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Antithrombotic, cytotoxic and antibacterial activities of methanol extract of *Antidesma ghaesembilla* Gaertn leaves.

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ABSTRACT

Extract from the leaves of *Antidesma ghaesembilla* were screened for their antithrombotic, cytotoxic and antimicrobial exercises. The cytotoxicity was surveyed with the brine shrimp lethality bioassay and antithrombotic impact with human blood. The brine shrimp lethality bioassay was utilized to assess cytotoxicity (LC_{50} = 432.13 µg/ml) contrasted with Vincristine sulfate (LC_{50} = 0.74 µg/ml). It was also assessed as antithrombotic activity when contrasted with streptokinase. It has significant antithrombotic movement (63.45±2.08%) contrasted with standard streptokinase (81.32±1.46%). The extract indicated zone of inhibition against Gram positive bacteria (*Staphylococcus aureus* and *Bacillus subtilis*) and Gram negative bacteria (*Salmonella typhi, Salmonella paratyphi, Escherichia coli, Pseudomonas aeruginosa*) 1000 µg/disc. Gram negative bacteria *Bacillus cereus* demonstrated no action against at both doses. *A. ghaesembilla* leaves extract and relative percentage inhibition of the extract also calculated. These results indicate that *A. ghaesembilla* have favorable Antithrombotic, cytotoxic and antibacterial effects of *A. ghaesembilla* extract to be processed for pharmaceutical use.

Key word: *Antidesma ghaesembilla,* antithrombotic, cytotoxic, antibacterial

INTRODUCTION

Medicinal plants have been used in healthcare since time immemorial. So the emphasis on the use of medicinal plants had hitherto been placed on the treatment rather than prevention of diseases. However, there exists in the literature considerable report in recent times on research work on the use of medicinal plants and their constituents in disease prevention. A World Health Organisation (WHO) Expert Group defined Traditional Medicine as the sum total of all knowledge and practices, whether explicable or not, used in diagnosis, prevention and elimination of physical, mental, or social imbalance and relying exclusively on practical experience and observation handed down from generation to generation, whether verbally or in writing (WHO, 1976) (1). For Africa, this may be extended further by including an expression, such as 'while bearing in mind the original concept of nature which includes the material world, the sociological environment whether living or dead and the metaphysical forces of the universe'

Bangladesh is home to a number of tribes or indigenous communities. Latest ethnographic

research suggests that the number of tribes within the country approximates 150 instead of the previously estimated about a dozen tribes (2)(3). Most of the indigenous communities and particularly the smaller ones (i.e. communities whose population is below 500 persons) are on the verge of disappearance because of decline in population, loss in tribal habitat, or because of merging with the mainstream Bengali-speaking population. As a result, the culture and knowledge possessed by these tribes are also fast disappearing, including their traditional medicinal practices. Adequate documentation of such knowledge, and especially traditional medicinal practices, is important because tribal medicinal practitioners or healers through long association with plants around their vicinity have acquired quite extensive knowledge on the medicinal properties of these various plant species (4).

Antidesma ghaesembilla Gaertn. Is commonly known as Black Currant Tree. *A. ghaesembilla* is a species of plant in the family Phyllanthaceae. It is endemic to northern Australia (5). It also found in Asia - southern China, Bangladesh, Indian subcontinent, Myanmar, Thailand, Cambodia, Laos, Vietnam, Malaysia, Indonesia, Philippines, New Guinea. *A. ghaesembilla* is a shrub or small tree with a dense crown, which can grow up to 20 meters tall, but is usually smaller. The bole is usually crooked and gnarled, branching from low down, though it can be unbranched for up to 8 metres, and up to 32cm in diameter. It is harvested from the wild for local use as food and medicine. The leaves are used as a poultice to treat headaches, scurf, abdominal swellings and fevers, the stems are emmenagogue, the fruit is purgative. The wood is cheap but soft and splits when dried. It is nevertheless used for roof construction. Another report says that it is hard and durable, but difficult to work because of the high silica content (6).

