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ISOLATION AND IDENTIFICATION OF *EISENIA FOETIDA* ASSOCIATED *PSEUDOMONAS AERUGINOSA* AND ITS CONTROL

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Abstract: Earthworm increases the soil fertility and is often referred to as a farmer's friend. The bacterial pathogen *Pseudomonas (P) aeruginosa* associated with the earthworm, penetrate into the body, cause diseases to the host and leads to the opportunistic infection. *Eisenia (E) foetida* collected from the local soil and *P. aeruginosa* spp. were isolated. The bacterial strain was confirmed through morphological and biochemical tests. In order to control the pathogenicity of *P. aeruginosa*, we used chloroform and isoamylalcohol extracts of medicinal plant including *Cinnnamomum zylanicum*, *Cuminum cyminum*, *Syzygium aromaticum*, *Curcuma long* Linn, *Trachyspermum ammi* as well as the n-Hexane, chloroform, ethanol, methanol and ethyl acetate extracts of *Momordica charantia* were investigated. On the other hand antibiotic sensitivity showed that isolated *P. aeruginosa* was highly sensitive to streptomycin, gentamycin and ciproflaxin. It was concluded that the extracts of these medicinal plants have considerable effect on the bacterial pathogens. Isolation and purification of different phytochemicals may further yield significant antibacterial agents.

Keywords: Earthworm, *Momordica charantia,* bacteria, antibiotic sensitivity

INTRODUCTION

Earthworms are very important soil creatures as they make up a large portion of the total biomass of invertebrates of the soil. Earthworms though well studied organisms all over the world, are badly neglected organism in Pakistan. Even the work on their taxonomy is far beyond completion. The most recent account of earthworm diversity comprises 3627 earthworm species described worldwide (Reynolds, 1994).

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Earthworms are diverse and more than 3,000 species are known worldwide (Morgan, 2002). They secrete enzymes such as proteases, lipases, amylases, cellulases and chitinases which bring about rapid biochemical conversion of the cellulosic and the proteinaceous materials in the variety of organic wastes which originate from homes, gardens, dairies and farms (Rajiv *et al*, 2004). Earthworm has been found to be a good source of protein (Kostecka and Pączka, 2006; Sogbesn and Ugwumba, 2008), and its usage as fish bait is well known in fishing (Omorinkoba *et al.*, 1985). Earthworms are major soil dwelling organisms and constitute larger biomass of soil invertebrates.

Worms themselves are not hosts for pathogens, but the materials they live in and consume can contain various disease-causing organisms. Not only are worms not pathogen-infested organisms, but there is actually a growing body of evidence to suggest that worms (specifically composting worms) can actually significantly reduce populations of pathogens in waste materials (Eastman *et al*., 2001). Similarly, Murry and Hinckley (1992) studied the fate of *Salmonella* in horse manure processed by *Eisenia (E) foetida*. These pathogens were also control by using various medicinal plants because plants are rich source of novel, powerful and potent drugs (Alam *et al.,* 2009; Anushia *et al.,* 2009). The aim of current research was isolation, screening and identification of bacterial pathogens associated with the surface of *E. foetida* and the screening of some medicinal plants, and standard antibiotics that possessed antimicrobial activity against these pathogens. The use of natural products as antibacterial compounds seems to be an interesting way to control the pathogenic bacteria.

MATERIALS AND METHODS

Collection and Identification of Eisenia foetida

Earthworms were collected from Muzaffarabad, Azad Jammu and Kashmir, Pakistan and brought in Biotechnology lab and preserved in 100 % absolute ethanol. Pictures of earthworm were taken by digital camera and identified by Lusty Istiqomah (Researcher) at Division of Feed and Animal Nutrition, Indonesian Institute of Sciences, Indonesia.

Isolation and preparation of test pathogens

The test pathogen *P. aeruginosa* was isolated from the *E. foetida*. Earthworms were brought in the Biotechnology laboratory, cut into small pieces with sterilized aseptic blades, washed with double distilled deionized water for 5 min to remove soil particles and later with 70% absolute ethanol. Washed pieces were placed on nutrient agar supplemented with methyl red and crystal violet. After the incubation of 24 h at 37°C, the small portion of growth area were picked with sterilized loop and again streaked on the different selected medium such as nutrient gar with crystal violet and methyl red, MacConky agar, xylose lysine deoxycholate agar and thiosulphate citrate-bile salts- sucrose agar, respectively for the isolation of single pathogen. Glycerol stock cultures were stored at -20°C before used.

