SCREENING AND CHARACTERIZATION OF ANTIBIOTIC PRODUCERS FROM SOIL AND HOT WATER SPRINGS

[1]I.Shivani, [1]K.Gupta, [1]M.Arya, [1]S.Rai, [2]R.Rane
Department of Biotechnology,
Sir Sitaram& Lady ShantabaiPatkar College of Arts & Science and V. P. Varde College Commerce & Economics, Mumbai – 400 104, India

Abstract: Antibiotic is one of the most important commercially exploited secondary metabolites produced by bacteria and fungi and employed in a wide range. With the increase in antibiotic resistance in microbial population the need for new antibiotics is increasing day by day. Extreme habitats are increasingly being recognized as sources of secondary metabolites which provide an encouraging source for development of novel natural pharmaceuticals. The present study is focused on screening of samples collected from under-explored ecological niches like marine environments &rhizosphere of plants. Screening of bacteria with potential antibiotic activity was carried out using Wilkins overlay technique. The isolates were identified based on their morphology & further confirmed through biochemical tests. Antibiotic producing ability was confirmed by Antimicrobial Susceptibility Testing (AST). A total of 11 isolates belonging to different genera were found. The production and extraction of antibiotics were done using synthetic fermentation broth method and methanol extraction method. The Minimal Inhibition Concentration (MIC) was determined by agar well diffusion method. UV spectrophotometric analysis was carried out to determine the class of antibiotics. Present research suggests that soil & hot water spring isolates having antibiotic producing capability can be further employed for industrial production of antibiotics after proper standardization.

Index Terms*:*Screening, Biochemical tests, Antimicrobial Susceptibility Testing (AST), Minimal Inhibition Concentration (MIC), UV spectrophotometry.

I. INTRODUCTION

Antibiotics are chemical substances that can inhibit the growth of or even destroy harmful microorganism.However, the over-prescription and misuse of antibiotics resulted in the development of resistant strains of various pathogens. Thus, extensive efforts have been carried out by many scientists in order to screen novel antibiotic producers. Through their efforts, many antibiotics have been discovered successfully that act against pathogens that cause diseases. But, the emergence of new diseases and re-emergence of multiple-antibiotic resistance pathogens have given rise to the need for the discovery of new antibiotics (Lihan*et al*, 2014).

Antibiotics can be classified according to their mode of actions. Antibiotics are classified as broad-spectrum antibiotics when they have the ability to affect a wide range of gram-positive and gram-negative bacteria while antibiotics that only effective towards certain group of bacteria are known as narrow-spectrum antibiotics (Samuel Lihan*et al*, 2014). Antibiotics that are currently of greatest use have been derived from a relatively small group of bacteria belonging to the genera *Streptomyces*, *Micromonospora* and *Bacillus* (Sethi*et al*, 2013).

It is feasible to screen antibiotic producing bacteria as they are very easy to isolate, culture and maintain (Lihan*et al*, 2014, Abdulkadir *et al*, 2012) and some ecosystems such as microbial plant endophytes, plant rhizospheres, microbial insect symbionts, lichens and most marine environments including hot water springs and thermal vents can be explored for presence of antibiotic producers (Nordenfjäll, 2014).However, soil and hot water springs, which are naturally occurring loose mixture of mineral and organic particles, still remains the most important target for most researchers in their efforts to discover novel antibiotics which have pharmaceutical values. This is because many microbes especially bacteria that are present in soil and hot water springs have the ability to produce biologically active secondary metabolites such as useful antibiotics. In their natural habitats, bacteria utilize the antibiotics they produce as protective substances by turning the invasion of other bacterial species ineffective (Lihan*et al*, 2014, Bavishi*et al*, 2017). Thus, according to Linares *et al*, 2006, protection is not the only function of antibiotics, they also act as signalling molecules that bacteria use as a means of communication between cells.

The present investigation has undertaken with an effort to isolate antibiotic producers from rhizosphere of medicinal plant and hot water springsfrom Mumbai, India and to optimise production, extraction and characterisation of antibiotic.

