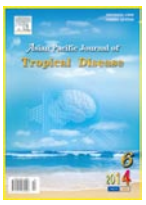
Contents lists available at ScienceDirect

Asian Pacific Journal of Tropical Disease

journal homepage: www.elsevier.com/locate/apjtd



Document heading doi:10.1016/S2222-1808(14)60611-3
© 2014 by the Asian Pacific Journal of Tropical Disease. All rights reserved.

Evaluation of antimicrobial study in *in vitro* application of *Clerodendrum infortunatum* Linn.

Talukdar Muhammad Waliullah[1*], Akter Mst Yeasmin[1], Islam Md Wahedul[2], Hassan Parvez[2]

[1]*Molecular and Cell Biology Laboratory, Bioscience Department, GSST, Shizuoka University 836-Oya, Suruga-ku, Shizuoka , 422-8529, Japan*

[2]*Institute of Biological Sciences, University of Rajshahi, Rajshahi-6205, Bangladesh*

PEER REVIEW

Peer reviewer

Dr. MD. Jahangir Alam, Associate Professor and Chairman, Department of Animal Production and Management, Faculty of Animal Science and Veterinary Medicine, Sher-e-Bangla Agricultural University, Sher-e-Bangla Nagar, Dhaka-1207, Bangladesh.
Tel: +880-2-9112665
Fax: 880-2-8155800
E-mail: jangalam@yahoo.com

Comments

This is a worthwhile work in which the author has established evaluation standards of a medicinal plant. These evaluations are carried out to ensure authentication of root, leaf and stem of *C. infortunatum* and results can be used for qualitative and quantitative analyses of this herbal drug. In this study, the authors provide evidence of its foremost antimicrobial activity.
Details on Page 487

ABSTRACT

Objective: To evaluate the antimicrobial potency of ethanol and chloroform extracts of root, leaf and stem of *Clerodendrum infortunatum* (Verbenaceae) and to explore a scientific data as this plant was randomly use in traditional medicine to cure common ailments such as intestinal disorder, diarrhea, tuberculosis and respiratory problems, *etc*.
Methods: The *in vitro* application was carried out by using disc diffusion, micro broth dilution and serial dilution techniques against clinically important life threatening organisms.
Results: All the extracts showed significant inhibitory activity over the bacteria and fungus comparable to the standard drug tetracycline and fluconazole. The maximum average diameter zone of inhibition was recorded to bacterial strains against *Bacillus megaterium*, *Salmonella typhi*, *Klebsiella pneumoniae* and to fungi against *Aspergillus niger* and *Candida albicans*. The minimum inhibitory concentration values of ethanol leaf extract were determined 64 μg/mL to *Bacillus megaterium*, *Salmonella typhi* and *Klebsiella pneumoniae*; 128 μg/mL to *Staphylococcus aureus*, *Streptococcus-β-haemolyticus* and *Escherichia coli*.
Conclusions: The findings evidently appear promising antibacterial and antifungal properties of *Clerodendrum infortunatum* against antagonistic pathogens. Leaf possesses quite potent activity than root and stem specially leaf extract>root extract>stem extract. This study serves as basis for further research to lead compounds to be isolated so that it may be as a template for the implications of these results for bioactivity and drug discovery potential of herbal products.

KEYWORDS

Plant, Ethnobotanical information, Bacterial strains, Fungal strains, Disc diffusion assay, Zone of inhibition, Minimum inhibitory concentration

1. Introduction

The use of indigenous knowledge traditional medical practitioners as leads provides a useful route employed in the search for novel drugs[1]. The antimicrobial potential of different medicinal plants is being extensively studied all over the world, but only a few studies have been carried out in a systematic manner[2]. Medical knowledge derived from traditional societies has motivated searches for new bioactive molecules derived from plants that show potent activity against bacterial pathogens[3]. Nowadays, some researchers are providing laboratory trials to justify for the

*Corresponding author: Talukdar Muhammad Waliullah, Molecular and Cell Biology Laboratory, Bioscience Department, GSST, Shizuoka University 836-Oya, Suruga-ku, Shizuoka, 422-8529, Japan.
Tel: +81-080-9486-2020
E-mail: olymwt@live.com

Foundation Project: Supported by Ministry of Science and Information and Communication Technology, Government of the Peoples Republic of Bangladesh under the project of Research and Development (R&D) (Grant No. Bitojopromo/sha-12/goupro-8/2008/268).