Identification of test pathogen through biochemical methods

The bacteria was identified and confirmed by conventional microbiology and biochemical procedures from microbiology lab of Combined Military Hospital (CMH), Muzaffarabad, Pakistan. The Gram's staining was aimed at differentiating gram reactions, sizes, shapes and arrangement of cells of the isolates. The other biochemical test such as oxidase, catalase, coagaulase were also used for the confirmation of test pathogen.

Preparation of plant extracts through Soxhlet extraction method

The medicinal plants *Cinnnamomum zylanicum* (Cinnamon; Dalchini) sample A, *Cuminum cyminum* (Cumin; Zeera) sample B, *Syzygium aromaticum* (Clove; Loang) sample C, *Curcuma long Linn* (Turmeric powder) sample D, *Trachiyspirum ammi* (Carom seeds; Ajwain) sample E were purchased from the super market of Muzafarrabad, Azad Jammu and Kashmir, Pakistan. The bitter gourd (*Momordica charantia)* was also purchased from local market. n-Hexane, chloroform, ethanol, methanol, isoamylalcohol and ethyl acetate solvents used for preparation of plant extracts through Soxhlet extraction method. After extraction solvent is typically removed by means of rotatory evaporation yielding the extracting compound. The non-soluble portion of extracted solid remains in the thimble and is usually discarded. The extracts of green part of *M. charantia* labeled as N6 to N10 and seed labeled as N1 to N5.

Sensitivity test of standard antibiotics

Antibiotic sensitivity of test pathogens was determined by the standard agar disc diffusion method as described by Baur *et al*. (1966). The potency of antibiotics per disc are as follows; norfloxacin (10µg), chloramphenicol (30µg), streptomycin (10µg), tobramycin (10µg), gentamycin (10µg), ciproflaxin (5µg), oxytetracycline (30µg), lincomycin (2µg), sulfomethoxyzol 25µg, neomycin (30µg), tetracycline (10µg), penicillin G (10µg), trimethobrim (5µg) and ampicillin (10µg).

Agar disc diffusion method

The agar diffusion method was followed for antibacterial susceptibility test (Baur *et al*., 1966). The nutrient agar was prepared, allowed to cool up to 40°C, mixed with freshly prepared overnight culture, poured on autoclaved Petri plates and allowed to solidify under aseptic conditions. After solidification, the discs (5 mm) with all the extracts of medicinal plants were placed on the surface of the plates with the help of sterilized pincers and gently pressed to ensure contact with the agar surface. Antibiotics were also used to check the susceptibility test. The methanol, ethanol, chloroform, ethyl acetate, n-Hexane and isoamylalcohol were used as blind controls. Finally the inoculated plates were incubated at 37°C for 24 h to allow the maximum growth of the microorganisms (Baur *et al*., 1966) and the zone of inhibition was observed and measured in millimeters. Experiment was repeated for three times in each case.

RESULTS

Taxonomist identification characteristics

The *E. foetida* is an Epigeic worm. Epigeic worms live on the surface of the soil or in the top 10 inches or so of the topsoil under the litter layer. The external features of *E. foetida* are given as: 1) length 35-130mm (generally >70mm); 2) diameter 3-5mm; 3) segments 80-120; a) first dorsal pore between segments 4/5 (sometimes 5/6); b) clitellum over segments 24, 25, 26-32; c) tubercula pubertatis on segments 28-30; d) seminal vesicles, four pairs on in 9-12; e) spermathecae, two pairs in 9/10 and 10/1.

Earthworms are hermaphrodites, meaning they possess both male and female reproductive organs. Sexually mature *E. foetida* have a swollen area approx. one-third of the distance between the head and the tail called

the clitellum. The average incubation period for *E. foetida* is between 32 and 73 days. Newly hatched worms take about 8 to 10 weeks to sexually mature and begin producing cocoons (Domı´nguez *et al*., 2004). Once it breeds and starts laying cocoons, it can lay two to three cocoons per week for 6 months to a year. All of this is dependent upon the environment, *i.e.* moisture, temperature, available food, etc. The optimum temperature for *E. foetida* is 68° to 77°F (20° to 25°C). They can tolerate temperatures from 40° to 80°F (4° to 27°C). They become stressed at 85°F and can die quickly when temperatures reach above 90°F (Domı´nguez, 2004). Common names for the *E. foetida* are: red worm, red wiggler, tiger worm, manure worm, stink worm, fish worm, dung worm, fecal worm, striped worm, angleworms, and bandlings.

