Journal of Pharmacognosy and Phytochemistry

Available online at www.phytojournal.com

E-ISSN: 2278-4136
P-ISSN: 2349-8234
JPP 2018; 7(2): 1756-1760
Received: 07-01-2018
Accepted: 08-02-2018

Ashish Malik
Department of Botany, Maharshi Dayanand University, Rohtak, Haryana, India

Suman Lata
Department of Chemistry, P.I.G Government College for Women, Jind, Haryana, India

Preeti Jain
Department of Biochemistry, Maharshi Dayanand University, Rohtak, Haryana, India

Correspondence
Preeti Jain
Department of Biochemistry, Maharshi Dayanand University, Rohtak, Haryana, India

Antibacterial activity of *Alternethera Punguns* plant extracts against some gram negative and gram positive bacterial strain

Ashish Malik, Suman Lata and Preeti Jain

Abstract
Bacterial infections treatment always remained a challenge due to development of resistance in bacterial species. Medicinal plants contain a wide range of diverse molecules that provide a platform to search novel anti-bacterial agents. *Alternethera punguns* plant material was sequentially extracted with five different solvents. Plant extracts were screened against gram +ve and gram –ve bacteria for their antibacterial activity using micro-broth dilution and thin layer chromatography (TLC) based bioassay. Plant extracts of *A. punguns* showed minimum inhibitory concentration (MIC) in a range of 0.098-5.0 mg/ml. A decreased MIC was observed along with the increased polarity of extraction solvents. TLC guided bio-assay of active plant extracts against different bacteria developed in different solvent systems. TLC of *A. punguns* chloroform extracts were developed in T:Ea:M::7:2:1 solvent system. Bio-autography of *A. punguns* chloroform extract showed one zone of bacterial inhibition (AP1). Plant extract exhibiting significant MIC does not necessarily shows zone of bacterial inhibition in TLC based bioassays. However, none of extract was found to be active against *Pseudomonas. aeruginosa* and *Escherichia coli*. Active compounds from *A. punguns* (AP1) was tentatively identified using mass spectrometry. AP1 was found to have two compounds i.e. D-cycloserine, and fortunellin.

Keywords: micro-broth dilution assay, TLC, bio-autography, anti-bacterial compounds, MIC

Introduction
Plants and herbs contribute to medicinal system since the early age of humankind and are still being used throughout the world to treat pathogenic diseases and for health promotion. Plants are earliest and richest source of bioactive compounds even then bacteria or fungi were also not used to extracts antibiotics. The discovery of antibiotics greatly improved the quality of healthcare system and human life in the nineteenth century. Because of the initial success of antibiotics, bacterial diseases were considered to be permanently eradicated from human life. However, clinical efficacy of these existing antibiotics is being threatened by the emergence of drug-resistant pathogens [1, 2]. It seems that pre-antibiotics era will return due to failure of antibiotics in prevention and control of various diseases.
Plants still being substantial source of bioactive compounds significantly contribute in the commercial drug preparation and form the basis of modern medicinal system [3]. Plants contain secondary metabolites of novel or known structures that lead to the production of semi-synthetic drug molecules of higher activity and lower toxicity. They are reliable source to isolate bioactive compounds for direct use as drugs [4]. Plant based drugs came into use through the screening study of medicinal plants because they showed few side effects, were cost effective and possessed better compatibility [5]. However, it is possible that recommending standardized herbal remedies with antibiotics for the treatment of infectious disease will proved a valid complementary approach in future if their safety and efficacy are clinically demonstrated. The aim of the present study is to evaluate the antimicrobial potential of *Alternethera punguns*, a local plant in Haryana, India used in ayurveda and traditional medicinal system for treatment of various infections caused by pathogenic microorganisms. Further, TLC based bio-assay was used which is highly efficacious due to target-directed isolation of the active compound even in a complex mixture of crude extract [6, 7 & 8].

Material and Methods
Chemicals and Apparatus: Hexane (hex), chloroform (chl), acetone (ace), methanol (met), dimethyl sulphoxide (DMSO) (Hi-media), HCl, ampicillin, nutrient broth, ethanol, resazurin dye, p-iodonitrotetrazolium violet (INT), autoclave (Hicon), laminar flow (Metrex), incubator shaker (Remi), Halo DB 20 spectrophotometer (Dynamica), spinx vortex shaker (Tarsons), water bath (Hicon), centrifuge (Remi) and 96-well plates.