The purpose of the present study focuses on the scientific investigation of Antithrombotic, cytotoxic and antibacterial activity of *Antidesma ghaesembilla* leaves.

MATERIAL AND METHOD

Plant material: Fresh leaves of *A. ghaesembilla* were collected from Bandarban, Chittagong, Bangladesh in the month of March 2015. It was authenticated by Dr. Shaikh Bokhtear Uddin, Professor, Department of Botany, University of Chittagong, Chittagong-4331, Bangladesh.

Preparation of Extract: The leaves were dried for a period of 10 days under shade and ground. The ground leaves (450 gm) were soaked in sufficient amount of ethanol for one week at room temperature with occasional shaking and stirring then the whole mixture was filtered and the filtrate thus obtained was concentrated using a water bath to get a viscous mass. The viscous mass was kept at room temperature under a ceiling fan to get a dried extract (yield value, 9%). The extract prepared was for pharmacological screening.

Chemicals and equipment: To the commercially available lyophilized streptokinase (SK) vial (Square Pharmaceuticals Ltd. Dimethyl sulfoxide) of 1500000 I.U., 5mL sterile distilled water was added and mixed properly. This suspension was used as a stock from which 100 μL (30,000 I.U.) was used for *in vitro* thrombolysis. Methanol purchased from Merck (Germany). Dimethyl sulfoxide (DMSO) and Vincristine sulfate (2mg/vial; Techno Drugs Limited Bangladesh). Kanamycin (30ìg/disc, Oxoid, England) was used as a standard antibiotic disc.

***In vitro* Antithrombotic activity**

Blood specimen: Whole blood (1.5 ml) was drawn from healthy human volunteers (n = 12) without a history of oral contraceptive or anticoagulant therapy. A new consent, approved by Mohammed Abu Sayeed, Assistant professor & Head of Department of Pharmacy, International Islamic University Chittagong, Bangladesh, for collection of blood samples from Human volunteers. Blood collection were conducted by Md. Shariful Islam (Lab technician, Department of Pharmacy, IIUC) and preservation were conducted by Abdul Karim (Lab technician, Department of Pharmacy, IIUC), who stored the clot containing Eppendorf tube in the refrigerator in Microbiology lab, Department of Pharmacy, IIUC. A 500 µl of blood was transferred to each of the three previously weighed Eppendorf tube tubes to form clots.

Statement on informed consent of the donors: The volunteer donors were supplied a consent form which informed the title of the research project, name and detail contact of investigators as well as purpose of the research. Description of the research mentioning step-by-step brief of the proposed research, inclusion and exclusion criteria of the donors, whether donors will receive any therapy or not, volume of blood to be taken, possible discomfort of the puncture sites, time required for the blood sampling. Benefits of the volunteer described. It was indicated to the consent form that the volunteers might refuse to donate blood at any time. Donor whether could withdraw his sample data was disclosed. The sample was restricted for that individual study not for future research

projects was presented in the consent form. Potential harm, injuries, discomforts or inconvenience associated with donors in this study was added as informed consent statement. If there was known harm to the donors, the potential harm, current knowledge regarding the probability of the occurrence of the harm, clinical importance of the harm; and any relevant knowledge regarding the probability of reversibility. Treatment alternative and possibility of the research was described. Confidentiality statement was included in the consent form in the way that "confidentiality will be respected and no information that discloses the identity of the participant will be released or published without consent unless required by law of states. Finally identification of investigators was provided in case of further query. The consent form was concluded with major questions on above disclosures in Yes/NO form followed by the signature (with date) of the donor.

