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ISOLATION AND CHARACTERIZATION OF *ACTINOMYCETES* FROM SOIL AND EVALUATION OF ANTIBACTERIAL ACTIVITIES OF *ACTINOMYCETES* AGAINST PATHOGENS

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ABSTRACT: *Actinomycetes* are one of the most attractive sources of antibiotics. In the present studies, total of 15 strains were isolated from Dr. Ram Manohar Lohia Hospital and RML Park in Lucknow, U.P India. Isolated strains were identified for their antibacterial activity but only six isolate showed good result, they were evaluated for their inhibitory activity on 3 strains of microorganism (*E. coli, P. aeruginosa* and *S. aureus*). Isolation of *Actinomycetes* strain was obtained by serial dilution method and grown on actinomycetes isolation agar. Antibacterial compounds were produced by submerged fermentation and activity of compounds were checked against bacterial culture by antibiogram analysis where intracellular and extracellular compounds showed positive result, compare to intracellular compounds, extracellular compounds was showing best result which was 30 mm zone of inhibition against *S. aureus* and MIC was found to be 0.0009 mg/ml.

Key word: Antibacterial compounds, *Actinomycetes,* MIC, Antibiogram analysis.

INTRODUCTION

Actinomycetes are the most widely distributed group of microorganisms in nature which primarily inhabit the soil (**Oskay *et al*, 2004)**. They have provided many important bioactive compounds of high commercial value and continue to be routinely screened for new bioactive compounds. These searches have been remarkably successful and approximately two thirds of naturally occurring antibiotics, including many of medical importance, have been isolated from actinomycetes (**Okami *et al*. 1988**). Almost 80% of the world's antibiotics are known to come from *Actinomycetes*, mostly from the genera *Streptomyces* and *Micromonospora (***Pandey *et al*, 2004)**. Present time most of the disaeases caused by bacteria have become resistance to most of the antibiotic **(Alanis 2005).** *Staphylococcus aureus* is commonly known pathogen that is responsible for infections like pneumonia diabetes, cancer, vascular disease, and lung disease have developed resistance to most classes of antibiotics (**Enright 2003)**, Physicians acquired methicillin-resistant *S. aureus* (MRSA), which also bears resistance too many antibiotics. During this time, vancomycin has been the therapeutic answer to MRSA, Vancomycin resistant strains have emerged clinically (**Hiramatsu 1998, Bozdogan *et al*. 2003, Chang *et al*. 2003, Anonymous 2004)**.

Vancomycin-resistant *S. aureus* (VRSA) challenges clinicians, not only because of vancomycin and methicillin resistance, but also because of resistance to many other antibiotics, including aminoglycosides, macrolides, and fluoroquinolones.certain undesirable side effects and the spread of pathogens with this new antimicrobial drug resistance emphasize the need for the development of other newer antimicrobial agents with activity against such gram positive bacteria (**Jevitt *et al*. 2003, Meka and Gold 2004, Nathwani 2005)**. Also the other cause is gram negative antibiotic-resistant opportunistic pathogens. Gram negative environmental and enteric organisms currently threaten patients in hospitals and communities with multi-drug resistance, including broad resistance to first, second, and third generations of penicillin's and cephalosporin's (**Urban *et al*. 2003, Obritsch *et al*. 2004, Paterson *et al*. 2004)**. These bacteria, like *Pseudomonas aeruginosa*, are common organisms which were found in environment, which act as opportunistic pathogens in clinical cases where the defense system of the patient is compromised (**Lyczak et al. 2000)**. Also other intrinsically antibiotic resistant organisms such as *Stenotrophomonas maltophilia* are emerging as opportunistic pathogens. The end result of this phenomenon is that many strains of bacteria have become resistant, and in many cases multi-resistant to these therapeutic agents, to overcome this problem a new antibiotic needed hich will show positive result **(Alanis 2005)**.

The present study is carried out by isolation of *Actinomycetes* culture and to check antibacterial activity of intracellular and extracellular compounds against different types of pathogens which were present in *Actinomycetes*, the pathogens were used *Staphylococcus aureus, Pseudomonas aeruginosa* and *E. coli.*

METHODOLOGY

Materials and methods

Actinomycetes used in this study were isolated from Dr. Ram Manohar Lohia Hospital (RMLH) and RML Park in Lucknow, U.P India. *Actinomycetes* were isolated by spread plate technique on actinomycetes isolation agar. After serial dilution method, colonies of *Actinomycetes* were selected for screening. Isolated colonies were preserved on actinomycetes isolation agar (Glycerol based media) and stored at -20°C. The sub culturing of *Actinomycetes* was done by streaking method on actinomycetes isolation agar and incubated at 28°C for 4-5 days.

