Microbiological Investigation of *Piper Chaba Hunter*

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Abstract - The Piperaceae is a family of flowering plants. It is a tropical family of five genera and 500 species which are distributed in tropical and subtropical regions. Stem and leaves were successively extracted with petroleum ether, chloroform, ethyl acetate and methanol. Antimicrobial investigations of petroleum ether, chloroform, ethyl acetate and methanol extract were carried out. All these extract showed mild to moderate activities against a number of gram positive and gram negative bacteria except shigella dysenteriae. Extracts of *piper chaba* stem and leaves were tested over six fungi. Viz; *Aspergillus fumugatous, Mucor, Penicillium sp, Human sp, Aspergillus nigar* and *Havous*. The extracts under examination exhibited no activity against the fungal strains.

Index Terms - Piperaceae, Flowering plants, *piper chaba*, Antimicrobial investigations, Antifungal activity, Extract, Stem and Leaves

1. INTRODUCTION

Plant products have been used from time immemorial for the treatment of several diseases. Treatment of diseases with extracts of stem, barks and leaves of plant has been a common phenomenon in our country. Use of decoction of indigenous plants for the recovery of health is still prevalent in our country. Most of the medicines used by millions of people in the different part of the world come directly or indirectly from plant sources. [1], [2], [3], [4]
The fight between man and diseases started in million years ago. In the humid tropical land of Bangladesh diarrhea, cholera, typhoid, malaria, diphtheria etc. diseases particularly in children during the last quarter of the 20 th century. Among all diarrhea diseases shigellosis is predominant and in Bangladesh about 80 to 85 of bacillary dysentery is due to shigella species. Generally organisms are non-motile, non-capsulated and gram-negative rod, the four strains are shigella dysenteriae, shigella flexneriae, shigella boydii and shigella sonnei. In 1970, only 0.6% of all diarrheal cases were associated with highland. This rate increased gradually 9% in 1972, 14% in 1973 to reach a peak 20% in 1974. During the dearly 1980's shigella incidence among the diarrhea patients was about 10% and mortality among these incidence was 17% during the last half century thousands of antibiotics were discovered; most of them are still used for the treatment of infectious diseases and thus chemotherapy is playing a major role in modern treatment. It has been noted that, many types of plant and plant products alleviate the sufferings of the body and mind form time immemorial. So a desperate need to search out new antibacterial agents from plants to relief this killer diseases in perspective of Bangladesh [5], [6], [7]. It has been expected that, the present work on antibacterial and antifungal activities in plant will lead to the scientists that continue work may have clinical success concerning the killer diseases.

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2. MATERIALS AND METHOD

2.1 Collection of plant material

The plant chui (*piper chaba*) was collected from rajshahi district (Motihar Thana). A voucher specimen of this plant was deposited in the Herbarium of the Botany Department of Rajshahi University. The leaves and stem of the plant were separated and cut into small pieces and dried under mild sunlight and then at 40°C in an oven [8], [9]. Afterwards the plant materials were powdered separately by grinding machine. The powder was used throughout the investigation.

2.2 Extraction of plant materials using solvents of various polarities

Extraction was carried out at room temperature with gentle stirring for nine days (three times within this period) from

the dried powdered material using petroleum ether (40-60°C), the resultant extracts were combined and the combined extract was filtered and concentrated under a vacuum condition to obtain semi-solid mass. Residues left after extraction [10], [11] with petroleum ether were dried in air and extracted with chloroform. Finally the same procedure was also followed for the extraction with ethyl acetate and methanol.

2.3 Test organisms used for the study

Twelve bacterial and six fungal strains were used to determine the antibacterial and antifungal activities of different solvent extracts of *piper chaba* [12], [13], [14]. The pure cultures were collected form the institute of Biological Science (IBSc), University of Rajshahi, Bangladesh. The bacterial and fungal strains used for this investigation at listed in the table-1 and table-2.

