There is evidence that the variety of phytoconstituents in plant extracts, such as flavonoids, alkaloids, terpenoids, and tannins, is responsible for the thrombolytic activity (Bhowmick et al., 2014; Dwivedi, 2007). As a result of its leaf and stem extracts, AO contains all the phytochemicals that are responsible for excreting thrombolytic effects, namely terpenoids, flavonoids, alkaloids, and tannins. This effect may be attributed to the presence of compounds called terpenoids, flavonoids, and alkaloids in the root extract of AO.

In-Vitro Anti Arthritic Effect

The symptoms of arthritis, such as joint pain and stiffness, are treatable with antiarthritic medicines. It may reduce inflammation, ease pain, or weaken the immune system, depending on the type of anti-arthritis drug. In certain arthritic circumstances, denaturation of proteins may be a factor in the generation of autoantigens. The denaturation mechanism most likely includes modifications to disulphide, hydrophobic, hydrogen, and electrostatic bonds (Deepika Arya, Mukesh Meena, Neha Grover, & Vidya Patni, 2014).
In-vitro anti-arthritic properties of ethanolic extracts of the leaf, stem, and root of AO were evaluated. **Figure 4** shows the antiarthritic activity of AO extracts from leaf, stem, and root. The standard indicated the maximum of 85.10±0.45%. The percent inhibition of protein denaturation of a 500 ppm plant extract of the leaf, stem, and root of AO was 37.93±2.08 %, 60.07±2.33 %, and 43.28±1.5 %, respectively. AO plant extract at a concentration of 250 ppm decreased protein denaturation by 28.80±0.68%, 47.22±1.43%, and 31.72±2.36%, respectively, compared to 73.79±0.73% for the standard.

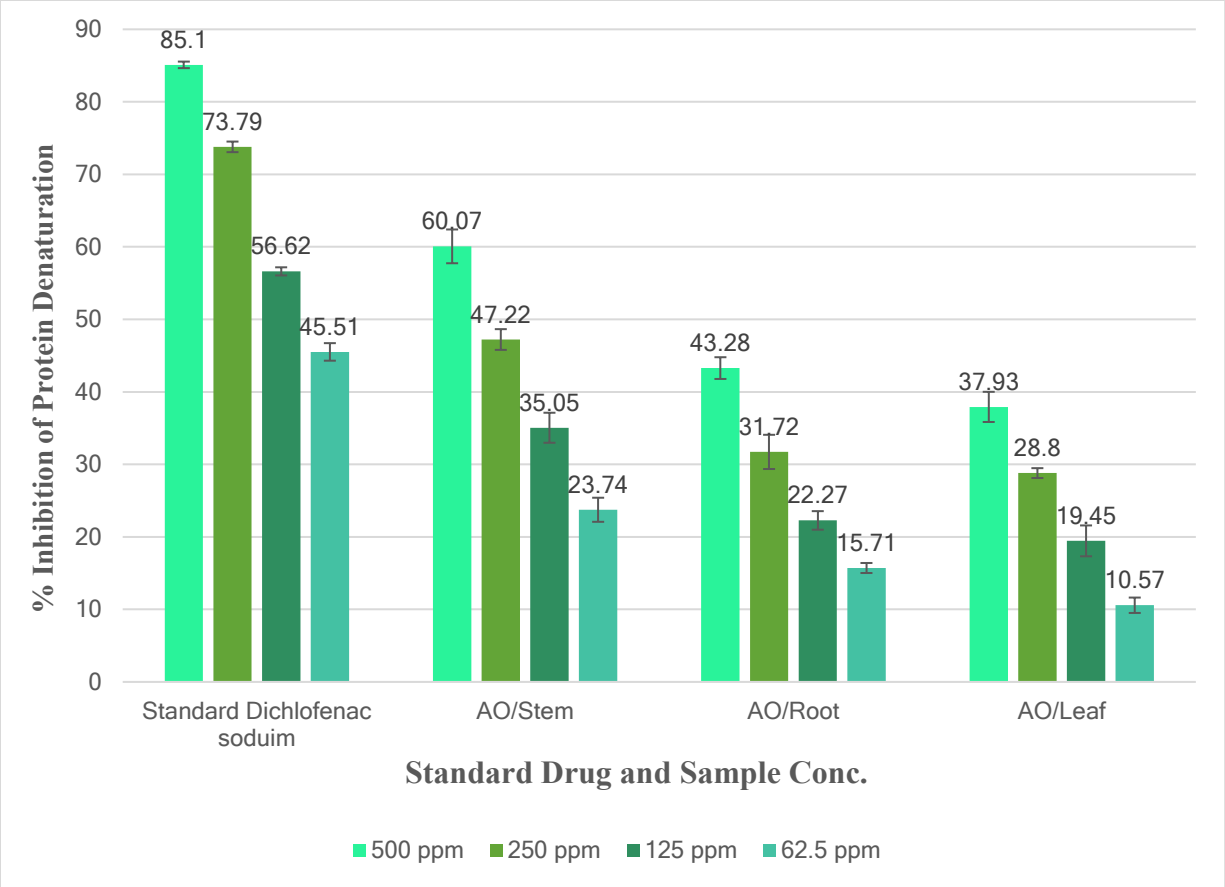

Figure 4: Comparison to the standard (Dichlofenac Sodium) and ethanolic extracts of several sections of AO demonstrated antiarthritic efficacy

The percentage of protein denaturation inhibition of a 125ppm plant extract of AO leaf, stem, and root was 19.45±2.13%, 35.05±2.07 %, and 22.27±1.28% chronologically, whereas the standard exhibited 56.62±0.56%. The plant extract of AO leaf, stem, and root at a concentration of 62.5 ppm was shown to prevent protein denaturation in the following ways: 10.57±1.06%, 23.74±1.66%, and 15.71±0.69%, respectively. A minimum of 45.51±1.21% was demonstrated by the standard.

