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Original Research Article

Evaluation of antimicrobial, thrombolytic and cytotoxic activities of ethanol flower extract of *Bauhinia acuminata*

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ABSTRACT

Background: To develop the herbal drug with the least side effects, there are superior opportunities to discover the medicinal and other biological properties. Natural products serve as sources of beneficial chemical molecules. For this study, Bauhinia acuminate (Family: Fabaceae) having some pharmacological activities was chosen. The present studies was designed for the antimicrobial, thrombolytic and cytotoxic activity of ethanolic flowers extract of Bauhinia acuminata.
Methods: The antimicrobial effect of the extracts was evaluated by Disc-Diffusion Assay Method and the cytotoxic activity was determined by brine shrimp lethality test. The thrombolytic activities of the plant extracts were evaluated by a method using streptokinase as a reference standard.
Results: The extract has mild antimicrobial activity against Staphylococcus aureus, Bacillus subtilis, enteropathegenic *Escherichia coli*, Salmonella typhi, Vibrio mimicus. The zone of inhibition of the extract was measured comparing with standard Tetracycline. Thrombolytic activity of flowers of Bauhinia acuminata showed mild (14.57%) clot lysis while the standard streptokinase showed (90%). Having moderate cytotoxic activities, the mortality rate of brine shrimp was observed in increasing order with the increasing of concentration of extract. In the present study of the ethanol extract LC50=3.251 µg/ml and LC90=286.57 µg/ml. However, Standard control LC50=0.0036 µg/ml and LC90=0.75 µg/ml were found respectively.
Conclusions: Through the studies, it can be concluded that the ethanolic extract of Bauhinia acuminata could be used in drug formulation or pharmaceutical application. Hence, the plant may further be explored for its various pharmacological activities.

Keywords: Antimicrobial, Thrombolytic, Cytotoxic, Bauhinia acuminata, Flower extract

INTRODUCTION

Pharmacological activity research on plant extracts has been a long-standing practice that has expanded rapidly over the past few decades on a global scale. New and emerging diseases have been inspired by nature, which has given rise to innovative drug concepts. The medicinal properties of the plant have been investigated because of their strong pharmacological activity, low toxicity, and economic viability.[1,2] Over 80% of the world's population receives primary healthcare through traditional medicine,

with the majority of these treatments requiring the use of herbal extracts and their active ingredients, according to estimates from the World Health Organization (WHO).[3] Approximately 374,000 plants are estimated to exist worldwide, compared to 28,187 human-used medicinal species.[4,5] The world health organization has also cataloged more than 20,000 species of medicinal plants and identified them as a possible source of novel pharmaceuticals.[6,7] There are laws governing medicinal plants in more than 100 nations. Throughout human history, people have chewed herbs to reduce pain or promote healing. They have also wrapped leaves around wounds, which is how phytotherapy first emerged.[8] A complex methodology combining botanical, phytochemical, biological, and molecular techniques is used in drug discovery, which begins with medicinal plants. The ongoing research into medicinal plants is yielding novel compounds to target various pharmacological targets such as pain, cancer, AIDS, Alzheimer's disease, and so on.[9] The chemical substance or group of compounds that cause a specific physiological action in the human body is what gives plants their medicinal value. Medicinal plants are thought to be an accumulation of many different kinds of bioactive substances with a wide range of therapeutic applications.[10] Numerous chemical compounds that have been found in medicinal plants shown to have a wide range of beneficial effects, such as vascular activity, cytotoxic antitumor activity, antimicrobial activity, antiallergic activity, oestrogenic activity, enzyme inhibition, and anti-inflammatory capabilities.[11]