TABLE 1
List of organisms used for determining antibacterial activity

	Gram Positive		Gram Negative
1.	*Bacillus megaterium*	1.	*Shigella sonnei*
2.	*Bacillus subtilis*	2.	*Pseudomonas aeruginosa*
3.	*Sarcina lutea*	3.	*Escherichia coli*
4.	*Staphylococcus aureus*	4.	*Shigella dysenteriae*
5.	*Streptococcus-β-haemoliticus*	5.	*Shigella shiga*
		6.	*Klebsiella sp.*
		7.	*Salmonella typhi*

TABLE 2
List of organisms used for determining antifungal activity

SL. NO.	Name of Organism
1	*Aspergillus fumugatous*
2	*Mucor sp.*
3	*Penicillium sp.*
4	*Human- 3 sp.*
5	*Aspergillus nigar*
6	*Havous*

2.4 Sterilization procedure

The antimicrobial screening was carried out on a laminar air flow unit and all types of precautions were highly maintained to avoid any type of contamination during the test. UV light was switched on for half an hour before working in the laminar hood to avoid any accidental contamination. Petridis and other glass were sterilized in the autoclave at 121°C temperature and a pressure of 15 Ib/sq. Inch for 15 minutes.

Micropipette, tips, culture media, cotton, blank discs etc. were also sterilized.

2.5 Culture media

Culture medium used for determining antibacterial activity for demonstrating the antibacterial activity and subculture of the test organisms, nutrient agar media was used. Composition of medium for 1000 ml as shown in the table-3

TABLE 3
Composition of culture medium for determining antibacterial activity

Ingredients	Amount
Bacto peptone	5 gm.
Sodium Chloride	5 gm.
Bacto yeast extract	10 gm.
Bacto agar	20 gm.
Distilled water	1000 ml
pH is maintained at about 7.2 ± 0.2 at 25˚ C	

2.6 Preparation of nutrient agar medium

To prepare the required volume of this medium, the amount of each of the constituent was calculated from the composition chart given for 1000 ml. peptone, sodium chloride and yeast extract of the required amount were taken in a conical flask after weighing corporately. Demineralized water (Volume was less than the required final volume) was added and the contents were heated in a water bath to make a clear solution. The pH was adjusted at 7.2 ± 0.2 using NaOH or HCl solution.

The required amount of agar was added to the solution and the demineralized water was added to make the final volume. Again the total volume was heated in a water bath to obtain a clear solution. The conical flask was plugged with cotton and then sterilized in an autoclave at 15-1 lbs. / inch^2 pressure for 15 minutes at 121˚ C.

2.7 Culture media used for determining antifungal activity

Potato Dextrose Agar (PDA) media was used to perform the antifungal activity test and for subculture of the test organism. The composition of the medium for 1000 ml was as shown in table-4

TABLE 4
Composition of culture medium for determining antifungal activity

Ingredients	Amount
Potato infusion	200 gm.
Dextrose	40 gm.
Agar	20 gm.
Distilled water	1000 ml
pH is maintained at about 7.2 ± 0.2 at 25˚ C	

2.8 Preparation of the potato dextrose agar medium

Potato infusion (sliced potato) was boiled with distilled water and filtered and dextrose of the required amount were taken in a conical flask after weighing separately. Demineralized water (Volume was less than the required final volume) was added and the contents were heated in a water bath to make a clear solution. The pH was adjusted at 5.6 ± 0.2 using NaOH or HCl solution. The required amount of agar was added to the solution and the demineralized water was added to make the final volume. Again the total volume was heated in a water bath to obtain a clear solution. The conical flask was plugged with cotton and the sterilized in an autoclave at 15-1 lbs. /inch^2 pressure for 15 minutes at 121˚ C.

2.9 Preparation of fresh culture

The nutrient agar medium was prepared and dispersed in a number of clean test tubes to prepare slants (5 ml in each test tube). The test tubes were plugged with cotton and sterilized in an autoclave at 15-1 lbs. /inch^2 pressure for 15 minutes at 121˚ C. After sterilization the test tubes were kept in an inclined position for solidification. These were then incubated at 37.5˚C to ensure the sterilization. The test organisms were transferred to the agar slants from the supplied pure cultures with the help of an inoculating loop in an aseptic condition. Burning the loop after each transfer of microorganism to avoid contamination very carefully. The inoculated slants were then incubated at 37.5˚C for 24 hours to assure the growth of test organisms. These cultures were used for the sensitivity test.

