Pharmacological Study

In vitro antimicrobial and brine shrimp lethality of *Allophylus cobbe* L.

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Abstract

Plants are rich source of pharmacologically active agents, which could be explored in disease management. Methanol, ethanol, and petroleum ether extracts of the whole plant of *Allophylus cobbe* L. were evaluated for antimicrobial and cytotoxic activities. *In vitro* antimicrobial sensitivity by disk diffusion method was conducted against four Gram-positive and seven Gram-negative pathogenic bacteria and seven fungi. In the antibacterial and antifungal sensitivity tests, growth inhibition was found to be within the range of 10.0–17.67 mm. Strong zone of inhibition by the ethanol extract of *A. cobbe* (EEAC) was found against *Trichophyton* spp. With some exceptions, a mild to strong antimicrobial activity was observed in this study. Significant minimum inhibitory concentration (MIC; 15.625 μg/ml) was found against *Trichophyton* spp. Other detected MICs were within the range of 31.25–125 μg/ml. The petroleum ether extract of the plant exhibited strong cytotoxicity in the brine shrimp lethality bioassay test.

Key words: *Allophylus cobbe*, antimicrobial, bioassay, brine shrimp lethality

Introduction

Many of the plant materials used in traditional medicine are readily available in rural areas and this has made traditional medicine relatively cheaper than modern medicine.¹ Medicinal plants contain pharmacologically active principles which, over the years, have been explored in traditional medical practice for the treatment of various ailments.² Bangladesh is a developing country and it covers a large number of poor people who are unable to access the modern medical support. Most of them are usually dependent upon the Kabiraj (traditional medicine practitioners) for their health problems.

*Allophylus cobbe* L. (Family: Sapindaceae), a herb, grows wild in the hilly region of Bangladesh. It has anti-feedant activity and is used as oxytocic and antidiarrheal agents by the traditional practitioners in the East-West region of Bangladesh.³ The aim of the present study is to evaluate the antimicrobial sensitivity and cytotoxic responses of the methanol, ethanol, and petroleum ether extracts of the targeted plant and to search logical evidence for its folk use and further exploitation.

Materials and Methods

Plant collection and identification

The plant under investigation was collected from the hilly area of Baluchara, Chittagong, Bangladesh, in January 2010 and was identified by the authority at Forest Research Institute, Chittagong, Bangladesh.

Extraction

The plant was subjected to shade dry. Then the crude dried plant was ground into coarse powder and subjected to hot extraction⁴ with methanol, ethanol, and petroleum ether by the Soxhlet apparatus. The extraction was carried out for about 18 h and the extract was filtered through a cotton plug followed by Whatman filter paper, no. 1. The extracts were then concentrated by using a rotary evaporator.

Antimicrobial screening

The antibacterial and antifungal activities of the crude extractives were evaluated by the disk diffusion method⁵ against four Gram-positive and seven Gram-negative pathogenic bacteria and seven fungi using ciprofloxacin (Ciprocin, 500 mg/tab., Square Pharmaceuticals Ltd.) and fluconazole (Flugal, 25 mg/cap., Square Pharmaceuticals Ltd., Dhaka, Bangladesh), respectively, as standards. The organisms were obtained as pure culture from the Faculty of Biology, University of Chittagong, Bangladesh. The antimicrobial activity of the test agents was expressed by measuring the diameter of zone of inhibition

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expressed in millimeters (mm). The experiments were carried out in triplicate.

Minimum inhibitory concentration
The minimum inhibitory concentrations (MICs) of the extractives were determined by the serial tube dilution test in nutrient broth medium containing graded concentrations (500, 250, 125, 62.50, 31.25, 15.625, 7.8125, 3.90625, 1.953125, and 0.9765625 μg/ml) of the crude extractives and inoculated test organisms. As the second highest MIC was found against Bacillus subtilis, Staphylococcus aureus, and Vibrio cholerae, respectively. But the extract EEAC was inactive against Bacillus megaterium, Bacillus cereus, Pseudomonas aeruginosa, Escherichia coli, Shigella dysenteriae, Shigella sonnei, Salmonella typhi, Aspergillus niger, Blastomycetes dermatitidis, Candida albicans, and Trichophyton spp.