Article history:
Received 15 May 2014
Received in revised form 20 May, 2nd revised form 24 May, 3rd revised form 30 May 2014
Accepted 14 Jun 2014
Available online 6 Jul 2014

use of the plants in folk medicine to treat various infectious diseases[4].

In the present study, *Clerodendrum infortunatum* (*C. infortunatum*) Linn. (Verbenaceae) is selected to assess its ethnobotanical importance due to randomly use as traditional medicine to cure common ailments such as intestinal disorder, diarrhea, tuberculosis and respiratory problems, *etc*. It is reported that Bangladesh has over 5 000 medicinal plants and uses of these plants for medicinal purposes are remarkable[5]. *C. infortunatum* is generally used in folk, hill tracts and rural slum dwellers as indigenous medicine, food supplement, decoration of home appliances and firewood. *Clerodendrum* is a very large and diverse genus and till now five hundred and eighty species of the genus have been identified and are widely distributed in Asia, Australia, Africa and America[6]. Roots and leaf extracts of *Clerodendrum indicum, Clerodendrum phlomidis, Clerodendrum serratum, Clerodendrum trichotomum, Clerodendrum chinense* and *Clerodendrum petasites* have been used for the treatment of rheumatism, asthma and other inflammatory diseases[7]. It was also reported that tribals use *Clerodendrum inerme* as an antidote of poisoning from fish, crabs and toads[8]. Roots, leaves and fresh juice of leaves of *C. infortunatum* were used in eliminating ascarids and tumors and also as a laxative[9]. Another study on *Clerodendrum chinense* and *Clerodendrum splendens* have been reported that chloroformic extracts of flower and stem were active against *Plasmodium falciparum* and *Trypanosoma cruzi* suggesting that leaf extracts showed promising activities against *Trypanosoma cruzi*[10].

To explore a scientific idea, this study was carried out *in vitro* evaluation of the antimicrobial potency against clinically important life threatening organisms using root, leaf and stem of ethanol and chloroform extracts.

2. Materials and methods

2.1. Plant collection and identification

The plant specimen was collected from Rajshahi University Campus, Bangladesh. Identification of voucher specimen was confirmed at the taxonomical section, Department of Botany, University of Rajshahi, Bangladesh.

2.2. Preparation of extracts

Root, leaf and stem were dried in shade and stored in cotton bags and then finely powdered (100 g) separately with the help of a grinder. Each ground material was soaked in 500 mL ethyl alcohol and chloroform separately for 24-72 h and filtered (Whatman No. 1). The filtered was then allowed to vaporize in rotary evaporator until completely dried and kept in a refrigerator. Then (100 mg and 50 mg) dried extract, for further study, was weighed and dissolved in 10 mL of respected solvents for dilution. The concentration of the final extract was 100 μg/10 μL and 50 μg/10 μL.

2.3. Microorganisms with strains No.

Six Gram-positive bacterial strains, *viz.*, *Staphylococcus aureus* (ATCC-259233) (*S. aureus*), *Sercinia lutea* (QL-166), *Bacillus subtilis* (QL-40) (*B. subtilis*), *Bacillus megaterium* (QL-38) (*B. megaterium*), *Bacillus cereus* (ATCC-14603) (*B. cereus*) and *Streptococcus-β-haemolyticus* (ATCC-10389) (*S.-β-haemolyticus*), nine Gram-negative bacterial strains, *viz.*, *Salmonella typhi* (ATCC-14028) (*S. typhi*), *Shigella dysenteriae* (AL-35587) (*S. dysenteriae*), *Escherichia coli* (FPFC-1407) (*E. coli*), *Shigella shiga* (ATCC-26107), *Shigella boydii* (AL-17313), *Shigella sonnei* (AJ-8992), *Proteus vulgaris* (*P. vulgaris*), *Klebsiella pneumoniae* (*K. pneumoniae*) and *Pseudomonus aeruginosa* (ATCC-27853) (*P. aeruginosa*) and seven fungal strains, *viz.*, *Aspergillus niger* (*A. niger*), *Aspergillus fumigatus, Aspergillus flavus, Candida albicans* (ATCC-2091) (*C. albicans*), *Fusarium vasinfactum* (*F. vasinfactum*), *Mucor* sp. and *Fusarium oxysporum* (*F. oxysporum*) were employed in this test. These species were obtained from the mother stock of the Enteric Microbiology Laboratory, ICDDR, Dhaka and the Molecular Biology Laboratory, Institute of Biological Sciences, University of Rajshahi, Bangladesh.