Test Microorganisms: Standard strains of the four gram-positive bacteria i.e. *Staphylococcus aureus* (NDCC-109), *Streptococcus pyogenes* (MTCC-1076), *Bacillus cereus* (NDCC-240) and *Bacillus subtilis* (NDCC-215) and four gram-negative bacteria, *Klebsiella pneumoniae* (NDCC-138), *Escherichia coli* (NDCC-135), *Pseudomonas aeruainosa* ATCC-10145) and *Salmonella typhi* (NDCC-71) were used in the study.

Collection of Plant Materials: *A. punguns* (Khaki) plant is traditionally well known for urinary tract disorder and other medicinal properties. Fresh and disease free leaves plant was collected from their natural habitat Tiliyar, Rohtak, Haryana. Identification of the plant material was done through the Botany department of Maharishi Dayanand University, Rohtak and samples of plant materials were kept in the herbarium. Cross authentication of selected plant was done with the help of flora of Haryana [9].

Preparation of Plant Extracts: Plant material was air dried by keeping in shade for 3 weeks. The properly dried plant material was crushed and grinded to fine powder. Afterward, 200 g of material was macerated three times for 72 h with five different solvents (100 ml each) in ascending order of polarity i.e. hex, chl, ace, met and water [10]. The combined extracts were filtered and solvents were evaporated to dryness in rota evaporator under reduced pressure below 45°C to yield a crude extract. Extracts were stored at -20°C until further use. The percent yield of the crude extract in different solvent was calculated by the following formula:

$$\text{Percent yield} = \frac{\text{Weight of the crude extract obtained in gm}}{\text{Total weight of plant powder in gm}} \text{ x } 100$$

Preparation of inoculums: Single colony of bacterial cultures from agar plate were transferred aseptically into flasks containing 100 ml of nutrient broth and placed in incubator at 35° C. After 15-18 h of incubation, the cultures were centrifuged at 4,000 rpm for 5 min. The supernatants were discarded. The pellets were re-suspended in 20 ml double distilled water and centrifuged again at 4,000 rpm for 5 min. This step was repeated until the supernatant was clear. The optical densities of bacterial suspensions were measured spectrophotometrically at 600 nm and diluted serially until the optical density 0.6 achieved. This process was repeated for all the eight bacteria. The actual number of colony forming units was determined from the viability graph to calculate the required dilution factor. The cultures were diluted to obtain a concentration of 10^6 cells/ml^{-1}.

Preparation of test material: Plant extracts 10 mg/ml concentrations were prepared in DMSO or sterile water as stock solutions. The hex, chl and ace extracts of different plants were dissolved in 5% (v/v) DMSO. Met extracts of different plants were dissolved in 2% (v/v) DMSO. Aqueous (aq) extracts were prepared in doubled distilled water. Ampicillin and streptomycin of 1.0 mg/ml concentration were used in the study as positive control.

***In-vitro* antimicrobial susceptibility assay:** The antibacterial activities of the extracts were determined by the micro broth dilution assay in 96-well plates [7]. Autoclaved 100 µl nutrient broth was added to the wells of the culture plates. The first row of microtiter plate was filled with 100 µl of test material dissolved in DMSO or sterile water. Two fold serial dilutions of extracts were done throughout the columns in the micro-well plates. Finally, 10 µl of bacterial inoculum was added to each well. Proper positive and negative controls were kept for each experiment. Two columns one with streptomycin and second with ampicillin was taken as positive control. Plates were incubated at 35° C for 12 h. After incubation, plated were removed from incubator and 10 µl of resazurin dye (4 mg/ml) added in each well. Resazurin is violet-blue dye irreversibly reduced to the pink colour in presence of viable bacterial cell. The extracts were considered to be active if violet colour appears in the well without any visible growth of bacteria. The results were expressed as minimum inhibitory concentration (MIC).

