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Evaluation of Antibacterial Effect of Ethanol Extract and its Different Fractions of *Anogeissus acuminata* [Roxb] Leaves by Disk Diffusion Technique

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ABSTRACT

Background: The reason of the present study was to assessment of antibacterial effect of leaves extract of *Anogeissus acuminata* [Roxb.] and its different fractions.

Methods: Leaves of *A. acuminata* was extracted with pure ethanol [EEAA], then methanol extract fractioned with n-hexane [NHFEEAA], chloroform [CHFEEAA], ethyl acetate [EAFEEAA] and methanol [MFEEAA]. Antibacterial activity was measured by observing zone of inhibition for each extract and fractions on both gram positive and negative bacteria.

Results: The highest antimicrobial potential observed for MFEEAA [12.0 ± 0.50] mm of zone of inhibition with 43.47% relative percentage of inhibition at 1000 µg/disc compared to Kanamycin 30 µg/disc and EEAA extract showing 41.33% of relative percentage of inhibition at 1000 µg/disc.

Conclusions: In a nutshell, results of the present experiments indicated significant Antibacterial action of the extract and fractions of leaves of *A. acuminata*. Moreover, this plant warrants additional research for other supreme pharmacological activities and inclusive research and isolation of the dynamic constituents accountable for these activities and set up the mechanism of action.

Keywords: *Anogeissus acuminata*, Antibacterial, Ethanol extract, Fraction

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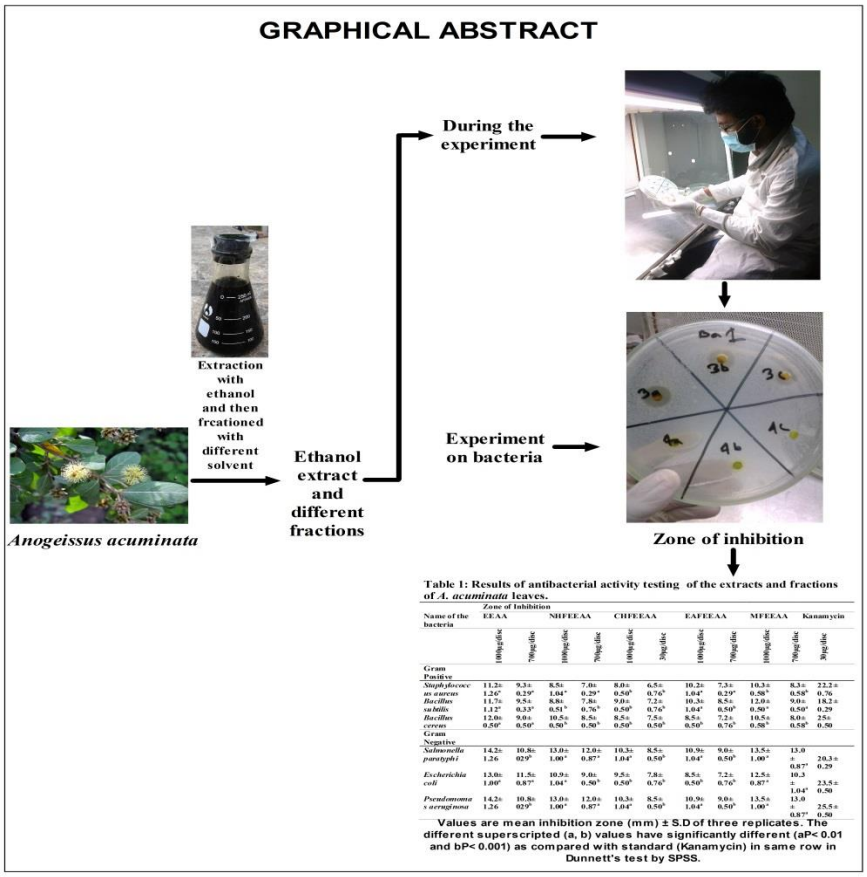

Table 1: Results of antibacterial activity testing of the extracts and fractions of *A. acuminata* leaves.