***In Vitro* Antithrombotic Study procedure:** Experiments for clot lysis were carried as reported earlier (7-9). Briefly, 1.5 ml venous blood drawn from the healthy volunteers was distributed in three different pre weighed sterile Eppendorf tube (0.5 ml/tube) and incubated at 37°C for 45 min. After clot formation, serum was completely removed without disturbing the clot and each tube having clot was again weighed to determine the clot weight (clot weight = weight of clot containing tube – weight of tube alone). To each Eppendorf tube containing pre-weighed clot, 100 µl of methanol extract of *A. ghaesembilla* leaves were added separately. As a positive control, 100 µl of SK and as a negative non-antithrombotic control, 100 µl of distilled water were separately added to the control tubes numbered. All the tubes were then incubated at 37°C for 90 min and observed for clot lysis. After incubation, fluid released was removed and tubes were again weighed to observe the difference in weight after clot disruption. Difference obtained in weight taken before and after clot lysis was expressed as percentage of clot lysis. The experiment was repeated with the blood samples of the 12 volunteers.

Cytotoxicity assay: Brine shrimp lethality bioassay was carried out with the method as described by Meyer *et al.*(10, 11) to investigate the cytotoxicity of methanol extract of *A. ghaesembilla* leaves. The dried extract preparations were re-dissolved in DMSO to obtain a solution of 10 mg/ml which was subjected to serial dilution to get the concentrations between 12.5 µg/ml- 400 µg/ml. Standard drug Vincristine Sulphate (VS) was used as positive control at concentrations of 5 µg/ml - 0.312 µg/ml. A 5.0 ml of artificial sea water was added into all the test tubes. Simple zoological organism (*Artemia salina*) was used as a convenient monitor for cytotoxic screening. The eggs of the brine shrimps were collected from local aquarium shop (Dhaka, Bangladesh) and hatched in artificial seawater (Prepared by using sea salt 38 g/L and adjusted to pH 8.5 using 1N NaOH) under constant aeration for 24 h under the light. The hatched shrimps were allowed to grow by 48 h to get shrimp larvae called nauplii. After 48 h, active nauplii were attracted to one side in a glass petri dish by using a micropipette. The nauplii were then separated from the eggs by aliquoting them in another glass petri dish containing artificial sea water and used for the assay. Suspension containing 10 nauplii was added into each test tube and was incubated at room temperature (25±1°C) for 12 h under the light. The tubes were then examined after 24 h and the number of surviving larvae in each tube was counted with the aid of a 3X magnifying glass. Experiments were conducted along with VS in a set of three tubes per dose. The concentration that would kill 50% of the nauplii (LC_{50}) was determined from a linear regression equation using the software "Microsoft excels 2007".

***In vitro* Antibacterial activity**

Microorganisms: Seven bacterial species, gram-positive *Staphylococcus aureus, Bacillus subtilis, Bacillus cereus* and gram-negative *Salmonella typhi, Salmonella paratyphi, Escherichia coli, Pseudomonas aeruginosa*. These microbes were obtained from the department of Pharmacy, International Islamic University Chittagong.

Media preparation and maintenance of bacteria: All of the bacterial strains were grown and maintained on Nutrient agar (Merck, India) media at 37 °C and pH (7.4±0.2). The bacteria were subculture overnight.

Preparation of concentration: In the study of the antibacterial activity, all the extracts were diluted in their solvent. So methanol extract diluted in methanol and other also. The concentrations corresponding to the extracts given in Table 2 are expressed in terms of µg/ disk.

Preparation of discs: The discs of about 5 mm in diameter were cut by punching machine from Whatman No.1 filter paper. The discs were taken in a petri dish and sterilized by autoclaving, dried in oven at 180^0C.

Antibacterial screening by disk diffusion technique: The antibacterial effects were tested by the disc diffusion method (12, 13) with some minor modification. The filter paper discs (5 mm in diameter) were individually impregnated with 24 µl of 800 µg/disk and 30 µl of 1000 µg/disk of leaves extract of *A. ghaesembilla* and then placed onto the

agar plates which had previously been inoculated with the test microorganisms (within 15 min). The Petri dishes were kept at 4 °C for 3 h before incubation at 37 °C for 24 h. The diameters of the inhibition zones were measured in millimeters. All the tests were performed in triplicate. Blank disc impregnated with distilled water was used as negative control and disc of Kanamycin (30 µg / disc) as positive control.