2.4. Medium

Nutrient broth, nutrient agar (NA) and Sabouraud dextrose agar (SDA) medium were used. All are the products of Mast Diagnostics, Mast Group Ltd. Merseyside, UK.

2.5. Disc diffusion assay

Antimicrobial activity was determined as diameter of inhibition zone using disc diffusion method[11]. NA and SDA were distributed in sterilized Petri dishes for bacteria and fungi respectively. This was accomplished by placing 10 μL of the extract on a small (6 mm) filter paper disc. This disc was placed on an agar growth medium containing a confluent lawn of microorganism. The concentration of the organism was also 10 μL/Petri dish. The absence of bacterial and fungal growth around the disc indicated that the plant extract contained antimicrobial properties against that particular organism.

2.6. Minimum inhibitory concentration (MIC)

The test was performed at ten concentrations (512, 256, 128, 64, 32, 16, 8, 4, 2 and 1 μg/mL) of the ethyl alcohol leaf extracts of *C. infortunatum*. The activity was tested against three Gram-positive and three Gram-negative bacterial strains: *S. aureus*, *B. subtilis*, *S.-β-haemolyticus*, *S. typhi*, *E. coli* and *K. pneumoniae* employing the nutrient broth

medium using by a serial dilution technique[12]. Each test was replicated in twice.

2.7. Standard disc

Standard disc of tetracycline (30 μg/disc) and fluconazole (25 μg/disc) obtained from Mast Diagnostics, Mast Group Ltd., Merseyside, UK was used as positive control.

2.8. Culture and inoculums

NA and SDA were prepared by dissolving 2.8 g and 6.2 g powder of agar respectively in 100 mL water. About 25 mL of medium was poured into a Petri dish. The inoculum was prepared by culturing a large number of organisms in a tube containing 10 mL liquid medium for bacterial strains and incubating over night at 37 °C. On the other hand, the inoculum of fungal strains was transferred directly into Petri dish and incubated at 27 °C for 72 h. The agar plates of the assay were prepared by labeling them with the date, the name of the microorganism and the name (code) of the discs. The inoculums of bacteria were transferred into Petri dish containing solid nutrient medium of agar using a sterile swab. The swab was used to spread the bacteria on the medium in a confluent lawn. It was done by rotating the Petri dish at 90 °C and continuing the spread of bacteria. One swab was used for one species of bacteria.

2.9. Placing test discs

Dried test discs were transferred on bacterial lawn under aseptic conditions using spirit-flame sterilized forceps each time. Each disc was placed gently on the agar surface, and platted with the forceps so that it sticks. The Petri dish was incubated upside down at 37 °C for 24 h. Resulting zones of inhibition were observed and measured in millimeters. Tests were repeated in twice and were performed to insure reliability of the results.

3. Results

In continuation of the study, keeping this in view, for substances of plant origin with microbiological effects, root, leaf and stem of *C. infortunatum* were screened out, and extracted into ethyl alcohol and chloroform. A correlation was found between the antibacterial activity observed by agar disc diffusion assay and MIC determination. It was note that these crude extracts showed more or less similar activity against a number of bacterial and fungal strains compared to the standard antibiotic tetracycline and antifungal fluconazole. All experiments were carried out in twice. To indicate the error, or the degree of uncertainty in a reported measurement error bars are used on graphs.

3.1. Antibacterial activity

The highest average zone of inhibition was found 12 mm against: *B. megaterium* and *K. pneumonia* by leaf extract; *S. typhi* and *K. pneumonia* by root extracts (Figures 1 and 2) in ethanolic fraction when compared to the tetracycline 21.5 mm in diameter against *S. dysenteriae*. Then 11.5, 11.0, 10.5, 10.0, 9.5, 9.0, 8.5, and 8.0 mm zone of inhibition were recorded respectively against Gram-positive and Gram-negative bacterial strains in both type of extraction either ethanol or chloroform by root, leaf or stem presented in Figures 1 and 2. Remaining of the lowest average zone of inhibition activity was observed 6.5 mm against *S. aureus* and *P. vulgaris* by stem extract of chloroform fraction (Figures 1 and 2) whereas the comparative study of tetracycline showed 17.5 mm in diameter against Gram-negative bacteria *P. aeruginosa* and 18.0 mm in diameter against Gram-positive bacteria *B. cereus*.