Native to tropical southeast Asia, Bauhinia acuminata is a species of flowering shrub whose leaves, bark, roots, flowers, and seeds are all used in traditional medicine. It's used to treat ulcers, skin conditions, and glandular swelling. The chemical constituents found in Bauhinia acuminata were vitamin C (leaves), beta-sitosterol, lupeol, Kampferol, 3,5,7-dehydroxy and 5,7 dimethoxy-flavanone-4-O-alpha-L-rhamnopyranosyl-beta- D-glucopyranosides. Bauhinia acuminata flavonoids were found to contain apigenin, quercetin, and kamepferol. Both species contained apigenin, quercetin, and kamepfrol. Quercetin derivatives, specifically Quercetin-3-glycoside, were found in Bauhinia acuminata and Quercetin-7-glycoside in Cassia oocidentailis.[12] The leaves of Bauhinia acuminate were found to contain a number of chemicals, including palmitic acid, three phthalic acid esters, phthalic acid, gallic acid, and ursolic acid.[13] The leaves and stems of Bauhinia acuminata were found to contain carbohydrates, phenolic compounds, saponins, flavonoids, oils, and fats, according to phytochemical screening. Their phenolic compounds are mostly responsible for their potential antioxidant activity.[14] The plant has a content of 23% crude protein, 20.8% crude fiber, 24.9% lipid, and 48% carbohydrates.[15] Alkaloids, anthocyanoside, phenolics, proteins, phlobatannins, steroids, tannins, flavonoids, anthraquinone, saponins, terpenoids, resins, balsams, amino acids, carbohydrates, sugars, and cardiac glycosides are among the chemical groups that the various plant parts contain, according to the phytochemical analysis.[16,17] Compounds derived from natural plants that exhibit antimicrobial activity have been extensively studied and employed as nutritional supplements in substantial amounts.[18] Compounds derived from natural sources have the potential to be used for safeguarding due to their antimicrobial properties against a broad range of pathogenic microbes.[19] However, the effectiveness of conventional antimicrobials has been considerably dropped due to the emergence of antimicrobial resistance. Antimicrobial resistance has been recognized by the world health organization (WHO) as one of the top ten global public health threats that humanity faces.[20] Therefore, more research is required to assess plant extracts' antimicrobial effects against drug-resistant pathogens. Numerous thrombolytic drugs are used to treat myocardial infarction. Streptokinase is one of the most notable and frequently used of them. Furthermore, compared to urokinase or streptokinase type activators, tissue-type Plasminogen activator is safer and more effective. All of the thrombolytic drugs currently on the market are noted to have serious drawbacks, such as the need for high dosages for maximum effectiveness, a restricted ability to target specific fibrin, and a notable tendency to bleed when used.[21] To reduce the shortcomings of the current thrombolytic medicines, attempts are therefore made to create better substitute medicinal substances.

Cancer is a significant public health concern in both developed and developing nations. Cancer is characterized by aberrant cell growth in the body, which may be fatal.[22] Artemia salina was used as the test organism in a cytotoxicity test that followed the brine shrimp lethality test (BSLT) protocol. Leach larvae are frequently used as a preliminary test to evaluate an agent's anti-tumor and anti-cancer efficacy.[23] Numerous studies have revealed a link between certain cancers and bacteria.[24] Antibiotics are the medications that are prescribed for the treatment of bacterial illnesses. The increased use of antibiotics as a treatment for disease, the administration of doses that are lower than the prescribed amount of medication and the consequences of discontinuing antibiotic treatment before bacteria have been completely eradicated by the medication all contribute to the development of antibiotic resistance in bacteria.[25,26] Because of this, there is a pressing need for alternative therapeutic compounds that can not only combat the issue of bacterial resistance but also have properties that make them effective against inflammation or cancer. Therefore, the present study is aimed to investigate antimicrobial, thrombolytic & cytotoxic activities of ethanol flower extract of *Bauhinia acuminata.*

METHODS

Plant material

In April 2019, fresh mature *Bauhinia acuminata* flowers were collected from Gollamari, Khulna, Bangladesh. The plant species was authenticated by experts from the

Bangladesh National Herbarium. After being cut into tiny pieces, they were sun-dried for a week. Using an appropriate grinder, the plant parts were reduced to a coarse powder. Until analysis started, the powder was kept in an airtight container in a cool, dry, and dark environment.

Animals

Swiss albino mice, of both sexes, were acquired from the pharmacology lab at Jahangirnagar University in Savar, Dhaka. They were kept in polypropylene cages with a temperature of 23±2°C and a relative humidity of 55±5%. The mice were kept on a 12-hour light/12-hour dark cycle to allow them to adjust to the laboratory environment in a systematic way. During the trial, standard pellets were used as the basal diet. The Disc-Diffusion Assay or Kirby-Bauer Method were used to examine the ethanol extract's antimicrobial activity.