Brine shrimp lethality bioassay
Brine shrimp lethality bioassay method was applied for determination of general toxic property of the plant extractives. Dimethyl sulfoxide (DMSO) solutions of the samples were applied against Artemia salina in a d ex vivo assay. For the experiment, crude extracts were dissolved in DMSO and solutions of varying concentrations (10.5, 9.0, 7.5, 6.0, 4.4, 5.0, 1.5, 0.75, 0.375, and 0.1875 μg/ml) were obtained by serial dilution. Vincristine sulphate (Vincristine, Richter Inj., powder for reconstitution, 1 mg vial, Gedeon Richter Ltd.) was used as a reference standard.

Statistics
Experimentally obtained primary data were manipulated as the source of responses. All experiments were performed in duplicate and replicated at least three times. Data were presented as mean ± standard deviation (SD) and were considered statistically significant when P values were <0.05.

Observations and Results
In the antibacterial and antifungal sensitivity test [Table 1], the highest zone of inhibition (17.67 ± 0.47 mm) was found against Methanol Extract of Allophylus cobbe (MEAC). This was followed by 11.67 ± 0.47, 11.0 ± 0.82, 11.67 ± 0.94, 10.33 ± 1.25, 10.33 ± 1.25, and 10.33 ± 0.47 mm by the same extract against Cryptococcus neoformans, Bacillus subtilis, Pityrosporum ovale, Salmonella paratyphi, Staphylococcus aureus, and Vibrio cholerae, respectively. But the extract EEAC was inactive against Bacillus megaterium, Bacillus cereus, Pseudomonas aeruginosa, Escherichia coli, Shigella dysenteriae, Shigella sonnei, Salmonella typhi, Aspergillus niger, Blastomyces dermatitidis, Candida albicans, and Trichophyton spp.

Table 1: Antimicrobial sensitivity of Allophylus cobbe extracts

| Test microorganisms | MEAC (50 μg/μl) | EEAC (50 μg/μl) | PEEAC (50 μg/μl) | STD (50 μg/μl) |
|---------------------|----------------|----------------|----------------|----------------|
| Gram-positive bacteria | CFN | CFN | CFN | CFN |
| Bacillus subtilis | ND | 11.00±0.82*** | 11.67±0.47** | 16.00±0.82 |
| Bacillus megaterium | 11.00±0.82*** | ND | 12.00±0.82** | 17.33±1.25 |
| Bacillus cereus | ND | ND | 10.33±0.47*** | 16.76±0.94 |
| Staphylococcus aureus | ND | 10.33±1.25*** | ND | 18.67±0.94 |
| Gram-negative bacteria | CFN | CFN | CFN | CFN |
| Pseudomonas aeruginosa | ND | ND | ND | 18.00±1.63 |
| Escherichia coli | 10.33±1.25** | ND | ND | 17.67±1.25 |
| Shigella dysenteriae | 15.00±0.82*** | ND | ND | 15.67±0.47 |
| Shigella sonnei | ND | ND | 12.33±0.47*** | 20.67±0.47 |
| Salmonella typhi | ND | ND | ND | 15.00±0.82 |
| Vibrio cholerae | ND | 10.33±0.47*** | 10.00±0.82*** | 19.33±0.47 |
| Salmonella paratyphi | ND | 10.33±1.25*** | 12.33±0.47*** | 17.33±0.47 |
| Fungi | CFN | CFN | CFN | CFN |
| Aspergillus niger | 10.33±0.47*** | ND | ND | 16.67±0.47 |
| Blastomyces dermatitidis | ND | ND | 10.00±0.82*** | 16.00±0.82 |
| Candida albicans | 15.33±1.25** | ND | 11.67±0.16*** | 16.67±0.47 |
| Pityrosporum ovale | ND | 11.67±0.47*** | ND | 17.33±0.47 |
| Trichophyton spp. | ND | ND | ND | 16.33±1.25 |
| Microsporum spp. | ND | 17.67±0.47*** | ND | 19.00±0.82 |
| Cryptococcus neoformans | 10.00±0.82*** | 11.67±0.94*** | 14.33±0.47* | 15.00±0.82 |

