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Full Length Research Paper

In vitro inhibition potential of *Lespedeza bicolor* Turcz against selected bacterial and fungal strains

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During the present study five concentrations (2.0, 1.5, 1.0, 0.5 and 0.025 mg/ml) of crude methanolic extract of *Lespedeza bicolor* was tested against infectious bacterial strains (*Escherichia* coli: ATCC 25922, *Bacillus subtilus*: ATCC 6059*, Pseudomonas aeruginosa*: ATCC 7221 *and Staphylococcus aureus*: ATCC 6538) and two fungal strains (*Candida tropicolie* and *Candida albican*). The highest concentration of 2 and 1.5 mg/ml of crude methanolic extract was found much effective against *E. coli, B. subtilus*, *P. aeruginosa*, *S. aureus, C. tropicolie* and *C. albicans* as compaired to the selected antibiotics Ampicillin, Chloramphenicol, Doxycyclin and fungicide (Terbinafine). Minimum inhibition concentration (MIC) of *L. bicolor* crude extract against *E. coli* and *B. subtilus* was found similar of 0.5 mg/ml whereas, it was 1.0 and 1.5 mg/ml against *P. aeruginosa* and *S. aureus,* respectively. The present study also supported that Doxycyclin have greater potential to inhibit the bacterial strains among the selected antibiotics used.

Key words: Crude extract, minimum inhibition concentration, antibiotics, doxycyclin.

INTRODUCTION

Lespedeza bicolor, commonly called shrub bush clover, bicolor lespedeza and shrubby lespedeza, is a semi-woody perennial legume (Family: Fabaceae) that is native to Japan. The shrub is shade-tolerant and planted as food for wildlife such as deer but more often for quail. *L. bicolor* may sometimes be used to prevent soil erosion. Purple flowers are present from mid to late summer and seed set is around the months of September to October. Many workers have reported the medicinal uses of halophytes while describing the economic importance of plants (Dagar, 1995). Halophytic plants are known to provide relief in the following diseases: cold, flu and cough: (*Achillea mellifolium, Phylla nodiflora, Portulaca quadrifida*), stomach ailments (*Juncus rigidus, Seriphidium quetenses, Thespesia populnea*), snake bite: (*Rumex vesicarius, Verbena officinalis, Zaleya pentandra*), heart disease: (*Ammi visnaga, Capparis decidua, Kochia indica*), skin diseases (*Centella asiatica, Salsola imbricata*) and asthma (*Evolvulous alsinoides*, *Solanum incanum*). In the present time, infections have increased to a great extent and resistance against antibiotics becomes an ever-increasing therapeutic problem (Austin et al., 1999). As natural products of higher plants may give a new source of antimicrobial agents, there are many research groups engaged in medicinal plants research (Samy et al., 1998; Motsei et al., 2003). Several groups of polyphenols (anthocyanins, tannins, flavanones, isoflavones, resveratrol and ellagic acid) are currently used in nutraceuticals industries and functional foods (Espin et al., 2007). Among the selected bacterial strains of *Escherichia coli* a causal agent for gastroenteritis, urinary tract infections and neonatal meningitis. *Bacillus subtilus* responsible for meningitis, urinary tract infections and anthrax while *Pseudomonas aeruginosa* induce pulmonary tract, urinary tract and blood infections.*Staphylococcus aureus* causes diabetes, cancer, vascular disease and lung diseases. The fungal strains selected *Candida tropicolie* and *Candida albican* responsible for oral and genital infections in humans. Ethanobotanical studies showed that *L. bicolor* has the ability to provide protection against cancer, arteriosclerosis, heart disease and several other diseases. *Lespedeza* was selected for the present study on the basis of the previous phytochemical studies mentioned in Table 1.

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Table 1. List of metabolites of *Lespedeza* species from different literature.