Screening of *Actinomycetes* for Antimicrobial activity:

The screening process were completed by two methods-

Primary and Secondary Screening:

In primary screening the antimicrobial activity of pure isolates were determined by perpendicular streak method on actinomycetes isolation agar. The test organisms used were; *Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas aeuginosa*. Secondary screening was performed by agar well diffusion method against the standard test organisms.

Characterization of Actinomycetes:

The *Actinomycetes* selected after secondary screening, were characterized by morphological and biochemical methods. Morphological characterizations were done by microscopic method. The microscopic characterization was done by cover slip culture method **(Kawato and Sinobu, 1979)**.

The mycelium structure, color and arrangement of conidiospore and arthrospore on the mycelium were observed through the oil immersion (100X). The observed structure was compared with **Bergey's Manual of Determinative Bacteriology, Ninth edition (2000)** and the organism was identified. Various biochemical tests performed for the identification of the potent isolates are as follows: casein hydrolysis, starch hydrolysis, urea hydrolysis, esculin hydrolysis, acid production from sugar, NaCl resistance, temperature tolerance.

Fermentation Process:

Fermentation was carried out in a 1L Erlenmeyer flask by following the procedure as described by **Liu *et al* (2011).**

Extraction of Intracellular and Extracellular compounds:

The intracellular compounds were extracted by using solvent as methanol and extracellular compounds were extracted by using solvent as chloroform.

Extraction of intracellular compounds:

Dried pellets were taken and dissolved in 0.5 ml of methanol and incubate at 0 ^{0} C for 1 hour then spin at 10,000 rpm for 10 min, then take supernatant and air dried properly and dissolve in tris (pH- 8.0).

Extraction of extracellular compounds:

0.5 ml supernatant were taken and dissolved in 0.5ml of chloroform and mix properly for 1 hour then spin at 10,000 rpm for 10 min, then take bottom layer and air dried properly and dissolve in tris (pH- 8.0).

Tested microorganisms:

Bacterial cultures were obtained from IMTECH, Chandigarh. Subcultures were maintained by MRD LifeSciences, Lucknow. *Staphylococcus aureus* (MTCC 2940) a gram positive and *Pseudomons aeruginosa* (MTCC 2453), *Escheriachia coli* (MTCC 739), both gram negative were used.

Antibiogram analysis:

The assay was conducted by agar well diffusion method **(Perez *et al.*, 1990).** Spreading method was done by using test organisms *i.e.* one gram positive and two gram negative on nutrient agar plates. The suspension was used to inoculate 90 mm diameter petriplates. Wells were punched in triangular form and filled with 50µl distilled water, 50µl antibacterial components (Intracellular and extracellular). Plates were incubated at 37^{0}C for 24 hours, antibacterial activity was evaluated by measuring the zone of inhibition in diameter. Distilled water was taken as control, leave the plates over night at 37^0 C in incubator, after 24 hours antibacterial intracellular and extracellular compounds showed inhibition zone, but bacteria show fully growth in distilled water, the activity of *Actinomycetes* were analyzed by inhibition zones. This result shows that isolated *Actinomycetes* is antibiotic producer, then the isolates were chosen for further tests.

Minimum Inhibitory Concentration (MIC):

Quantitative assay was done by agar dilution method which was used to determine MIC of extract against test bacteria. The minimum inhibitory concentration is the minimum concentration of the antibacterial agent in a given culture medium below which bacterial growth is not inhibited. MIC provides an idea of effectiveness an active extract or compound against a microorganism.

MIC means the lowest concentration of extract at which the test microorganism did not show any visible growth was taken as its MIC. During study of MIC 3ml nutrient broth was taken in 6 test tubes, **(Kirby-Bauer method)**. Then added 1.0 ml antibacterial compound into first test tube and made a serial dilution, then 20µl of pathogen were added into each test tube & incubate at 120 rpm in shaker incubator for overnight. Next day least concentration of MIC test tubes was observed by taking the optical density (OD) at 600 nm by using the colorimeter.