2.10 Preparation of the test plates

The test organism were transferred from the subculture to the test tube containing 20 ml autoclaved media with the help of an inoculating loop in an aseptic area. The test tube was shaken by rotation to get a uniform suspension of the organism. The suspensions were immediately transferred to the sterile Petridis in an aseptic area. The Petridis were rotated several times, first clockwise and then anti clockwise to assure homogeneous distribution of the test organisms [15], [16], [17], [18]. The medium was poured into Petridis in such a way as to give a inform depth of approximately 4 mm. after the medium was cooled to room temperature, it was stored in a refrigerator at 4˚C.

2.11 Preparation of discs

Two types of discs were prepared for antimicrobial screening. These are as follows:

i) Sample discs: Petroleum ether, chloroform and methanol extracts of bark and leaves of *piper chaba* were dissolved in sufficient amount of respective solvents so that, each 20 µl of solutions contained 400 µg of the test materials. Sterilized filter paper discs (5 mm diameter) were taken in a blank Petridis and 20 ml solution of the extract was applied on it with a micropipette and left for sufficient time for complete evaporation of the solvent.

ii) These were used to compare the antibacterial and antifungal activities of the test materials. Standard antibiotic discs of amoxicillin (30 µg/disc) for antifungal activity were used for comparison.

2.12 Measurement of the zones of inhibition on plates

The antibacterial and antifungal activities of the crude extracts were determined by the disc diffusion method by measuring the diameter of the inhibitory zone in mm by a transparent scale. After 12 to 18 hours of incubation each of the oblates was examined. The diameter of the zones of inhibition was measured by naked eyes using a scale in terms of millimeter [19], [20]. The diameter of the zones of inhibition of the sample was taken compared with the diameter of the zone of inhibition produced by standard antibiotic disc used.

3. Results and Discussion

In order to detect the antibacterial activity (In vitro antibacterial screening) of a new leading compound for development as potential new drug is a useful technique. The different crude extracts (400 µg/disc) were tested for their antibacterial activities against a number of gram-positive and gram-negative bacteria. The results were compared with standard antibiotic amoxicillin (30 µg/disc) and furnished in the table-5 and table-6.

As observed in table-5, all the extracts obtained from *piper chaba* stem bark showed mild activities against most of the tested bacteria. The organism staphylococcus aureus was resistant to all the extracts whereas all the extract showed no activity against shigella dysenteries Pet. ether extract was found to be enactive against all the organism except staphylococcus aureous.

As depicted in table-6, all the crude extract of the *piper chaba* bark exhibited little to mild activity against most of the bacteria, but pet. ether extract was mild action against only the organism staphylococcus aureus. All the extracts were inactive against shigella dysenteries.

It is evident that, all the extract of leaves (Except pet. ether) exhibited mild activity against most of bacteria, the organism shigella dysenteries was resistant to all the extracts of leaves, but petroleum ether extract displayed activity against only the organism staphylococcus aureus.

TABLE 5
Antibacterial activities of crude extracts obtained from stem of *piper chaba*

Name of bacteria	Diameter of the zone of inhibition in mm				
	Pet. Ether extract 400µg / disc	Ethyl acetate extract 400µg / disc	Chloroform extract 400µg / disc	Methanol extract 400µg / disc	Standard Amoxicillin 30µg / disc
Gram-positive					
Bacillus megaterium	Inactive	Inactive	8	6	25
Bacillus subtilis	Inactive	6	7	7	21
Sarcina lutea	Inactive	7	6	8	20
Staphylococcus aureus	9	9	12	12	23
Streptococcus-B haemoliticus	Inactive	8	7	11	23
Gram-negative					
Shigella sonnei	Inactive	6	6	6	24
Pseudomonas aeruginosa	Inactive	7	Inactive	7	20
Escherichia coli	Inactive	Inactive	7	6	25
Shigella dysenteriae	Inactive	Inactive	Inactive	Inactive	23
Shigella shiga	Inactive	6	6	7	21
Klebsiella sp	Inactive	7	Inactive	6	19
Salmonella typhi	Inactive	7	8	6	22