The diameter of the zone of inhibition was measured in millimeters to ascertain the crude extract's antimicrobial activity. The average zone of inhibition was computed after the experiment was run in triplicate. Streptokinase (SK) was used as the standard material in Daginawala's method to assess the thrombolytic activity of all extractives.[27] Each plant's extractive (100 mg) was suspended in 10 millilitres of distilled water and left overnight. Brine shrimp, or Artemia salina, were used in the study to determine the ethanol extract's cytotoxic properties. Eighteen to twenty-four hours were given to the Artemia cyst brine shrimp eggs to hatch and develop into Nauplius larvae. To gather the hatchling shrimp for bioassay, they were drawn to light.

Bacterial strains

For the antimicrobial activity test, two Gram positive bacteria, such as *Staphylococcus aureus* and *Bacillus subtilis*, and three Gram negative bacteria, including enteropathegenic *Escherichia coli* (EPEC), *Salmonella typhi*, and *Vibrio mimicus*, were utilized. These organisms were gathered from the Microbiology Lab. of pharmacy discipline, University of Dhaka.

Preparation of agar media and equipment sterilization

A dehydrated product containing all the ingredients was combined with water to create nutrient agar media. A precise weight of 2.8 gm of nutrient agar was measured and transferred into a 250 ml volumetric flask along with 100 ml of distilled water. After thoroughly dissolving agar over a hot plate while shaking it occasionally, a clear medium was produced. The final volume was then adjusted to 100 ml. Glassware such as petridishes and agar medium were autoclaved for 15 minutes at 121°C and 15 pounds per square inch of pressure. Before spending an hour working in a laminar hood, the UV light was turned on and the loop and forceps were stored inside to prevent unintentional contamination.

Chemical and drugs

Approximately 99.5% of the plant material was macerated using ethanol as the solvent. The degree of cytotoxicity for which sea salt (sodium chloride Crystal GR; Merck Ltd., Mumbai, India) was used as the hatching medium for shrimp eggs was assessed using the brine shrimp lethality assay method. For the antimicrobial activity test, Tetracycline antibiotic (Beximco Pharmaceutical Ltd., Bangladesh) was utilized. And for the thrombolysis activity test, streptokinase (Popular Pharmaceutical Ltd., Bangladesh) was used. All of the analytical grade chemicals were bought from various Bangladeshi pharmacies.

Antimicrobial activity test

In a sterile 5-milliliter glass vial, 1000 microliters of ethanol were used to dissolve 100 mg, or 100,000 µg, of the ethanolic crude extract of Bauhinia acuminata flowers to create the ethanol fraction stock solution. An autoclave was used to heat sterilize a 250 mL sterilized nutrient agar solution that had been prepared in an agar bottle. After that, the sterilized agar solution was added to the sterilized Petri dish to create an agar plate with an approximate 4 mm agar thickness. The agar plates were left inside the laminar hood to solidify. An autoclave was used to heat sterilize the 100 ml isotonic NaCl solution, which was made by dissolving 0.9 g of iodine-free NaCl salt in 100 ml of distilled water. Next, bacteria from the test microorganism subcultures and the sterile isotonic NaCl solution were sampled and used to create microbial suspensions in Eppendorf tubes. The suspensions were then vortexed using a vortex mixer and allowed to turbidity for a short while. Following the process of solidification, a single microorganism was added to each Agar plate to ensure uniform inoculation. Filter paper discs with a diameter of 5 mm that had been dried and sterilized were then put on the agar plates. Subsequently, 20 µl of extract solution were applied to the impregnated filter discs using a Micropipette (2-20 µl Grade), resulting in 2 mg of crude extract per disc. The solvents that remained were allowed to evaporate. The standard method was to use Tetracycline discs. To ensure that the organisms could grow to their full potential, the plates were then incubated for 24 hours at 37 degrees Celsius in an incubator. The test material's antimicrobial activity prevented the microorganisms from growing, and this resulted in the formation of a distinct, clear zone of inhibition around the disc. The diameter of the zone of inhibition in millimeters was used to calculate the crude extract's antimicrobial activity. The average zone of inhibition was determined after the experiment was run in triplicate.

