# *In vitro* antimicrobial, antiprotozoal activities and heavy metals toxicity of different parts of *Ballota pseudodictamnus* (L.) Benth

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**Abstract**: The study was done to check the antimicrobial and antiprotozoal activity of different parts of *Ballota pseudodictamnus* (L.) Benth. These activities were then compared with the heavy metals toxicity of different parts, which plants accumulate in different concentrations in different parts. In *in-vitro* antileishmanial results ethanolic extract, chloroform and ethyl acetate fractions in roots of *Ballota pseudodictamnus* (L.) Benth showed antileishmanial activity. The ethanol, *n*-butanol and ethyl acetate fraction in stem revealed inhibition of amastigote form of leishmania. The ethanolic extract, chloroform, and *n*-butanol fraction in leaves showed inhibition of leishmanial parasite. In heavy metals study, Chromium was above permissible value in all parts except in leaves. Nickel was above WHO limit in roots. Cadmium and lead were beyond permissible limits in entire plant parts. Results revealed that different parts of the plant have different inhibition properties. So each part of plant should be checked for antimicrobial and antiprotozoal assay separately. It is concluded that various metals accumulates with miscellaneous concentrations in different plant parts.

**Keywords**: Antimicrobial, antileishmanial, *Ballota pseudodictamnus,* heavy metals, WHO limits.

## INTRODUCTION

Medicinal plants represent the main source of substances that are active against different microbes as a result, we can say that medicinal plants showed antimicrobial activity. This significant use of medicinal plants results in screening of plants for bioactive compounds is, therefore, very important. Due to the appearance of many drug resistant pathogenic strains and varieties they are especially important (Dildar *et al*., 2009). There is a continuous need to search new and effective drugs, because of the continuous increase in resistance of microbes against drugs. In different regions of the world, many screening studies have been performed. All these screening studies indicated that herbal extracts have antimicrobial activity (Sankar *et al*., 2010). Trypanosomatid parasite (Family Protozoa) causes a disease, which is called Leishmaniasis. Leishmania is the genus of this parasite and transmitted to humans by the bite of a sand fly called Phlebotomine. Visceral, cutaneous and mucocutaneous is the three major forms of Leishmania, in which cutaneous is the most lethal form of parasite. About ten million people are affected by this parasite these days. It is estimated that globally, one in every twenty individuals is affected by this parasite (Bero *et al*., 2011). Therefore, different bioactive compounds of medicinal plants can be widely used as a therapeutic source against these parasites. Medicinal plants importance amplified due to the presence of these potent bioactive compounds, which could be used against various microbes (Edeoga *et al*., 2005). In the environment heavy metals concentration above the permissible or high value can be damaging to an immense diversity of living species. Excessive intake of these heavy metals through either ingestion, or inhalation, can cause serious complications like accumulative poisoning, damage nervous system, cancer and ultimate death (Onundi *et al*., 2010). Heavy metals enter the food chain either by inhalation from the air or ingestion of polluted food by the inhabitants. As a result, persistent heavy metals can be bio-accumulated through these biologic chains. Since heavy metals are extremely toxic, so presence of high concentration of heavy metals in atmosphere indicates a possible threat for human population and also for other environment constituents. This is the reason, that precise monitoring of their concentration is required (Gabriela *et al*., 2005).

The present research was therefore designed to investigate the antimicrobial and antiprotozoal activities of various parts of *Ballota pseudodictamnus*. The appraisal of heavy metal toxicity was also the key focus of the study.

## MATERIALS AND METHODS

### *Collection and drying of plant materials*

Plants sample of *Ballota pseudodictamnus* (2Kg each part) was collected from Latamber, District Karak, Khyber Pakhtunkhwa and was identified by Dr. Nisar Ahmad Lecturer Department of Botany, KUST, Kohat, Pakistan. The plant samples were shade dried at 25-30$^{o}$C.

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The dried parts (root, stem and leave) were powdered separately and then stored in clean, dried plastic bags for further processing.