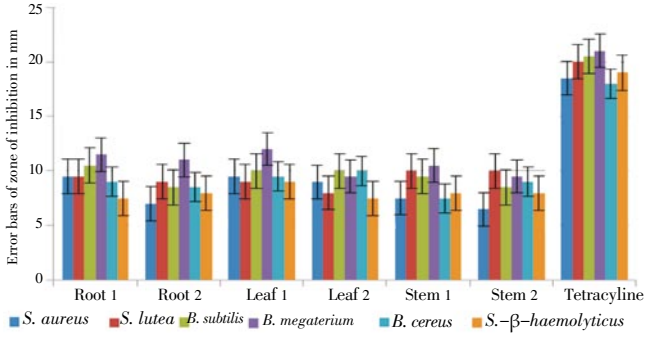


Figure 1. Antibacterial activity of *C. infortunatum* extracts against Gram-positive bacterial strains and antibiotic tetracycline.
1=ethanol fraction, 2=chloroform fraction, *Sercinia lutea*: *S. lutea*.

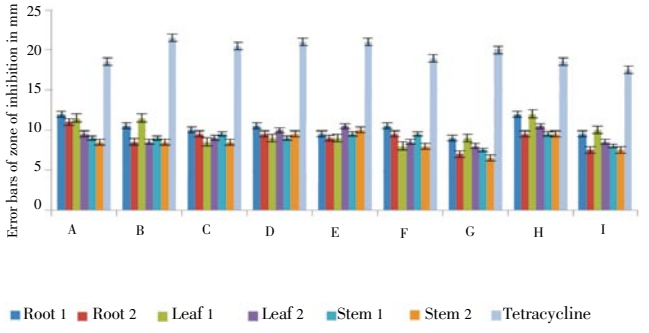


Figure 2. Antibacterial activity of *C. infortunatum* extracts against Gram-negative bacterial strains and antibiotic tetracycline.
1=ethanol fraction, 2=chloroform fraction. A: *S. typhi*, B: *S. dysenteriae*, C: *E. coli*, D: *Shigella shiga*, E: *Shigella boydii*, F: *Shigella sonnei*, G: *P. vulgaris*, H: *K. pneumoniae*, I: *P. aeruginosa*.

3.2. Antifungal activity

The maximum average inhibitory activity was determined 10.5 mm by root extract of ethanol fraction against *A. niger* and *C. albicans* when compared to standard drug fluconazole

22.0 mm in diameter against *C. albicans*. Then 10.0, 9.5, 9.0, 8.5, 8.0, 7.5, 7.0 and 6.5 mm zone of inhibition in using both type of extraction either ethanol or chloroform by root, leaf or stem are presented in Figure 3. On the other hand, the lowest average zone of inhibition activity was observed 6.5 mm against *A. niger* by stem extract of chloroform fraction and in comparison to fluconazole showing 17.5 mm in diameter against *F. vasinfactum* and *F. oxysporum* (Figure 3).

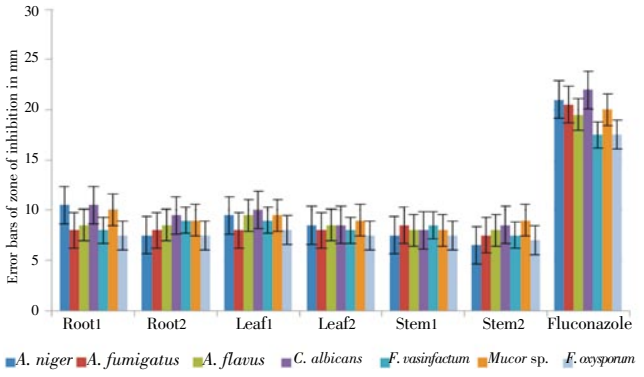


Figure 3. Antifungal activity of *C. infortunatum* extracts against common fungal strains and antifungal drug fluconazole.
1=ethanol fraction, 2=chloroform fraction.

3.3. Minimum inhibitory concentration activity

From the ten concentrations (512, 256, 128, 64, 32, 16, 8, 4, 2 and 1 μg/mL), the MIC activity 64 μg/mL against *B. subtilis* (Gram-positive), *S. typhi* and *K. pneumoniae* (Gram-negative); 128 μg/mL against *S. aureus*, *S.-β-haemolyticus* (Gram-positive) and *E. coli* (Gram-negative) were observed and presented in the Figure 4 as relatively good antibacterial compounds.

