ANTIBACTERIAL EFFICACY OF FOUR SELECTED MEDICINAL PLANTS AGAINST *XANTHOMONAS ORYZAE* PV. *ORYZAE*

A. K. Mondal[1], M. M. Hasan[2*], S. Ananya[1], M. M. Begom[1] and A. T. M. Shafiqul Islam[2]
[1]MS student, [2]Professor, Department of Plant Pathology, Hajee Mohammad Danesh Science and Technology University, Dinajpur
*Email of corresponding author: mhasan@hstu.ac.bd

ABSTRACT

Mondal, A. K., Hasan, M. M., Ananya, S., Begom, M. M. and Islam, A.T.M. S. 2017. Antibacterial efficacy of four selected medicinal plants against *Xanthomonas oryzae* pv. *oryzae*. Bangladesh J. Plant Pathol. 33 (1&2): 37-42

Bacterial leaf blight (BLB) caused by *Xanthomonas oryzae* pv. *oryzae* is an important disease of rice causes heavy yield losses. The bacterium was isolated and identified through different biochemical test and pathogenicity test. Methanol and aqueous Leaf extract of some plant leaves including *Azadirachta indica* (Neem), *Citrus limon* (Lemon), *Annona squamosa* (Ata) and *Aegle marmelos* (Bael) were evaluated for their antibacterial efficacy against the isolated pathogen using disc diffusion method. Water extracts (2.42 cm) of all the plant showed higher antibacterial efficacy against the tested bacterium compared to methanol extract (1.48 cm) and control (0.00 cm). Among medicinal plants, Neem leaf water extract showed the higher zone of inhibition (2.60 cm) than methanol extract (1.90 cm). Water extract of Ata leaf showed the higher zone of inhibition (2.57 cm), followed by methanol (1.67 cm). On the other hand, water extract of Bael leaf showed the higher zone of inhibition (2.47 cm) compared to methanol (1.70 cm)., Lemon water extract showed the maximum inhibition zone (2.13 cm) comparing to methanol (1.60 cm). MIC and MBC of water extract were found best at 62.5 µg/ ml using Neem and Ata leaf extract where Lemon and Bael leaf extract gave 125 µg/ ml. In cell viability test, all plants with water extract showed 100% cell death at 240 min time of exposure.

Key words: Antibacterial efficacy, Medicinal plants, *Xanthomonas oryzae* pv. *oryzae*

INTRODUCTION

Rice (*Oryza sativa*) is a staple food crop for more than half of the world's population including Bangladesh. However, the yield of rice in Bangladesh is less in compared to other major rice growing countries (BBS 2016). Among the constraints of low yield of rice, diseases play a great role (Fakir 1982). Among the diseases, bacterial leaf blight (BLB) caused by *Xanthomonas oryzae* pv. *oryzae* is one of the major threat to the low yield of rice in Bangladesh and it may cause up to 30% yield loss (Shahjahan *et al*. 1991; Alam and Quayum 2008; Kini *et al*. 2017).

Various medicinal plants contain a lot of bioactive compounds including alkaloids, flavonoids, tannin, and phenolic compounds which have antimicrobial properties (Kawaii *et al*. 2000, Tyagi and Malik 2010). The indigenous medicinal plants in Bangladesh including Neem, Lemon, Ata and Bael also possess antimicrobial bioactive compounds including alkaloids, flavonoids, phenols, saponins, terpenoids, and tannins (Ranjan 1999, Kawaii *et al*. 2000, Biswas *et al.* 2002, Badam *et al*. 2002, Burt 2004; Ortuno *et al*. 2006, Patel *et al.* 2008). Chemical control of plant diseases are very costly, cause environmental pollution and health hazard. Despite of the harmful effect, peoples are still using chemicals or antibiotics for the management of bacterial diseases, however; the management practices are not effective to control the bacterial diseases properly. Hence, it is urgent to develop an effective, economical and eco-friendly management practices to control such a devastating disease like bacterial leaf blight of rice. Regrettably, limited works have been carried out to find out the suitable management practices using the indigenous medicinal plants in Bangladesh. Therefore, the study was aimed to evaluate the antibacterial efficacy of some important medicinal plants including Neem, Lemon, Ata and Bael against *X. oryzae* pv. *oryzae* causing bacterial leaf blight.