Lespedeza species	Metabolite name	References
Lespedeza bicolor	Isoliquiritigenin	Maximov et al. (2004)
Lespedeza bicolor	Daidzein	Maximov et al. (2004)
Lespedeza davurica	Isoorientin 2"-O-xyloside	Matsuzaki (1990)
Lespedeza capitata	Isocarlinoside	Jay et al. (1984)
Lespedeza capitata	Isocarlinoside	Linard et al. (1982)
Lespedeza capitata	Neocarlinoside	Besson et al. (1985)
Lespedeza capitata	Neocarlinoside	Linard et al. (1982)
Lespedeza davidii	Lespedezaflavanone C	Li et al. (1989)
Lespedeza cyrtobotrya	Xanthoangelol	Kozawa et al. (1977)
Lespedeza cyrtobotrya	Xanthoangelol	Miyase et al. (1980)
Lespedeza cyrtobotrya	Lespeol	Miyase et al. (1980)
Lespedeza davidii	Euchrestaflavanone A	Shirataki et al. (1985)
Lespedeza formosa	Lespedezaflavanone F	Li et al. (1992)
Lespedeza formosa	Lespedezaflavanone G	Li et al. (1992)
Lespedeza penduliflora	Petunidin 3-glucoside	Pomilio (1973)
Lespedeza bicolor	Daidzein	Maximov et al. (2004)
Lespedeza cyrtobotrya	Lespedeol C	Miyase et al. (1981)
Lespedeza homoloba	Lespedeol A	Ueno et al. (1973)
Lespedeza homoloba	Lespedeol B	Ueno et al. (1973)
Lespedeza cyrtobotrya	Haginin D	Miyase et al. (1981)
Lespedeza homoloba	Haginin D	Miyase et al. (1999)
Lespedeza cyrtobotrya	Haginin B	Miyase et al. (1980)
Lespedeza cyrtobotrya	Haginin C	Miyase et al. (1981)
Lespedeza cyrtobotrya	Haginin A	Miyase et al. (1980)
Lespedeza homoloba	Lespein	Ueno et al. (1973)

Objectives of the study

During the present investigation *L. Bicolor* leaves extract were checked for their potentials against several virulance microorganisms for example *E. coli*, *B. subtilus*, *P. aeruginosa*, *S. aureus*, *C. tropicolie* and *C. albican.*

MATERIALS AND METHODS

Collection of plant samples

In the present study *L. bicolor* were collected from different locations of District Mardan (altitiude of 400 to 1,700 m). The arial parts of *L. bicolor* were used to study the inhibition potential against bacterial strains for example *E. coli*, *B. subtilus, P.* aeruginosa and *S. aureus* and fungal strains for example *C. tropicolie* and *C. albican*.

Extraction

Fresh arial parts of *L. bicolor* were collected, rinsed with distilled water and air dried for 12 days. The plant parts were grounded into powder form using homogenizer then soaked in 80% methanol, incubated for two weeks at room temperature (25°C). After 14 days of extraction, mixture was filtered twice, using Whatman-41 filter paper.

The extract was reduced to dryness by removing methanol through rotary film evaporator. 2.0, 1.5, 1.0, 0.5 and 0.025 mg/ml of the plant extracts were used against the selected bacterial and fungal strains.

Antibacterial activity of the plant extract

Preparation of media for bacteria

Nutrient broth medium was prepared by dissolving 0.4 g of nutrient broth per 50 ml of distilled water for the growth of bacterial inocula, pH was adjusted at 7.0 and was autoclaved. Nutrient agar medium was prepared by dissolving 2.3 g agars in 100 ml of distilled water; pH was adjusted at 7.0 and was autoclaved at 121°C.

Mcfarland 0.5 barium sulphate turbidity standard

The standard was prepared by adding 0.5 ml of 0.048 M barium chlorides to 99.5 ml of 0.36 N sulphuric acid. Barium sulphate turbidity standard (4 to 6 ml) and was taken in screw capped test tube.

Bacterial strains used

Four strains of bacteria were used in the study. Two were gram positive, *S. aureus* (ATCC 6538) and *B. subtilis* (ATCC 6059) and

two were gram negative which were *E. coli* (ATCC 25922) and *P. aeruginosa* (ATCC 7221). The organisms were maintained on nutrient agar medium at 4°C. extract was prepared, which were used for positive control. Slants without extract were used for negative control. The test tubes were incubated at 28°C for 7 days. Cultures were examined twice weekly

Preparation of inocula

Centrifuged pallets of bacteria from 24 h old culture in nutrient broth (SIGMA) of selected bacterial strains were mixed with physiological normal saline solution until a Mcfarland turbidity standard was obtained. Then this inoculum was used for seeding the nutrient agar.