Determination of relative percentage inhibition: The relative percentage inhibition of the test extract with respect to positive control was calculated by using the following formula (14).

Relative percentage inhibition of the test extract:

$$\frac{100 \times (x - y)}{(z - y)}$$

Where,

x = total area of inhibition of the test extract

y = total area of inhibition of the solvent (Methanol)

z = total area of inhibition of the standard drug

The total area of the inhibition was calculated by using area = πr^2; where, r = radius of zone of inhibition.

Statistical analysis: The results were expressed as mean±SD from triplicate experiment for zone of inhibition from triplicate experiments for Antibacterial activity. All other results are expressed as mean ± standard error of the mean (SEM). Data were analyzed using one way factorial ANOVA tests using SPSS Data Editor for Windows, Version 22.0 (SPSS Inc., USA) followed by Dennett's tests on each group except negative control for antibacterial activity. The results obtained were compared with the control groups for antithrombotic activity by using Tukey test and $P < 0.01$, $P < 0.001$ and $P < 0.0001$ was considered to be statistically significant in Dennett's and Tukey tests. GRAPHPAD PRISM® (version 6.00; GraphPad Software Inc., San Diego, CA, USA) was used for graphical presentation.

RESULTS

***In Vitro* Antithrombotic activity:** In antithrombotic activity assay, addition of 100µl streptokinase as positive control (30,000 I.U.) to the clots and subsequent incubation for 90 minutes at 37°C, showed 81.32±1.46 % lysis of clot. On the other hand, distilled water treated as negative control exhibited a negligible percentage of lysis of clot (6.81±0.97%). The mean difference in clot lysis percentage between positive and negative control was found statistically very significant (P<0.0001). In this study, the crude methanol extract of *A. ghaesembilla* exhibited antithrombotic activity (63.45±2.08%) (Figure 1).

