Bangladesh Pharmaceutical Journal ***18****(2): 103-109, 2015*

Evaluation of Antibacterial, Antifunfgal and Cytotoxic Potentials of Crude Metabolite of ANAM-39, a Marine Bacterium Isolated from Sundarbans, Bangladesh

Ashish Kumar Sarker[1], Md. Anwarul Haque[1], Urmi Saha[2], Md. Ajijur Rahman[3] and Md. Anwar Ul Islam[3]

[1]Department of Pharmacy, Pabna University of Science and Technology, Pabna-6600, Bangladesh
[2] Eastern Medical College and Hospital, Comilla, Bangladesh
[3]Department of Pharmacy, University of Rajshahi, Rajshahi-6205, Bangladesh

Received: April 05, 2015; Accepted: April 30, 2015; Published (Web): July 21, 2015

Abstract

The present study was undertaken to screen the antimicrobial, antifungal and cytotoxic potentials of crude metabolite of a marine bacterium isolated from a soil sample collected from Sundarbans, Bangladesh. Primary and secondary screenings for antimicrobial activity were conducted by cross streak and agar well diffusion method, respectively. The antifungal potency of the crude extract was also tested by agar well-diffusion method. The brine shrimp lethality test was conducted to determine the cytotoxic nature of crude metabolites, which showed that the degree of lethality of metabolite was directly proportional to the concentration (LC_{50} 30.19 µg/ml). Further studies are needed to isolate and characterize the active principles present in the crude metabolite and determine their biological activity. The results of this investigation suggested that the soils of Sundarbans are rich sources of microorganisms with potent biological activities and systematic screening programs need to be conducted to explore the presence of pharmacologically active marine bacterial metabolites.

Key words: Antibacterial, antifungal, cytotoxic, marine bacteria, Bangladesh.

Introduction

The prevalence of antimicrobial resistance among key microbial pathogens is increasing world-wide at an alarming rate. The problem is not just antibiotic resistance but also multidrug resistance**.** In 2001, more than 70% of pathogenic bacteria were estimated to be resistant to at least one of the currently available antibiotics (Cragg *et al*., 2001). So, the incidence of multidrug resistant organisms is increasing and compromising the treatment of a growing number of infectious diseases. The appearance of multidrug resistant pathogenic strains caused substantial morbidity and mortality especially among the elderly and immunocompromised patients (Barsby *et al*., 2001; Parungao *et al*., 2007). As a result, there is an urgent need for developing new drugs which are safe and effective against current antibiotic resistant pathogens.

Actinomycetes have been proven as a potential source of bioactive compounds and the richest source of secondary metabolites (Suthindhiran *et al*., 2009). Actinobacteria are fruitful producers of thousands of biologically active secondary metabolites. Terrestrial sources have been studied and screened since the 1950s for actinomycetes producing many important antibiotics, anticancer, antitumor and immunosuppressive agents (Manivasagan *et al*., 2014). But in the recent years, the rate of discovery of novel drugs from terrestrial actinomycetes has been decreased and repetition of the same compounds from the terrestrial actinomycetes has made them less attractive for screening programs. So much attention has been focused now on screening of microorganisms from diverse environments to evaluate new sources for the isolation of microorganisms and to discover new bioactive compounds.

Correspondence to: Dr. Md. Anwar Ul Islam, Phone: +88-0721-711110; Fax: +88-0721-750064; Email: profanwarulislam@yahoo.com

Marine habitat has been proven as an inexhaustible, outstanding and fascinating resource for innovating new and potent bioactive metabolites producing microorganisms. It is estimated that less than 1% of potentially useful chemicals from marine environment has been screened so far, with microbial products representing approximately 1% of the total number (Neidleman *et al*., 2000). Marine bacteria are efficient producers of new secondary metabolites that show a range of biological activities including antibacterial, antifungal, anticancer, insecticidal and enzyme inhibitors. Bioactive compounds from marine bacteria possess distinct chemical structures that may form the basis for synthesis of new drugs which could be used to combat resistant pathogens (Solanki *et al*., 2008). So, the exploration of microbial secondary metabolites from marine environment has led to the discovery of hundreds of biologically active compounds.

In Bangladesh, no significant studies have been conducted so far to isolate and evaluate marine bacteria from the Sundarbans habitats that could produce useful bioactive compounds. Therefore, the present study is intended to isolate and screen marine bacteria producing bioactive secondary metabolites from of Sundarbans, Bnagladesh.