***Extraction Procedure***

The dried root, stem and leaves (2Kg each) of *Ballota pseudodictamnus* were soaked in ethanol for 18 days, extracted and then filtered. The filtrate was evaporated by Vacuum Rotary Evaporator at 35$^{o}$C to give crude extracts. These extracts were made dried and weighed, which were suspended in $H_2O$ and successively partitioned with *n*-hexane, chloroform, ethyl acetate and *n*-butanol to obtain *n*-hexane-soluble, chloroform-soluble, ethyl acetate-soluble, *n*-butanol soluble and $H_2O$ fractions, respectively. The crude ethanolic extracts and succeeding other solvent fractions were then subjected to antimicrobial and antileishmanial bioassays.

***Antimicrobial Assay***

In this study, extracts and various fractions of *Ballota pseudodictamnus* were studied for antimicrobial assay using Gram negative, Gram positive bacteria and against fungi. Six bacterial strains *Proteus mirabilis, Escherichia coli, Staphylococcus aureus, Klebsiella pneumoniae, Enterococcus faecalis* and *Salmonella typhi* were used by agar well diffusion susceptibility method, and four fungal strains *Aspergillusniger, Aspergillus flavis, Aspergillus fumigatus* and *Fusariums olani* were tested by Test Tube Dilution Susceptibility Method. Microorganisms were provided by Department of Pharmacy, KUST, Kohat.

***Preparation of the test compound***

In Dimethyl Sulfoxide Oxide (DMSO) solutions of extracts and fractions at concentration of 2µg/µL were prepared. DMSO was used as a solvent, because it does not show any activity against bacteria and fungi, therefore DMSO was selected for the present study (Khan *et al*., 2008a; Khan *et al*., 2008b). In separate conical flasks, solution of concentration of 28g/L nutrient agar was prepared. Media solution, Petri plates and borer were sterilized for 15 minutes at high pressure and at 121$^{o}$C in autoclave. Agar medium was poured in Petri dishes in laminar flow.

***Antibacterial bioassay***

The antibacterial bio screening was studied by Agar Well Method, by measuring inhibition zones against the selected microorganisms using Ashish *et al.* (2006) method.

***Methodology***

Ashish *et al.* (2006) customized methodology was followed. Wells of 6mm were dug in media by using sterile plastic borer. Each well was given a specific number. Bacterial culture corresponding to $10^4$ to $10^6$cfu (colony forming unit) was streaked on solidified media surface. Solutions of crude extracts and fractions of concentration 2µg/µL were prepared. From each stock solution 3mL was added into relevant wells. The inhibition zones were measured after one day of incubation at 37$^{o}$C in the incubator. Amoxicillin (5µg/µL) and Levofloxacin (5µg/µL) were used as standard positive control and DMSO was as a negative control. The zones of inhibition of crude extracts and other solvent soluble fractions were compared with standard drugs zones of inhibition i.e. Amoxicillin and Levofloxacin. The amount of growth in each well was measured.

***Antifungal bioscreening***

The antifungal bioassay was determined by Agar Test Tube Dilution Method by using modified Rehman *et al*. (2001) method.

***Methodology***

Four fungal strains, i.e. *Aspergillusflavus, Fusariunsolani, Aspergillusfumigatus* and *Aspergillusniger* were used for antifungal activities. To refresh fungal strains, nutrient broth solution of concentration 13g/L in distilled water was prepared. Sterilized in an autoclave and four flasks of 250mL were filled from broth. To each flask fungal colonies were inoculated separately. Three days, these flasks were then placed in incubator at 30°C for refreshing fungal strains. In a 1Liter conical flask, 28 grams of nutrient agar was taken and dissolved in 1Liter of water (distilled). The flask was uncontaminated in autoclave at 121°C for about 15 minutes at 1.5pounds pressure. An antibiotic, Clotrimazole was taken as a positive control standard and dissolved it in (2µg/µL) in distilled water, while DMSO (6µL/disc) was taken as negative control. About 9mL of medium was added to clean, dry and sterilized test tubes. Solutions of crude extracts and sub fractions were prepared each of 2µg/µL concentration. One mL of sample (2µg/µL) was also added to each test tube, the test tube was kept in tending point to make a slope. The same process was repeated for other test tubes. After cooling and solidifying, the fungi inoculums suspension was spread over the agar medium uniformly using sterile cotton swabs. After that the test tubes were kept in incubator for 3 days at 30°C.After 3 days the fungal growth was observed in each test tube.