Name of the bacteria	Zone of Inhibition EEAA 100µg/disc	EEAA 70µg/disc	NHFEEAA 100µg/disc	NHFEEAA 70µg/disc	CHFEEAA 100µg/disc	CHFEEAA 30µg/disc	EAFEEAA 100µg/disc	EAFEEAA 70µg/disc	MFEEAA 100µg/disc	MFEEAA 70µg/disc	Kanamycin 30µg/disc
Gram Positive											
Staphylococcus aureus	11.2± 1.26^a	9.3± 0.29^a	8.5± 1.04^a	7.0± 0.29^a	8.0± 0.50^b	6.5± 0.76^b	10.2± 1.04^a	7.3± 0.29^a	10.3± 0.58^b	8.3± 0.58^b	22.2± 0.76
Bacillus subtilis	11.7± 1.12^a	9.5± 0.33^a	8.8± 0.51^a	7.8± 0.76^b	9.0± 0.50^b	7.2± 0.76^b	10.3± 1.04^a	8.5± 0.50^b	12.0± 0.50^a	9.0± 0.50^a	18.2± 0.29
Bacillus cereus	12.0± 0.50^a	9.0± 0.50^a	10.5± 0.50^a	8.5± 0.50^b	8.5± 0.50^b	7.5± 0.50^b	8.5± 0.50^b	7.2± 0.76^b	10.5± 0.58^b	8.0± 0.58^b	25± 0.50
Gram Negative											
Salmonella paratyphi	14.2± 1.26	10.8± 029^b	13.0± 1.00^a	12.0± 0.87^a	10.3± 1.04^a	8.5± 0.50^b	10.9± 1.04^a	9.0± 0.50^b	13.5± 1.00^a	13.0 ± 0.87^a	20.3± 0.29
Escherichia coli	13.0± 1.00^a	11.5± 0.87^a	10.9± 1.04^a	9.0± 0.50^b	9.5± 0.50^b	7.8± 0.76^b	8.5± 0.50^b	7.2± 0.76^b	12.5± 0.87^a	10.3 ± 1.04^a	23.5± 0.50
Pseudomonas aeruginosa	14.2± 1.26	10.8± 029^b	13.0± 1.00^a	12.0± 0.87^a	10.3± 1.04^a	8.5± 0.50^b	10.9± 1.04^a	9.0± 0.50^b	13.5± 1.00^a	13.0 ± 0.87^a	25.5± 0.50

Values are mean inhibition zone (mm) ± S.D of three replicates. The different superscripted (a, b) values have significantly different (aP< 0.01 and bP< 0.001) as compared with standard (Kanamycin) in same row in Dunnett's test by SPSS.

INTRODUCTION

Antibacterial also known as antibiotics are the type of antimicrobials use in the treatment and prevention of bacterial infection [1]. A wide range of antibacterial drugs have been discovered over the few decades which fundamentally enhanced the quality of life and extremely benefited world populations.

Though, the wellbeing benefits are under danger as generally used antibiotics have turn into less effective against certain sickness not only they create toxic reactions but also due to emergence of drug resistant strain [2].

In 2012, WHO gave statement a gradual increase in resistance to HIV drugs, albeit not reaching dangerous levels. Ever since then, additional augments in resistance to first-line treatment drugs were reported. In 2013, there were about 480 000 new cases of multidrug-resistant tuberculosis [MDR-TB].

Extensively drug-resistant tuberculosis [XDR-TB] has been identified in 100 countries. WHO's 2014 report on global surveillance of antibacterial resistance exposed that antibiotic resistance is no longer a forecast for the future; it is occurring right now, across the world, and is putting at risk the ability to treat general infections in the population and hospitals. Without urgent, coordinated action, the world is heading towards a post-antibiotic era, in which common infections and minor injuries, which have been treatable for decades, can once again kill [3]. A widespread and broad research is very important to find out resource of antibacterial drugs and acquire the results and collection them in statistical data. Infectious illnesses caused by bacteria and fungi are a major cause of death and morbidity in humans. Even though numerous antibiotics have been developed to handle these diseases with best possible efficacy, their misconduct and

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maladministration, as well as microbial alteration have led to the coming out of drug-resistant strains. So, over the last decades, antibiotics that are recognized to heal precise diseases have lost their usefulness. Consequently, the hunt for newantimicrobial drugs from natural sources is warranted.

Natural products of higher plants may give another wellspring of antimicrobial operators with potentially novel instruments of activity [4,5]. Plants are rich in a wide assortment of optional metabolites, for example, tannins, terpenoids, alkaloids, flavonoids, glycosides, and so on., which have been found *in vitro* to have antimicrobial properties [6,7]. Hence, logical examinations have been completed on the antimicrobial exercises of plant separates against various sorts of microorganisms, which have brought about the advancement of option plant-based antimicrobial medications [8].