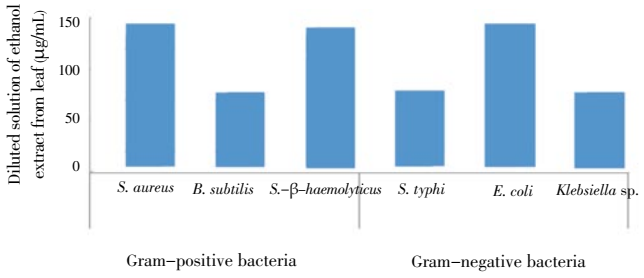


Figure 4. MIC of ethanol leaf extract of *C. infortunatum*.

4. Discussion

The ethanol and chloroform crude extracts of root, leaf and stem showed in variable average zone of inhibition (6.5-12.0 mm against Gram-positive and Gram-negative bacterial strains whereas the standard antibiotic tetracycline showed 17.5-21.5 mm in diameter). Ethanolic fraction of leaf and root extracts exhibited highest antimicrobial activity against *B. megaterium*, *K. pneumonia* and *S. typhi*. The crude antifungal activity was also observed significantly average 6.5-10.5 mm zone of inhibition whereas standard antifungal drug fluconazole showed 17.5-22.0 mm zone of inhibition. Root extract of ethanol fraction indicated the maximum antifungal activity against *A. niger* and *C. albicans*. The MIC test also exposed a potential activity.

The result of antimicrobial activity is in accord with former studies executed on *Clerodendrum viscosum*[13]. This finding also coincides with the study conducted by Steane *et al.*[14,15] who investigated the antimicrobial activity of the same species of *Clerodendrum*. Praveen *et al.* reported that the chloroform extract of *Clerodendrum paniculatum* Linn had great *in vitro* potential for antimicrobial activity against tested microorganism used during the analysis[16]. Similar results have been reported[17] in some medicinal plants used in folklore remedies in Southwestern core, in some *Allium* species from Hamedan, Iran[18]. Our previous studies have demonstrated the reliable antimicrobial potentiality of *C. infortunatum*[19]. Thus, we believe that *C. infortunatum* will be a possible source for new antimicrobial substances against important pathogens of medical and veterinary importance.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgements

Institute of Biological Sciences, University of Rajshahi has provided the lab facility. This paper was supported by Ministry of Science and Information and Communication Technology, Government of the Peoples Republic of Bangladesh under the project of Research and Development (R&D) (Grant No. Bitojopromo/sha-12/goupro-8/2008/268).

Comments

Background

Medicinal plants are frequently used in traditional medicine either curative or preventive in different types of ailments not only in Bangladesh but also all over the world. Evaluations which are carried out in *in vitro* application studies provide us basis to establish the microbiological and pharmacognostic aspect.

Research frontiers

The present antimicrobial study provides basis to establish standards of different parts of *C. infortunatum* against antagonistic pathogens as a herbal remedy. This research

is helpful to select plants' part and to estimate the dose of drug. It can also be used to screen adulteration which makes a template for bioactivity and drug discovery.

Related reports

In this investigation, the authors have followed standard protocols to assess the antimicrobial activity. The investigated data showed that ethanol extracts of root and leaf may be new potential source as natural antimicrobial applied in pharmaceutical and food industries. The results suggest isolating plant micro-molecule should be used for the discovery of potential herbal products.

Innovations & breakthroughs

Medicinal value of *C. infortunatum* has been reported for the treatment of rheumatism, asthma and other inflammatory diseases, *etc*. In my knowledge, there is no work for antimicrobial activity with the fraction of ethanol and chloroform. The present report serves as the first hand information based on chemical and morphological evaluations. Therefore, this research is a key step in this regard.

Applications

This research is helpful for establishing the correct identification of a plant material. It will be a diagnostic tool for standardization and characterization of *C. infortunatum*. Crude extracts have been evaluated for their effects on the growth of life threatening pathogenic microorganisms, including Gram-positive and Gram-negative bacteria and fungi. It will also be helpful for other researchers to maintain the standards of this plant for their research projects.

Peer review

This is a worthwhile work in which the author has established evaluation standards of a medicinal plant. These evaluations are carried out to ensure authentication of root, leaf and stem of *C. infortunatum* and results can be used for qualitative and quantitative analyses of this herbal drug. In this study, the authors provide evidence of its foremost antimicrobial activity.

