Academic Sciences International Journal of Pharmacy and Pharmaceutical Sciences

ISSN- 0975-1491 Vol 5, Issue 2, 2013

Research Article

CHARACTERIZATION OF *BACILLUS SP.* FROM SOIL ISOLATES AND COMPARATIVE STUDY OF *COCCINIA GRANDIS* (L.) DIFFERENT LEAF EXTRACTS ON ANTIBACTERIAL STATUS AGAINST *BACILLUS SP.*

SAIFUR RAHMAN[1,3], MD. MORSHEDUL ALAM[2,3*], MUSLIMA JAHAN[3] MD. REZUANUL ISLAM[3]

[1]Center for Cognitive Sciences, University of Bremen, Bremen, Germany, [2]Institute of Development, Aging and Cancer, Tohoku University, Sendai, Japan, [3]Dept. of Biotechnology & Genetic Engineering, Islamic University, Kushtia 7003, Bangladesh. Email: morshedbt@gmail.com

Received: 09 Jan 2013, Revised and Accepted: 03 Mar 2013

ABSTRACT

Objective: *Bacillus* sp. is now broadly responsible for various human infection and food spoilage. At present, identification and molecular characterization of major types of *Bacillus* sp. are important for human being.

Methods: Antibacterial activities of six different concentrations each of ethanol, ethyl acetate and chloroform extracts of *Coccinia grandis* (L.) leaf on soil bacteria were assessed on the basis of disk diffusion method and the zone of inhibition was measured in mm unit. Different *Bacillus sp.* was characterized by using 16S rDNA sequencing.

Results: Six different types of *Bacillus sp.* were identified depending on 16S rDNA sequencing and NCBI database information. It has also been shown that the best extract was ethyl acetate due to its highest zone of inhibition (*Paenibacillus sp*. BF38 (23.0±0.41), *Terribacillus sp*. 3LF (21.0±0.00), *Bacillus simplex* (23.0±0.43), *Bacillus cereus* (23.5±0.70), *Paenibacillus sp*.L32 (22.5±0.37), *Bacillus megaterium* (23.0±0.04)) as compared to other two extracts.

Conclusion: The efficacy of *Coccinia grandis* (L.) leaf extracts as natural antibacterial agent was found ethyl acetate extract > ethanol extract > chloroform extract.

Keywords: *Bacillus sp*., *Coccinia grandis* (L.), 16S rDNA, Antibacterial activity.

INTRODUCTION

Natural sources are the vital fountain-head of medicinal agents and so many modern drugs have been isolated from natural sources, many of which are based on their use as folklore medicine. It has been noted that the raw source of many pioneering pharmaceuticals currently in use have been plants that was used by the indigenous population [1]. Among the total global population, reliability on medicinal plants for provision of health-care is about 64% has been recorded [2].

Coccinia grandis L., of the family Cucurbitaceae, is distributed in Indian sub-continent, Eastern Africa, and Central America [3]. *C. grandis* is a climber and trailer [4]. It is widely available in Bangladesh and is locally known as Telakachu. In English, the plant is known as Ivy gourd. In the folk medicinal system of Bangladesh, the leaves and fruits of the plant are predominantly used to treat leprosy, fever, asthma, bronchitis, jaundice, and diabetes [5].

Due to the emergence of multi-drug resistance to human pathogenic infections, it has become essential to search alternative antimicrobial substances from natural sources such as plants. Many plants also show versatile medicinal effects as well [6, 7] Phytochemical screening of *Coccinia grandis* revealed the presence of saponins, cardenolides, flavonoids and polyphenols that may be attributed to antibacterial activity. It provides the basis for further investigation on these plants to isolate active constituents and drug development. It has been reported that phenolic compounds are responsible for antimicrobial activities [8]. In view of the importance of *Coccinia grandis* L. in ethanobotany as health remedy, the present work was planned to investigate the antibacterial activity of the leaf extracts against some pathogenic bacteria from soil isolate. Although numerous studies have shown the antimicrobial effect of this plant [9, 10] there still remains ample scope for further in depth research to seek out this effect on some specific bacterial species. So far, for the first time an attempt was taken to investigate the comparative antimicrobial activity of this plant against Bacillus sp. from soil isolate that was screened through 16S rDNA sequencing.