Preparation of seeded agar plates

Nutrient agar medium was prepared by adding nutrient agar (MERCK) 2.3 g in 100 ml of distilled water; pH was adjusted at 7.0 and was autoclaved. It was allowed to cool up to 45°C. Petri plates were prepared by pouring 75 ml of seeded nutrient agar and allowed to solidify. 4 wells per plate were made with sterile cork borer (5 mm).

Pouring of test solutions, incubation and measurement of zone of inhibitions

Agar well diffusion method was used for antibacterial activity (Carron et al., 1987), using micropipette, 100 µl of test solutions was poured in respective wells. These plates were incubated at 37°C after 24 h of incubating the diameter of the clear zones of inhibitions was measured by a ruler. Antibacterial activity of two dilutions of each plant extract was determined against four bacterial strains.

Antifungal activity of the plant extract

The agar tube dilution method is used for determination of antifungal activity of extract (Washington and Sutter, 1980).

Fungal strains used

The following fungal strains were used in this study:

1) *C. albicans*.
2) *C. tropicolie*.

Each fungal strain was maintained on sabouraud dextrose agar (SDA) medium at 4°C.

Assay procedure for antifungal activity

The samples for antifungal assay were prepared from initial stock of 100 mg of extract per ml of dimethyl sulfoxide (DMSO). Media for fungus was prepared by dissolving 6.5 g of SDA per 100 ml in distilled water, pH was adjusted at 5.6. Test tubes were marked to 10 cm mark. The Sabouraud dextrose agar (MERCK) dispensed as 4 ml volume into screw capped tubes or cotton plugged test tubes and was autoclaved at 121°C for 21 min. Tubes were allowed to cool to 50°C and non-solidified SDA was loaded with 67 µl of compound pipette from the stock solution. This would give the final concentration of 200 µg/ml of the pure compound in media. Tubes were then allowed to solidify in slanting position at room temperature. One slant of the extract sample was prepared for each fungus species. The tubes containing solidified media and test compound were inoculated with 4 mm diameter piece of inoculums, taken from a seven days old culture of fungus. One sample of each during the incubation. Reading was taken by measuring the linear length of fungus in slant by measuring growth (mm) and growth inhibition was calculated with reference to negative control. Percentage inhibition of fungal growth for each concentration of compound was determined by the following formula:

$$\text{Percentage inhibition of fungal growth} = 100 - \frac{\text{Linear growth in test (mm)}}{\text{Linear growth in control (mm)}} \times 100$$

Statistical analysis

The data were analyzed statistically by Analysis of variance technique (Steel and Torrie, 1980) and comparison among treatment means was made by Duncan's multiple range test (DMRT) (Duncan, 1955).

RESULTS

Antibacterial activity of *Lespedeza bicolor* crude extract

Figure 1 revealed that *L. bicolor* leaves extract of 2.0 and 1.5 mg/ml showed maximum inhibition against *E. coli* among all the five selected concentrations similar to antibiotic Ampicillin. Plant extract of 0.5 mg/ml was considered the minimum inhibition concentration as it showed the least zone of inhibition of 7 mm against *E. coli*. Chloramphenicol among the three selected antibiotics showed maximum zone of inhibition of 22 mm. Results in Figure 2 showed that inhibition potential of 2.0 and 1.5 mg/ml plant extract against *B. subtilus* was found maximum whereas, 0.5 mg/ml extract was considered the minimum inhibition concentration showed the least inhibition of 7 mm against *B. subtilus*. Antibiotics for example Ampicillin, Doxycyclin and Chloramphenicol was not an effective inhibitor against *B. subtilus* in comparison with plant extracts. In Figure 3 the plant extracts of 2.0 and 1.5 mg/ml against *P. aeruginosa* showed similar inhibition like Ampicillin. The plant extract of 1.0 mg/ml was considered the minimum inhibition concentration showing the least inhibition of 4 mm against *P. aeruginosa*. Among the antibiotics used Doxycyclin showed maximum inhibition of 33 mm followed by Chloramphenicol. Figure 4 revealed that *S. aureus* was found much resistant against the plant extract used. Only 2.0 and 1.5 mg/ml of the plant extracts among the selected concentration were effective. Plant extract of 1.5 mg/ml was considered the minimum inhibition concentration against *S. aureus*. Ampicillin and Chloramphenicol showed similar inhibition whereas, Doxycyclin showed maximum inhibition of 35 mm against *S. aureus*.