Standardization of TLC Plate for Different Plant Extracts: Different solvent systems were prepared by mixing defined ratio of different organic solvents and acids. Solvent systems were poured into the chambers and cover until saturation occurs. Plant extracts (chl, ace, met and aq extracts) were dissolved in enough organic solvent to have a concentrated solution instead of a turbid suspension. The 2 µl of plant extracts (5 mg/ml) was applied to TLC silica gel 60 F^{254} (Merck EMD Millipore, KGaA, Darmstadt, Germany) plates as narrow bands with a capillary, leaving a 0.5 cm border on the sides of the plates and developed in suitable solvent systems. TLC plates were dried overnight under the stream of air to remove residual solvent and mark the height of the solvent front & position of bands with a pencil. TLC plates were visualized under visible and UV light and stored at -20 °C till further use.

Qualitative antibacterial activity assay by bio-autography: Bio-autography was done with agar overlay method. Inoculums were prepared by suspending the microorganism in nutrient agar media before it solidifies with an approximate concentration of 10^6cell/ml. TLC plates were placed in sterile petri-dishes and covered with 4.5 ml of inoculum. Plates were kept at low temperature for some time, until the media solidifies and incubated for 15-18 h at 35 °C. After incubation, plates were sprayed with 2.0 mg/ml aq solution of p-iodo-nitrotetrazolium (INT) violet. Plates were incubated about 1 h at 36°C. Clear zones on chromatograms indicate inhibition of growth and positions of active compounds in extracts [7].

HPLC-MS

The band at the position of active fraction was scratched from TLC silica gel plate and isolated compounds were identified by Waters Q-Tof mass spectrometer equipped with electrospray ionization (ESI) source. To resolve the compound(s), a reverse phase column (C18, 4.5 mm × 250 mm, and 5 μm particle size) was used in HPLC. The mobile phase used for HPLC was combination of solvent A (0.1% formic acid in water) and solvent B (0.1% formic acid in acetonitrile). The gradient was varied linearly 5–10% in 15 min, 10–45% in 22 min, 45–65% in 30 min, 65–90% in 35 min, and finally to 5% B at 45 min (Gahlaut *et al*., 2013). Solvent flow rate was maintained to 0.2 ml/min. Sample volume of 20 μl was injected to HPLC C-18 column by auto-sampler. Mass spectrophotometer was operated in positive ion polarity mode.

Result

Yield of plant material: Total five plant extracts were prepared from powdered materials of plant. Different organic

solvents were used in the increasing polarity order (hex, chl, ace, met and water) for the initial partitioning of plant metabolites. Overall, percent yield of plants extracts varied from 0.07 to 18.01%. Percent yield of hexane extract of *A. punguns* was negligible however highest in aqueous extract.

***In vitro* antibacterial activity of plant extracts:** MIC of plant extracts obtained by microbroth dilution assay against different bacteria is summarized in Table 1. MIC of ampicillin (positive control) varies in the range 0.0078 to 0.125 mg/ml against different bacterial strains. Plant extracts showed selective antibacterial potential against different bacterial strain. Hexane extract showed lowest MIC against gram +ve bacteria in comparison to gram -ve bacteria. Hex and chl extracts of *A. punguns* leaves were exhibited MIC in range of 0.098 to.5.0 mg/ml except for *E. coli*, whereas ace extract was moderately active and showed MIC in range of 0.078 to 5.0 mg/ml. Aq extract didn't show reasonable activity. However, considerable better activities of this plant were observed against gram +ve as compared to gram –ve bacteria. Extracts of this plant screened in this study were least active against *P. aeruainosa.*

Table 1: MIC of plant extracts against the bacteria by micro broth dilution assay (mg/ml) in 96-well plates

	B. cereus **(mg/ml)**	***B. subtilis*** **(mg/ml)**	***S. aureus*** **(mg/ml)**	***S. pyogens*** **(mg/ml)**	***E. coli*** **(mg/ml)**	***P. aeruainosa*** **(mg/ml)**	***S. typhi*** **(mg/ml)**	***K. pneumoniae*** **(mg/ml)**
A. *Punguns chl*								
Hex	0.098	0.098	0.098	0.098	2.5	2.5	1.25	0.156
Chl	0.78	0.098	0.78	0.312	5.0	2.5	2.5	2.5
Ace	0.156	0.078	0.156	0.625	5.0	2.5	2.5	2.5
Met	2.5	2.5	5.0	1.25	-	5.0	-	-
Aq	-	-	-	5.0	-	-	-	-