II. MATERIAL AND METHODS

Collection of sample:

The soil sample was collected from rhizosphere of various medicinal plants like Neem, Tulsi, Insulin plant, Kapur, Bhang and Seeta-ashoka from Kandivali and water sample was collected from hot spring water from 4 kunds of Vajreshwari temple in vasai, Mumbai, India.

Isolation and screening of antibiotic producers:
Isolation and screening of antibiotic producing bacteria was done using crowded plate technique and the identification of antibiotic producing colonies was done by Wilkins overlay method. Test culturesused were *S.aureus* and *E.coli*(Bavishi*et al*, 2017).

Characterization of Pure Antibiotic Producing Bacterial Isolates:
Macroscopic and microscopic characteristics of bacterial isolates were studied (Torome, 2011,Abdulkadir*et al*, 2012) and the identification of bacterial isolates wasdone based on Bergey's Manual of Determinative Bacteriology.

Production and Extraction of Secondary Metabolites from Pure Isolates:
Production and extraction of secondary metabolites was carried out using two different methods. Agar plates containingpure antibiotic producer were dried in hot air oven at 40°Cand then grinded before immersionin absolute methanol. After 4 days of immersion, the solvent was filtered and then concentrated by evaporation at room temperature. The collected crude methanol extracts were kept in refrigerator for further usage (Samuel Lihan*et al*, 2014).
The second method involved the enrichment of pure Antibiotic producer in tryptic soy broth medium and then transferred in synthetic fermentation broth medium. After inoculation, medium was kept at shaking condition for 96hr and then bymedium centrifugation supernatant containing crude antibiotic was separated and stored for further analysis (Sethi*et al*, 2013).

Antimicrobial Susceptibility Testing (AST):
AST was carried out via agar well diffusion method on Muller Hinton agar medium seeded with test culture for the antibiotics extracted by Methanol extraction and Synthetic Fermentation broth extraction. Test cultures used were *S.aureus, E.coli, B.subtilis and S.typhi* (Sethi*et al*, 2013, Abdulkadir*et al*, 2012).

Determination of Minimum Inhibitory Concentration (MIC) of extracted antibiotic:
Determination of MIC for crude extracted antibiotic was carried by agar well diffusion method. The antibiotic concentrations used were in the range of 20-70mg/ml of the antibiotic extract that exhibited superior activity against the test organisms(Lihan*et al*, 2014).

UV spectrophotometric analysis:
All the methanolic extracts were scanned at UV-Visible spectrum to determine theclass of antibiotics (Severino*et al* 2015, Balyejjusaa*et al* 2002, Abdulghani*et al* 2013, Herriott 1946, Krzek*et al* 2011, Hadi 2014, Tilinca*e.et al*, 2017). Standard antibiotics belonging to different classes were used as standards.

III. RESULTS

Isolation and screening of antibiotic producers:
Soil samples from rhizosphereof medicinal plant and water sample from hot water spring were chosen as samples for analysis in this study as these native microorganisms have higher probability to produce novel antimicrobial substances. Total of 11 bacterial isolates from different soil and water samples were isolated via preliminary screening and selection. The first seven isolates were obtained from rhizospheres of plants like Tulsi, Bhang and Insulin and were numbered 1-7 respectively. Four isolates were obtained from hot water springs and were numbered 8-11 respectively. All bacterial isolates were subjected to secondary screening (Figure 1).

Characterization of Pure Antibiotic Producing Bacterial Isolates:
Gram nature of pure antibiotic producing isolates was determined and 4 isolates were found to be Gram-negative and 7 isolates were found to be Gram-positive.Various Biochemical tests (Table 1) were performed to classify bacterial isolates and based on Bergey's manual isolateswere classified under the Genus*Micromonospora*(Thawai*et al*, 2018),*Actinomycetes* (Salim*et al*, 2017), *Pseudomonas*(Darabpour*et al*, 2010), *Bacillus* (Kuta*et al*, 2009).