Materials and Methods

Isolation and selection of marine bacteria: The marine bacterial strain of this study was isolated from a soil sample of Sundarbans, Bangladesh (08-16 August, 2010). A total number of nine marine soil samples were collected carefully from various depth of the earth, ranging from layers just beneath the upper surface to 1.5 meter depth in sterile pouch. By using spread plate technique **(**Bernard, 2007), isolation of the marine bacteria from these soil samples were done. A total of thirty nine marine bacterial strains [ANAM-1 to ANAM-39] were isolated and purified as pure culture from these soil samples. All of these purified isolates were preserved on yeast-extract-glucose-agar slants at 4 ^{o}C. Then by using streak plate technique **(**Alcamo *et al*., 2004) on yeast-extract-glucose-agar medium, all of these pure isolates were preliminary screened for antibacterial activity. The isolate ANAM-39, a brownish yellow colored marine microorganism (Sarker *et al*., 2015) was chosen for further study based on testing antibacterial, antifungal and cytotoxic activities of metabolites of the strain since it showed highest antibacterial activity in preliminary antibacterial screening, visibility of the metabolite as well as excellent growth properties.

Fermentation and extraction: The fermentation of marine bacteria in liquid media is very important for the production of secondary metabolites and small scale liquid fermentation is the most frequent fermentation process (Shepherd *et al*., 2010; Demain *et al*., 1999). To obtain sufficient quantity of the active secondary metabolites, fermentation was carried out in yeast-extract glucose broth medium. A loopful of the inoculums of the strain ANAM-39 was added to 500 ml flask containing yeast-extract glucose broth medium (seed culture). The flask was then incubated at 30 °C for three days. This seed culture was used to inoculate a number of 500 ml conical flasks containing 200 ml broth medium. These flasks were then incubated at 30 °C for 7 days. After incubation, the contents of the flasks were filtered through sterile Whatman filter paper no. 1 aseptically. For extraction, at first step the cell biomass were separated by filtration and in the second step the products were isolated from the filtrate by solvent extraction. The extraction of the metabolites was carried out by ethyl acetate on the basis of best solubility and maximum antimicrobial activities. At first, 200 ml of culture filtrate was taken in a 500 ml separating funnel. Then, 100 ml of ethyl acetate was added into the separating funnel and shaken for 15 minutes. On standing, the ethyl acetate layer was separated as upper layer from the aqueous layer. The ethyl acetate fraction was evaporated under reduced pressure in a rotary vacuum evaporator at 45 ^{o}C until a reddish orange solid mass was obtained. After evaporation of ethyl acetate, the weight of the solid extract was determined. This was called “crude extract”. The crude metabolic extract was subjected to screenings for antibacterial, antifungal and cytotoxic activities.

Minimum inhibitory concentration (MIC) determination: The MIC was determined by serial dilution method (Tyler *et al*., 1988) against *Bacillus cereus, Streptococcus agalactiae, Escherichia coli, Shigella dysenteriae* and *Pseudomonas aeruginosa.* Sterile nutrient broth tubes containing different dilutions of extract (0.05 mg to 1.0 mg/ml) were specifically inoculated with 0.1 ml of standardized inoculums (10^{9}cfu/ml). The tubes were incubated aerobically at 37^{o}C for 18-24 h. Two separate

control tubes for each organism were maintained. The lowest concentration (highest dilution) of the extract that produced no visible bacterial growth (no turbidity) when compared with the control tubes was regarded as the MIC (Hassan *et al*., 2009).

Antibacterial activity: Antimicrobial activity of the crude extract of ANAM-39 was determined by disc diffusion method (Bauer *et al*., 1966). Nutrient agar was sterilized in a flask and cooled to 45-50 °C and then taken in sterilized petridishes with a diameter of 120 mm. The filter paper discs (6 mm in diameter) were impregnated with the crude extract at 300 and 500 µg/disc and then placed onto the previously inoculated agar plates with the test microorganisms (*Streptococcus agalactiae*, *Bacillus cereus*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Shigella dysenteriae*, *Shigella sonnei* and *Agrobacterium* sp.). Kanamycin was used as standard at the dose of 30 µg/disc. The petridishes were kept at 4 °C for 2 h. The plates were incubated at 37 °C for 16 h to allow the growth of the microorganisms. The diameters of the zone of inhibition were measured in millimeter using a calibrated scale. The experiment was carried in triplicate to get average reading.