*Hex- Hexane, Chl- Chloroform, Ace- Acetone, Met- Methanol, Aq- Aqueous

Bio-autography assay: TLC based bio-assay identifies the active band of antibacterial fractions from different plant extract. All five extract of this plant were screened against eight bacteria through bio-assay for identification of active band/compound. R_f values of active band, solvent system, susceptible bacteria and relative zone of inhibition are summarized in Table 2. The inactive plant extracts were not included in bioautographic assays. Bio-autography of *A. punguns* chloroform extract showed one zones of bacterial inhibition AP1 at Rf 0.37±0.001. API inhibits six bacterial strains out of eight namely *S. pyogens* (A.1), B. *subtilis* (A.2), *B. cereus* (A.3), *S. aureus* (A.4), *K. pneumonia* (A.5) and *S. typhi* (A.6) (Fig. 1)..On the other hand gram –ve bacteria *P. aeruainosa* and *E. coli* did not show any active band instead of having moderate MIC.

Table 2: TLC guided bio-assay of active plant extracts of A. *Punguns* against different bacteria developed in organic solvent systems

Plant Extract	**Solvent system**	**Compound**	**Rf value**	**BC**	**BS**	**SA**	**SP**	**EC**	**KP**	**PA**	**ST**
A. punguns chl	T:Ea:M: 7:2:1	AP1	0.37±0.001	+++	++	++	+++	-	+++	-	+++

*Chl- Chloroform, T- Toluene, Ea- Ethyl acetate, M- Methanol, BC- *Bacillus cereus*, BS-*Bacillus subtilis*, SA- *Staphylococcus aureus*, SP- *Streptococcus pyogenes*, EC- *Escherichia coli*, KP- *Klebsiella pneumonia*, PA- *Pseudomonas aeruainosa* and ST- *Salmonella typhi*

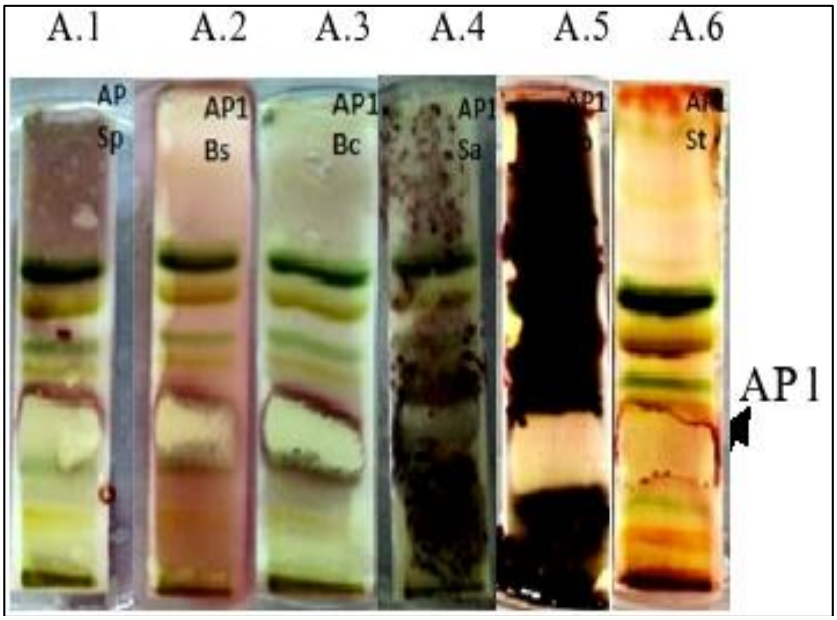

Fig 1: Bio-autography of chloroform extract of A. *Punguns* against pathogenic bacteria. Chloroform extract of *A. punguns* (AP) active against *S. pyogens* (A.1), B. *subtilis* (A.2), *B. cereus* (A.3), *S. aureus* (A.4), *K. pneumonia* (A.5) and *S. typhi* (A.6).

Characterization of AP1 and LA3

HPLC resolved the active compounds i.e. AP1 at RT 2.70 (Fig. 2A & Fig. 2B). The total ion chromatogram (TIC) of AP1 was found to have two compounds of m/z 103.97 and 593.29. (Fig.2. B). Other minor compounds of m/z 453.50, 475.47, 679.68 and 701.21 were appeared in TIC due solvent system, as these were disappeared after base line correction and TIC subtraction of controls from tests. Empirical

formulas for the compounds were calculated by MassHunter Qualitative Analysis software and Pubchem compound database was searched for tentative identification of compounds. Analysis showed m/z 103.98 and 593.29 represents D-cycloserine ($C_3H_6N_2O_2$) and fortunellin ($C_{28}H_{32}O_4$) in AP1.