Extraction of antibiotic and Antimicrobial Susceptibility Testing (AST):
Antibiotic extraction was carried out using methanol extraction and fermentation broth method. AST of methanol crude extracts and broth extract, obtained from all isolates, was analyzed using Agar well diffusion method. Crude extracted antibiotic exhibited the ability to inhibit the growth of the test organism used in this study. This result was in parallel with the result obtained during secondary screening, where the isolate exhibited strong anti-bacterial activities. Extract from these isolates were active against *S.aureus*, *B.subtilis*(Figure 2) (Gram-positive bacterium) and *E.coli*, *S.typhi*(Gram-negative bacterium) whereby the size of inhibition zone was approximatelysame. If compared between Gram-positive and Gram-negative, it gives the better result against Gram-positive organisms. All the antibiotics extracted from methanol extraction method were found to be active and efficient in inhibiting the growth of test organisms (Graph 1, Graph 2). Hence, methanol extraction method was preferred to be used for MIC determination as it is also possible to obtain crystals of antibiotic in this method that can be used to prepare different concentrations.

Minimum Inhibitory Concentration (MIC) of extracted antibiotic:
Minimum inhibitory concentration of antibiotic extracts were estimated against test organisms *S.aureus*, *B. subtilis*(Gram-positive bacterium) and *E. coli*, *S. typhi*(Gram-negative bacterium), in the range from 20mg/ml – 70mg/ml. Crude antibiotic extracted from isolates 3 and 4 were selected for MIC determination as they showed superior activity and effect against test organisms during AST. Antibiotic extract from isolate 3 and 4(Figure 3) showed inhibitory activity against *S.aureus*and*B. subtilis*(in case of isolate 4)even in low concentrations while its MIC values is 60mg/ml as it was seen to be inhibiting all the test organisms at this concentration (Graph 3, Graph 4). Extracted antibiotics show much more efficient activity against Gram-positive organisms at lower concentrations than Gram-negative organisms, but can inhibit Gram-negative organisms at higher concentrations.