MATERIALS AND METHODS

Collection of the Plant Material

The experiment was carried out from September, 2011 to April, 2012. The leaves of *C. grandis* were collected from Kushtia, Bangladesh. The plant was identified by the Department of Biotechnology & Genetic Engineering, Islamic University, Kushtia, Bangladesh and the specimens were stored in there for the further reference.

Extraction of the plant leaf

The plant leaves were selected for the study. They were sun dried for five days and heated through oven to be fully dried at below 40^0c for 24 hours. Then the fully dried leaves were grinded into coarse powder with the help of a mechanical grinder. The whole powders were extracted by cold extraction with three solvents (ethanol, ethyl acetate, and chloroform) and kept for a period of 3 days accompanying occasional shaking and stirring. The whole mixture were then underwent a coarse filtration by a piece of clean, white cotton material. Then these were filtered through whatman filter paper. The filtrates (ethanol, ethyl acetate, chloroform and n-hexane) obtained were evaporated by Rotary evaporator (Bibby RE-200, Sterilin Ltd., UK) at 5 to 6 rpm and at 68ºc temperature. It rendered a gummy concentrate of chocolate black color. The gummy concentrate was designated as crude extract. Then the crude extracts were dried by freeze drier (HETOSICC, Heto Lab Equipment, Denmark) and preserved at 4ºC.

Collection of sample and preparation of test sample

The microorganisms used in this study were soil isolates of *Bacillus sp*. Soil (20g) were suspended in sterile NaCl (0.9%) and maintained on a rotary shaker for 45 min at the maximum speed and were serially diluted.

0.1ml aliquots of the soil serial dilution (1:10, 1:100) were dropped and spread onto the solid media plate count agar (PCA) and incubated aerobically at 30 °C. After 14 days the number and the morphology (shape, size, color etc.) of colonies were recorded [11]. Representative colonies of different morphologies were isolated and stored in glycerol at -20° for molecular analyses. Finally 6 six

variants were used for the study. Stock cultures were maintained at 4°C on nutrient agar medium.

DNA extraction from colonies

DNA was extracted from isolated colonies which were cultured in liquid broth by the alkaline lysis method [12], with the following modifications. After incubation, cells from 2 ml of each culture was collected in 2 ml Eppendorf tubes by centrifugation at 10000 × g for 1 min at room temperature. The supernatant was discarded and the bacterial pellet suspended in 400 µl of Solution 1 (50 mM glucose, 25 mM Tris·HCl, pH 8.0, 10 mM EDTA, pH 8.0). After incubation at room temperature for 10 min, 400 µl of Solution 2 (1% [w/v] SDS, 0.2 N NaOH) was added and the tubes were incubated on ice for 10 min. Following the addition of 300 µl of 7.5 M ammonium acetate (pH 7.6), the tubes were incubated on ice for 10 min, and then centrifuged at 13 000 × g for 5 min at room temperature. The plasmid DNA was precipitated from the supernatant by the addition of 650 µl isopropanol for 10 min at room temperature. The precipitated plasmid DNA was collected at room temperature by centrifugation at 12 000 × g for 10 min and the supernatant discarded before addition of 100 µl of 2 M ammonium acetate (pH 7.4). The tubes were incubated on ice for 10 min. Following ambient centrifugation at 12 000 × g for 5 min, 110 µl of isopropanol was added to the supernatant and the tubes incubated at room temperature for 10 min. Precipitated DNA was collected and the pellets washed with 70% ethanol to remove residual salts from the DNA. The DNA was air-dried and resuspended in UHQ Millipore water. DNA thus prepared was stored at -20°C.