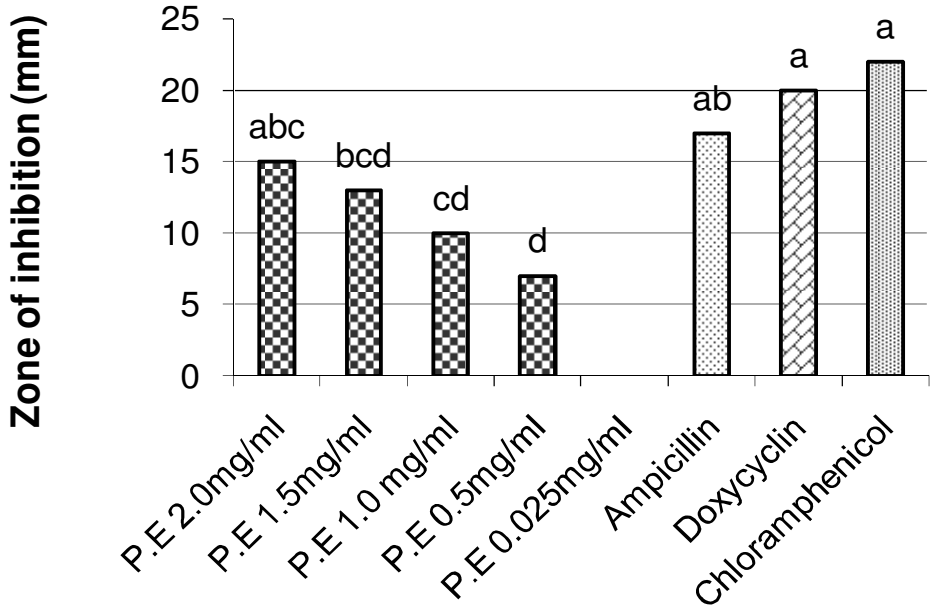

Figure 1. Inhibition potential of *Lespedeza bicolor* leaves extract against *Escherichia* coli. All bars which share same letters are non-significantly different at 5% level of significance.

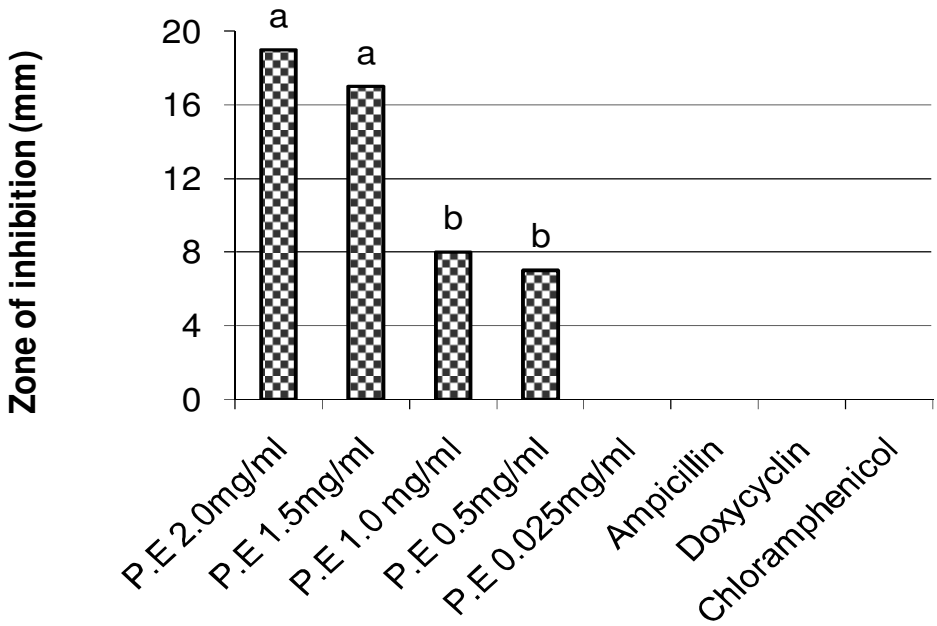

Figure 2. Inhibition potential of *Lespedeza bicolor* leaves extract against *Bacillus subtilus.* All bars which share same letters are non-significantly different at 5% level of significance.