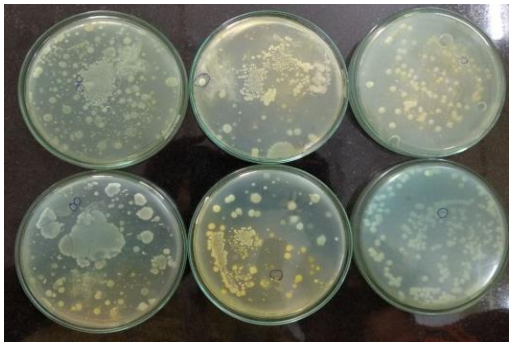


Figure 1. Screening of Antibiotic producers from soil and water sample

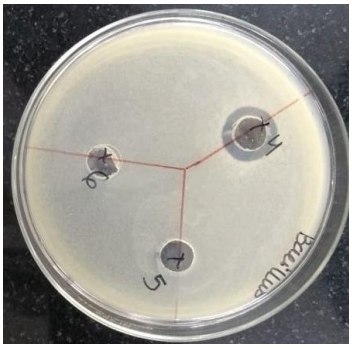


Figure 2. AST of crude methanolic extract

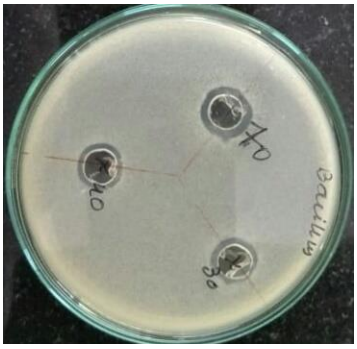


Figure 3. MIC determination of crude methanolic extract

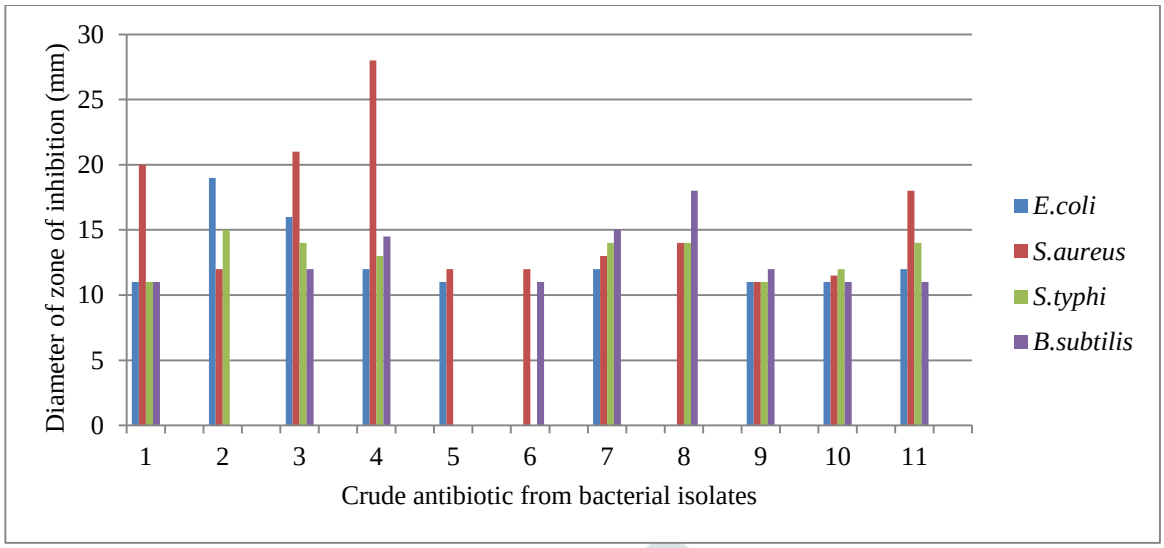
Table 1. Biochemical analysis of isolated antibiotic producers

Test\Sample no.	1	2	3	4	5	6	7	8	9	10	11
Sugar fermentation											
Glucose	+	+	+	-	+	+	+	+	+	-	-
Maltose	+	+	+	-	+	+	+	+	+	-	-
Sucrose	+	+	-	-	+	+	+	+	+	+	-
Lactose	+	+	-	-	+	+	+	+	+	+	-
Mannitol	+	+	-	-	+	+	+	+	+	-	-
Xylose	+	-	-	-	+	+	+	+	+	-	-
Triple Sugar Iron test	NA	NA		NA	NA				NA	NA	NA
Butt			Acd			Acd	Alk	Acd			
Slant			Alk			Alk	Alk	Acd			
Gas			-			-	-	-			
H_2S			-			-	-	-			
IMViC Test	NA	NA		NA	NA				NA	NA	NA
Indole			-			-	-	-			
Methyl Red			+			+	+	+			
Vogus Proskauer			-			-	-	-			
Citrate			-			-	-	+			
Urease	-	-	-	-	-	-	-	-	-	-	-
Catalase	+	-	-	+	+	+	+	+	+	-	+
Oxidase	+	-	+	-	+	+	+	-	+	-	+
Motility	-	-	-	-	-	+	-	-	-	-	-
Gram Nature	+	+	-	+	+	-	-	-	+	+	+

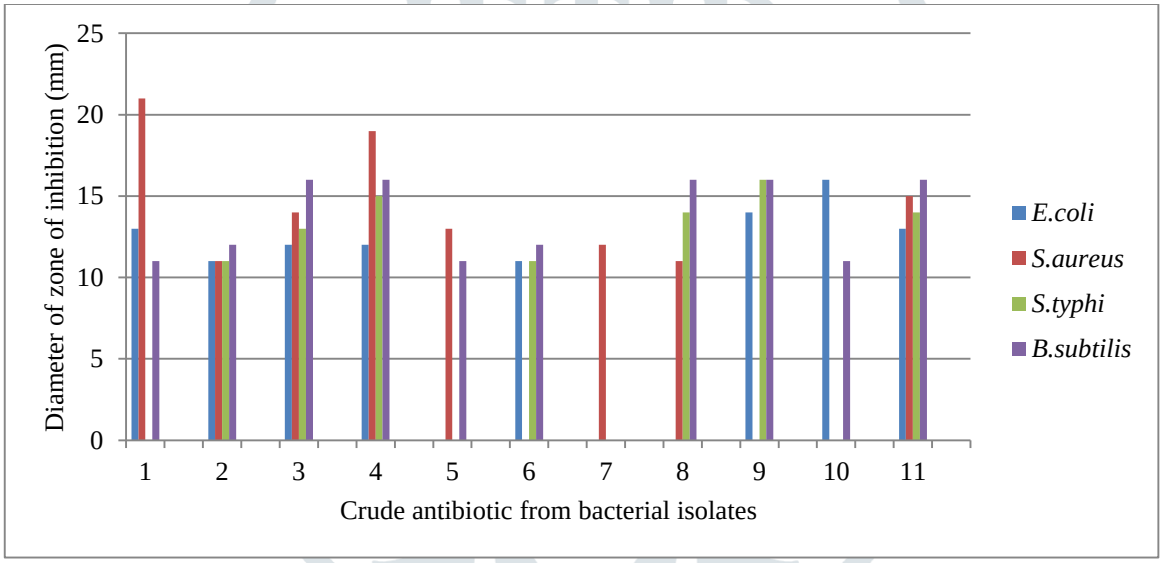
Key: +=Positive, - = Negative, Acd = Acidic, Alk = Alkaline