Thrombolytic activity test

Each plant's extractive (100 mg) was suspended in 10 millilitres of distilled water and left overnight. After that, the soluble supernatant was decanted and passed through a syringe filter with a 0.22-micron filter. Venous blood was

extracted from healthy individuals and placed (1 millilitre per tube) in separate pre-weighed sterile micro centrifuge tubes for clot lysis. The tubes were then incubated for 45 minutes at 37° C. The serum was totally removed without affecting the clot after it had formed, and the weight of each tube holding the clot was once more measured to ascertain the clot weight (clot weight=weight of clot containing tube-weight of tube alone). The institutional ethical review committee granted the experiment ethical clearance, and it was carried out in accordance with the safe animal handling guidelines. Each micro centrifuge tube containing the pre-weighed clot received a separate addition of 100 µl aqueous solution containing various partitionates and crude extract. The positive and negative control tubes were then filled with 100 µl of streptokinase and 100 µl of distilled water, respectively. After 90 minutes of incubation at 37°C, all tubes were checked to see if any clots had dissolved. Following incubation, the fluid that had been released was removed, and tubes were weighed once more to see how the weight had changed following clot disruption. Difference obtained in weight taken before and after clot lysis was expressed as percentage of clot lysis as shown below:

$$\%\ \boldsymbol{of\ clot\ lysis,} = (\boldsymbol{wt\ of\ clot\ after\ release\ of\ fluid\ /clot\ wt}) \times \mathbf{100}$$

Five millilitres of sterile distilled water were added to a commercially available vial of lyophilized Streptokinase (Popular Pharmaceuticals Ltd.) containing fifteen million IU, and the mixture was thoroughly mixed. For in vitro thrombolytic experiments, 100 µl (30,000 IU) of this suspension was used as a stock.

Cytotoxicity test

Artemia salina was used as the test organism in a cytotoxicity test that followed the brine shrimp lethality test protocol.[23] Thirty milligrams of brine shrimp (Artemia salina) eggs are added to saltwater in a closed container. An air hose is inserted into the base of the container to facilitate hatching. The eggs of A. salina will hatch and turn into larvae in 24 hours. Subsequently, ten larvae of every species were gathered and put into a container holding a sample solution containing 0; 10; 100; and 250 mg/l of concentration. The A. salina larvae in the samples and controls had obviously perished after a day. After a few seconds of observation, the larvae of A. salina stop moving, which is the indicator of whether or not they have died. Following the determination of the percentage of A. salina larvae that perished, the data underwent linear regression after the probit value was located via a search of the probit table.

Preparation of control group

In order to validate the test procedure and guarantee that the results obtained were solely attributable to the test agent's activity and that the effects of any other potential factors were eliminated, control groups were used in cytotoxicity studies. There were two different kinds of control groups used: positive control and negative control. Vincristine sulfate was used as a positive control in the present study. Since vincristine is an extremely cytotoxic alkaloid, very low concentrations were examined. As a negative control, however, eight premarked test tubes containing 10 ml of simulated sea water and ten shrimp nauplii to be used as control groups were each filled with 50µl of DMSO. The remaining brine shrimp in these vials are deemed invalid if they exhibit a high rate of mortality since the nauplii may have perished for reasons unrelated to the compounds' cytotoxicity.

RESULTS

The ethanol crude extract and different fractions of the flower parts of Bauhinia acuminata were screened against antimicrobial, cytotoxic and thrombolytic activity.

Table 1: Result of Antimicrobial Activity of the flowers of the *Bauhinia acuminata.*

Microorganisms	Species	Zone of Inhibition in mm	Zone of Inhibition in mm
		Ethanol extract (2 mg/Disc)	Standard (Tetracycline)
Gram negative bacteria	*Salmonella Typhi*	10	21
	Vibrio mimicus	9	22
	E. coli	11	19
Gram positive bacteria	*Staphylococcus aureus*	8	23
	Bacillus subtillis	12	24

Antimicrobial activity test

By observing the zone of inhibition around the paper disc, antimicrobial activity may be gauged. In order to observe the zone of inhibition, the agar diffusion technique was performed. The test material extract was tested for its activity against *Staphylococcus aureus, Bacillus subtilis, Enteropathogenic Escherichia coli, Enteropathegenic Salmonella typhi and Vibrio mimicus* (Table 1).

It is cleared that the extract has mild activity against *Staphylococcus aureus, Bacillus subtilis, Enteropathogenic Escherichia coli, Enteropathegenic Salmonella typhi and Vibrio mimicus* (Figure 1).