*P<0.5, **P<0.1, ***P<0.01, the diameter of zone of inhibition is expressed as Mean±SD (n=3), a diameter less than 8 mm was considered inactive. ND - Not detected; STD: Standard drug. CFN: Ciprofloxacin, FCN: Fluconazole, MEAC: Methanol extract of Allophylus cobbe, EEAC: Ethanol extract of Allophylus cobbe, PEEAC: Petroleum ether extract of Allophylus cobbe.
against Sh. sonnei, Sa. paratyphi, Ba. megaterium, Ba. subtilis, Ca. albicans, Ba. cereus, Vi. cholerae, and Bl. dermatitidis, respectively. Also, the extract was found to be inactive against St. aureus, Ps. aeruginosa, Ei. coli, Sh. dysenteriae, Sa. typhi, As. niger, Pi. ovale, Trichophyton spp., and Microsporum spp.

In MIC by serial tube dilution method, a potent MIC (15.625 μg/ml) was shown by MEAC against Microsporum spp. [Table 2]. The MIC of 62.50 μg/ml was found against Ba. subtilis, Pi. ovale, Cr. neoformans and 125 μg/ml was found against St. aureus, Vi. cholerae, and Sa. paratyphi, respectively, with the same extract. MICs for the other test organisms were not detected.

For the extract MEAC, the MIC of 31.25 μg/ml was found against Sh. dysenteriae and Ca. albicans. This was followed by 62.50 μg/ml for Ba. megaterium, and 125 μg/ml for Ei. coli, As. niger, and Cr. neoformans. MICs for the other test organisms were not detected.

Again, with some exceptions, the PEEAC produced mild to moderate MICs against the test organisms. The MIC of 31.25 μg/ml was found against Sh. sonnei, Sa. paratyphi, and Cr. neoformans. This was followed by 62.50 μg/ml for Ba. subtilis, Ba. megaterium, and Ca. albicans. On the other hand, the pathogens Ba. cereus, Vi. cholerae, and Bl. dermatitidis were inactivated at the MIC of 125 μg/ml. But the extract was unable to inhibit the growth of other nine pathogens.

The growth of the test pathogen, Microsporum spp. was strongly inhibited by the ethanol extract of Al. cobbe. From the study, it was observed that mild to moderate MICs were produced by the extractsives (with some exceptions), where the standard drugs were ciprofloxacin and fluconazole for the test bacteria and fungi, respectively.

In the cytotoxicity test [Table 3], the extract PEEAC showed significant cytotoxicity in comparison to the other two extractives. The LC_{50} and LC_{90} of the PEEAC, MEAC, and EEAC were 0.79 and 1.31, 6.15 and 10.32, and 6.51 and 12.50 μg/ml, respectively. The standard, vincristine sulfate, produced a potent LC_{50} and LC_{90} of 0.47 μg/ml and 0.67 μg/ml, respectively, which were compared with the extractives.

### Discussion

A mild to moderate zone of inhibition was observed by all the extractives against Cr. neoformans. All the extractives were inactive against Trichophyton spp. and Ps. aeruginosa. A wide spectrum of antimicrobial activity was observed with the PEEAC. PEEAC was found to have a moderate antibacterial activity for the test Gram-positive species. Among the three extractives, PEEAC was found to be more active against Ba. subtilis, Ba. megaterium, Ba. cereus, Sh. sonnei, Sa. paratyphi, Bl. dermatitidis, Ca. albicans, and Cr. neoformans. The extractive MEAC was more active than the other two extractives against E. coli, Sh. dysenteriae, As. niger, and Ca. albicans. The test pathogens, St. aureus, Vi. cholerae, Pi. ovale, and Microsporum spp. were found to be more sensitive to the extractive EEAC than the others.