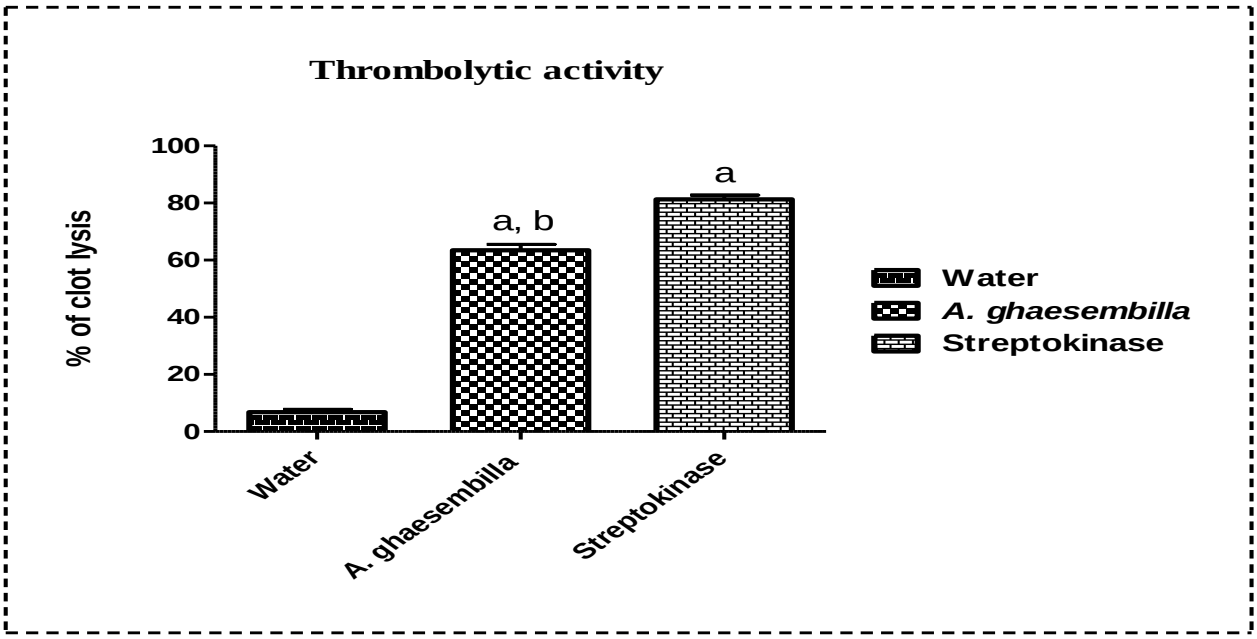

Fig. 3: Antithrombotic activity of methanol extract of *A. ghaesembilla* leaves. Values are mean ± SEM (n = 12); [a]P <0.0001, Tukey test as compared to negative control (Water), [b]P < 0.001, compared to positive control (Streptokinase). Statistical representation of the effective clot lysis percentage by drugs preparations, positive thrombolytic control (streptokinase), and negative control (sterile distilled water) processed by Tukey test by using SPSS for windows, version 22.0.

***In vitro* Brine Shrimp Lethality Bioassay:** In brine shrimp lethality bioassay, the methanol extract of *A. ghaesembilla* leaves showed optimistic result in comparison with the positive control vincristine sulphate. By plotting the log of concentration (log C) versus percent (%) of mortality for all test samples showed an approximate linear correlation. From the graph, the median lethal concentration (LC_{50}) Cytotoxic effect of the extract is summarized in the Figure 2. The LC_{50} for ethanol extract of *A. ghaesembilla* leaf were found to be 432.13 µg/ml respectively, and that of Vincristine Sulphate was 0.74µg/ml. DMSO was used as negative control to validate the test method.