Antifungal activity: The antifungal efficacy of the crude extract was tested by agar well-diffusion method against two human pathogenic fungi namely *Candida albicans* and *Cryptococcus neoformans* (Vinayaka *et al*., 2009). Here, 48 hours old dextrose broth cultures of test fungi were swabbed uniformly on solidified sterile dextrose agar plates using sterile cotton swab. Then, wells of 6 mm diameter were bored in the inoculated plates with the help of sterile cork borer and the extract (20 mg/ml in sterile water) and standard (Amphotericin B, 1 mg/ml) were added separately into labeled wells. The plates were incubated at 37 °C for 24 hours and the zone of inhibition formed around the well was measured with a ruler. The experiment was carried out thrice to get the average reading.

Cytotoxic activity: To determine cytotoxic nature of the crude extract obtained from ANAM-39, the brine shrimp lethality test was conducted using *Artemia salina* (Ullah *et al*., 2013). The eggs were hatched in seawater. A constant oxygen supply was maintained throughout the process. Mature nauplii were used for this study. After 36-48 hours, the phototropic shrimps were collected by pipette for bioassay. The different concentrations of extract (10, 50, 100, 1000 µg/ml) were tested in vials containing 5 ml of brine and 25 shrimp in each of three replicates, also having a control. The vials were incubated at 25°C and surviving shrimps were counted after 24 hours. Based on these data, percentage of lethality of the brine shrimp nauplii was calculated. From this value, the LC_{50} of the sample was determined.

Results and Discussion

Marine sediment is an inexhaustible resource that has not been properly explored. It is estimated that less than 1% of potentially useful chemicals from marine environment has been screened so far, with microbial products representing approximately 1% of the total number (Neidleman *et al*., 2000). In this study, we explored the presence of microbes producing antimicrobial compounds in Sundarbans soils, the largest tidal halophytic mangrove forest in the world. The soils samples were collected from different regions of mangrove forest that is frequently inundated with moderate saline water. From nine marine soil samples, 39 pure isolates were separated and screened for antibacterial activity. Due to potent antibacterial activities of one isolate namely ANAM-39 was selected for further large scale fermentation to determine antibacterial, antifungal and cytotoxic activities of the metabolite secreted by the selected marine bacteria.

The results of MIC determination of the crude extract of ANAM-39 are shown in the table 1. It was determined against two gram-positive bacteria such as *Bacillus cereus*, *Staphylococcus agalactiae* and three gram-negative bacteria namely *Pseudomonas aeruginosa*, *Escherichia coli* and *Shigella dysenteriae*. The MIC value of extracts varied between 16-64 µg/ml. The lowest MIC value was found 16 µg/ml against *B. cereus* and *P. aeruginosa*. The MIC value of 32 µg/ml was found against *S. agalactiae* and *E. coli* whereas highest MIC value of the crude extract was 64 µg/ml against *S. dysenteriae*.

The antibacterial activity of extract was investigated against four bacteria by agar well diffusion method. Results were recorded as presence or absence of zones of inhibition around the well. The inhibitory zone around the well indicated the absence of test bacterial growth and is reported as positive and the absence of zone as negative. The results of antibacterial screening of the crude extract are given in the table 2 and figure 1.

\

Table 1. Minimum inhibitory concentration of the crude extract of ANAM-39.

Name of bacteria	MIC (μg/ml) ± Standard deviation (n=3)
Bacillus cereus	16 ± 0.21
Streptococcus agalactiae	32 ± 0.35
Escherichia coli	32 ± 0.27
Pseudomonas aeruginosa	16 ± 0.41
Shigella dysenteriae	64 ± 0.17

Table 2. Zone of inhibition of crude extract against a series of test bacteria.

Test organisms	Zone of inhibition (in mm) ± Standard deviation (n=3)		
	Crude Extract (500 μg/disc)	Kanamycin (30 μg/disc)	Crude Extract (300 μg/disc)
Gram-positive bacteria			
Bacillus cereus	21 ± 0.15	27 ± 0.65	18 ± 0.14
Streptococcus agalactiae	18 ± 0.34	27 ± 0.29	16 ± 0.19
Agrobacterium sp.	20 ± 0.16	25 ± 0.75	17 ± 0.17
Gram-negative bacteria:			
Pseudomonas aeruginosa	21 ± 0.42	26 ± 0.45	16 ± 0.37
Escherichia coli	20 ± 0.22	25 ± 0.74	18 ± 0.31
Shigella dysenteriae	22 ± 0.25	28 ± 0.58	18 ± 0.25
Shigella sonnei	22 ± 0.37	28 ± 0.51	17 ± 0.46