***Antileishmanial assay***

*In vitro* antileishmanial activity of crude extracts and other fractions of *Ballota pseudodictamnus* were evaluated.

***Collection of sample specimen***

In order to carry out antileishmanial bioassay, specimens were collected in Latamber, Karak, Khyber Pakhtunkhwa and some specimens were provided by Habib Laboratory near KDA Hospital Kohat from many patients having *Cutaneous leishmaniasis*. The area of infection and the adjacent normal looking skin around the infection were washed and sterilized with ethanol. With the help of

lancet or sterilized surgical blade skin cuts were made till blood oozes out from the infected part in one periphery. In the margin of scratch, blood is collected and stored in eppindorf containing 0.9% saline solution. Small quantity of sample was applied to slide and then seen under the microscope to observe non flagellated amastigote, whereas in the other part buffer solution of pH 7.2 were mixed and placed in incubator for further process. The sample, in which amastigotes were observed, was further processed.

***Culturing of parasite***

For Cultivation process Hamid *et al.* (2012) method was used with some modifications. From the samples *leishmanial* parasite was cultured in the RPMI 1640 (Sigma. USA) culture media. The quantity of 0.3g/30mL of RPMI 1640 media was dissolved in (10g/ 1000mL of distilled water) distilled water and added to 20 separate small screwed caped tubes each having 4mL of the media. To avoid any bacterial and fungal contamination, the antibiotic/s Cefixime or Penicillin G and Kanamycin were mixed. To the test tubes 1mL of that sample was added in which amastigote was observed. At 25$^{o}$C the test tubes were placed in incubator (Memmert type Inb 500, Germany). After 11 days of incubation period, promastigote of *Leishmaniatropica* culture was observed with Giemsa staining and seen under Olympus Microscope at different magnifications 10X, 40X and 100X.

***Leishmanicidal assay***

In laminar flow 4mL of RPMI (1640) was taken in each test tube. Then 1mL from solutions of extracts and fractions of concentration 25µg/mL were added to these test tubes which contain media and shake well for complete mixing. About 20µL of *leishmanial* colony was added to each test tube along with positive control except to the test tube containing negative control. For avoiding any contamination of microbes 25µg of antibiotic Cefixime or Penicillin-G and Kanamycin were added. Then tubes were placed in the incubator for 96 hours at 26$^{o}$C. The activity of ethanolic extracts and other fractions was analyzed in non-treated control (DMSO without plant extracts) and other test tubes after time intervals of 24 hours by microscope.

***Heavy metals detection***

*Dry digestion*

For dry digestion Khan *et al.* (2008) modified method was used as standard method. Specified weights (2gm) of the grinded and powdered plant samples of different parts were placed in china dish. Heat it in oven at 105$^{o}$C for 5 hours to remove moisture. Then samples were positioned in the furnace. It's inside temperature was increased steadily from 25$^{o}$C to 550$^{o}$C in one hour. Here plants powder samples were ashed for five hours so that a white residual ash was obtained. The white ashed samples were placed in desiccators to cool to 25$^{o}$C. Solution (3ml) of $HNO_3$ of concentration 6 M was supplemented into the china dish and then heated to dissolve the content of the china dish. The solutions were filtered into 25mL graduated cylinder and were diluted up to the mark. Different heavy metals like Ni, Cr, Fe, Zn, Cd, Mn and Pb assessment was done on "Flame Atomic Absorption Spectrophotometer (Parkin Elmer 400) (Rehman*et al*., 2001; Onocha and Ali, 2010).