UV spectrophotometric analysis:

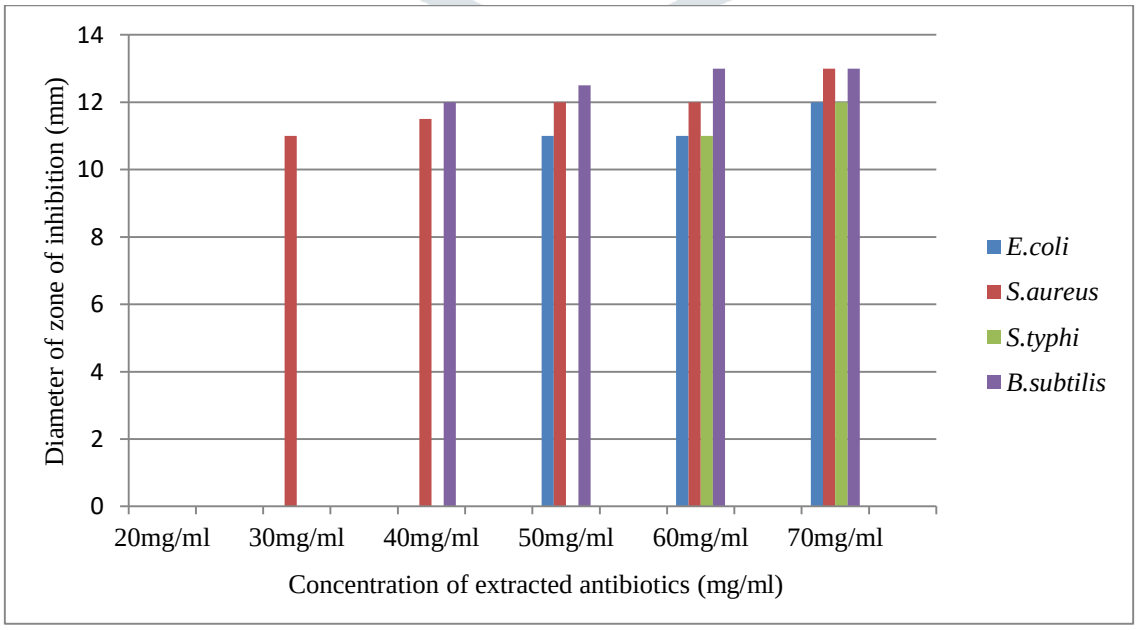
Extracted antibiotics were analyzed using UV Spectrophotometer for determination their class. On comparison of λmax of extracted antibiotic with that of standard antibiotic, antibiotics were found to be belonging to Anti-mycobacterial and Antimetabolite class.