Minimum inhibition concentration (MIC)

Minimum inhibition concentration (MIC) of *L.bicolor* crude methanolic extract against *E. coli* and *B. subtilus* was found 0.5 mg/ml, whereas, MIC of 1.0 and 1.5 mg/ml was found against *P. aeruginosa* and *S. aureus,* respectively.

Antifungal activity of *Lespedeza bicolor* crude extract

Antifungal activity of the selected five concentrations (2.0, 1.5, 1.0, 0.5 and 0.025 mg/ml) of *L. bicolor* crude extract against *C. tropicolie* and *C. albicans* was shown in Figures 5 and 6. In comparison with fungicide

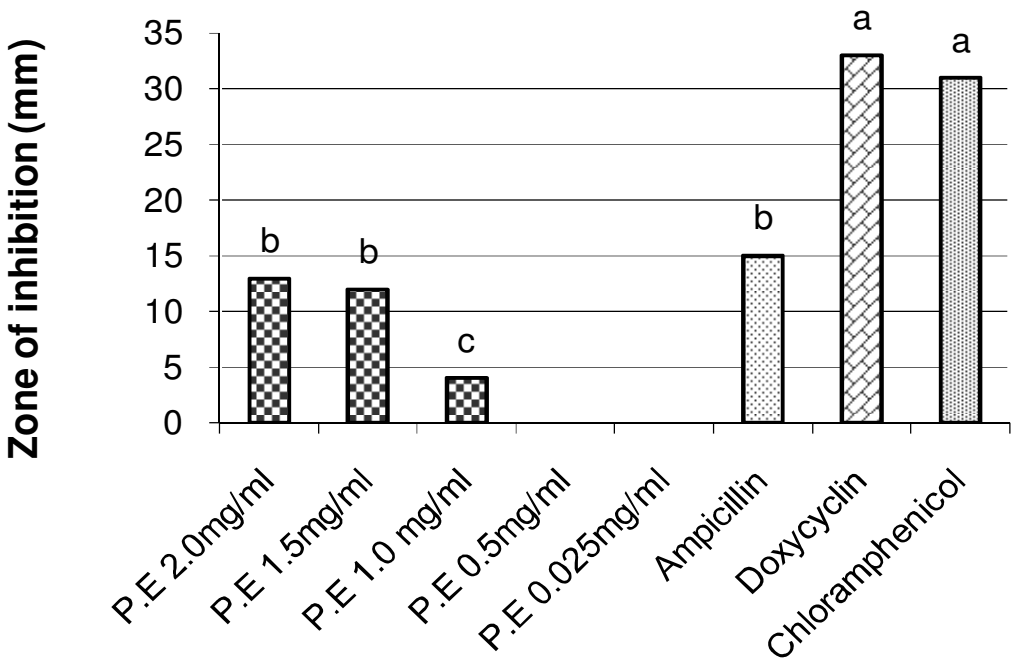

Figure 3. Inhibition potential of *Lespedeza bicolor* leaves extract against *Pseudomonas aeruginosa*. All bars which share same letters are non-significantly different at 5% level of significance.