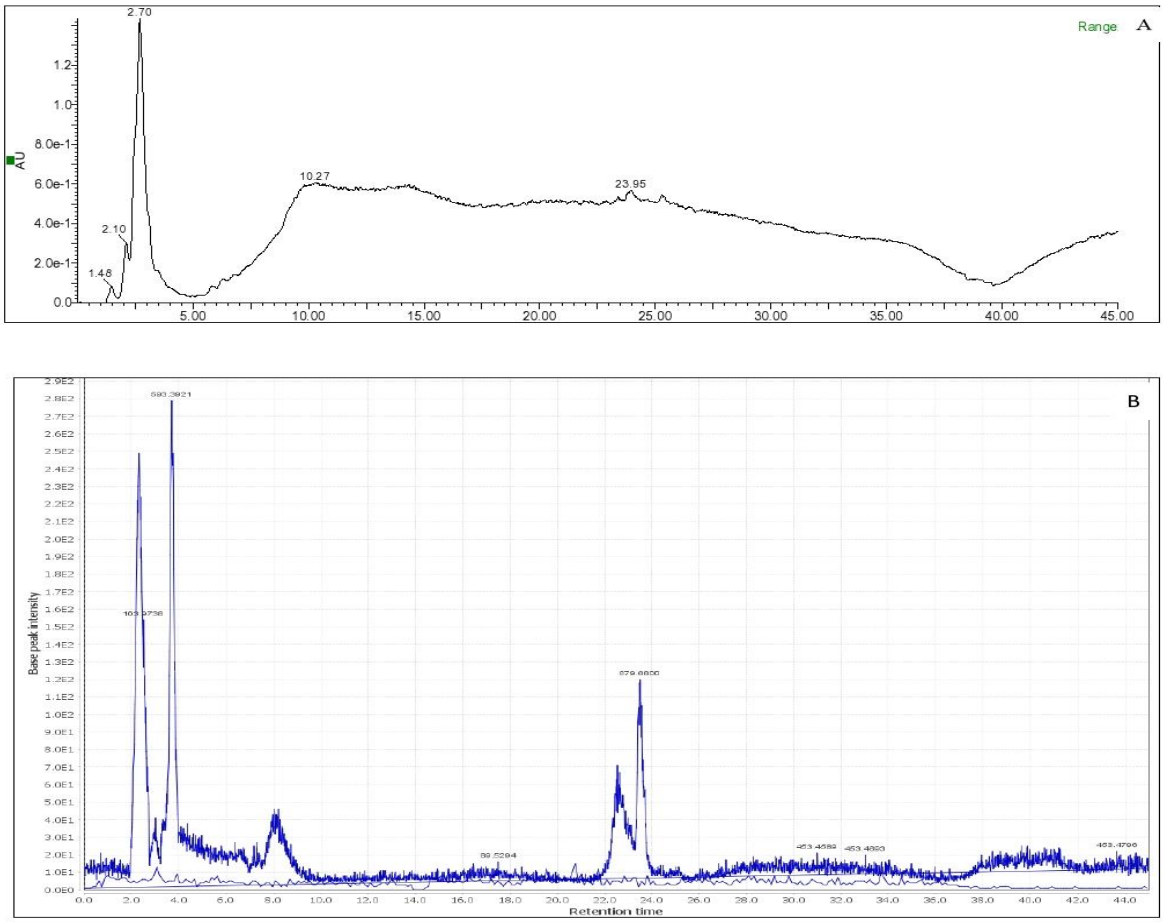

Fig 2: (A) UV diode array of AP1 showing separation at 2.70. (B) Mass spectra of AP1 show separation of compounds at m/z 103.98, 593.39 and 679.68. Other minor compounds of m/z 453.50, 475.47, 679.68 and 701.21 were appeared in TIC due solvent system