Isolation of Bacterial pathogen

Earthworm plays a foremost role in the proper functioning of the soil ecosystem. It acts as forager and helps in recycling of dead and decayed plant material by feeding on them. Earthworm increases the soil fertility and is often referred to as a farmer's friend.

Earthworms though well studied organisms all over the world, are badly neglected in Pakistan. *E. foetida* collected from the local soil of the Azad Jammu and Kashmir, Muzaffarabad. Two strains of *P. aeruginosa spp.* were isolated. From previous results it was observed that worms themselves are not hosts for pathogens, but the materials they line in and consume can contain various disease causing organisms (Eastman *et al*., 2001).

Pathogen was grown on nutrient agar supplemented with crystal violet and methyl red to select the Gram negative bacterium and MacCkonky agar (Fig. 1E and 1F). The bacteria were grown on various differential and selected medium such as MacCkonky agar, eosin methylene blue and nutrient agar medium. Whereas the growth was not observed on the xylose lysine deoxycholate agar and thiosulfate-citrate-bile salts-sucrose agar after overnight incubation at 37 °C (Fig. 1). A selective medium selected for the growth of some organisms, while inhibiting the growth of others. In the case of MacConkey agar, the presence of bile salts and crystal violet inhibits the growth of most Gram positive bacteria.

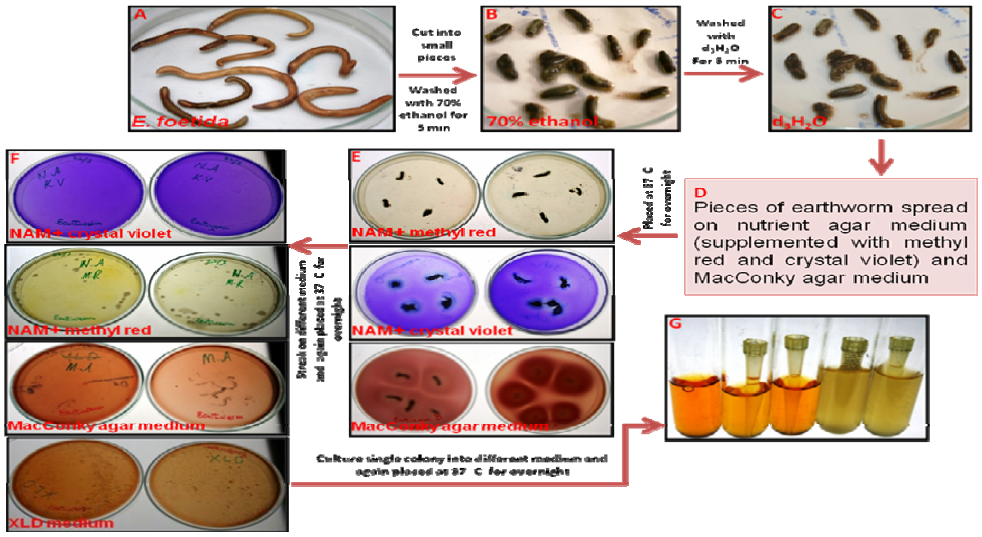

Figure 1. Isolation of earthworm associated bacterial pathogen, *Pseudomonas aeruginosa.*

Biochemical and morphological tests for bacterial identification

The morphological and biochemical tests were performed according to Bergey's manual of systematic bacteriology (Elizabeth, 2005). *P. aeruginosa* is Gram (-) rod shaped bacteria and it showed positive characteristics in case of various tests like citrate utilized, urease, nitrate reduction and oxidase test while negative results were obtained in case of methyl red, indole, voges prosteur and catalase tests, respectively (Dhayanithi *et al.* 2010) as indicated in Table I. Two strains of *P. aeruginosa* indicated with the EA and EB for further study.

Antibacterial activity of herbs against Pseudomonas aeruginosa

The antibacterial activity of chloroform (Chl) and isoamylalcohol (ISO) extracts of medicinal plants *viz*., *C. zylanicum*, *C. cyminum*, *S. aromaticum*, *C. long Linn*, and *T. ammi*, the n-Hexane, chloroform, ethanolic, methanol and ethyl acetate extracts of *M. charantia* (both seeds and green parts Bitter gourd) were screened against opportunistic pathogen

using agar disc diffusion assay. Negative control discs containing n-Hexane, chloroform, ethanol, methanol, isoamylalcohol and ethyl acetate showed no inhibition zone. Growth inhibition (zone of inhibition) was recorded as very high (++++), high (+++), medium (++) and low (+), which indicated zones of inhibition between 40-49, 20-35, 12-20, and below 12mm, respectively (Figure 2). It was observed that isoamylalcohol extracts of sample B, sample C and sample D showed significant results (19, 15 and 19 mm) against *P. aeruginosa* (EA) while chloroform and isoamylalcohol extracts of sample E had no effect on *P. aeruginosa* (EA).