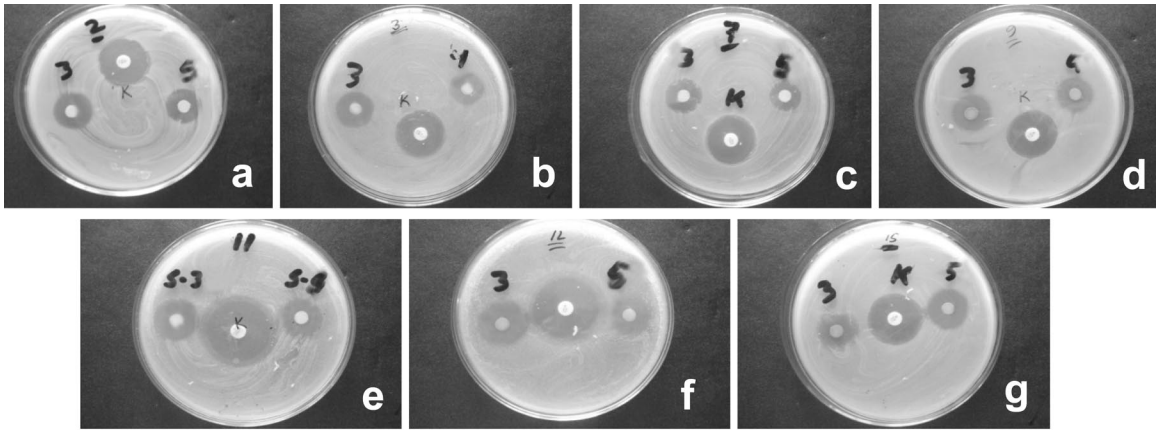

Figure 1. Antibacterial activity test of the crude extracts (500 μg/disc and 300 μg/disc) against (a) *S. agalactiae*, (b) *B. cereus*, (c) *P. aeruginosa*, (d) *E. coli*, (e) *S. dysenteriae*, (f) *S. sonnei* and (g) *Agrobacterium* sp.

The result showed that the crude extract (500 μg/disc) was moderately active against both gram-negative and gram-positive bacteria. The zone of inhibition of the extract was roughly close to kanamycin at 30 μg/disc against gram-negative bacteria. In case of gram-positive bacteria, the zone of inhibition of this extract was lower than standard kanamycin at 30 μg/disc. It was found that the extract caused marked inhibition of Gram negaitive bacteria when compared to Gram positive bacteria. Among bacteria, *S. dysenteriae* and *S. sonnei* were inhibited to high extent followed by *P. aeruginosa*, *B. cereus*, *E. coli*, *Agrobacterium* sp. and *S. agalactiae*. The result indicates that the extract is more active against gram-negative bacteria than gram-positive bacteria.

The result for the crude extracts (300 µg/disc) showed that it is moderately active against both gram-negative and gram-positive bacteria. The zone of inhibition of this extracts was significantly lower than standard kanamycin at 30 µg/disc against both gram-positive bacteria and gram-negative bacteria. In a study, Oskay *et al.* (2004) reported 32 mm (50 µg/disc) inhibition zone against *Klebsiella pneumoniae*. Another study reports that the extract of *Streptomyces tanashiensis* strain A_2D showed antibacterial activity against *B. subtilis* (15 mm), *Staphylococcus aureus* (25 mm), *E. coli* (21 mm) and *Klebsiella pneumoniae* (23 mm) (Singh *et al*., 2009). The observed minimal inhibitory concentration of the crude metabolite against the human pathogenic bacteria showed that it has moderate antibacterial potency.

The antifungal activity of the crude extract was tested by agar well diffusion method. Among the fungi, the growth of *C. albicans* was inhibited by extract to higher extent when compared to *C. neoformans*. Here also, inhibition caused by standard amphotericin B was higher than that of extract (Table 3).

Table 3. Antifungal activity of crude extracts of ANAM-39.