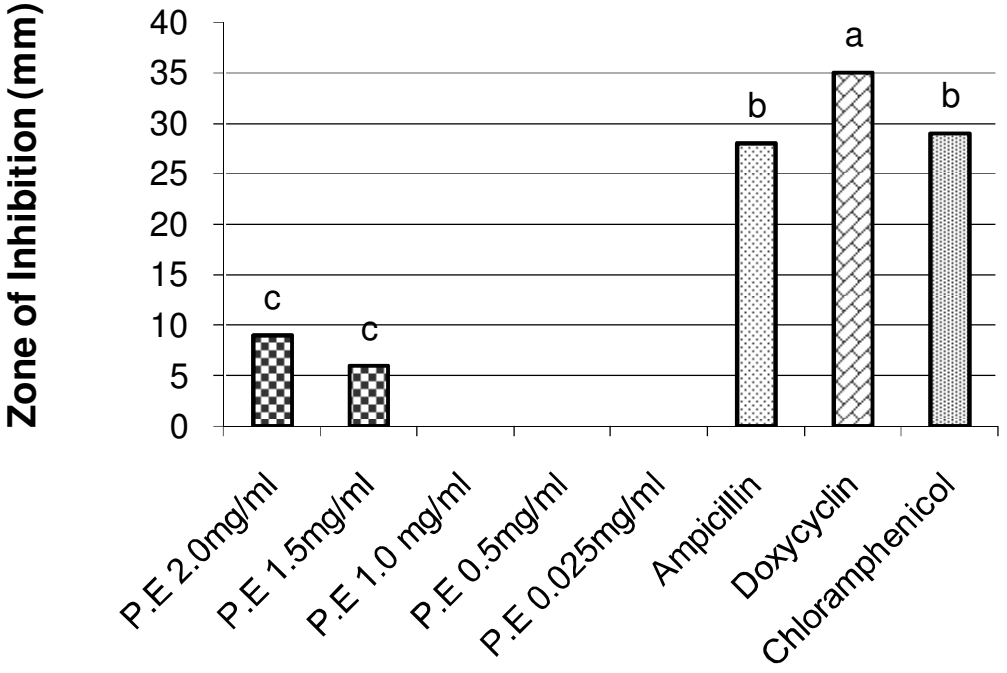

Figure 4. Inhibition potential of *Lespedeza bicolor* leaves extract against *Staphylococcus aureus.* All bars which share same letters are non-significantly different at 5% level of significance.

(Terbanifine) all the five concentrations was found effective against *C. tropicolie* and *C. albicans* but the potential of inhibition of 2.0 mg/ml plant extract was found almost similar to Terbanifine. Among the two fungal strains *C. albicans* showed greater resistance against *L. bicolor* plants extract.

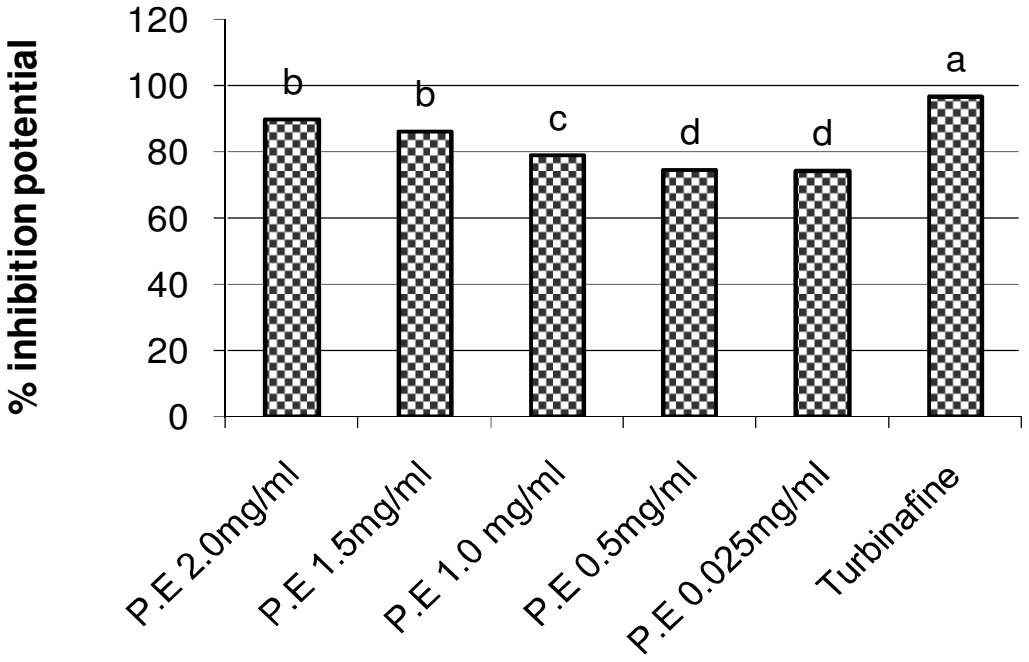

Figure 5. Inhibition potential of *Lespedeza bicolor* leaves extract against *Candida tropicolie.* All bars which share same letters are non-significantly different at 5% level of significance.