Discussion

In most of the plant materials, it was observed that yield percent increases with increase in polarity of solvents. In all the plants hex extract showed lowest and aq extracts showed highest yield percent. Plant extracts were found to be selectively active against bacterial spp. and activity decreased along with the increased polarity of extraction solvents. Moreover, plant extracts showed low MICs toward gram +ve then gram -ve. This difference was due to the ability of the compounds to penetrate inside the bacterium through the membrane. Gram +ve bacteria have a relatively loose outer wall and being hydrophobic nature of membrane, compounds of less polarity can diffuse through it easily than polar compounds [11]. In other case, gram-ve bacteria have a complex outer membrane that prevents the passage of many larger and non-polar compounds. Only a few compounds from the crude mixture of plant extracts can cross gram -ve membrane with low rate of penetration. It explains the reason for higher MICs in gram -ve. Moreover some compounds like standard drug penicillin G are selectively acts on a class of bacteria thereby it effectively kills gram +ve bacteria, but not gram -ve bacteria [12, 13].

MICs in case of *A. pungens*, were lower toward all gram +ve and gram -ve bacteria. This may be due to the common targets the bacteria. It was also observed plants extracts did not show any band against gram -ve and didn't show activity against *E. coli* and *P. aeruginosa*. These results indicated the specific and different targets in bacteria for these extracts/compounds to kill bacteria. Isoniazid, a popular drug to treat tuberculosis targets specifically to the mycobacteria, because it prevents the synthesis of mycolic acids, specifically found in the cell walls of mycobacteria and not in the most of other types of bacteria [14, 15]. In the present study, 25 µl plant extract from a solution of 50 mg/mL was applied to the TLC plates. Hence, almost 12.5 µg extract containing thousands of compounds was applied to the TLC plate. Therefore, the compounds exhibited either of broad range or of specific antibacterial activity toward selective bacterial strains and formed clear zone of inhibitions in bio-autographic assays are of importance. Their activity was better or equivalent to the standard drug ampicillin. However, in some cases crude extracts exhibited activity that was not observed in bio-autographic assays. It indicated the synergistic action of some compounds present in the extracts. These active fractions AP can be further purified by column chromatography and identified by MS/MS.

In order to identify antibacterial compounds present in chl extract of *A. pungens* (AP1), extracts were chromatographed and active bands were fractionated after repeated TLC, to get purified compounds. Analysis of mass spectra showed the shows the presence of D-cycloserine and fortunellin in AP1. D-cycloserine exhibited anti-bacterial activity against *S. aureus* in silkworm larva infection model [16]. It is a broad range antibiotics and competitive inhibitor of D-Ala-D-Ala

ligase and alanine racemase enzyme involved in peptidoglycan biosynthesis [17, 18]. It was reported to be second antibiotics to treat tuberculosis; hence, the active fractions or plants extracts of *A. pungens* may be useful to treat tuberculosis. However, further toxicity studies are required. A second active compound from *A. pungens* has been identified as fortunellin, primarily an antioxidant compound having weak antibacterial activity [19, 20]. This compound is widespread in plants of different family for e.g. reported in *Mentha piperita* of lamiaceae, *Citrus limon* of rutaceae and species of rubiaceae family [20, 21]. *A. pungens* have been reported for its antibacterial activity [22], however, the antimicrobial components of the plant have been reported first time in this study. However, the active antibacterial compound from the plant has been identified first time**.**

A. pungens contains antimicrobial compounds of broad range activity including antibacterial activity. Compounds identified also have broad activity and can be studied further in order to explore their clinical except D-cycloserine being already known broad range antibiotics. Other extracts showed selective antibacterial activity may be explored for to identify the active compounds as activities in auto-biographic assays ware equivalent to standard drugs. The selective activity of these extracts or compounds may lead toward the identification of novel and bacteria specific targets.

Acknowledgements

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors. Authors are thankful to the Botany Department, Maharshi Dayanand University, Rohtak for identification and cross-authentification of plants used in this study.

Declaration of interest

Authors declare that they have no conflict of interest. All persons designated as authors are qualified for authorship.

Ethical approval

This article does not contain any studies with animals performed by any of the authors.