Thrombolytic activity test

Addition of 100 µl of streptokinase of 30,000 IU concentrations to tubes showed clot lysis of 88.49% respectively comparing with 5.70% clot lysis of distilled water considered as a negative control. Flower extract of *Bauhinia acuminate* displayed mild effect of ethanol extract is 14.57% (Table 2).

Table 2: Thrombolytic activity (in terms of % clot lysis) of *Bauhinia acuminata.*

Sample	Blank tube weight (g)	1st clot + tube weight (g)	1st clot weight (g)	2nd clot + tube weight (g)	2nd clot weight (g)	% of lysis
Standard (Streptokinase)	0.838	1.663	.825	.9335	.0955	88.49
Control (Distilled water)	0.824	1.472	0.648	1.4355	0.6115	5.70
***Bauhinia acuminata* extract**	0.848	1.651	0.803	1.534	0.686	14.57

Cytotoxic activity test

The results of the cytotoxic effect of the ethanol extract of *Bauhinia acuminata* is given in (Table 3). The mortality rate of the brine shrimp (Artemia salina) was found to be increased with the increasing of concentration of the extract and plotting of % mortality versus log concentration on the graph paper produced an approximate linear correlation between them (Figure 3). The concentration at which 50% mortality (LC_{50}) and 90% mortality (LC_{90}) of brine shrimp nauplii caused by the test extract were calculated from the graph by extrapolation and was found LC_{50} 3.251µg/ml and LC_{90} 286.57µg/ml respectively (Figure 4).

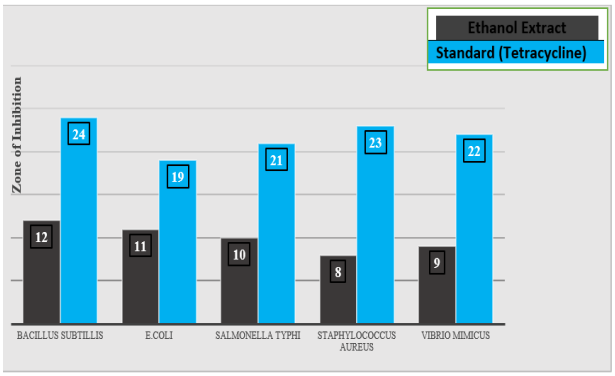

Figure 1: Zone of inhibition of standard and extract.

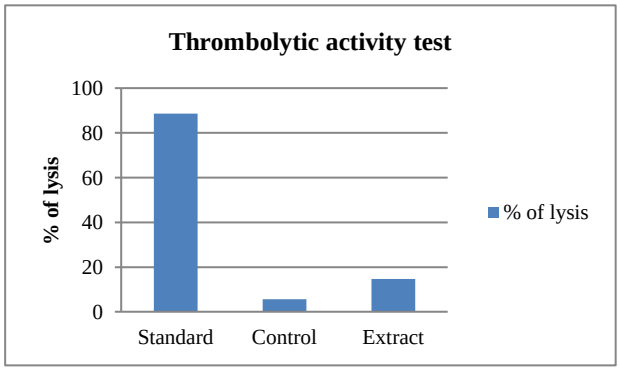

Figure 2: % lysis of standard and extract.

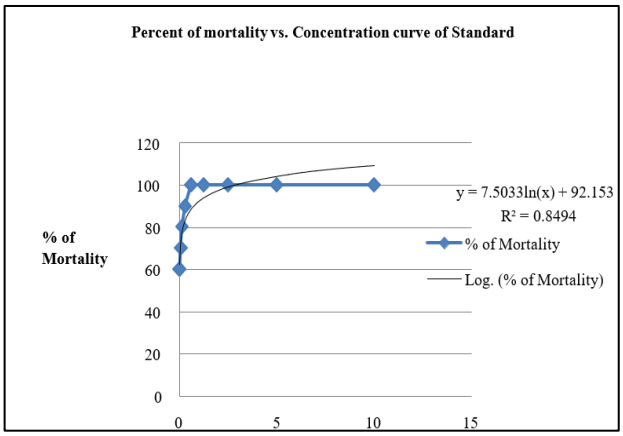

Figure 3: % of mortality of standard.