## STATISTICAL ANALYSIS

All values were presented as the mean ± standard error of mean and analyzed for TWO Way-ANOVA and ONE-Way ANOVA. Differences between groups were considered significant at $P<0.05$. Statistical analysis was carried out on GraphPad PRISM 6.

## RESULTS

***Antimicrobial activity***

Different extracts and fractions of *Ballota pseudodictamnus* root, stem and leaves with concentration (2µg/µL) showed different zone of inhibitions. *In vitro* antibacterial activities of crude extract and other solvent fractions of *Ballota pseudodictamnus* were performed and they were compared with the standard antibiotics (Amoxicillin and Levofloxacin). For antibacterial activity six bacterial strains *Escherichia coli, Staphylococcus aureus, Proteus mirabilis, Klebsiella pneumoniae, Enterococcus faecalis and Salmonella typhi* were tested by Agar Well Diffusion Susceptibility Method. Crude ethanolic extract of plant root was completely active and aqueous fraction was completely inactive against all bacterial strains (table 1). Hexane and chloroform fractions showed no activity against *S. aureus, E. coli* and *E. faecalis.* Ethyl acetate fraction was inactive against *K. pneumoniae.* Butanol fraction was inactive against *S. aureus* and *K. pneumoniae.* Crude extract of plant stem revealed activity against all bacterial strains except *S. aureus* and showed maximum inhibition (23±0.95mm) against *E.faecalis*. Hexane and chloroform fractions were active against *E. coli* and *E. faecalis*. All fractions were inactive against *S. aureus*. Ethyl acetate and butanol fractions showed inhibition against all except *S. aureus* and maximum of 21±0.74mm and 22±0.73mm against *E. faecalis*, respectively (table 2). *Ballota pseudodictamnus* all fractions and crude ethanolic extract were inactive against *S. typhi*. All fractions except crude extract and butanol fraction were inactive against *E. coli*. Butanol fraction showed maximum inhibition of 19±0.48mm and ethyl acetate of 18±0.49mm against *E. faecalis.* Crude extract shows maximum inhibition of 16mm against *E. coli.* Aqueous fraction was inactive against all except *K. pneumoniae* (table 3).

**Table 1**: Antibacterial activity of Root extracts of *Ballota pseudodictamnus* (L.) Benth.

| Test Micro-Organisms | Zone of Inhibition (mm) ± Standard Error Mean | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Plant Extract (µg/ µL) | | | | | | Standards (µg/ µL) | |
| | Crude | *n*-hexane | Chloroform | Ethyl acetate | *n*-butanol | Aqueous | Amx$^{\alpha}$ | Lev$^{\beta}$ |
| *Salmonella typhi* | 12±0.49* | _ | 11±0.46 | 08±0.52 | 09±0.50 | _ | _ | 28±0.82 |
| *Staphylococcus aureus* | 11±0.38 | _ | _ | 13±0.48* | _ | _ | _ | 29±0.59 |
| *Proteus mirabilis* | 10±0.42 | 09±0.62* | 12±0.57 | 09±0.38 | 12±0.58 | _ | _ | 26±0.64 |
| *Klebsiellapneumoniae* | 13±0.27 | 10±0.50* | 11±0.71 | _ | _ | _ | _ | 28±0.47 |
| *Escherichia coli* | 10±0.50 | _ | _ | 12±0.70 | 16±0.47* | _ | _ | 22±0.36 |
| *Enterococcus faecalis* | 17±0.64* | _ | _ | 11±0.61 | 11±0.26 | _ | _ | 29±0.52 |