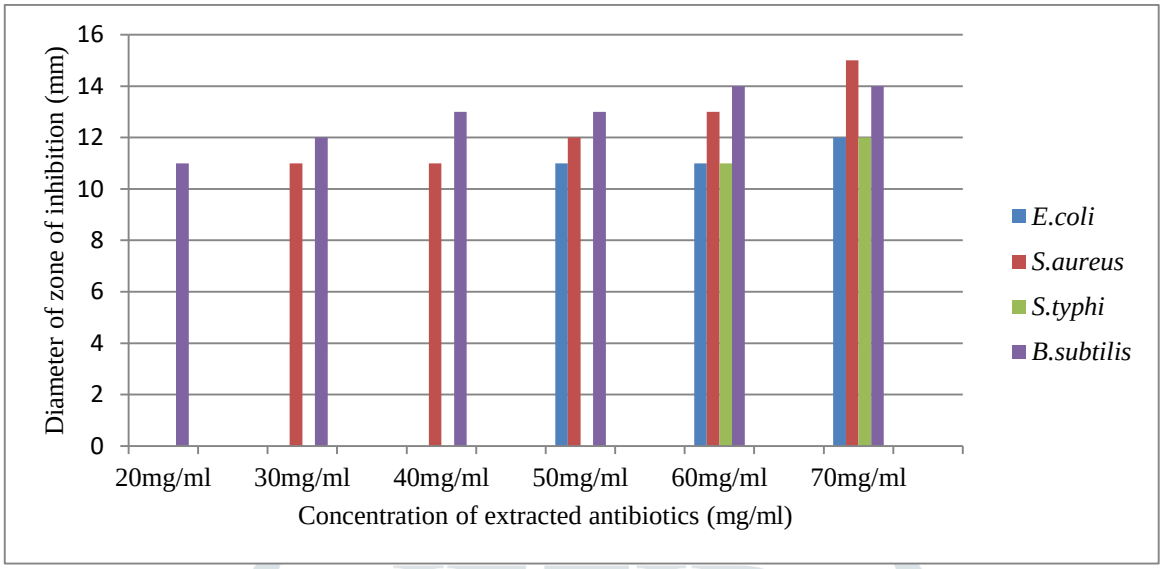
Graph 1. AST of Methanolic extract against Gram positive and Gram negative test organisms



Graph 2. AST of antibiotic from fermentation broth against Gram positive and Gram negative test organisms



Graph 3. MIC determination of crude methanolic extract from isolate 3



Graph4. MIC determination of crude methanolic extract from isolate 4

IV. CONCLUSION:

Soil samples are commonly evaluated for isolation of the antibiotic producing organisms, since soil microorganisms produce diverse antibiotics as they have to compete against each other for their nutrition, space and survival. The aim of this study isto isolate microorganisms from different ecological niches like soil from Rhizospheres of Medicinal plants – Tulsi, Neem and Insulin plant, hot water springs.

The antimicrobial potential of crude extracts of isolated micro-organisms from hot water springs and rhizosphere of plants that act against selected test organisms – *E.coli, S.aureus, S.typhi, B.subtilis*is demonstrated in this work based on results from AST assays. The extracts showed antimicrobial activity and are likely to be potential candidates for discovery of novel secondary metabolites for application as antibiotics. Importantly, this study shows that extreme habitats may harbour antibiotic producing organisms which could be useful as sources of antimicrobial compounds especially in this era of increasing antimicrobial resistance of microbes to the existing antibiotics. The MIC determination of the extracted antibiotics that exhibited superior activity against test organisms during AST analysis (sample 3 and sample 4) was carried out which showed 20mg/ml concentration of antibiotic can effectively inhibit growth of Gram positive and Gram negative organisms.

Purification of the extracted antibiotics can be done using various chromatographic techniques as well as counter current extraction methods as solventsolventextraction needs to be optimized separately for each antibiotic extract.

UV spectrophotometric analysis of the extracted antibiotics was carried out to comment on the class of antibiotics.Antibiotic extracted from isolate 1, 3, 4, 6 and 7 showed lambda max nearby the values of standard antibiotics. The other antibiotics can be purified further for determination of its class by UV spectrophotometric analysis. The antibiotic extracted from isolate 1 may belong to class of Antimetabolites and 3, 4, 6 and 7 may belong to class antimycobacterials.

Although the *in vitro* evaluation of the extracts for antibacterial activity may not give any correlation with *in vivo* assays, they may aid in screening studies to provide positive antagonists for further testing by in vivo assays. Furthermore, the eleven isolates that were active on all the test organisms could potentially be developed as producers of novel metabolites.