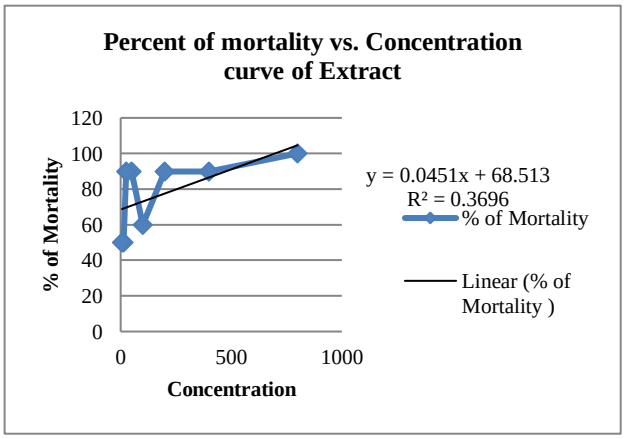

Figure 4: % of mortality of ethanol extract.

DISCUSSION

The plant's ethnobotanical uses for microbial infections led to the selection of the crude extract for antimicrobial activity assessment. A mild spectrum of activity was seen in the extract. *Bacillus subtillis* exhibited the largest zone of inhibition, measuring 12 mm. Following a 90-minute incubation period at 37°C and the addition of 100 µl SK, a positive control containing 30,000 IU, the clots exhibited

88.49% clot lysis. When 100 µl of sterile distilled water (negative control) was applied to clots, there was very little clot lysis (5.70%). It was discovered that there was a statistically significant mean difference in the percentage of clot lysis between the positive and negative controls. According to the in vitro thrombolytic activity investigation, *B. acuminata* exhibited 14.57% clot lysis.

Table 3: Results of standard.

Serial No	Concentration	Alive	Death	% of Mortality
01	10	0	10	100
02	5	0	10	100
03	2.5	0	10	100
04	1.25	0	10	100
05	0.625	0	10	100
06	0.312	01	09	90
07	0.156	02	08	80
08	0.078	03	07	70
09	0.039	04	06	60
10	0.019	04	06	60

Standard- LC_{50}=0.0036 µg/ml, LC_{90} = 0.751 µg/ml.

Table 4: Results of ethanolic extract.

Serial No	Concentration	Alive	Death	% of Mortality
01	800	00	10	100
02	400	01	09	90
03	200	01	09	90
04	100	04	06	60
05	50	01	09	90
06	25	01	09	90
07	12.5	05	05	50
08	6.25	05	05	50

Extract (Ethanol), LC_{50}=3.251 µg/ml, LC_{90}=286.57 µg/ml.

After applying the extract solution, the percentage of the clot's weight that was lost was considered the functional indicator of thrombolytic activity. In order to predict significant pharmacological activities such as enzyme inhibition, ion channel interference, antimicrobial activity, and cytotoxic activity, the brine shrimp lethality bioassay is a simple and straightforward bench top screening method.[28] The extract demonstrated LC50 at a low concentration in the present study, suggesting that it is highly effective. Determining whether the detected cytotoxic and antibacterial activities are caused by the same compound or compounds is an interesting question. Any substance that can be used to treat cancer should ideally not be hazardous to healthy cells. Anticancer drugs, however, are frequently harmful to healthy cells as well, especially to those that are growing quickly.[29] It is crucial to conduct experiments on a range of cancer and normal cell lines in order to validate the viability of additional research into the anticancer properties of this plant extract. Since pharmacologically active compounds frequently tend to be toxic in high doses, the toxicity may also be caused by compounds with a different pharmacological activity.[30,31] Further investigations are required to find the responsible compound (s) for the cytotoxic activity observed for *B. Acuminata.*

CONCLUSION

The present study was aimed to investigate in vitro thrombolytic activity and antimicrobial activity & in vivo cytotoxic activity of ethanolic extract of Bauhinia acuminata. As a positive and negative control for thrombolytic activity, streptokinase and water respectively were used. The antimicrobial test used tetracycline as a standard drug. From our data, it is clear that the scientific community may benefit from our discoveries in the development of novel thrombolytic, antimicrobial, and cytotoxic compounds that have fewer extract-related side effects. Further studies are necessary to isolate and characterize the compounds and to explore the possible mechanism of action for in vitro and in vivo activity.

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Ethical approval: The study was approved by the Institutional Ethics Committee

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