16S rDNA Amplification

For amplification of 16SrDNA from cultural bacteria PCR was performed in a final volume of 20 µl (1ul template DNA, 2.5ul of 10x buffer, 0.5ul of *Taq* DNA polymerase (Amersham Biosciences), 2ul of 2.5mM each of dNTPs, 0.5ul of each primer 63F 5'-CAGGCCTAACACATGCAAGTC-3' and 1389R 5'-ACGGGCGGTGTGTACAAG-3', 13ul ddH2O) [13]. The thermal cycler (Bio Rad lCycler 170-8740) was programmed for the initial denaturation step (95°C) of 5 min, followed by 44 cycles of 30 sec denaturation (94°C) along with 30 sec primer annealing (55°C) and 1 min primer extension (72°C), followed by the 10 min primer extension (72°C) step and finally stopped by 4°C. The PCR products were analyzed by 2% agarose gel electrophoresis and visualized with UV irradiation after staining with ethidium bromide. The PCR product was then purified.

Sequencing and Phylogenetic Analysis

After purification of the amplified PCR product, it was sequenced commercially using the BigDye Terminator ver1.1 commercial kit (Applied Biosystems, CA, USA). The 16S rDNA sequence was analyzed using Chromas LITE (Version 2.01); the most similar bacterial species was found in the GenBank by using BLAST search. Neighbor-joining phylogenetic trees were constructed based on 16S rDNA sequences using BioNumerics software version 4.5 (Applied Maths, Sint-Martens-Latem, Belgium). The nucleotide sequences were aligned with known sequences from GenBank using the CLUSTAL-W software. Individual sequences are compared with those available in the database in order to get the identification of bacterial species that make up the community analysis. Sequence analysis confirmed that the DNA isolated from the desired bacteria tentatively identified as species of *Bacillus.*

Antibacterial Assay

The antimicrobial activity for different extracts was determined by the disc diffusion method [14]. Isolated bacterial strains were used for the test. The bacterial strains used for the investigation are listed in Table 1. Solutions of known concentration (ug/10ul) of the test samples were made by dissolving measured amount of the samples in calculated volume of solvents. Dried and sterilized filter paper discs (6 mm diameter) were then impregnated with known amounts of the test substances using micropipette. Discs containing the test material were placed on nutrient agar medium uniformly seeded with the test microorganisms. Standard antibiotic discs (Cloxaciline 10 µg/µl/disc) and blank discs (impregnated with solvents) were used as a positive and negative control. These plates were then incubated at 37ºC for 24 h to allow maximum growth of the organisms. The test materials having antibacterial activity inhibited the growth of the microorganisms and a clear, distinct zone of inhibition was visualized surrounding the medium. The antimicrobial activity of the test agents was determined by measuring the diameter of zone of inhibition expressed in millimeter (mm). The experiment was carried out three times and the mean of the reading is required.

Determination of Minimum Inhibitory Concentration (MIC)

The method of Thongson *et al.* [15] was applied with slight modification. The MIC of the leaf extract was determined by micro dilution method using two-fold dilution for the dilution series. Both the ethanol and ethyl acetate extracts were tested to determine the Minimal Inhibitory Concentration (MIC) for each bacterial strain. Freshly, grown bacterial strains 100 µl (10^6cells/ml) in nutrient broth was inoculated in tubes with nutrient broth supplemented with different concentrations (7.80 – 1000.00 µl) from the stock extract (1 mg/ml) and antibiotic, respectively and incubated for 24 h at 37 °C. Presence of turbidity denoted presence of microorganism in the test tube after the period of incubation where as the complete absence of any turbidity indicates complete inhibition of microbial growth. The test tube with the lowest dilution with no detectable growth by visual inspection was considered the MIC. The MIC was calculated for the individual bacterial species**.**

RESULTS

Molecular Characterization of Test Microorganism Based On 16S rDNA Sequence

The 16S rDNA of each test strain was amplified using the respective primers and then all of the PCR products were analyzed by 2% agarose gel electrophoresis and visualized with UV irradiation after staining with ethidium bromide. The amplified DNA all isolates were 1300bp in length because only partial sequence of the 16S rDNA Gene was amplified (Fig. 1).