MATERIALS AND METHODS

Isolation and Identification of *X. oryzae* pv. *oryzae*

Infected leaves of rice were cut into small pieces, washed and surface sterilized by using 70% ethanol for 3 min., and 10% Clorox followed by three repeated washing with sterilized distilled water. Then the sterilized leaf pieces were placed on NA media and incubated at 28 ˚C and maintained as pure culture of the bacterium. Some biochemical tests including Gram's staining test (Ryan K.J. and Ray 2004),

Potassium hydroxide test (Suslow *et al*. 1982), tween 80 hydrolysis test (Ishaq *et al*. 2015), starch hydrolylis test (Swings *et al*. 1990), anaerobic growth test (Hugh and Leifson 1953) and lecithinase activity test (Mc Clung and Toabe 1947) were carried out to identify the bacterium. Pathogenicity test was also done using leaf clipping method followed by Kauffman *et al*. (1973).

Preparation of leaf extracts

Leaf extracts of four (4) indigenous medicinal plants viz. neeem (*Azadirachta indica*), lemon (*Citrus limon*), ata (*Annona squamosal*) and bael (*Aegle marmelos*) were selected to study their antibacterial efficacy in *in-vitro*. The collected leaves were dried by electric drier at 45 °C followed by air drying and were powdered using an electric grinder. Then the solvent (methanol and water) and the leaf powder were mixed at 3:1 ratio and placed in an electrical shaker for 24 h (Ayogu and Amadi 2009). Extracts were filtered by using whatman filter paper no.1 and finally concentrated by using a rotary evaporator (Salam *et. al.* 2014). The crude extracts were then stored at 4 ^{0}C until use. Some sensitivity discs (6.0 ± 1.0 mm) of whatman filter paper no. 1 were impregnated with the extract for the determination of antibacterial activity of the plant leaves at a concentration of 16.67 (0.5 g extract in 3 ml solvent, respectively).

Antibacterial efficacy assay

The antibacterial efficacy of the leaf extract with different solvent was carried out by using disc diffusion method (Heatley 1944). A 100 µL of the *X. oryzae pv. oryzae* suspension containing 1×10^8 CFU /ml inoculum was overlaid uniformly on petriplates with 20 mL of sterile nutrient agar medium and allowed to dry for 5 min. A sterile Whatman No. 1 filter paper disc (6 mm diameter) was impregnated with 10 µL (500 µg/ml) of plant extracts dissolve in the solvent used during the extraction and placed on the NA medium containing bacterial suspension. A control was prepared and placed on the same petriplates using the same solvent employed to dissolve the plant extracts. After incubating the petriplates at 28 °C for 2-3 days (Salam *et. al.* 2014). The antibacterial efficacy was evaluated by comparing the diameter of zones of inhibition produced by the effect of plant extracts impregnated paper disc against the isolated plant pathogen.

Minimum inhibitory (MIC) and minimum bactericidal (MBC) concentration

The MIC of the plant extracts was evaluated by standard two-fold serial dilution method (NCCLS 1997). The plant extracts dissolved in water were incorporated into NB medium to obtain a concentration of 2000 µg/ml, and then serially diluted to achieve 1000, 500, 250, 125, 62.5 and 31.25 µg/mL. A 10 µL standardized bacterial suspension of the isolated bacterium (10^7 CFU/ml) was transferred to the each of eppendorf tube containing different concentration of plant extract and incubated at 28±2 °C for 24 h with shaking using a mechanical shaker. The MIC of the extract was calculated. Further, the concentration showing inhibition of the bacterial visual growth was determined, and 50 mL of each diluted culture broth transferred on to the NA plates, and the plates were incubated for 2-3 days at 28±2 °C. The complete absence of growth on the media surface in the lowest concentration of sample was defined as MBC.