The plant under examination *Anogeissus acuminata* [Family: *Combretaceae*]. It is a native plant disseminated in Chittagong Hill Tracts and Cox's Bazar and with tribal name Phul jhumuri gaas [Chakma]. The plant is rich in tannins and flavonoids. The plant material used for this study was collected from Bandarban district, Bangladesh. The tannoid principles of the plant possess antioxidant activity which was proven to reduce microbial infection [9,10].

As a piece of determined examination of various important restorative plants in Bangladesh, the ethanol extract of *A. acuminata* [EEAA] and its various fractions were studied for the potential of antibacterial effect.

MATERIALS AND METHODS

Plant materials

The leaves of *A. acuminata* were collected from Bandarban, Bangladesh in March, 2015 at mature stage. The leaves were cut into small pieces and then dried in shade at 21-30ºC for 7 days. Then the materials were dried in an oven at low temperature to improve grinding. Then the pieces were ground by a mechanical grinder and then passed through a size 60 mesh screen to obtain a fine powder of the leaf material. This was stored in an air-tight container.

Preparation of sample

The fine powder of leaves of *A. acuminata* [800 g] was taken in a clean round-bottom flask [5 L] and soaked in 4 L of Ethanol for 15 days at room temperature with occasional shaking and stirring. Then the mixture was first filtered with cotton plug followed by Whatman No. 1 filter paper. The filtrate is evaporated to dryness in Heidorph rotary evaporator at 45ºC to obtain a concentrated extract. This was then air dried to obtain solid residue. Thus the Ethanolic extract of the leaves of *A. acuminata* was prepared and then four solvents chloroform, n-hexane, ethyl acetate and methanol was used for solvent-solvent partitioning from ethanol solution.

CHEMICALS AND REAGENTS

The chemicals used were: ethanol, methanol, n-hexane, chloroform [Merck, Germany]. Dimethylsulfoxide [DMSO] was from Sigma-Aldrich and rests of the chemicals used were analytical grade. Kanamycin [30 µg/disc, Oxoid, England] was used as a standard antibiotic disc.

In vitro antibacterial activity

Antibacterial screening by disk diffusion technique

EEAA, NHFEEAA, CHFEEAA, EAFEEAA and MFEEAA were screened at two concentrations [700 and 1000 µg/disc] against three gram-positive [*Staphylococcus aureus, Bacillus subtilis, Bacillus cereus*] and three gram negative bacteria [*Salmonella paratyphi, Escherichia coli, Pseudomonas aeruginosa*] using the disc diffusion method [11-13]. Solutions of known concentration [33.3 mg/mL] of the test samples were prepared. Dried and sterilized filter paper discs [about 5 mm diameter] were then impregnated with known amounts [30 µl for 1000 µg/disc, 21 µl for 700 µg/disc] of the test substances using a micropipette. Discs containing the test material were placed on nutrient agar medium [Merck, India] uniformly seeded with the pathogenic test microorganisms. The prepared inoculum size was approximately 106 CFU/mL. Standard antibiotic discs [kanamycin, 30 µg/disc] and blank discs [impregnated with solvents] were used as positive and negative controls, respectively. These plates were then, kept at 4°C for a 1-h diffusion of the test material. There was a gradual change in concentration surrounding the discs. The plates were then, incubated at 37°C for 24 h to allow organism growth. The test materials having antibacterial activity inhibited microorganism growth, and a clear, distinct zone of inhibition surrounding the

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discs was visualized [14]. The antibacterial activity of the test agents was determined by measuring the diameter of the zone of inhibition expressed in millimeters [mm].

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 [15-17].

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

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. Relative percentage inhibition was calculated by Microsoft Excel 2007. Data were analyzed using one way ANOVA tests using SPSS Data Editor for Windows, Version 22.0 [SPSS Inc., USA] followed by Dennett's tests. The results obtained were compared with the positivetive control group for antibacterial activity and $P<0.01$ and $P<0.001$ was considered to be statistically significant in Dennett's tests. GRAPHPAD PRISM® [version 6.00; Graph Pad Software Inc., San Diego, CA, USA] was used for graphical presentation.