In the MIC test, EEAC strongly inhibited the growth of the test species, Microsporum spp. Moderate MICs (31.25–62.50 μg/ml) were found against Sh. dysenteriae, Ca. albicans, Sh. sonnei, Sa. paratyphi, Cr. neoformans, Ba. subtilis, Ba. megaterium, and Pi. ovale by the all extractives. Also, the test extractives produced a mild growth inhibition (125 μg/ml) against Ba. cereus, St. aureus, Ei. coli, Vi. cholerae, Sa. paratyphi, As. niger, Bl. dermatitidis, and Cr. neoformans. All the extractives produced no inhibition to Ps. aeruginosa, Trichophyton spp., and Sa. typhi.

The extract PEEAC was found to be potent cytotoxic agent to the brine shrimps as it produced strong LC_{50} and LC_{90}. The extractives MEAC and EEAC also produced a moderate cytotoxic activity in comparison to the standard, vincristine sulfate.

### Conclusion

From the study it is evident that the extractives of Al. cobbe L. showed mild to strong antimicrobial activity and moderate to strong cytotoxicity. A significant antimicrobial and cytotoxic profile was observed by the crude PEEAC. Further investigation is required to isolate the bioactive moieties.

### Table 2: Minimum inhibitory concentrations (MICs) of Allophylus cobbe extracts

| Test organisms          | Minimum inhibitory concentrations (MICs) (μg/ml) | PEEAC | EEAC | MEAC |
|-------------------------|-------------------------------------------------|-------|------|------|
| Bacillus subtilis       | 62.50                                           | 62.50 | ND   | ND   |
| Bacillus megaterium     | 62.50                                           | ND    | 62.50| ND   |
| Bacillus cereus         | 125                                             | ND    | ND   | ND   |
| Staphylococcus aureus   | ND                                              | ND    | 125  | ND   |
| Pseudomonas aeruginosa  | ND                                              | ND    | ND   | nd   |
| Escherichia coli        | ND                                              | ND    | 125  | ND   |
| Shigella dysenteriae    | ND                                              | ND    | 31.25| ND   |
| Shigella sonnei         | 31.25                                           | ND    | ND   | ND   |
| Salmonella typhi        | ND                                              | ND    | ND   | ND   |
| Vibrio cholerae         | 125                                             | ND    | 125  | ND   |
| Salmonella paratyphi    | 31.25                                           | ND    | 125  | ND   |
| Aspergillus niger       | nd                                              | nd    | 125  | ND   |
| Blastomyces dermatitidis| 125                                             | nd    | 31.25| ND   |
| Candida albicans        | 62.50                                           | nd    | 62.50| ND   |
| Pityrosporum ovale      | nd                                              | nd    | nd   | ND   |
| Trichophyton spp.       | nd                                              | nd    | nd   | ND   |
| Microsporum spp.        | nd                                              | 15.63 | ND   |      |
| Cryptococcus neoformans | 31.25                                           | 62.50 | 125  |      |

### Table 3: Cytotoxic response of Allophylus cobbe extracts

| Sample               | LC_{50} (μg/ml) | LC_{90} (μg/ml) |
|----------------------|-----------------|-----------------|
| VS                   | 0.47            | 0.67            |
| MEAC                 | 6.13            | 10.32           |
| EEAC                 | 6.51            | 12.50           |
| PEEAC                | 0.79            | 0.31            |

VS: Vincristine sulfate; MEAC: Methanol extract of Allophylus cobbe; EEAC: Ethanol extract of Allophylus cobbe; PEEAC: Petroleum ether extract of Allophylus cobbe
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