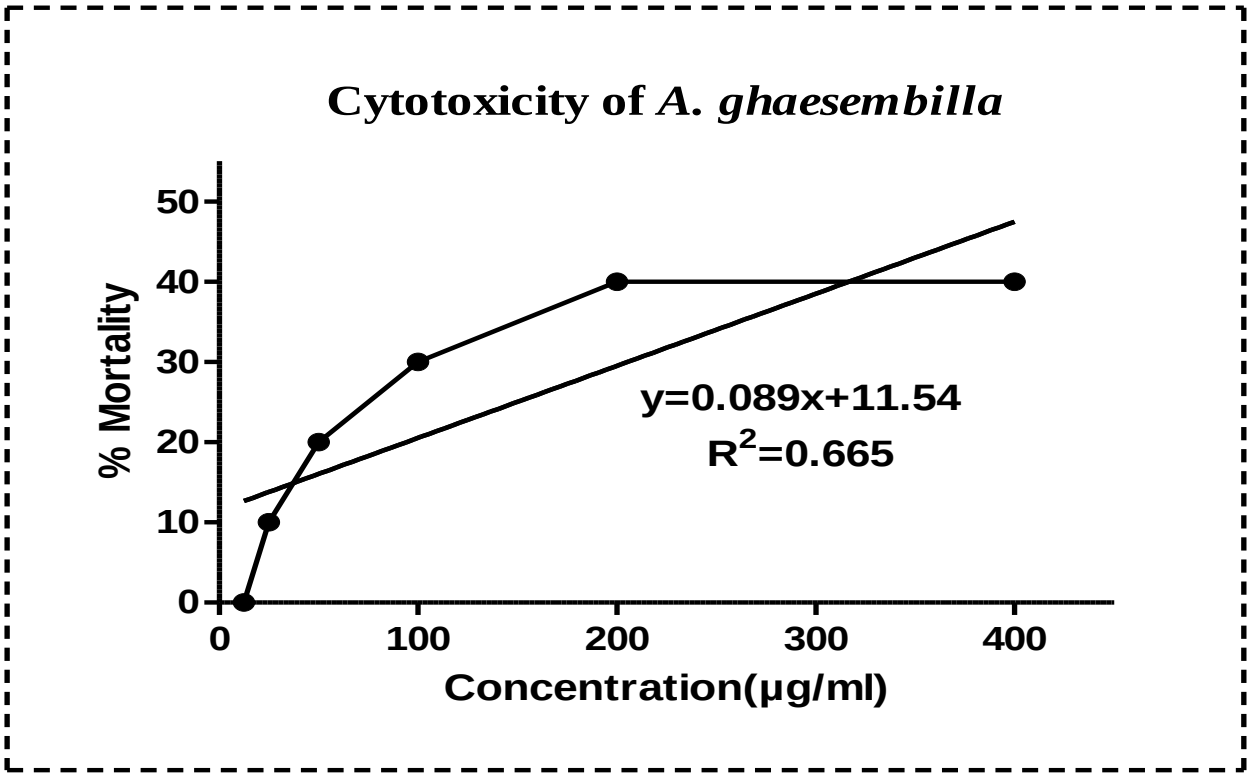

Fig.2: Effects of various concentrations of methanol extract of *A. ghaesembilla* leaves on the viability of brine shrimp nauplii after 24 hrs incubation. Percent mortality of brine shrimps of methanol extract of *A. ghaesembilla*. Data are shown as mean ± SEM of ten shrimps for each concentration.

***In vitro* Antibacterial activity**

Determination of zone of inhibition: Antibacterial activity results of *A. ghaesembilla* leaves extract are given in Table 1. The extract at two different concentrations 1000 µg/disc and 800 µg/disc showed significant ($P < 0.01$ and $P < 0.001$) as compared with standard Kanamycin 30µg/disc showed zone of inhibitions against Gram positive *Staphylococcus aureus* (Nil), *Bacillus subtilis* (9.0±0.50, 7.0±0.50), *Bacillus cereus* (9.3±0.58, 7.3±0.58), *Salmonella typhi* (10±0.50, 8.5±0.50), *Salmonella paratyphi* (8.8±0.29, 7.3±0.58), *Escherichia coli* (11.2±1.26, 9.3±0.29), *Pseudomonas aeruginosa* (12.5±0.50, 9.5±0.50) respectively. The extract showed the highest zone of inhibition against the Gram negative *Pseudomonas aeruginosa* (12.5±0.50) at concentration 1000 µg/disc. However, *Staphylococcus aureus* showed the no antibacterial activity to the extract *A. ghaesembilla* leaves. Relative percentage inhibition of the tested extract presented in Table 2.

Table 1: Results of antibacterial activity testing of *A. ghaesembilla* leaves.

Name of the bacteria	Negative control (Methanol)	Methanol extract of *A. ghaesembilla* leaves		Kanamycin
	30µl/disc	1000µg/disc	800µg/disc	30µg/disc
Gram Positive				
Staphylococcus aureus	-	8.5±0.50[a]	-	22.2±0.76
Bacillus subtilis	-	9.0±1.00[a]	6.5±0.50[b]	18.2±0.29
Bacillus cereus	-	-	-	25±0.50
Gram Negative				
Salmonella typhi	-	7.8±0.29[b]	-	25.3±0.58
Salmonella paratyphi	-	8.5±0.50[b]	6.5±0.50[b]	20.3±0.29
Escherichia coli	-	11.0±1.0[a]	9.0±0.50[a]	23.5±0.50
Pseudomonas aeruginosa	-	12.5±0.50[b]	8.5±0.50[b]	25.5±0.50

Values are mean inhibition zone (mm) ± S.D of three replicates. The different superscripted (a, b) values have significantly different ([a]P < 0.01 and [b]P < 0.001) as compared with standard (Kanamycin) in same row in Dunnett's test by SPSS. - - - = no zone of inhibition.

Table 2: Relative percentage inhibition of Methanol extract of *A. ghaesembilla* leaves with their doses compare to standard anibiotics.