**Table 2**: Antibacterial activity of Stem extracts of *Ballota pseudodictamnus* (L.) Benth.

| Test Micro-Organisms | Zone of Inhibition (mm) ± Standard Error Mean | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Plant Extract (µg/ µL) | | | | | | Standards (µg/ µL) | |
| | Crude | *n*-hexane | Chloroform | Ethyl acetate | *n*-butanol | Aqueous | Amx$^{\alpha}$ | Lev$^{\beta}$ |
| *Salmonella typhi* | 17±0.53 | | | 16±0.63 | 24±0.50 | | | 28±0.82 |
| *Staphylococcus aureus* | _ | _ | _ | _ | _ | _ | _ | 29±0.59 |
| *Proteus mirabilis* | 08±0.37* | _ | _ | 11±0.47* | 14±0.65 | _ | _ | 26±0.64 |
| *Klebsiellapneumoniae* | 16±0.46 | _ | _ | 13±0.84 | 16±0.71* | _ | _ | 28±0.47 |
| *Escherichia coli* | 21±0.62 | 13±0.59 | 11±0.47 | 18±0.66* | 20±0.82 | 12±0.45 | _ | 22±0.36 |
| *Enterococcus faecalis* | 23±0.95* | 15±0.74 | 14±0.63 | 21±0.74 | 22±0.73* | _ | _ | 29±0.52 |

**Table 3**: Antibacterial activity of Leaves extracts of *Ballota pseudodictamnus* (L.) Benth.

| Test Micro-Organisms | Zone of Inhibition (mm) ± Standard Error Mean | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Plant Extract (µg/ µL) | | | | | | Standards (µg/ µL) | |
| | Crude | *n*-hexane | Chloroform | Ethyl acetate | *n*-butanol | Aqueous | Amx$^{\alpha}$ | Lev$^{\beta}$ |
| *Salmonella typhi* | _ | _ | _ | _ | _ | _ | _ | 28±0.82 |
| *Staphylococcus aureus* | 14±0.56 | 16±0.62 | 15±0.71* | 16±0.46 | 12±0.82* | _ | _ | 29±0.59 |
| *Proteus mirabilis* | 15±0.69* | _ | 17±0.62 | 16±0.63 | 11±0.50 | _ | _ | 26±0.64 |
| *Klebsiellapneumoniae* | 09±0.41 | 18±0.39 | 12±0.37* | 17±0.84 | 14±0.83 | 12±0.37 | _ | 28±0.47 |
| *Escherichia coli* | 16±0.74 | _ | _ | _ | 13±0.42* | _ | _ | 22±0.36 |
| *Enterococcus faecalis* | 14±0.55* | 12±0.60 | 17±0.53 | 18±0.49 | 19±0.48 | _ | _ | 29±0.52 |

(-): No inhibition zone (7mm), $^{\alpha}$Amoxicillin: 5µg/µL, $^{\beta}$Levofloxacin: 5µg/µL, *p<0.05 was considered statistically significant.

**Table 4**: Antifungal activity of *Ballota pseudodictamnus* (L) Benth root

| Fungal Strains | Crude | *n*-hexane | Chloroform | Ethyl acetate | *n*-butanol | Water | Standard |
|---|---|---|---|---|---|---|---|
| | | | | | | | Clotrimazole* |
| *Aspergillusniger* | _ | _ | _ | _ | _ | + | _ |
| *Aspergillusfumigatus* | _ | + | _ | _ | + | + | _ |
| *Aspergillusflavus* | _ | _ | + | + | _ | + | _ |
| *Fusariumsolani* | _ | _ | _ | + | + | + | _ |

(-): Inhibition, (+): No Inhibition, *Clotrimazole (2µg/µL)

**Table 5**: Antifungal activity of *Ballota pseudodictamnus* (L) Benth stem

| Fungal Strains | Crude | *n*-hexane | Chloroform | Ethyl acetate | *n*-butanol | Water | Standard |
|---|---|---|---|---|---|---|---|
| | | | | | | | Clotrimazole* |
| *Aspergillusniger* | _ | + | _ | + | _ | _ | _ |
| *Aspergillusfumigates* | _ | + | + | + | + | + | _ |
| *Aspergillusflavus* | _ | + | _ | _ | _ | _ | _ |
| *Fusariumsolani* | + | _ | _ | _ | _ | _ | _ |

(-): Inhibition, (+): No Inhibition, *Clotrimazole (2µg/µL)