RESULTS

***In vitro* antibacterial activity**

Determination of zone of inhibition

The antibacterial activity of EEAA, NHFEEAA, CHFEEAA, EAFEEAA and MFEEAA were tested against pathogenic bacteria and all the extract and fractions exhibited a significant antibacterial activity against both gram positive and gram negative bacteria at the concentration of 700 and 1000 µg/disc which is shown in Table 1. The inhibitory activities showed the test samples were compared with standard broad spectrum antibiotic Kanamycin [30 µg/disc]. The zones of inhibition produced by EEAA, NHFEEAA, CHFEEAA, EAFEEAA and MFEEAA against gram-positive bacteria were found to be 6.5 ± 0.76 mm to 12.0 ± 0.50 mm and against gram-negative bacteria was found to be 7.2 ± 0.7 6 mm to 14.2 ± 1.00 mm at different concentration. EEAA exhibited wide zone of inhibition of 11.7 ± 1.12 mm against gram-positive bacteria *Bacillus subtilis* at 1000 µg/disk and 14.2 ± 1.26 for gram negative bacteria *Escherichia coli* at 1000 µg/disk. MFEEAA resulted 12.0 ± 0.50 mm zone of inhibition for *Bacillus subtilis* at 1000 µg/disk and 13.5 ± 1.00 mm for *Escherichia coli* at the same concentration and hence significant relative % of inhibition. On the other hand, Kanamycin [30 µg/disc] showed a zone of inhibition against gram-positive bacteria in the range of 18.2 ± 0.29 mm to 25 ± 0.50 mm and against gram-negative bacteria in the range of 20.3 ± 0.29 mm to 25.5 ± 0.50 mm.

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Table 1: Results of antibacterial activity testing of the extracts and fractions of *A. acuminata* leaves

	Zone of Inhibition [mm]										
Name of the bacteria	**EEAA**		**NHFEEAA**		**CHFEEAA**		**EAFEEAA**		**MFEEAA**		**Kanamycin**
	1000 µg/disc	**700 µg/disc**	**1000 µg/disc**	**700 µg/disc**	**1000 µg/disc**	**30 µg/disc**	**1000 µg/disc**	**700 µg/disc**	**1000 µg/disc**	**700 µg/disc**	**30 µg/disc**
Gram Positive											
Staphylococcus aureus	11.2 ± 1.26[a]	9.3 ± 0.29[a]	8.5 ± 1.04[a]	7.0 ± 0.29[a]	8.0 ± 0.50[b]	6.5 ± 0.76[b]	10.2 ± 1.04[a]	7.3 ± 0.29[a]	10.3 ± 0.58[b]	8.3 ± 0.58[b]	22.2 ± 0.76
Bacillus subtilis	11.7 ± 1.12[a]	9.5 ± 0.33[a]	8.8 ± 0.51[b]	7.8 ± 0.76[b]	9.0 ± 0.50[b]	7.2 ± 0.76[b]	10.3 ± 1.04[a]	8.5 ± 0.50[b]	12.0 ± 0.50[a]	9.0 ± 0.50[a]	18.2 ± 0.29
Bacillus cereus	12.0 ± 0.50[a]	9.0 ± 0.50[a]	10.5 ± 0.50[b]	8.5 ± 0.50[b]	8.5 ± 0.50[b]	7.5 ± 0.50[b]	8.5 ± 0.50[b]	7.2 ± 0.76[b]	10.5 ± 0.58[b]	8.0 ± 0.58[b]	25 ± 0.50
Gram Negative											
Salmonella paratyphi	14.2 ± 1.26	10.8 ± 029[b]	13.0 ± 1.00[a]	12.0 ± 0.87[a]	10.3 ± 1.04[a]	8.5 ± 0.50[b]	10.9 ± 1.04[a]	9.0 ± 0.50[b]	13.5 ± 1.00[a]	13.0 ± 0.87[a]	20.3 ± 0.29
Escherichia coli	13.0 ± 1.00[a]	11.5 ± 0.87[a]	10.9 ± 1.04[a]	9.0 ± 0.50[b]	9.5 ± 0.50[b]	7.8 ± 0.76[b]	8.5 ± 0.50[b]	7.2 ± 0.76[b]	12.5 ± 0.87[a]	10.3 ± 1.04[a]	23.5 ± 0.50
Pseudomomas aeruginosa	14.2 ± 1.26	10.8 ± 029[b]	13.0 ± 1.00[a]	12.0 ± 0.87[a]	10.3 ± 1.04[a]	8.5 ± 0.50[b]	10.9 ± 1.04[a]	9.0 ± 0.50[b]	13.5 ± 1.00[a]	13.0 ± 0.87[a]	25.5 ± 0.50

Values are mean inhibition zone [mm] ± S.D of three replicates. Bold text indicates the highest antibacterial activity of extracts on each test bacteria. 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.