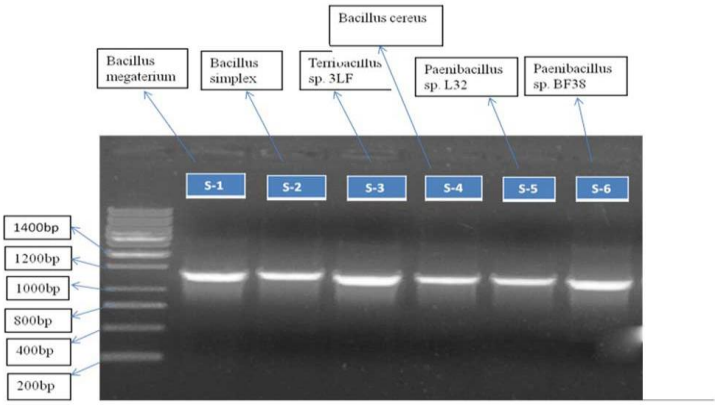

Fig. 1: Visualization of amplified DNA by agarose gel electrophoresis.

The amplified PCR products were sequenced and compared with the known sequences in the public databases in NCBI and BLAST. Based on the 16S rDNA sequences, the selected bacteria were considered as the *Bacillus sp.* (Table. 1). The sequences are showing maximum similarity of 100% for *B. megaterium*, *B. simplex*, *B. cereus* and *Paenibacillus* BF38.

Table 1: **Identification and similarity of test strain with database.**

Strain	GenBank accession No.	Original species	Sequence similarity (%)
Hb42	FJ614260	*Bacillus megaterium*	100
Xb17	FJ225298	*Bacillus simplex*	100
Hb21	EU741083	*Bacillus cereus*	100
Db26	AM934687	*Paenibacillus sp.* BF38	100
Db8	DQ196465	*Paenibacillus sp*.L32	99
Xb10	AM931170	*Terribacillus sp.*3LF22T	96

Antimicrobial Screening

The different extracts of the *C. grandis* were screened against the isolated bacteria to check antibacterial activities by disc diffusion method which showed valuable zone of inhibition (Table 2, 3 & 4). Depending on 500µg/10µl concentration, the order of the zone of inhibition from higher to lower for ethanol extract was *Paenibacillus sp*.L32 (22.0±0.00), *Terribacillus sp*. 3LF (21±0.18), *Bacillus simplex* (21±0.41), *Bacillus megaterium* (21.0±0.88), *Bacillus cereus* (20.0±0.44), Paenibacillus *sp.* BF38 (19.5±0.10); for ethyl acetate extract was *Bacillus cereus* (23.5±0.70), *Bacillus megaterium* (23.0±0.04), *Paenibacillus sp.* BF38 (23.0±0.41), *Bacillus simplex* (23.0±0.43), *Paenibacillus sp*.L32 (22.5±0.37), *Terribacillus sp*. 3LF (21.0±0.00); and for chloroform extract was *Paenibacillus sp*.L32 (21.0±0.00), *Terribacillus sp*. 3LF (21.0±00.00), *Bacillus megaterium* (21.0±0.14), *Bacillus simplex* (21.0±0.18), *Bacillus cereus* (20.0±0.18), *Paenibacillus sp.* BF38 (19.5±0.44).