Table I: The morphological and biochemical tests of *Pseudomonas aeruginosa*

Characteristics and morphology		*P. aeruginosa*
1.	Gram staining	-
2	shape	Rod
3	Motility	M
4	Indole test	-
5	Methyl red test	-
6	Voges proskeur tes	-
7	Citrate utilization test	+
8	Urease test	+
9	H2S	-
10	Gas	-
11	Nitrate redction test	+
12	Catalase test	-
13	Oxidase test	+
	Carbohydrate test	
14	Glucose	+
15	Maltose	+
16	sucrose	-

On the other hand isoamylalcohol extracts of sample A was not applied whereas chloroform extracts of sample A, Sample B, Sample C, and Sample D showed significant results (25, 21, 21, and 18 mm) against *P. aeruginosa* (EA). Sample E had no effect on *P. aueroginosa.* Such results are very interesting, because this bacterium was opportunistic pathogen and its control is very difficult by therapeutic means. More hence our results are related to the previous literature that its growth inhibited by the extracts from clove, jambolan, pomegranate and thyme (Chandler *et al.*, 1982; Bisset, 1994). Such results are very interesting, because this bacterium was opportunistic pathogen and its control is very difficult by therapeutic means. The highest zone of inhibition was recorded by the extract of carom seeds (Sample D) such as 45 mm against *P. aeruginosa* (EB). Similarly, the high zone was also recorded by choloroform extracts of sample A and C (33 and 33 mm) against *P. aeruginosa* (EB) whereas isoamyl alocohol extracts of sample B, C, and D had no effect. Studies regarding the mode of action for these compounds in the bacterial cell should be done. Cinnamon and clove are used by the several people due to antibacterial and antifungal properties (Prabuseenivasn *et al.*, 2006). Beside this, Cinnamon oil possesses anti-diabetic and anti-inflammatory activity (Mitra *et al*., 2000).

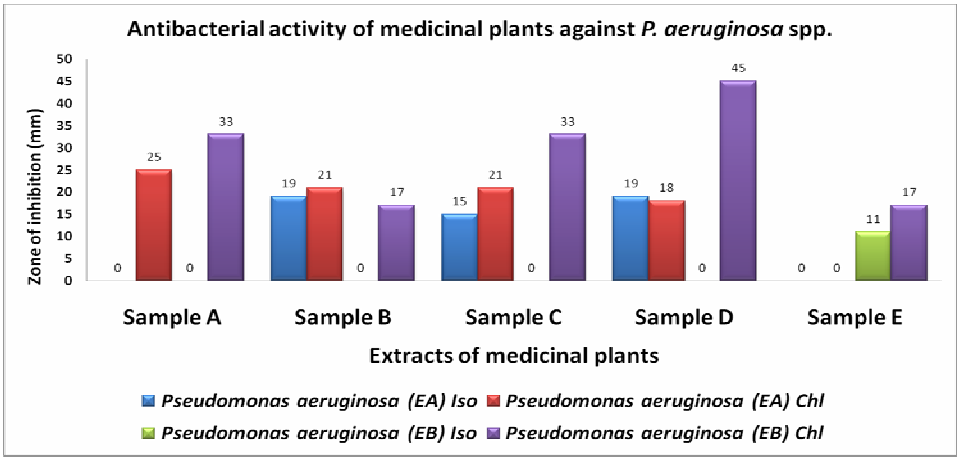

Figure 2. Zone of inhibition of chloroform and isoamyl alcohol extracts of medicinal plants against *Pseudomonas aeruginosa* (EA) and (EB).