Name of the bacteria	Relative percentage inhibition (%)	
	Methanol extract of *A. ghaesembilla* leaves	
	1000µg/disc	800µg/disc
Gram Positive		
Staphylococcus aureus	14.7	-
Bacillus subtilis	24.5	12.8
Bacillus cereus	-	-
Gram Negative		
Salmonella typhi	9.5	-
Salmonella paratyphi	17.5	10.2
Escherichia coli	21.9	14.7
Pseudomomas aeruginosa	24.0	11.1

Values calculated from their mean values.

DISCUSSION

Thrombus formed in blood vessels lead to atherothrombotic diseases such as myocardial or cerebral infarction. Thrombolytic agents are used to dissolve the already formed clots in the blood vessels; however, these drugs sometimes cause serious and fatal consequences (15). Coronary artery thrombosis has been treated by urokinase (UK), streptokinase (SK) or tissue plasminogen activators (t-PA) which are widely used clinical thrombolytic agent for the treatment of severe or massive deep venous thrombosis, pulmonary embolism, myocardial

infarction, and occluded intravenous or dialysis cannulas (16). Although UK and SK are widely used in India, Bangladesh and other developing countries due to lower cost (17) as compared to other thrombolytic drugs but, the use is associated with high risk of bleeding intracranial hemorrhage, severe anaphylactic reaction and lacks specificity (18). However, herbal drugs are wide-spoken as green medicine for their safe and dependable health care paradigms. The traditional herbal medicines increased an uprising interest since couple of decades due to their incredible pharmacological activities, economic viability and less side effects in different healthcare management system (19). Thus, tremendous efforts have also been directed towards the discovery and development of natural products with antiplatelet (20), anticoagulant (21), antithrombotic (22) and thrombolytic activity of the plants not documented.

Antithrombotic activity of leaf of *A. ghaesembilla* was measured and compared with streptokinase (the positive control) and sterile distilled water (the negative control). In the study, methanol leaves extract was found to provide very well clot lysis activity. However, the percentage of clot lysis produced by the commercially available positive control was greater than this produced by the extract. Cytotoxicity screening models provide important preliminary data to help select plant extracts with potential antineoplastic properties for future work (23). Brine shrimp lethality test is carried out in order to reveal new anticancer compounds.

The effects of leaf extract of *A. ghaesembilla* on the rate of mortality of brine shrimps larvae are presented in Figure 2. The methanol extracts of *A. ghaesembilla* leaves displayed moderate cytotoxic potential against *Artemia salina* (Brine shrimps larvae) in that the LC_{50} of the extract was 432.13 µg/ml.

It was watched that the antimicrobial impact of plant extract fluctuates starting with one plant then onto the next in distinctive inquires about completed in diverse areas of the world. This may be because of numerous components, for example, the impact of atmosphere, soil structure, age and vegetation cycle stage, on the quality, amount and arrangement of extricated item, diverse bacterial strains (24, 25). Moreover, different studies found that the type of solvent has an important role in the process of extracting (26, 27).

The leaves extract of *A. ghaesembilla* indicated zone of inhibition against Gram positive bacteria (*Staphylococcus aureus* and *Bacillus subtilis*) and Gram negative bacteria (*Salmonella typhi*, *Salmonella paratyphi*, *Escherichia coli*, *Pseudomonas aeruginosa*) at 1000 µg/disc. Gram negative bacteria *Bacillus cereus* demonstrated no action against *A. ghaesembilla* leaves extract at both doses.

CONCLUSION

Methanol extract of *A. ghaesembilla* leaves indicated antithrombotic, cytotoxic and antibacterial impact, it can be expected that distinctive dynamic auxiliary metabolites were available in this concentrate and maybe some of these mixes may work in a synergistic way. On the other hand, further studies are important to illustrate the component lying with these impacts. On the other hand, this is the first write about this example and it may serve as a stride with respect to the natural and pharmacological exercises of this specimen.

CONFLICT OF INTEREST STATEMENT

The authors declare that they have no conflict of interest.

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