Table 2: Determination of antibacterial activity of Ethanol extract of *C. grandis* against various bacteria

Bacterial strain	Diameter of the zone of inhibition (mm) (Cloxaciline 10 µg/µl)	Negative Control	Ethanol extract concentration (µg/10µl)			
			500	250	125	62.5
Bacillus megaterium(Hb42)	26.00±0.00	-	21.0±0.88	20.0± 0.7	18.0±1.0	17.0±0.5
Bacillus simplex (Xb17)	25.7±0.49	-	21±0.41	18.0±1.4	15.5±0.8	12.0±0.6
Bacillus cereus (Hb21)	27.00±0.00	-	20.0±0.44	18.5±0.7	17.0±1.2	14.5±1.2
Paenibacillus sp. BF38(Bb26)	24.16±0.23	-	19.5±0.10	19.0±1.2	18.0±1.2	14.5±1.5
Paenibacillus sp.L32	25.23±0.32	-	22.0±0.00	18.5±1.2	17.0±1.1	16.5±0.9
Terribacillus sp. 3LF(Xb10)	28.66±0.47	-	21±0.18	19.0±1.5	16.0±1.2	14.5±1.2

Data were measured in mm and presented as Mean ± SD of triplicate. (-) denotes no zone of inhibition.

Table 3: **Determination of antibacterial activity of Ethyl Acetate extract of *C. grandis* against various bacteria.**

Bacterial strain	Diameter of the zone of inhibition (mm) (Cloxaciline 10 µg/µl)	Negative Control	Ethyl Acetate extract concentration (µg/10µl)			
			500	250	125	62.5
Bacillus megaterium(Hb42)	26.00±0.00	-	23.0±0.04	20.0±1.6	19.0±0.6	00.0±0.0
Bacillus simplex (Xb17)	25.7±0.49	-	23.0±0.43	21.0±0.6	19.0±1.1	18.0±0.6
Bacillus cereus (Hb21)	27.00±0.00	-	23.5±0.70	22.5±1.1	22.0±1.6	21.010±1.5
Paenibacillus sp. BF38(Bb26)	24.16±0.23	-	23.0±0.41	22.0±1.1	21.0±0.7	19.0±1.2
Paenibacillus sp.L32	25.23±0.32	-	22.5±0.37	21.0±1.4	20.5±0.7	20.0±0.7
Terribacillus sp. 3LF(Xb10)	28.66±0.47	-	21.0±0.00	18.0±1.2	17.0±0.5	16.5±0.6

Data were measured in mm and presented as Mean ± SD of triplicate. (-) denotes no zone of inhibition.

Table 4: **Determination of antibacterial activity of Chloroform extract of *C. grandis* against various bacteria.**

Bacterial strain	Diameter of the zone of inhibition (mm) (Cloxaciline 10 µg/µl)	Negative Control	Chloroform extract concentration (µg/10µl)			
			500	250	125	62.5
Bacillus megaterium(Hb42)	26.00±0.00	-	21.0±0.14	20.0±0.6	18.0±0.7	17.0±0.5
Bacillus simplex (Xb17)	25.7±0.49	-	21.0±0.18	18.0±0.5	15.5±1.3	12.5±0.8
Bacillus cereus (Hb21)	27.00±0.00	-	20.0±0.18	18.5±0.7	17.0±0.5	14.0±0.6
Paenibacillus sp. BF38(Bb26)	24.16±0.23	-	19.5±0.44	19.0±1.6	18.0±0.5	14.5±1.2
Paenibacillus sp.L32	25.23±0.32	-	21.0±0.00	18.5±1.0	17.0±1.2	16.5±0.5
Terribacillus sp. 3LF(Xb10)	28.66±0.47	-	21.0±00.00	19.0± 0.7	16.0±1.0	14.5±0.6

Data were measured in mm and presented as Mean ± SD of triplicate. (-) denotes no zone of inhibition.