The volatile oil of ajwain and cinnamon was found to be highly effective against *P. aeruginosa, E. coli, B. subtilis* and *Staphylococcus aureus.* while the turmeric oil was found to be inactive against *Salmonella typhi* and *P. aeruginosa* (Singh *et al*., 2007). A drug based on cinnamon was used against infections caused by *Candida* in AIDS patients. Similarlay, it was illustrated that essential oil extracted from *C. zeylanicum* demonstrated strong antifungal activity on both the species of *Aspergillus* and *in vitro* antimicrobial activity of *C. zelyanicum* (bark) was used against human pathogenic fungi and commensally bacteria was studied by Matan *et al*., 2006). The antimycotic activity of cinnamon bark due to presence of cinnamaldehyde is well known (Viollon and Chaumont, 1994). In the same way, *P. aeruginosa* indicated that it was resistant microbial strains in the presence of both isoamylalcohol and chloroform extracts of turmeric (Figure 2). Resistance exhibited by *P. aeruginosa* may be attributed to the differences in the structural integrity of the cell wall that is, the lack of "binding material" and hence interaction between the cellular lipoproteins (present in the peptidoglycan dense cell walls of Gram-positive microorganisms providing a greater target surface for the active components to attach to and initiate its antimicrobial action) and the active compounds present in turmeric extracts. It was observed that individual compounds such as thyme from ajwain, ar-turmerone from turmeric, eugenol, taninns and flavonoids from clove have antimicrobial activities (Bisset, 1994; Evans, 1996; Juglal *et al*., 2002).

Antibacterial activity of M. charantia against P. aeruginosa

The antibacterial activity of n-Hexane, chloroform, ethanol, methanol and ethyl acetate extracts of *M. charantia* (both seeds and green parts Bitter gourd) were screened against opportunistic pathogen *P. aeruginosa* using agar disc diffusion assay. Growth inhibition was recorded (Figure 2). The seeds extracts of *M. charantia* showed very less anti-bacterial activity and growth inhibition ranged from 5-8 mm.. While the extracts of green parts of *M. charantia* showed greater anti-bacterial activity against *P. aeruginosa* species and showed growth inhibition ranged from medium (12-20) to very high (40-62). Cucurbits are among the most essential plants supplying humans with edible fruits and useful seeds. Plants have high genetic diversity for fruit, shape and other characteristics, resulting in a variety of uses. The most important cultivated

genera *M. charantia* L. (bitter gourd) is the summer vegetable grown extensively throughout the country and covers an area of about 5697 ha with an annual production of about 52099 tons in the country (Anonymous *et al*., 2005) which serve as the main source of nutrition, energy, valuable vitamins and minerals. In present research it was observed that the extracts of green part of *M. charantia* (N6, N7, N8, N9 and N10) have antibacterial activity as compared to the seeds (N1, N2, N3, N4, and N5; Figure 3).

The ethanol (N9) and methanol (N10) extracts of green part of *M. charantia* showed greater antibacterial activity (30 and 28 mm) against *P. aeruginosa* (EA) and the etanolic extract showed 30 mm against *P. aeruginosa* (EB) while the ethylacetate (N8), and n-Hexane (N6) extract of green part showed no activity.(Figure 3). On the other hand the seed extracts of n-Hexane (N1), chloroform (N2), ethanolic (N4) of *M. charantia* had no effect on the growth of *P. aeruginosa* (EA and EB). Similarly methanol (N5) seed extracts of *M. charantia* also had no effect while N3 showed low antibacterial activity against both *P. aeruginosa* (EA and EB; 5 and 8 mm). On the other hand the chloroform (N7) extract of green part indicated slightly susceptible results (8, 10 mm). The lowest zone was also measured by N10 against *P. auerоginosa* (EB), respectively (Figure 3).

Sensitivity test against antibiotics against Pseudomonas aeruginosa

Sensitivity test revealed that gentamycin, tobramycin, ampicillin indicated more significant and very high zone of inhibition against *P. auerоginosa* (45, 55, and 51 mm) against *P. aeruginosa* (EA) while (45, 50 and 49 mm) against *P. aeruginosa* (EB) and high zone of inhibition was also shown by sulfomethoxyzole, and streptomycin (25, 25, 25, and 20 mm) against both *P. aeruginosa* (EA and EB), respectively. On the other hand lowest zone of inhibition was recorded by trimethobrin, pencillin, ciproflaxin, tetracycline and amoxyline (7, 7, 8, 8, and 8 mm) against *P. aeruginosa* (EA) and (8, 8, 6, 6, and 8 mm) against *P. aeruginosa* (EB), respectively (Figure 4). Our result is somewhat similar to that reported by Sadasivan *et al*. (1977) who observed that *P. aeruginosa* is sensitive to chloramphenicol, streptomycin and gentamycin (Figure 4). These antibiotics involved in the inhibition of peptidoglycan, protein synthesis, DNA replication, folic acid metabolism and murien assembly. Our study is

consistent with (Walker *et al.*, 2002) who found that gentamycin was most effective for the organism including *P. aeruginosa*.