References

1. Allen HK, Donato J, Wang HH, Cloud-Hansen KA, Davies J, Handelsman J. Call of the wild: antibiotic resistance genes in natural environments. Nat Rev Microbiol. 2010; 8(4):251-259.
2. Bartoloni A, Pallecchi L, Rodríguez H, Fernandez C, Mantella A, Bartalesi F, *et al*. Antibiotic resistance in a very remote Amazonas community. Int J Antimicrob Agents. 2009; 33(2):125-129.
3. Cowan MM. Plant products as antimicrobial agents. Clin Microbiol Rev. 1999; 12(4):564-582.
4. Balandrin MF, Kinghorn AD, Farnsworth NR. Plant-derived natural products in drug discovery and development: an overview. In ACS symposium series (USA). 1993, 2-12.
5. Kinghorn AD, Balandrin MF, eds. Human medicinal agents from plants. American Chemical Society (ACS), 1993, 1-6.
6. Sasidharan S, Chen Y, Saravanan D, Sundram KM, Latha LY. Extraction, isolation and characterization of bioactive compounds from plants' extracts. Afr J Tradit Complement Altern Med. 2011; 8(1):1-10.
7. Khond M, Bhosale JD, Arif T, Mandal TK, Padhi MM, Dabur R. Screening of some selected medicinal plants extracts for in-vitro antimicrobial activity. Middle-East J Sci Res. 2009; 4(4):271-278.
8. Masoko P, Eloff JN. The diversity of antifungal compounds of six South African *Terminalia* species (Combretaceae) determined by bioautography. Afr J Biotechnol. 2005; 4(12):1425-1431.
9. Jain SP, Verma DM, Singh SC, Singh JS, Kumar S. Flora of Haryana. Lucknow: Central Institute of Medicinal and Aromatic Plants, En Icones, Keys. Geog. 2000; 6:266.
10. Dabur R, Gupta A, Mandal TK, Singh DD, Bajpai V, Gurav AM, *et al*. Antimicrobial activity of some Indian medicinal plants. Afr J Tradit Complement Altern Med. 2007; 4(3):313-318.
11. Silhavy TJ, Kahne D, Walker S. The bacterial cell envelope. CSH Perspect Biol. 2010; 2(5):000414.
12. Warnke PH, Becker ST, Springer IN, Haerle F, Ullmann U, Russo PA, *et al*. Penicillin compared with other advanced broad spectrum antibiotics regarding antibacterial activity against oral pathogens isolated from odontogenic abscesses. J Craniomaxillofac Surg. 2008; 36(8):462-467.
13. Toku-E. Penicillin G, 2012.
14. Marrakchi H, Lanéelle G, Quémard A Inh. A target of the antituberculous drug isoniazid, is involved in a mycobacterial fatty acid elongation system, FAS-II. Microbiol. 2000; 146(2):289-296.
15. Quemard A, Sacchettini JC, Dessen A, Vilcheze C, Bittman R, Jacobs Jr WR, *et al*. Enzymic characterization of the target for isoniazid in Mycobacterium tuberculosis. Biochem. 1995; 3(26):8235-8241.
16. Kurokawa K, Hamamoto H, Matsuo M, Nishida S, Yamane N, Lee BL, *et al*. Evaluation of target specificity of antibacterial agents using Staphylococcus aureus ddlA mutants and D-cycloserine in a silkworm infection model. Antimicrob Agents Chemother. 2009; 53(9):4025-4027.
17. Lambert MP, Neuhaus FC. Mechanism of D-cycloserine action: alanine racemase from Escherichia coli WJ Bacteriol. 1972; 110(3):978-987.
18. Neuhaus FC, Lynch JL. The Enzymatic Synthesis of D-Alanyl-D-alanine III on the Inhibition of D-Alanyl-D-alanine Synthetase by the Antibiotic D-Cycloserine. Biochem. 1964; 3(4):471-480.
19. Lou SN, Lai YC, Hsu YS, Ho CT. Phenolic content, antioxidant activity and effective compounds of kumquat extracted by different solvents. Food Chem. 2016; 197:1-6.
20. Xu HX, Lee SF. Activity of plant flavonoids against antibiotic-resistant bacteria. Phytother Res 2001; 15(1):39-43.
21. Saleh NA, El Sherbeiny AE, El Sissi HI. Local plants as potential sources of tannins in Egypt. Part VII (Rubiaceae to Vitaceae). Qual Plant Mater Veg. 1971; 20(4):311-328.
22. Cheikna Z, Savadogo A, Somda MK, Koudou J, Traore AS. *In vitro* evaluation of the antimicrobial and antioxidant properties of extracts from whole plant of Alternanthera pungens HB & K. and leaves of *Combretum sericeum* G. Don. Int J Phytomed. 2011; 3(2):182-191.