In comparative study, the highest zone of inhibition to *Bacillus cereus* for ethyl acetate, ethanol and chloroform were 23.5±0.70, 20.0±0.44 and 20.0±0.18 respectively and the lowest zone of inhibition to *Terribacillus sp*. 3LF for ethyl acetate, ethanol and chloroform were 21.0±0.00, 21±0.18 and 21.0±00.00.

Minimum Inhibitory Concentration (MIC)

Minimum inhibitory concentration (MIC) was observed with almost all of the bacterial strains against different extracts (Table 5).

Table 5: Comparison of MIC of ethanol and ethyl acetate extract of C. *grandis* leafs.

Bacterial strains	Ethanol extract (µg/10µl)	Ethyl Acetate extract(µg/10µl)
Bacillus megaterium (Hb42	62.5	250
Bacillus simplex (Xb17)	62.5	62.5
Paenibacillus sp. BF38(Bb26)	62.5	62.5
Terribacillus sp. 3LF(Xb10)	62.5	250

DISCUSSION

Through the identification of ribosomal RNA as a premier molecule, the evaluation of evolutionary relationships and the application of molecular techniques to microbial systematics have revolutionized the concept of phylogenetic relationships among bacteria [16, 17]. Moreover, the application of this phylogenetic knowledge to microbial ecology has contributed enormously to microbial diversity studies. Prior to the era of phylogenetic revolution and the development of culture-independent molecular approaches, actual estimation of diversity of microbial communities could not be made [18]. Such studies are becoming common and many bacteria have been described based on their 16S rRNA (rDNA) gene sequences.

The goal of this study was to evaluate the potential of 16S rRNA sequencing to rapidly identify *Bacillus* in cultures. Depending on the basis of 16S rDNA sequencing, we found six different *Bacillus sp.* having almost 100% sequence similarities for *Bacillus megaterium, Bacillus simplex, Bacillus cereus, Paenibacillus sp.* BF38, *Paenibacillus sp*.L32, and *Terribacillus sp*. 3LF. Strains having <4% difference between their sequence 16S rRNA genes of database were considered as the same species.

In the present work, *in vitro* studies concluded that the plant extract inhibited bacterial growth but their effectiveness varied. The antimicrobial activity has been attributed to the presence of some active constituents in the extracts. This antibacterial study of the plant extracts demonstrated that folk medicine can be as effective as modern medicine to combat pathogenic microorganisms. The millenarian use of these plants in folk medicine suggests that they represent an economic and safe alternative to treat infectious diseases [19]. These findings support the traditional knowledge of local users and it is a preliminary, scientific, validation for the use of these plants for antibacterial activity to promote proper conservation and sustainable use of such plant resources [20].

Antimicrobial activity of the extracts on the bacteria revealed inhibition of growth, though the susceptibility pattern to the extracts and the highest order of sensitivity of the *Bacillus sp.* was recorded as ethyl acetate, ethanol and chloroform accordingly. The growth inhibitory effect was concentration dependent [21]. This is important in considering dosage and rate at which the extract inhibits the growth of organism which means that the extracts were as effective as standard antibiotic used. The present study showed that ethyl acetate extract of the leaf of C. *grandis* generally has potent antimicrobial properties against the test organisms, especially different species of *Bacillus* in comparison to other two extracts.

CONCLUSION

Thus it can be concluded that, C. *grandis* (leaf) has got promising antibacterial effect in respect to different *Bacillus sp.* and the accordance of its effect of efficacy can be explained as ethyl acetate extract > ethanol extract > chloroform extract.

ACKNOWLEDGEMENT

We are thankful to all of the stuffs of the Department of Biotechnology & Genetic Engineering, Islamic University, Kushtia, Bangladesh.