Cell viability assay

To assay the viable count, each of the eppendorf tubes containing re-suspended bacterial isolates suspension (approximately 10^7 CFU/mL) was inoculated with 62.5-125 µg/mL concentration of the plant extracts in 10 mL NB broth, and incubated at 28 °C. To assay the viable cell count, samples were taken out at 0, 40, 80, 120, 160, 200 and 240 min time intervals, and the viable bacterial cells were counted as follows: following the incubation, one mL of the re-suspended bacterial culture was added into 9 mL buffer peptone water, there by diluting 10 times. From each treatment, 0.1 mL sample was diluted further and laid on the surface of NA agar plates. Following incubation at 28 °C, the colonies were counted after 2-3 days. The controls were constituted of bacterial inoculums without the plant extracts with the same experimental conditions. Each set of viable count assay in this experiment was replicated for three times.

RESULTS AND DISCUSSIONS

Identification of the isolated bacterium from diseased rice plant

The isolated bacterium from the infected leaf of rice plant was identified through different biochemical test. The gram staining test showed reddish pink or red color in the bacterium which confirms the isolated bacterium as gram negative. Potassium hydroxide (KOH) test also showed the elastic thread of the bacterial suspension which re-confirms the gram negative nature of the isolated bacterium (Suslow *et al.* 1982). Gram negative bacteria have relatively fragile cell walls bounded by an outer membrane which easily disrupted after exposing to the diluted alkali solutions and become viscous because of the releasing of relatively defragmented threads of DNA (Halebian *et al.* 1981). A turbid zone was observed

surrounding the bacterial colony when the NA plate impregnated with iodine potassium iodide (IKI) solution. Starch is a complex carbohydrate (polysaccharide) composed of glucose molecules linked together by α-1, 4 and α-1, 6 glycosidic bonds. The tested isolate possess the ability to degrade starch producing amylase. Amylase is an exo-enzyme that hydrolyzes i.e. break down a polysaccharide starch into monosaccharide and disaccharides like glucose, maltose produce the turbid zone instead of a clear zone (Samanta *et al.* 2014, Swings *et al.* 1990). The bacterium also showed positive result by changing the color from blue to yellow confirms the aerobic nature (Hugh and Leifson 1953). Moreover, a very clear white opaque edge surrounding the *X. oryzae* pv. *oryzae* colony, indicated the test was positive result of the isolates. The enzyme lecithinase could break down the phospholipid emulsion of egg yolk, liberating turbid zone of free fats around the colonies i.e. white opacity, which extends beyond the edge of growth (Mc Clung and Toabe 1947). After inoculation, the isolated bacterium cause leaf blight symptoms in the rice showing its pathogenic ability to cause the disease (Rafi *et al.* 2013). However, the pathogenicity test of the isolated bacterial strain along with the entire biochemical test confirmed the isolated bacterium as *X. oryzae* pv. *oryzae* causing bacterial leaf blight of rice.

Antimicrobial effect of the plant extracts

The antibacterial efficacy of selected plant extracts were calculated by the presence of zone of inhibition. All the plant extract (500 µg/disc) used in this study exhibited potent antibacterial efficacy against the isolated *X. oryzae pv. oryzae* (Table 1). Among the solvent used in this study, the water (2.42 cm) showed significantly higher performance for extracting the potential metabolites showing antibacterial activity as zone of inhibition in compare with methanol (1.48 cm) (Table 1). As high polar solvent, water can dissolve more astringent antipyretics and tannins than other polar solvents which might help to extract and dissolve more antimicrobial compounds.

Table 1. Performance of different solvent in the aspect of showing zone of inhibition against the tested bacterium.