TABLE 6
Antibacterial activities of crude extracts obtained from leaves of *piper chaba*

Name of bacteria	Diameter of the zone of inhibition in mm				
	Pet. Ether extract 400µg / disc	Ethyl acetate extract 400µg / disc	Chloroform extract 400µg / disc	Methanol extract 400µg / disc	Standard Amoxicillin 30µg / disc
Gram-positive					
Bacillus megaterium	Inactive	6	7	6	22
Bacillus subtilis	Inactive	7	6	8	20
Sarcina lutea	Inactive	6	7	7	21
Staphylococcus aureus	8	10	14	12	24
Streptococcus-B haemoliticus	Inactive	7	8	10	23
Gram-negative					
Shigella sonnei	Inactive	Inactive	7	6	25
Pseudomonas aeruginosa	Inactive	7	6	8	23
Escherichia coli	Inactive	10	9	11	22
Shigella dysenteriae	Inactive	Inactive	Inactive	Inactive	20
Shigella shiga	Inactive	6	7	Inactive	21
Klebsiella sp	Inactive	8	Inactive	6	24
Salmonella typhi	Inactive	6	6	7	22

The activities of the crude extracts were measured by the zone of inhibition which indicated the activity against fungi tested. The zone of inhibition of the samples were then compared with the zone of inhibition produced by the standard antibiotic disc used, the results obtained are given in table-7 and table-8.

TABLE 7
Antifungal activities of crude extracts obtained from bark of *piper chaba*

Name of the fungi	Diameter of the zone of inhibition in mm				
	Pet. Ether extract 400µg / disc	Ethyl acetate extract 400µg / disc	Chloroform extract 400µg / disc	Methanol extract 400µg / disc	Standard Amoxicillin 50µg / disc
Aspergillus fumugatous	-	-	-	-	14
Mucor	-	-	-	-	16
Penicilium sp.	-	-	-	-	12
Human sp.	-	-	-	-	11
Aspergillus nigar	-	-	-	-	13
Havous	-	-	-	-	11

TABLE 8
Antifungal activities of crude extracts obtained from leaves of *piper chaba*

Name of the fungi	Diameter of the zone of inhibition in mm				
	Pet. Ether extract 400µg / disc	Ethyl acetate extract 400µg / disc	Chloroform extract 400µg / disc	Methanol extract 400µg / disc	Standard Amoxicillin 50µg / disc
Aspergillus fumugatous	-	-	-	-	15
Mucor	-	-	-	-	14
Penicilium sp.	-	-	-	-	16
Human sp.	-	-	-	-	12
Aspergillus nigar	-	-	-	-	13
Havous	-	-	-	-	10

As shown table-5, the crude extract of *piper chaba* stem displates mild activity against most of the tested bacteria. The petroleum ether extract was active only on the gram positive organism *Staphylococcus aureus.*

All the extract did not show any activity against shigella dysenteriar comparatively the extracts chloroform and methanol showed exhibited better activity against *Staphylococcus aureus.* As given in table-5, all the extracts where mild active against most of the tested bacteria.

4. CONCLUSION

Piper chaba belongs to the genus piper and family piperaceae and is locally known as Chui. Different parts of the plant are being used for the treatment of different diseases. All part of the plant are being used for the treatment of different diseases. Every part of the plant like roots, barks and leaves contain various compounds like alkaloid saponin, ester, sugar, steroids, acid and minerals.

The petroleum extract displayed activity against only *Staphylococcus aureus.* The organism shigella dysenteriae was a resistance to all the crude extracts and all the crude extract comperatively (except pet-ether) displayed higher activity against *Staphylococcus aureus.*

Table-7 and table-8 demonstrated the antifungal activities of petroleum ether, chloroform, ethyl acetate, methanol, but all crude extracts (400 µg / disc) were inactive against the tested fungi.

Fresh stem of piper chaba was extracted with petroleum ether (60-80°C). Then the extract was triturated with chloroform.

In antibacterial screening petroleum ether, ethyl acetate, chloroform and methanol extracts exhibited mild to moderate activity against most of the tested bacteria except shigella dysenteriae. On the other hand all these extracts of *piper chaba* stem and leaves were found inactive against the fungi tested.

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