Results:

Microbes from soil were isolated by serial dilution method and mixed culture was obtained by spreading as shown below-

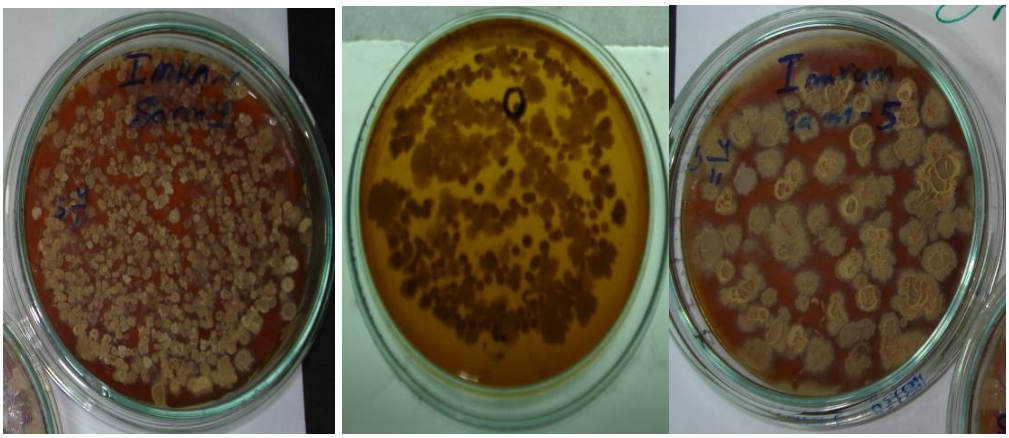
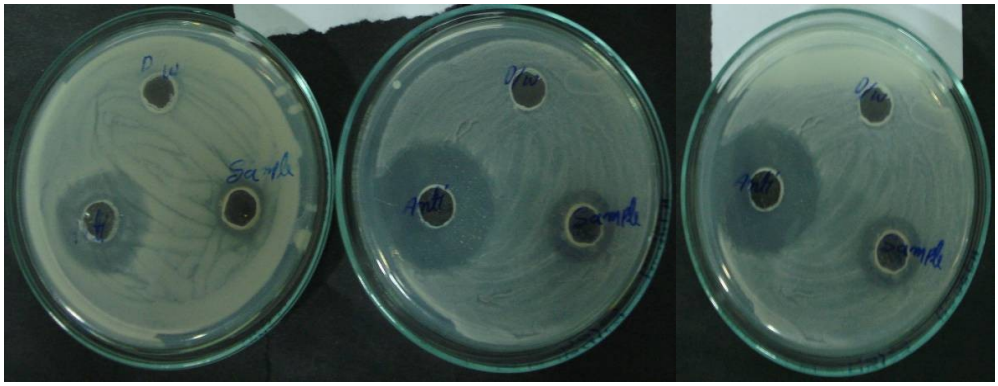


Figure-1 Mixed colony in spread plate (RMLH -Sample), 10^{-1}, 10^{-3}, 10^{-5}

Antibiotic sensitivity test (Antibiogram of purified culture):

Antibiogram of purified culture of (RMLH) was performed against various pathogens. There were zones of inhibition obtained in each culture which showed positive result against pathogens.



E. coli ***P. aeruginosa*** ***S. aureus***

Figure 2- Antibiogram analysis of culture (RMLH) against various pathogens

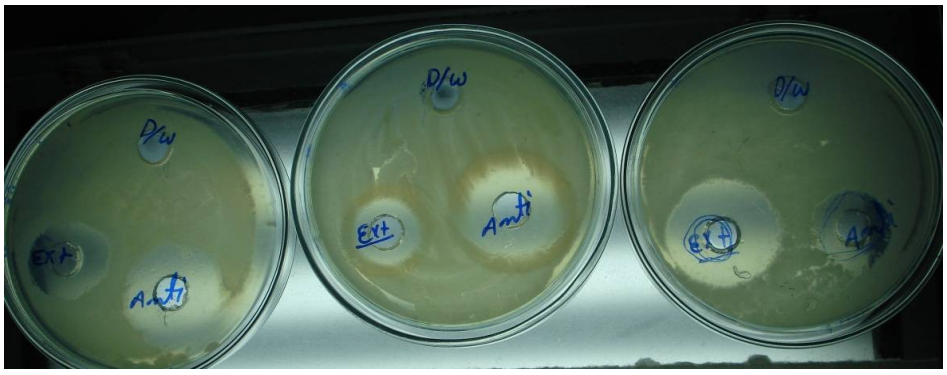
(Figure indicated that *Actinomycetes* were showing best result in the form of zone of inhibition against *P. aeruginosa* and *S. aureus*) as mentioned in table 1.