Determination of relative percentage inhibition

The consequences of antimicrobial action of plant remove were contrasted and the positive control [Standard drugs] for assessing their relative percentage inhibition. The extract and fractions display most extreme relative percentage inhibition against the tried microscopic organisms are exhibited in Table 2.

DISCUSSION

Plants are critical wellspring of possibly valuable structures for the advancement of new chemotherapeutic specialists. The initial move towards this objective is the *in vitro* antibacterial action measure [18]. It seems very likely, therefore, that the extract of *A. acuminata* may inhibit bacteria by a different mechanism than that of currently used antibiotics and may have therapeutic value as an antibacterial agent against multi-drug resistant bacterial strains [19]. Because infections caused by *Pseudomonas aeruginosa*, especially those with multi-drug resistance, are among the most difficult to treat with conventional antibiotics. From the results obtained, it appears that the antibacterial action of the extract and fractions are more pronounced on gram-negative than on gram-positive bacteria in most cases or is even equal [20,21].

The antimicrobial effect of the compound of plant oils and extracts has framed the premise of numerous applications, including crude and prepared nourishment protection, pharmaceuticals, option prescription and common treatments [22,23]. It is widely accepted that plants are good sources of novel antimicrobial agents. Screening of antimicrobial activities to find which type of bacteria are susceptible to plant extracts is useful, however the investigation of underlying mechanism is also crucial for drug development [24]. The extracts of *A. acuminata* exhibited significant *in vitro* antibacterial activity against both gram positive and gram negative bacteria and susceptible to these extracts (Table 1). The relative percentage of inhibition for methanol extract [1000 µg/disc] for *B. subtilis* was the highest, such results were not totally unexpected since these bacteria form resting spores and are more resistant to environmental conditions than any

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other tested bacteria [25]. Furthermore, the fractions showed antibacterial activity against *S. aureus, B. cereus, E. coli and P. aeruginosa. S. paratyphi*is likely to have shown resistance against the phytochemicals found in *A. acuminata* but notable zone of inhibition is observed against standard Kanamycin 30 µg/disc.

Table 2: Relative percentage inhibition of different extracts with their doses compare to standard antibiotics

	Relative percentage inhibition [%]									
Name of the bacteria	**EEAA**		**NHFEEAA**		**CHFEEAA**		**EAFEEAA**		**MFEEAA**	
	1000 µg/disc	**700 µg/disc**	**1000 µg/disc**	**700 µg/disc**	**1000 µg/disc**	**700 µg/disc**	**1000 µg/disc**	**700 µg/disc**	**1000 µg/disc**	**700 µg/disc**
Gram Positive										
Staphylococcus aureus	25.45	17.55	14.66	9.94	12.99	8.57	21.11	10.81	21.53	13.98
Bacillus subtilis	41.33	27.25	23.38	18.37	24.45	15.65	32.03	21.81	43.47	24.45
Bacillus cereus	23.04	12.96	17.64	11.56	11.56	9	11.56	8.29	17.64	10.24
Gram Negative										
Salmonella paratyphi	48.8	28.2	40.9	34.8	25.7	17.5	28.7	19.6	44.1	40.9
Escherichia coli	36.51	21.12	30.6	26.08	19.21	13.08	21.51	14.67	33	30.6
Pseudomomas aeruginosa	25.99	20.34	18.27	12.46	13.88	9.36	11.11	7.97	24.03	16.32

Values calculated from their mean values

CONCLUSIONS

In a nutshell, this present study evaluated significant antibacterial activity of Ethanol extract and its different fractions of *A. acuminata*. Although, all pharmacological profile were not performed for a solitary plant promote enhancements on such strategies and headways in the technique could be obtained through watchful and fundamental strategies and consequently requires progress new supplementary methodologies. More accurate studies are wanted to clarify their mechanism of actions for individual phytochemical if possible.

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COMPETING INTERESTS

The authors declare that they have no competing interests.

Employment or leadership: None declared.

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