Name of the solvent	Inhibition zone (cm)
Control	0±0.00 c
Water	2.42±0.24 a
Methanol	1.48±0.22 b

*Means with the same letter are not significantly different

However, water extracts of all the plant extracts including neem (2.60 cm), lemon (2.13 cm), ata (2.57 cm) and bael (2.40 cm) showed statistically significant higher zone of inhibition over methanol (1.67-1.90 cm) and control (0.00 cm) (Table 2). Higher antimicrobial activity of aqueous extract of *A. indica* in compare with other solvents including methanol, ethanol and chloroform against the gram negative organism including *E. coli* and human pathogenic bacterium *Staphylococcus aureus* was also observed (Sukanya *et al.* 2008). However, the active ingredient like, azardirachtin, 1-maliantriol, salannin, nimbin, nimbdin, triterpenoids, phenolic compounds etc. present in *A. indica* leaf are soluble in water which might gave the better antibacterial activity (Ibekwe *et al.* 2001). The aqueous extract of *C. limon* alos found to show highest antibacterial activity than ethyl acetate against *Escherichia coli, Pseudomonas aeruginosa, Bacillus subtilis, and Staphylococcus aureus* (Sapkota *et al.* 2012, Rizwan and Aziz 2016). The water extract of ata and bael leaf also showed improved antibacterial activity against *E. coli* and *S. typhi* than another solvent like methanol as it contains some bio- compounds like alkaloids, oils, tannins, phenols, saponins, glycosides, and flavonoids (Chavda *et al.* 2012, Gowdhami *et al.* 2014, Victoria *et al.* 2014). However, bioactive compounds of leaves are soluble at different degree varying with the solvent polarity (Nenaah and Ahmed 2011).

Table 2. Antibacterial efficacy of different plant extracts against the tested bacterium

Plant extract	Solvent	Inhibition Zone (cm)
Control	--	0±0.00 c
A. indica (Neem)	Water	2.6±0.21 a
	Methanol	1.9±0.06 b
C. limon (Lemon)	Water	2.13±0.09 a
	Methanol	1.67±0.03 b
A. squamosa (Ata)	Water	2.57±0.39 a
	Methanol	1.67±0.03 b
A. marmelos (Bael)	Water	2.4±0.21 a
	Methanol	1.7±0.21 b

*Means with the same letter are not significantly different

MIC and MBC

All the plant extracts used in this study showed potent inhibitory effect as MIC and MBC values against the isolated bacterium. The MIC and MBC values of the water extract (as water showed best performance) of all the plant extracts against the tested bacterium were found in the range of 62.5 to 125 µg/ml (Table 3). As different plant contain different types of secondary metabolites, and so their

performance in terms of MIC and MBC against the tested bacterium might be different.

Table 3. Minimum Inhibitory Concentration and Minimum Bactericidal Concentration of *X. oryzae* pv. *oryzae*

Plant Extracts	MIC (µg/ml)	MBC (µg/ml)
Neem (*A. indica)*	62.5	62.5
Lemon (*C. limon)*	125	125
Ata *(A. squamosa)*	62.5	62.5
Bael *(A. marmelos)*	125	125

Efficacy of plant extracts on cell viable count

To assess the antibacterial effect of the plant extracts on the tested bacterium, a cell viable count assay was also executed. The water extracts of all the plant used in this study had a negative effect on the growth of the tested plant pathogenic bacterium (Fig 1). About 90% inhibition of the bacterial growth was observed against the isolated bacteria at 200 min exposure for all four plants extract and at 240 min exposure time, the water extract of four leaves showed 100% growth inhibition, and no colony forming unit was observed.