Table 1- Antibiogram of tetracycline, distilled water and RMLH sample isolate against various pathogens.

S.NO.	Pathogens	Tetracycline Inhibition zones (mm)	Distilled Water	RMLH sample Inhibition zones (mm)
1	*E. coli*	12	-	10
2	*P. aeruginosa*	16	-	11
3	*S. aureus*	16	-	12

(Table showed that maximum zone of inhibition was observed best in *S. aureus*)

Antibiogram analysis of extracellular compounds from RMLH samples were performed against various pathogens:



E .coli ***P. aeruginosa*** ***S. aureus***

Figure 3- Antibiogram analysis of extracellular antimicrobial compounds against various pathogens (RMLH)

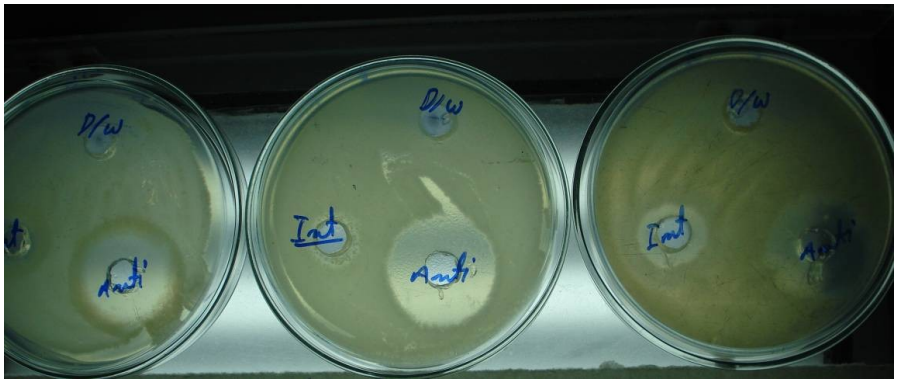
(Figure indicate that Actinomycetes extracellular compounds were showing best result in the form of zone of inhibition against *S. aureus* followed by *E. coli* and *P. aeruginosa***)** as mentioned in table 2.

Table 2:- Antibiogram of extracellular antimicrobial compounds against various pathogens (RMLH)

S.NO	Pathogens	Tetracycline Inhibition zones (mm)	Extracellular compounds Inhibition zones (mm)
1	*E. coli*	30	28
2	*P. aeruginosa*	30	28
3	*S. aureus*	18	30

(Table showed that maximum zone of inhibition was observed against *S. aureus*)

Antibiogram analysis of intracellular compounds from RMLH samples were performed against various pathogens:



E. coli *P. aeruginosa* *S. aureus*

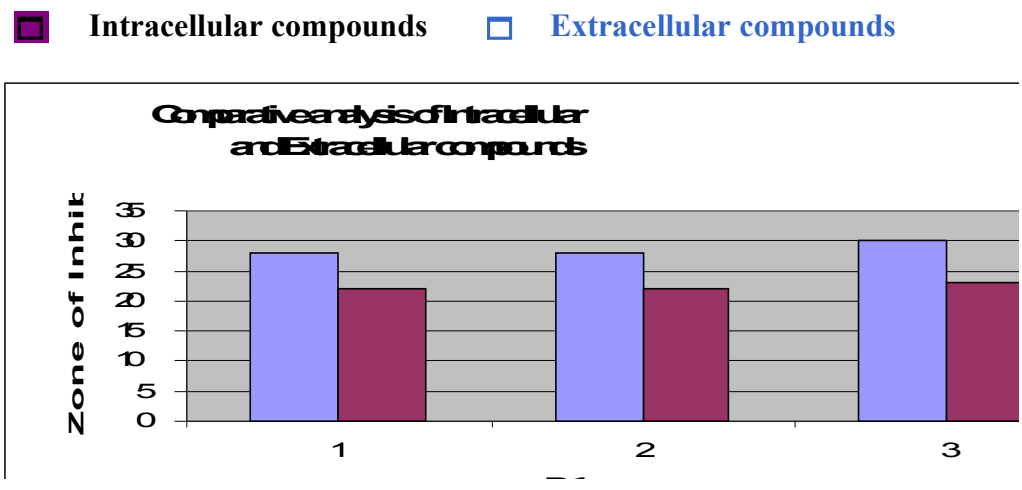
Figure 4- Antibiogram analysis of intracellular antimicrobial extract against various pathogens (RMLH)

(Figure indicate that *Actinomycetes* intracellular compounds were showing best result in the form of zone of inhibition against *S. aureus* followed by *E .coli* and *P. aeruginosa*) as mentioned in table 3.