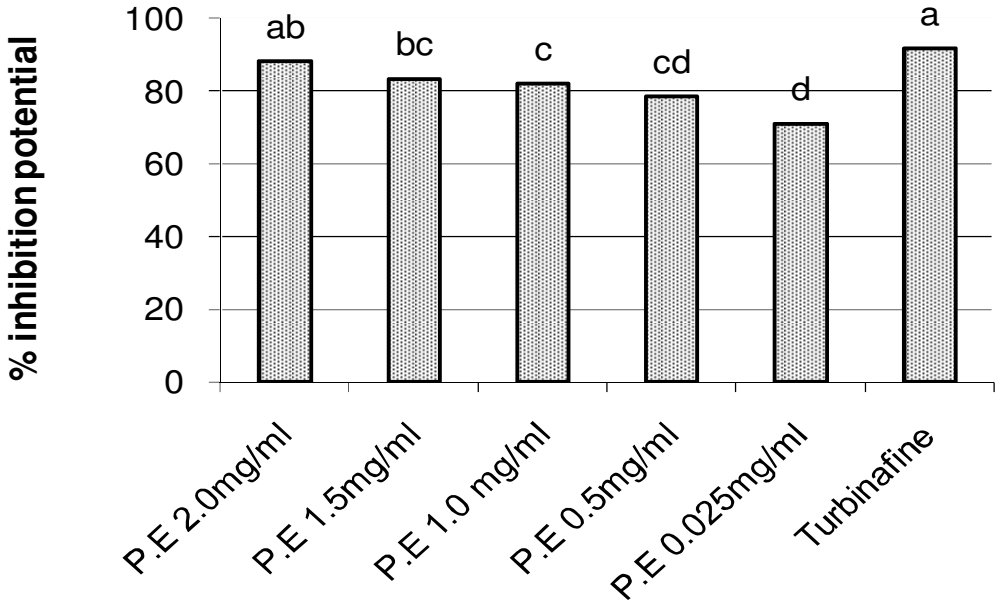

Figure 6. Inhibition potential of *Lespedeza bicolor* leaves extract against *Candida albicans.* All bars which share same letters are non-significantly different at 5% level of significance.

DISCUSSION

Resistance against antibiotic has been reported previously in the food born pathogens like *E. coli, S. aureus and Clostridium perfringens.* Some of the medicinal plants have been studied for their inhibition potential against 6 bacterial strains in India. The rural population of Asia and Africa depend on medicinal plants to cure diseases; India and Pakistan are the extensive users (Atta-ur-Rehman, 1979). The phytochemicals

derived from root, stem, leaves, fruits, flowers and seeds of medicinal plants include phenolics compounds, essentials oils, proteins and antioxidants, which serve as biocontrol agents (Cragg et al., 1996). The inhibition potential of plant extract against the growth of microbes was demonstrated due to the presence of antioxidants in plants (Cutter, 2000; Puupponen-Pimia et al., 2001). Crude methanolic extract has been used in the present study because most of the antimicrobial agents in plants are soluble in methanol (Chandrasekaran and Venkatesalu, 2004). All the concentrations of *L. bicolor* plant extracts inhibited the growth of *B. subtilus* whereas; none of the selected antibiotic inhibits *B. subtilus.* The plant extract being least inhibitory against *S. aureus.* Against *E .coli* the % inhibition ranked as, Chloramphenicol > Doxycyclin > Ampicillin > 2.0 mg/ml plant extract > 1.5 mg/ml plant extract > 1.0 mg/ml plant extract > 0.5 mg/ml plant extract. For *P. aeruginosa* Chloramphenicol = Doxycyclin > Ampicillin = 2.0 mg/ml plant extract = 1.5 mg/ml plant extract. Against the fungal strain of *C. tropicolie* % inhibition ranked as, Terbinafine = 2.0 mg/ml plant extract > 1.5 mg/ml plant extract > 1.0 mg/ml plant extract > 0.5 mg/ml plant extract > 0.025 mg/ml plant extract.The % inhibition against *C. albicans* ranked as, Terbinafine = 2.0 mg/ml plant extract >1.5 mg /ml plant extract = 1.0 mg/ml plant extract = 0.5 mg/ml plant extract > 0.025 mg/ml plant extract.

Conclusion

From the present work it has been revealed that *L. bicolor* have the ability to provide protection against *E. coli*, *Bacillus subtilus*, *P. aeruginosa*, *S. aureus*, *C. tropicolie* and *C. albican.* The leaves extract of 2.0 and 1.5 mg/ml showed maximum inhibition against all the bacterial and fungal strains similar to antibiotic Ampicillin used.

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