REFERENCES:

1. Abdulghani A, Jasim H, Hassan A (2013)Determination of Tetracycline in Pharmaceutical Preparation by Molecular and Atomic Absorption Spectrophotometry and High Performance Liquid Chromatography via Complex Formation with Au(III) and Hg(II) Ions in Solutions, International Journal of Analytical Chemistry, 1 – 11
2. Abdulkadir M and Waliyu S (2012), Screening and Isolation of the Soil Bacteria for Ability to Produce Antibiotics, European Journal of Applied Sciences 4 (5): 211 – 215
3. Balyejjusaa S, Adome R, Musoke D (2002), Spectrophotometric determination of sulphamethoxazole and trimethoprim (co-trimoxazole) in binary mixtures and in tablets, African Health Sciences, 2(2): 56–62.
4. Bavishi A, Dalal R, Singh S (2017), Screening, isolation and identification of antibiotics producing microorganisms from potential soil samples of Western regions of Mumbai, Journal of Global Biosciences, 6(6): 5070 – 5076
5. Darabpour E,Ardakani M, Motamedi H, Ghezelbash G, RonaghM(2010), Isolation of an antibiotic producer *Pseudomonas*sp. from the Persian Gulf, Asian Pacific Journal of Tropical Medicine, 318 – 321
6. Hadi H (2014), Spectrophotometric Determination of Vancomycin Hydrochloride in Pharmaceutical Preparations through Diazotization and Coupling Reactions, Iraqi Journal of Science, 55(4B):1684 – 1693

7. Herriott M (1946) A spectrophotometric method for the determination of penicillin, Journal of Biological Chemistry, 164:725 – 736
8. Krzek J and Piotrowska J (2011) Spectrophotometric determination of bacitracin in bulk drug as dabsyl derivative in a range of visible light, ActaPoloniaePharmaceutica – Drug Research, 68(6): 853 – 858
9. Kuta F, Nimzing L, Orka'a P (2009), Screening of *Bacillus* Species with Potentials of Antibiotics Production, Applied Medical Informatics, 24(1-2): 42 – 46
10. Lihan S, Lin C, Ahmad I, Sinang F, Hua N, SallehinA(2014), Antimicrobial producing microbes isolated from soil samples, European Journal of Experimental Biology, 4(1):494 – 501
11. Linares J, Gustafsson I, Baquero§ F, Martinez J (2006) Proceedings of the National Academy of Sciences, 103(51): 19484 – 19489
12. Nordenfjäll E (2014), Isolation of antibiotic producing microorganisms by screening for antibiotic resistance, Faculty of Natural Resources and Agricultural Sciences, 7
13. Salim F, Sharmili S, Anbumalarmathi J, Umamaheshwari K (2017), Isolation, Molecular Characterization and Identification of Antibiotic Producing *Actinomycetes*from Soil Samples, Journal of Applied Pharmaceutical Science, 7(09): 069 – 075
14. Sethi S, Kumar R, Gupta S(2013), Antibiotic Production by microbes isolated from soil, International Journal of pharmaceutical sciences and research (IJPSR),4(8): 2967 – 2973
15. Severino P, Silveira E, Vazzana M, Chaud M, Santana M, Souto E (2015), Validation of an UV spectrophotometric assay for the quantification of polymyxin B in solid lipid nanoparticles,Pharmazie, 70(11)
16. Thawai C, Kanchanasin P, Ohkuma M, Kudo T, Tanasupawat S (2018), Identification and antimicrobial activity of *Micromonospora* strains from Thai peat swamp forest soils, Journal of Applied Pharmaceutical Science,8(04): 119 – 125
17. TilincaM, Hancu G, Mircia E, Iriminescu D, Rosu A, Vlad R, BarabasE(2017), Simultaneous determination of isoniazid and rifampicin by uv spectrophotometry, Farmacia, 65(2): 219 – 224
18. Torome T (2011), Isolation and characterization of antibiotic producing thermophilic*Bacillus* in selected hotspringsalong lake bogoria, Kenya, A thesis submitted in partial fulfilment of requirements for the award of the degree of master of science (biotechnology) of Kenyatta university, I56/CTY/PT/24751/2011
19. Yunus F, Khalid Z, Rashid F, Ashraf A, Iqbal M, Hussain F (2016),Isolation and Screening of Antibiotic producing Bacteria from Soil in Lahore City, PSM Microbiology, 1(1): 1 – 4