Test fungi	Zone of inhibition (in mm) ± Standard deviation (n=3)	
	Crude Extract (20mg/ml)	Standard (1mg/ml)
Candida albicans	15 ± 0.28	22 ± 0.37
Cryptococcus neoformans	14 ± 0.44	20 ± 0.33

Bacteria isolated from cultivated field soil, garden soil, decaying organic matters and stored agricultural products was evaluated for antifungal activity (Jain *et al*., 2003). In their studies, out of 287 isolates, a total of 166 isolates were active against *C. albicans*, while 164, 134 and 132 showed antagonistic properties against *Aspergillus niger*, *Microsporium gypseum* and *Trichophyton* sp., repectively. The antifungal activity of heptane group of antibiotics from *Streptomyces sampsonii* was determined against four fungi namely *C. albicans*, *A. niger*, *M. gypseum* and *Trichophyton* sp. (Jain *et al*., 2007). They found strong antifungal activity of antibiotics. In our study, it was found that the crude extract of ANAM-39 caused marked inhibition of test fungi.

The cytotoxic activity of different concentrations of extract, in terms of mortality of brine shrimps, is presented in table 4. The degree of lethality of extract was directly proportional to the concentration of the extract i.e., at higher concentration, the mortality was higher and a plot concentration versus percent morality on graph showed an almost linear correlation. Highest lethal effect was observed at extract concentration of 1000 µg/ml at which the mortality was 100%. Lethal effect was lesser (31%) in case of 10 µg/ml extract concentration. LC_{50} values greater than 1000 µg/ml were considered inactive (non-toxic). The LC_{50} of the crude extract was found to be 30.19 µg/ml and hence the extract is toxic.

Table 4. Cytotoxic activity of crude extract of ANAM-39.

Concentration (µg/ml)	Mortality of shrimps (%)	LC_{50} value (µg/ml)
0.0	0.0	30.19
10.0	31.0	
50.0	60.0	
100.0	75.0	
1000.0	100.0	

A number of studies have been carried out on cytotoxic nature of actinomycete extracts and purified metabolites on brine shrimp. According to Safaeian *et al*., (2005) actinomycetes isolated from Persian gulf showed marked cytotoxic effect on two *Artemia* species namely *A. urmiana* and *A. franciscana*. From ethyl acetate extract of a *Streptomyces* strain, Sultan *et al*. (2002) isolated three active metabolites from and tested their toxicity against brine shrimp. The metabolites as well as extract showed marked lethal effect on brine shrimp. The ethyl acetate extract and a purified compound of *Streptomyces rajshahiensis* exhibited potent lethal effect against brine shrimp (Ripa *et al*., 2010). In our study, the extract caused dose dependent lethal effect on brine shrimp, thus the

extract is toxic and purified metabolite can have antitumor properties.

Conclusion

To identify and discover various novel drug molecules, marine bacteria producing diverse natural compounds play a significant role. The biologically active compounds obtained from these marine bacteria have diverse applications. Hence, these compounds can serve as basic platform to find out new antibacterial, antifungal and other pharmacologically active natural compounds. In this context, the present study highlighted the antibacterial, antifungal and cytotoxic potentials of the crude extract of a marine bacterium, ANAM-39 isolated from the Sundarbans, Bnagladesh. The metabolites of marine bacteria from Sundarbans may prove to be useful. To isolate and characterize the active principles further chemical and biological studies are required.

Acknowledgement

This study was supported by Pharmaceutical Microbiology Laboratory, Department of Pharmacy, University of Rajshahi, Rajshahi-6205, Bangladesh.

References

Alcamo, E. and Pomerville, J.C. 2004. *Alcamo's laboratory fundamental of microbiology*. 7th Edn, Jones & Bartlett Publishers, London.

Bauer, A.W., Kirby, W.M., Sherris, J.C. and Turck, M. 1966. Antibiotic susceptibility testing by a standardized single disk method. *Am. J. Clin. Pathol.* **45**, 493- 496.

Barsby, T., Kelly, M.T., Gagne, S.M. and Andersen, R.J. 2001. Bogorol a produced in culture by a marine *Bacillus* sp. reveals a novel template for cationic peptide antibiotics. *Org. Lett*. **3**, 437-440.

Bernard, B. 2007. Access excellence @ the national health museum, Isolation of antibiotic strains from soils. (www. Access excellence. Org.).

Cragg, G.M. and Newman, D.J. 2001. Medicinals for the millennia: The historical record. Ann. *N. Y. Acad. Sci*. **953**, 3-25.

Demain, A.L. and Davies, J.E. 1999. Manual of industrial Microbiology and Biotechnology 2nd edition (Chapter 1, page 3-20), American Society for Microbiology, Washington.