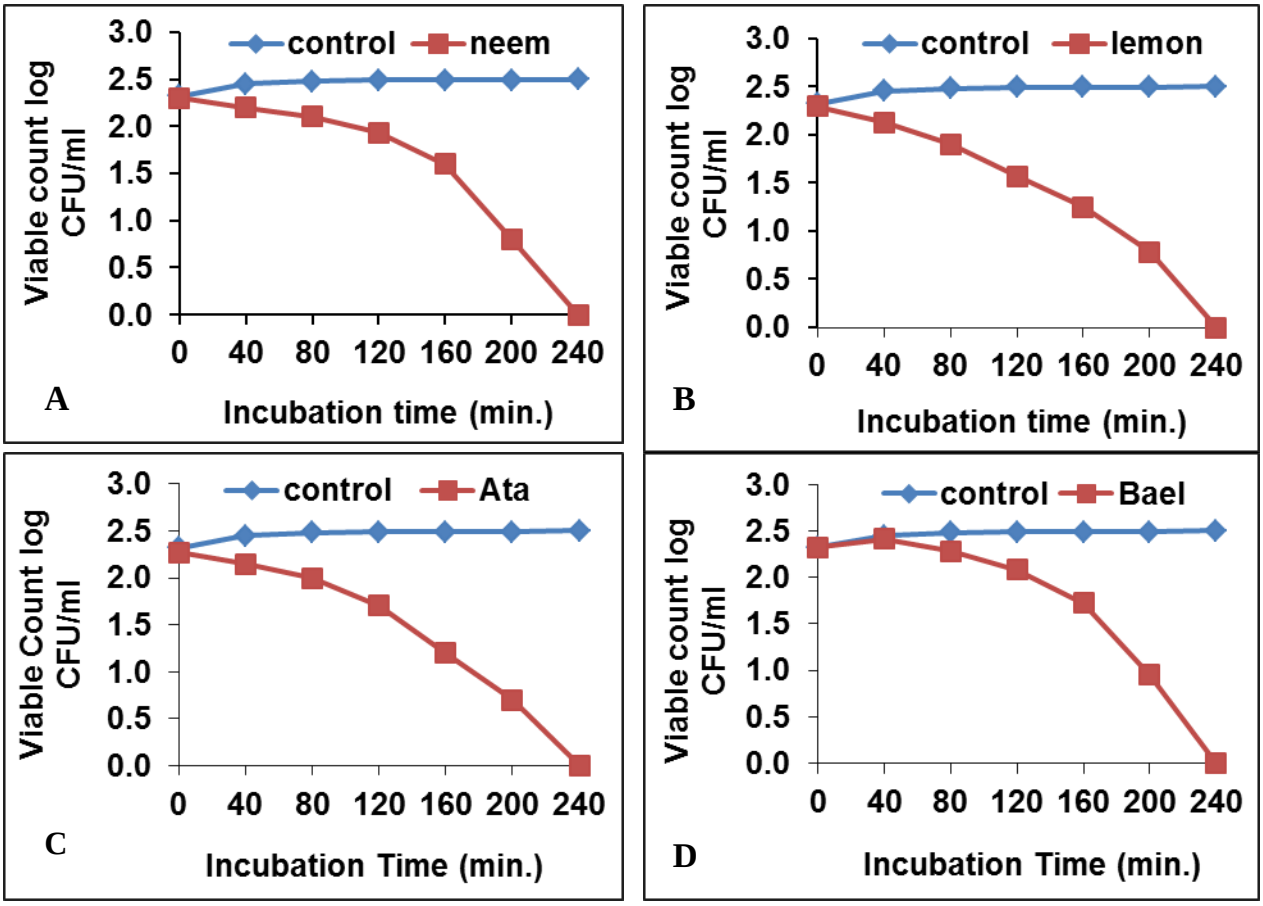

Fig.1. Cell viability counting of *X. oryzae* pv. *oryzae* using A) Neem, B) Lemon, C) Ata and D) Bael leaf extract

CONCLUSION

Water extract of all the selected plants had potential antibacterial effect against *X. oryzae* pv. *oryzae*. However, the antibacterial compounds in the indigenous medicinal plants have ability to retard the infection of plant pathogens. Therefore, there is a chance to formulate the water extract of Neem, Lemon, Ata and Bael to control BLB disease of rice.

LITERATURE CITED

Alam, M.S. and Quayum, M.A. 2008. Shifting from Rice to Non-rice Crop Production in Bangladesh: An Analysis of Farmers' View. The Agriculturist. 6(1&2): 124-131.