Table 3:- Antibiogram of extracellular antimicrobial compounds against various pathogens (RMLH).

S.NO	Pathogens	Tetracycline Inhibition zones (mm)	Intracellular compounds Inhibition zones (mm)
1	*E. coli*	28	22
2	*P. aeruginosa*	28	22
3	*S. aureus*	28	23

(Table showed that maximum zone of inhibition was observed against *S. aureus)*



Pathogens: **1. *E. coli*** **2. *P. aeruginosa*** **3. *S. aureus***

(Chart showed that extracellular compounds were having maximum antibacterial activity against *S. aureus).*

MIC (Minimum Inhibitory Concentration) for extracellular and intracellular compounds:

Table 4:- MIC for extracellular compounds from RMLH

Test tube no	Concentration (mg/ml)	*P. aeruginosa* at 600nm (O.D.)	*E. coli* at 600nm (O.D.)	*S. aureus* at 600nm (O.D.)
T2	0.25	0.37	0.48	0.25
T1	0.06	0.20	0.52	0.30
T3	0.015	0.34	0.62	0.40
T4	0.0037	0.50	0.72	0.49
T5	**0.0009**	**0.67**	**0.41**	**0.23**
T6	0.00022	0.80	0.83	0.56

(Least conc. were obtained **0.06** mg/ml for *P. aeruginosa* and **0.0009** mg/ml for *E.coli* and *S. aureus*)

Table 5:- MIC for extracellular compounds from RMLH

Test tube no	Concentration (mg/ml)	*P. aeruginosa* at 600nm (O.D.)	*E. coli* at 600nm (O.D.)	*S. aureus* at 600nm (O.D.)
T2	0.25	0.30	0.32	0.19
T1	**0.06**	**0.17**	**0.31**	0.27
T3	0.015	0.42	0.40	0.28
T4	**0.0037**	0.52	0.54	**0.24**
T5	0.0009	0.70	0.62	0.32
T6	0.00022	0.78	0.73	0.47

(Least conc. were obtained **0.06** mg/ml for *P. aeruginosa* and *E. coli* and **0.0037** mg/ml for *S. aureus*)

Discussion

The putative isolates of primary screening when subjected to secondary screening, showed different activity from that of primary screening; some of the active isolates didn't show the activity in the secondary screening while some showed little activity and some showed improved activity. According to **Bushell (1997),** during the screening of the novel secondary metabolite, *Actinomycetes i*solates are often encountered which show antibiotic activity on agar but not in liquid culture. The result of primary and secondary screening revels that most of the active isolates were active against gram positive bacteria (*S. aureus*) than gram negative bacteria (*E. coli* and *P. aeruginosa*). The reason for different sensitivity between gram positive and gram negative bacteria could be explain to the morphological differences between these microorganisms, gram negative bacteria having an outer polysaccharide membrane carrying the structural lipopolysaccharide components. This makes the cell wall impermeable to lipophilic solutes, The gram positive should more susceptible having only an outer peptidoglycan layer which is not an effective permeability barrier **(Waksman,1961, Shirling and Gottlieb,1966**).

Although various biochemical tests were performed, to identify the *Actinomycetes* up to species level, for proper identification of genera and species of *Actinomycetes*, besides morphological and physiological properties, various other biochemical properties such as cell wall chemo type, whole-cell sugar pattern, peptidoglycan type, phospholipids type and G+C% of DNA should be determined. The minimum inhibitory concentration (MIC) for the intracellular antimicrobial extracted from *S. aureus* was 0.0009mg/ml and for extracellular antimicrobial extracted from *S. aureus* was 0.0037 mg/ml, this showed that the extracellular antimicrobial from *S. aureus* was more active than from intracellular antimicrobials.

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REFERENCES

Alanis AJ .**2005**. Resistance to antibiotics: are we in the post-antibiotic era? Archives of Medical Research **36**.697-705.

Anonymous.**2004**.Brief report: vancomycin-resistant *Staphylococcus aureus*-New York. MMWR **53**. 322-323.