Some phytochemical constituents have the same properties, such as flavonoids, which have anti-rheumatism, antihypertensive, antimicrobial, diuretic, and antioxidant properties. Some phytochemicals have the same effects. For example, flavonoids treat rheumatism, high blood pressure, infections, make you pee, and fight free radicals (Hossain, Al-Raqmi, Al-Mijizy, Weli, & Al-Riyami, 2013; Kamtekar, Keer, & Patil, 2014). All extracts contain flavonoids but their effectiveness may vary because of the concentration of the phytochemicals.

We demonstrate in our research that AO extracts are effective antimicrobial agents, can be used to dissolve blood clots, and can halt the production of autoantibodies. Ethanol extracts from all three parts of AO showed cytotoxic, thrombolytic, and antiarthritic properties. The leaf extracts showed the strongest antimicrobial activity.

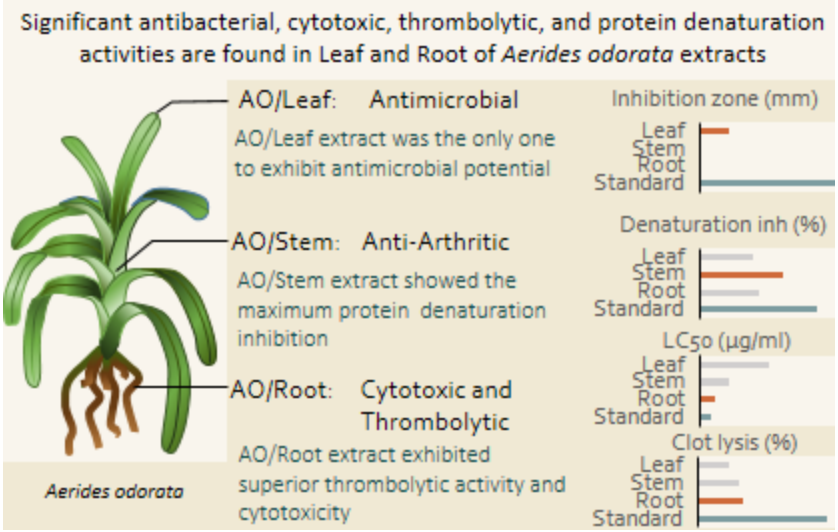

Figure 5: Graphical Abstract

Even so, these might serve as a significant source of antibacterial, antifungal, anticancer, anti-trauma, thrombolytic, and antiarthritic properties. So far no animal trial has been carried out. In the future, in vivo research will be done and may help find corresponding drugs responsible for the activities as mentioned earlier using high-performance liquid chromatography.

CONCLUSIONS

The antimicrobial study employed fourteen different bacteria; for orchid samples, ciprofloxacin proved to be the most effective drug. In contrast to AO/Stem and AO/Root, only AO/Leaf exhibited antibacterial activity. We assessed plant extracts' cytotoxicity based on their LC50 values. In comparison to the extracts of AO/Leaf and AO/Stem, the AO/Root extract exhibited the highest level (1.124 µg/ml) of cytotoxicity. To evaluate the extracts' thrombolytic activity, two incubation times (1.5 hours and 24 hour) two concentrations (one thousand and one hundred ppm) were employed. When compared to AO/Leaf and AO/Stem extracts, AO/Root extract had greater thrombolytic activity. Additionally, the denaturation of proteins by the herbal extract was examined at four distinct concentrations. With respect to the other sections, the highest protein denaturation values for the AO/Stem extract were found to be, for 500–62.5 ppm, 60.07±2.33%, 47.22±1.43%, 35.05±2.07%, and 23.74±1.66%, respectively. In view of the rise in drug-resistant bacteria, cancer, blood clot-related stroke, autoimmune diseases, etc., our research will be useful in identifying novel phytochemicals. This study may be very useful in discovering several sources to design medications for micro-organism related diseases, cancer, thrombosis, and arthritis as prospective future chances for pharmaceutical development.

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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, Paper highlights; **Shah Md. Marzuk Hasnine and Tanvir Ahmad:** Formal Analysis, Writing-Original Draft + Review & Editing, Visualization, Graphical abstract; **Md. Abdul Quaiyum Bhuiyan:** Writing - Review & Editing; Visualization; **Salma Sultana:** Formal Analysis, Resources, Visualization; **Md. Ismail Hossain:** Formal Analysis, Visualization; **Mubarak Ahmad Khan:** Critical review, commentary, or revision – including pre-or post-publication stages.

Data Availability Statement

The data used to support the findings of this investigation are included in the publication. The corresponding author can provide additional data or information upon request.

Supplementary Materials

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

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