The lowest results showed by the antibiotics because opportunistic pathogen has low permeability membrane barriers and thereby intrinsically resistant to many antibiotics (Figure 4).

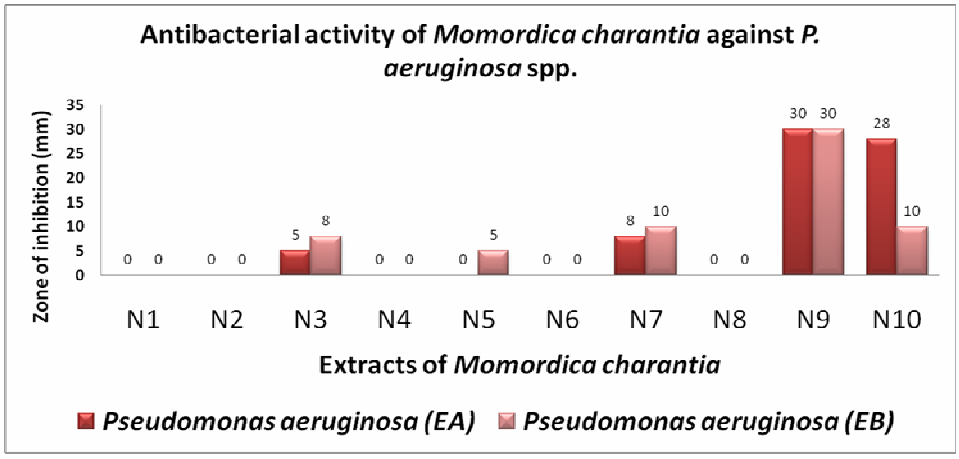

Figure 3. Zone of inhibition of extracts of *Momordica charantia* against *Pseudomonas aeruginosa*. Green part extracts of *M. charantia* indicated by (N6-N10) and seeds extracts indicated by (N1- N5).

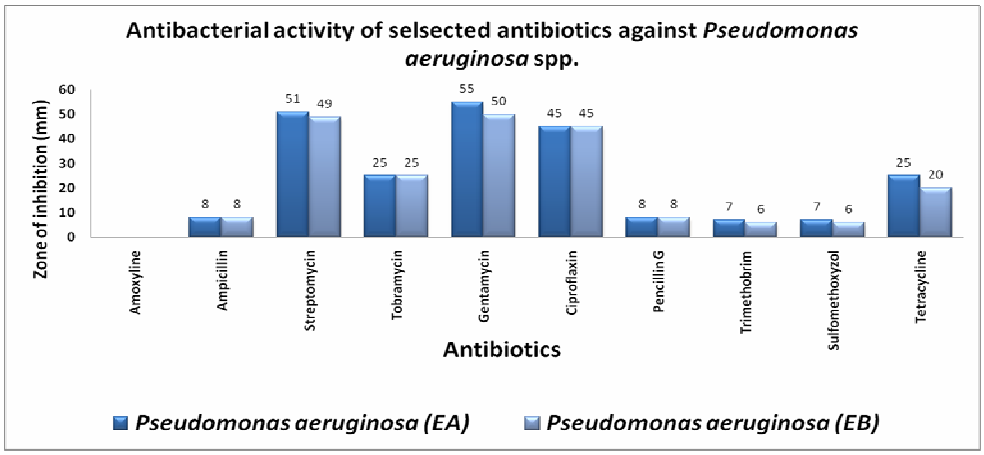

Figure 4. Antibacterial activity of selected standard antibiotics against *Pseudomonas aeruginosa* (EA and EB).

In this situation there are many evidences that may be 1) *P. aeruginosa* producing an enzyme capable of inactivating the antibiotic; 2) altering the target site receptor for the antibiotic to reduce or block its binding; 3) preventing the entry of the antibiotic into the bacterium and using an efflux pump to transport the antibiotic out of the bacterium; 4) modulating gene expression to produce more of the bacterial enzyme that is being tied up or altered by the antibiotic respectively. All sample were used to inhibit the bacterial growth.

Conclusions

It is concluded from the present study that, the extracts of medicinal plants can be effectively used as a potential antimicrobial agents to overcome the problem of bacterial infection, so as to enable to enhance the market revenue throughout the world. The phytochemicals such as thyme from ajwain, ar-turmerone from turmeric, eugenol, taninns and flavonoids from clove have antimicrobial activities and could be used as a new potential source of therapeutic drugs against pathogens.

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