REFERENCE

1. Balick M.J. and Cox P.A. Plants, People and Culture: the Science of Ethnobotany. Scientific American Library, New York 1996. p. 228.
2. Cotton C.M. Ethnobotany: Principle and Application. John Wiley and Sons, New York 1996. P. 399.
3. Sastri B.N. *The Wealth of India*, A Dictionary of Raw Material and Industrial Products, Publication and Information Directorate CSIR New Delhi 1950, 2 & 8: p. 257 & 285-293.
4. Nasir E. and Ali S.I. *Flora of West Pakistan*, Cucurbitaceae, No. 154, Botany Department, University of Karachi; 1973.
5. Abbasi A.M., Khan M.A., Ahmad M., Zafar M., Khan H., Muhammad N., Sultana S. Medicinal plants used for the treatment of jaundice and hepatitis based on socio-economic documentation. Afr. J. Biotechnol 2009; 1643-1650.
6. Morshed M. Alam, Haque Anwarul, Rokeya Begum, Ali Liaquat Anti-hyperglycemic and lipid lowering effect of *Terminalia arjuna* Bark extract on Streptozotocin induced Type-2 Diabetic Model Rats. Int J Pharm Pharm Sci 2011; 3(4): 449-453.
7. Neelavathi P., Venkatalaksmi P., Brindha P. Antibacterial activities of aqueous and ethanolic extracts of *Terminalia catappa* leaves and bark against some pathogenic bacteria. Int J Pharm Pharm Sci 2013; 5(1): 114-120.
8. Evans W.C., Trease, Evans Pharmacognosy. 13th Edn. E.LBS with Bailliere Tindall, 1989. p. 388 - 546.
9. Sivaraj A., Jenifa B.P., Kavitha M., Inbasekar P., Senthilkumar B., Panneerselvam A. Antibacterial activity of *Coccinia grandis* leaf extract on selective bacterial strains. J App Pharm Sci 2011; 1:120-123.
10. Bhattacharya B., Samanta M., Pal P., Chakraborty S., Samanta A. In vitro evaluation of antifungal and antibacterial activities of the palnt Coccinia grandis (L.) Voigt. (Family - Cucurbitaceae). J Phytology 2010; 2:52-57.
11. NCCLS Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically; approved standard—Fifth edition. NCCLS document M7-A5. USA, 2000. p. 1887-1898.
12. Birnboim H C & Doly J. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucl. Acids Res 1979; 7:1513-1523.
13. Akel H. and Al-Quadan F. Phenotypic and Genotypic Characterization of Three Novel Halophilic *Bacillus* Strains from Jordanian Hot Springs. Jordan J Biol Sci 2008; 1:19-25.
14. Bauer A.W., Kirby W.M. M., Sherries J.C., Tuck M. Antibiotic susceptibility testing by a standardized disc diffusion method. American J Clin Path 1966; 45:493-496.
15. Thongson C., Davidson P.M., Mahakarrchanakul W., Weiss J. Antimicrobial activity of ultrasound – assisted solvent – extracted spices. Lett App Micr 2004; 39: 401-406.
16. Woese CR Bacterial evolution. Microbiol. Rev 1987; 51:221-271.
17. Olsen GJ, Woese CR and Overbeek R. The winds of (evolutionary) change: breathing new life into microbiology. J. Bacteriol 1994; 176:1-6.
18. Amann RI, Ludwig W and Schleifer KH Phylogenetic identification and *in situ* detection of individual microbial cells without cultivation. Microbiol. Rev 1995; 59:143-169.
19. Toama M.A., El-Alfy T.S., El-Fatatry H.M. Antimicrobial activity of the volatile oil of *Nigella sativa* Linneaus seeds. Antimicrobial Agents and Chemotherapy 1974; 6:225-226.
20. Li X.Z., Livermore D.M., Nikaido H. Role of efflux pump(s) in intrinsic resistance of *Pseudomonas aeruginosa*: resistance to tetracycline, chloramphenicol and norfloxacin. Antimicrobial Agents and Chemotherapy 1994; 38:1732-1741.
21. Achi OK Composition and antibacterial activities of *Tetrapleura tetraptera* Taub. pod extracts. Res J Micro 2006; 5:416-422.