Hassan, A., Rahman, S., Deeba, F. and Mahmud, S. 2009. Antimicrobial activity of some plant extracts having hepatoprotective effects. *J. Med. Plants Res*. **3**, 20-23.

Jain, P.K. and Jain, P.C. 2003. Antifungal activity of some actinomycetes isolated from various habitats. *Hindustan Antibiot. Bull*. **45-46**, 5-10.

Jain, P.K. and Jain, P.C. 2007. Isolation, characterization and antifungal activity of *Streptomyces sampsonii* GS 1322. *Ind. J. Experi. Biol*. **45**, 203-206.

Manivasagan, P., Venkatesan, J., Sivakumar, K. and Kim, S.K. 2014. Pharmaceutically active secondary metabolites of marine actinobacteria. *Microbiol. Res*. **169**, 262-78.

Neidelman, S.L. and Laskin, A.L. 2000. Advances in applied microbiology, *Elsevier*. **47**, 88-90.

Oskay, M., Tamer, A.U. and Azeri, C. 2004. Antibacterial activity of some actinomycetes isolated from farming soils of Turkey. *Afr. J. Biotechnol*. **3**, 441-446.

Parungao, M.M., Maceda, E.B.G. and Villano, M.A.F. 2007. Screening of antibiotic producing actinomycetes from marine, brackish and terrestrial sediment of Samal Island, Philippines. *J. Res. Sci. Comput. Eng*. **4**, 29-38.

Ripa, F.A., Nikkon, F., Rahman, B.M. and Khondkar, P. 2010. *In vitro* antibacterial activity of bioactive metabolite and crude extract from a new *Streptomyces* sp. *Streptomyces rajshahiensis*. *Int. J. Pharm.Tech. Res*. **2**, 644-648.

Safaeian, S., Alsadat, N.A. and Oryan, S. 2005. Studies on cytotoxic activity of marine Actinomycetes from Persian gulf on *Artemia franciscana* and *Artemia urmiana*. *J. Agricul. Sci. Nat. Resour*. **11**, 133-139.

Sarker, A.K., Haque, M.A., Islam, M.S., Rahman, M.A. and Islam, M.A.U. 2015. Isolation and characterization of a marine bacterium from Sundarbans, Bangladesh. *Bri. Micro. Res. J*. **6**, 348-357.

Shepherd, M.D., Kharel, M.K., Bosserman, M.A. and Rohr, J. 2010. Laboratory Maintenance of *Streptomyces* Species, UNIT10E.1.1.

Singh, L.S., Mazumder, S. and Bora, T.C. 2009. Optimisation of process parameters for growth and bioactive metabolite produced by a salt-tolerant and alkaliphilic actinomycete, *Streptomyces tanashiensis* strain A_2D. *J. Mycol. Med*. **19**, 225-223.

Sultan, M.Z., Sathi, Z.S., Biswas, M.H.U., Choudury, M.A., Rahman, M.A.A. and Gafur, M.A. 2002. *In vitro* toxicological studies of metabolites of *Streptomyces* species on brine shrimp. *J. Med. Sci*. **2**, 240-242.

Suthindhiran, K. and Kannabiran, K. 2009. Cytotoxic and antimicrobial potential of actinomycete species *Saccharopolyspora salina* VITSDK4 isolated from the Bay of Bengal Coast of India. *Am. J. Infect. Dis*. **5**, 90-98.

Solanki, R., Khanna, M. and Lal, R. 2008. Bioactive compounds from marine actinomycetes. *Ind. J. Microbiol*. **48**, 410-431.

Tyler, V.E., Brady, L.R. and Robbers, J.E. 1988. Pharmacognosy, 9th edition, Lea and Fabiger, Philadelphia, pp. 312-318.

Ullah, M.O., Haque, M., Urmi, K.F., Zulfiker, A.H., Anita, E.S. and Begum, M. 2013. Anti-bacterial activity and brine shrimp lethality bioassay of methanolic extracts of fourteen different edible vegetables from Bangladesh. *Asian. Pac. J. Trop. Biomed*. **3**, 1-7.

Vinayaka, K.S., Swarnalatha, S.P., Preethi, H.R., Surabhi, K.S., Kekuda, T.R.P. and Sudharshan, S.J. 2009. Studies on *in vitro* antioxidant, antibacterial and insecticidal activity of methanolic extract of *Abrus pulchellus* Wall (Fabaceae). *Afr. J. Bas. Appl. Sci*. **1**, 110-116.