References

[1] Amusan OO, Sukati NA, Dlamini PS, Sibandze FG. Some Swazi phytomedicines and their constituents. *Afr J Biotechnol* 2007; **6**: 267-272.

[2] Arora DS, Kaur GJ, Kaur H. Antibacterial activity of tea and coffee: their extracts and preparations. *Int J Food Prop* 2009; **12**: 286-294.

[3] Bocanegra-Garcia V, Del Rayo Camacho-Corona M, Rmirez-Cabrera M, Rivera G, Garza-González E. The bioactivity of plant extracts against representative bacterial pathogens of the lower respiratory tract. *BMC Res Notes* 2009; **2**: 95.

[4] Shihabudeen MS, Hansi Priscilla H, Kavitha Thirumurugan D. Antimicrobial activity and phytochemical analysis of selected Indian folk medicinal plants. *Int J Pharm Sci Res* 2010; **1**(10): 430-434.

[5] Biswas KR, Ishika T, Rahman M, Khan T, Swarna A, Monalisa MN, et al. Medicinal plants used for preventive medicinal purposes: a survey in Muktipara village, Chuadanga district, Bangladesh. *Am Eurasian J Sustain Agric* 2011; **5**(2): 247-251.

[6] Venkatanarasimman B, Rajeswari T, Padmapriya B. Antibacterial potential of crude leaf extract of *Clerodendrum philippinum* Schauer. *Int J Pharm Biol Arch* 2012; **3**(2): 307-310.

[7] Panthong A, Kanjanapothi D, Taesotikul T, Wongcomea T, Reutrakul V. Anti-inflammatory and antipyretic properties of *Clerodendrum petasites* S. Moore. *J Ethnopharmacol* 2003; **85**: 151-156.

[8] Pandey R, Verma RK, Singh SC, Gupta MM. 4α-Methyl-24β-ethyl-5α-cholesta-14,25-dien-3β-ol and 24β-ethylcholesta-5, 9(11), 22E-trien-3β-ol, sterols from *Clerodendrum inerme*. *Phytochemistry* 2003; **63**: 415-420.

[9] Ambasta SP. *The useful plants of India*. New Delhi, India: Publication and Information Directorate, CSIR; 1992.

[10] Abouzid SF, Wahba HM, Elshamy A, Cos P, Maes L, Apers S, et al. Antimicrobial activity of some *Clerodendrum* species from Egypt. *Nat Prod Res* 2013; **27**(11): 1032-1036.

[11] Bauer AW, Kirby WM, Sherris JC, Turck M. Antibiotic susceptibility testing by a standardized single disk method. *Am J Clin Pathol* 1966; **45**: 493-496.

[12] Reiner R. *Antibiotics: an introduction*. Basle, Switzerland: F Hoffman La Roche and Co.; 1982, p. 70-71.

[13] Oly WT, Islam W, Hasan P, Parween S. Antimicrobial activity of *Clerodendrum viscosum* (Verbenaceae). *Int J Agric Biol* 2011; **13**: 222-226.

[14] Steane DA, Scotland RW, Mabberley J, Olmstead RG. Molecular systematics of *Clerodendrum* (Lamiaceae): its sequences and total evidence. *Am J Bot* 1999; **86**: 98-107.

[15] Hsiao JY, Lin ML. A chemotaxonomic study of essential oils from the leaves of genus *Clerodendrum* (Verbenaceae) native to Taiwan. *Bot Bull Acad Sin* 1995; **36**: 247-251.

[16] Praveen M, Radha K, Hari Kumar R, Padmaja V, Mathew A, Ajith Kumar P. Preliminary phytochemical, antimicrobial and toxicity studies on *Clerodendrum paniculatum* Linn leaves. *Hygeia J Drugs Med* 2012; **4**: 41-50.

[17] Akinpelu DA, Onakoya TM. Antimicrobial activities of medicinal plants used in folklore remedies in south-western. *Afr J Biotechnol* 2006; **5**: 1078-1081.

[18] Chehregani A, Azimishad F, Alizade HH. Study on antibacterial effect of some *Allium* species from Hamedan-Iran. *Int J Agric Biol* 2007; **9**(6): 873-876.

[19] Waliullah MT, Yeasmin MA, Ashraful A, Islam W, Hassan P. Antimicrobial potency screening of *Clerodendrum infortunatum* Linn. *Int Res J Pharm* 2014; **5**(2): 57-61.