Bauer AW., Kirby WM., Sheris JC., Turck M .**1966**. Antibiotic susceptibility testing by a standardized single disc method.Am J Clin Pathol. 45: 149-158.

Bergey's manual of determinative bacteriology.**2000**. Actinomycetales. 9th edition.

Bozdogan B., Esel D., Whitener C.**2003.** Antibacterial susceptibility of a vancomycin-resistant Staphylococcus aureus strain isolated at the Hershey Medical Center. Journal of Antimicrobial Chemotherapy 52. 864-868.

Bushell M. E., Smitht v and Lynchs H.C.,**1997**. A physiological model for the control of erythromycin production in batch and cyclic fed batch culture. Microbiology. 143.475-480.

Chang S., Sievert DM, Hageman JC. **2003**.Infection with vancomycin resistant *Staphylococcus aureus* containing the vanA resistance gene. The New England Journal of Medicine, **348**, 1342- 1347.

Enright MC.**2003.** The evolution of a resistant pathogen-the case of MRSA.*Current Opinion in Pharmacology* **3 (5)**, 474-479.

Hiramatsu K .**1998**. Vancomycin resistance in *Staphylococci*. Drug Resist Updates **1**.135-150.

Hugh R., Ryschenkow E. **1961**.*Pseudomonas maltophilia*, an alcaligenes-like species. Journal of General Microbiology **26**.123-132.

Jevitt LA., Smith AJ., Williams PP., Raney PM., McGowan JE., Tenover FC. **2003**.In vitro activities of daptomycin, linezolid, and quinupristin-dalfopristin against a challenge panel of *Staphylococci* and *Enterococci*, including vancomycin-intermediate *Staphylococcus aureus* and vancomycin- resistant *Enterococcus faecium*. *Microbial Drug Resistance* .**9**.389-393.

Kawato, M., Shinobu., R.**1959.** A simple technique for the microscopical observation, memoirs of the Osaka University Liberal Arts and Education.114.

Liu.QG.,Ren.PG., Wang.LX., Zhao.Y.**2011**.Statistical optimization of key medium components by response surface methodology to promote ganoderic acid formation by medicinal mushroom Ganoderma sinense in submerged culture. Journal of Medicinal Plant research .**5(3).** 425-431.

Lyczak JB., Cannon CL., Pier GB.**2000**. Establishment of *Pseudomonas aeruginosa* infection: lessons from a versatile opportunist. Microbes and Infection **2,(9).** 1051-1060.
Meka VG., Gold HS .**2004**. Antimicrobial resistance to linezolid. Clinical Infectious Diseases **39**. 1010-1015.
Nathwani D.**2005.** Tigecycline: clinical evidence and formulary positioning.*International Journal of Antimicrobial Agents* .**25**.185-192.
Obritsch MD., Fish DN., MacLaren R., Jung R.**2004**. National surveillance of antimicrobial resistance in *Pseudomonas aeruginosa* isolates obtained from Intensive Care Unit Patients from 1993 to 2002. *Antimicrobial Agents and Chemotherapy* .**48**. 4606-4610.
Okami Y and Hotta K.**1988** editors, Goodfellow M.,Williams S.T. and Mordarski M. *Academic Press Inc, New York*, 33-67.
Oskay M., Tamer A.U. and Azeri C.**2004**.*African J Biotechnol* .**3 (9).**441- 446.
Pandey B., Ghimire P. and Agrawal V.P. **2004**. *International Conference on the Great Himalayas: Climate, Health, Ecology, Management and Conservation, Kathmandu, Organized by Kathmandu University and the Aquatic Ecosystem Health and Management Society, Canada*.
Paterson DL., Ko WC., Von Gottberg A., Mohapatra S., Casellas JM., Goessens H., Mulazimoglu L., Trenholme G., Klugma KP., Bonomo RA., Rice LB., Wagener MM., McCormack JG., Yu V.**2004.**
Perez C., Pauli M., Bazerque P.1990. An antibiotic assay by the agar-well diffusion method. Acta Biol Med Exper **15:** *113-115.*
*Shirling E B .,Gottlieb D .1966. Methods,Classifications , Identfications and sescription of of genera and species .The William and Wilkin copmpany, Baltimoe***.2**.61-292.
Urban C., Segal-Maurer S., Rahal JJ. **2003**.Considerations in control and treatment of nosocomial infections due to multi-drug resistant *Acinetobacter baumannii*. Clinical Infectious Diseases **36**.1268-1274.