Ayogu, T. and Amadi, E. 2009. Studies on the antibacterial activities of medicinal plants on

typhoid fever organisms. Int. J. Third World Med. 7(2): 32-36.

Badam, L.; Bedekar, S.S.; Sonawane, K.B. and Joshi, S.P. 2002. J. Communicable Dis. 34(2): 88-99.

BBS (Bangladesh Bureau of Statistics). 2016. Statistical Year Book of Bangladesh. Agriculture Statistical Wing. Ministry of Planning, Govt. of the People's Republic of Bangladesh, Dhaka, Bangladesh.

Biswas, K., Ishita, C., Ranajit, K.B. and Uday, B. 2002. Biological activities and medicinal properties of *A. indica*. Current Sci. 82: 1336-1345.

Burt, S.A. 2004. Essential oils: Their antibacterial properties and potential applications in foods: A review. Int. Jo. Food Microbiol. 94: 223-253.

Chavda, N.; Adarsh, M., Mehta, S.K. and Dodia, P.P. 2012. Primary identification of certain phytochemical constituents of *Aegle marmelos* L. Corr. Serr. Responsible for antimicrobial activity against selected vegetable and clinical pathogen. Int. J. Physical and Social Sci. 2(6): 190-206.

Fakir, G.A. 1982. An annotated list of seed borne diseases in Bangladesh Agricultural Information Service. Dhaka, Bangladesh. pp. 15-22.

Gowdhami, M., Sarkar, B.L. and Ayyasamy, P.M. 2014. Screening of phytochemicals and antibacterial activity of *A. squamosa* extracts. Int. J.Pharma. Sci. Inven. 3(7): 30-39.

Halebian, S., Harris, B., Finegold, S.M. and Rolfe, R.D. 1981. Rapid method that aids in distinguishing gram-positive from gram-negative anaerobic bacteria. J. Clin. Microbiol.13: 444-448.

Heatley, N.G. 1944. A method for the assay of penicillin. Biochem. J.. 38: 61-65.

Hugh, R. and Leifson, E. 1953. The taxonomic significance of fermentative versus oxidative metabolism of carbohydrates of various Gram-bacteria. J. Bact. 66: 24-26.

Ibekwe, V.I. Nnanyere, N.F. and Akujobi, C.O. 2001. Studies on antibacterial activity and phytochemical qualities of extracts of orange peels. Int. J Environ., Health and human Development. 2 (1):41-46.

Ishaq, A. Rehman. A. Zakaria, M. and Munir, A. 2015. Biochemical characterization of *X. oryzae* pv. *oryzae* populations from Kallar Belt of Punjub, Pakiatan. Int. J. Agric. Innov. Res. 3(4): 2319-1473.

Kauffman, H.E. Reddy, A.P.K. Hsieh, S.P.Y. and Merca, S.D. 1973. An improved technique for evaluating resistance of rice varieties to *Xanthomonas oryzae* pv. *oryzae*. Plant Dis.. Rep. 57: 537-541.

Kawaii, S.; Yasuhiko, T. Eriko, K. Kazunori, O.; Masamichi, Y. Meisaku, K. Chihiroito and Hiroshi, F. 2000. Quantitative study of flavonoids in leaves of *Citrus* plants. J. Agric. Food Chem. 48: 3865-3871.

Kini, K. Agnimonhan, R. Afolabi,O. Milan, B.; Soglonou, B. Koebnik, R. and Silué, D. 2017. First Report of a New Bacterial Leaf Blight of Rice Caused by *Pantoea ananatis* and *Pantoea stewartii* in Benin. Plant dis. 101 (1): 242.

Mc Clung, L.S. and Toabe, R. 1947. The egg yolk plate reaction for the presumptive diagnosis of Clostridium sporongenes and certain species of gangrene and botulinum groups. J. Bacter. 53: 139-147.

NCCLS (National Committee for Clinical Laboratory Standards). 1997. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically. Approved Standards M7-A4, Wayne, Pa.

Nenaah, E.G. and Ahmed, M.E. 2011. Antimicrobial activity of extracts and latex of *Calotropis procera* (Ait) and synergistic effect with reference. Antimicrob. Res. J. Medicin. Plant. 5(6): 706-716.

Ortuno, A.A. Baidez, P. Gomez, M.C. Arcas, I. Porras, A.G. and Delrio, J.A. 2006. *Citrus paradise* and *Citrus sinensis* flavonoids: Their influence in the defence mechanism against *Penicillium digitatum*. Food Chem. 98(2): 351-358.

Patel, D.J. and Vipin, K. 2008. Phytochemical analysis and antimicrobial screening of *A. squamosa* L. J. Pharm. Res. 1(1): 34-38.

Rafi, A. Hameed, A. Akhtar, M.A. Farhatullah, S.K.; Shahid, M. and Fahim, M. 2013. Identification and characterization of *X. oryzae* pv.*oryzae*in north-west of Pakistan. J. Agric. 29(3): 423-432.

Ranjan, V. 1999. Exotic medicinal plants of district Lalitpur, Uttar Pradesh. Bulletin of Medico-Ethno- Botanic. Res. 20(1-4): 54-65.

Rizwan, A. and Aziz, A. 2016. Evaluation of antibacterial activity of *Morus nigra* & *C. limon*. J. Ayurvedic and Herbal Med. 2(6): 213-217.

Ryan, K.J. and Ray, C.G. 2004. Sherris Medical Microbiology (4th ed.). McGraw Hill. Pp. 232.

Ryu, E. 1940. A simple method of differentiation between Gram-positive and Gram-negative organisms without staining. Ixilasato Archives of Hxpcr. Med. 17: 58-63.

Salam, R.; Khokon, J.U. Baidya, S. and Mussa, M.T. 2014. Effect of neem and betel leaf against oral bacteria. Int. J. Nat. Soc. Sci. 1. 52-57.

Samanta, T.T.; Prodip, S. and Das, A. 2014. Isolation and characterization of *X. oryzae* isolates from different regions of Midnapore district of West Bengal and their ecofriendly management by some medicinal plant extracts. Int. J. Phytomed. 6: 29-42.

Sapkota, R., Dasgupta, R., Nancy and Rawat, D.S. 2012. Antibacterial effects of plants extracts on human microbial pathogens & microbial limit tests. Int. J. Res. Pharm. Chem. 2(4).

Shahjahan, A.K.M. Ahmad, H.U. Mia, M.A.T. Sharma,M.A. and Nahar, N.S. 1991.Outbreak of leaf blight in rice crop in Bangladesh.16: 21.

Sukanya. S.L. Sudisha, J. Hariprasad, P. Niranjana, S.R.; Prakash, H.S. and Fathima, S.K. 2008. Antimicrobial activity of leaf extracts of Indian medicinal plants against clinical and phytopathogenic bacteria. Afr. J. Biotec. 8 (23): 6677-6682.

Suslow, T.V. Schroth, M.N and Isaka. M. 1982. Application of a rapid method for Gram differentiation of plant pathogenic and saprophytic bacteria without staining. Phytopathol. 72: 917-918.

Swings, J. Mooter, M.V. Vauterin, L. Hoste, B. Gillis, M. Mew, T.W. and Kersters, K. 1990. Reclassification of the causal agents of bacterial blight of rice (*X. campestris* pv. *oryzae*) and bacterial leaf streak (*X. campestris* pv. *oryzicola*) of rice as pathovars of *X. oryzae* (ex Ishiyama 1922) sp. nov., nom. rev. Int. J. Sys.c Bacteriol. 40:309-311.

Tyagi, A.K. and Malik, A. 2010. Liquid and vapour phase antifungal activities of selected essential oils against *Candida albicans*. BMC Complementary and Alternative Med..10: 65.

Victoria, D. Rao, T.K. and Samrot, A.V. 2014. Antibacterial activity and phytochemical screening of Aegle marmelos. Int. J. Pharm. Bio. Sci. 5(4): (B) 895-902.