**Table 6**: Antifungal activity of *Ballota pseudodictamnus* (L) Benth leaves

| Fungal Strains | Crude | *n*-hexane | Chloroform | Ethyl acetate | *n*-butanol | Water | Standard |
|---|---|---|---|---|---|---|---|
| | | | | | | | Clotrimazole* |
| *Aspergillusniger* | _ | + | _ | + | _ | _ | _ |
| *Aspergillusfumigates* | _ | _ | _ | + | _ | + | _ |
| *Aspergillusflavus* | + | _ | _ | + | + | + | _ |
| *Fusariumsolani* | _ | _ | _ | _ | _ | + | _ |

(-): Inhibition, (+): No Inhibition, *Clotrimazole (2µg/ µL)

**Table 7**: Leishmanial parasites in RPMI 1640 media and Negative Control (DMSO)

| Media | Culture Growth After Days | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1st | 2nd | 3rd | 4th | 5th | 6th | 7th | 8th | 9th | 10th | 11th |
| RPMI(1640) | - | - | - | - | + | + | + + | ++ | ++ | ++ | ++ |
| Negative Control | - | - | - | - | - | - | - | - | - | - | - |

(-): sign indicates amastigote form of Cutaneous Leishmaniasis: (+): indicates initial conversion of amastigote into promastigotes: (++): shows promastigotes growth at peak level

**Table 8**: Antileishmanial activity of *Ballota pseudodictamnus* (L) Benth root

| Extracts | Day1-2 | Day3 | Day4 | Day5 | Day6 | Day7 | Day8 | Day9 | Day10 | Day11 |
|---|---|---|---|---|---|---|---|---|---|---|
| Crude | + | + | + | + | _ | _ | _ | _ | _ | _ |
| *n*-hexane | + | + | + | + | + | ++ | _ | _ | _ | _ |
| Chlorofom | + | + | + | + | _ | _ | _ | _ | _ | _ |
| Ethyl acetate | + | + | + | + | _ | _ | _ | _ | _ | _ |
| *n*-butanol | + | + | + | + | + | ++ | ++ | ++ | ++ | ++ |
| Aqueous | + | + | + | + | + | ++ | ++ | ++ | ++ | ++ |

**Table 9**: Antileishmanial activity of *Ballota pseudodictamnus* (L) Benth stem

| Extracts | Day1-2 | Day3 | Day4 | Day5 | Day6 | Day7 | Day8 | Day9 | Day10 | Day11 |
|---|---|---|---|---|---|---|---|---|---|---|
| Crude | + | + | + | _ | _ | _ | _ | _ | _ | _ |
| *n*-hexane | + | + | + | + | + | ++ | _ | _ | _ | _ |
| Chloroform | + | + | + | + | + | ++ | _ | _ | _ | _ |
| Ethyl acetate | + | + | + | + | + | _ | _ | _ | _ | _ |
| *n*-butanol | + | + | + | + | + | _ | _ | _ | _ | _ |
| Aqueous | + | + | + | + | + | ++ | ++ | ++ | ++ | ++ |

**Table 10**: Antileishmanial activity of *Ballota pseudodictamnus* (L) Benth leaves

| Extracts | Day1-2 | Day3 | Day4 | Day5 | Day6 | Day7 | Day8 | Day9 | Day10 | Day11 |
|---|---|---|---|---|---|---|---|---|---|---|
| Crude | + | + | + | _ | _ | _ | _ | _ | _ | _ |
| *n*-hexane | + | + | + | + | + | ++ | _ | _ | _ | _ |
| Chloroform | + | + | + | _ | _ | _ | _ | _ | _ | _ |
| Ethyl acetate | + | + | + | + | + | _ | _ | _ | _ | _ |
| *n*-butanol | + | + | + | + | _ | _ | _ | _ | _ | _ |
| Aqueous | + | + | + | + | + | + + | + + | ++ | + + | + + |

(-): Indicates absence of amastigote and promastigote, (+): Indicates presence of amastigote, (++): Indicate the presence of promastigote.

**Table 11**: Heavy Metals Concentration (mg/kg) in *Ballota pseudodictamnus* (L) Benth

| S. No | Heavy Metals (mg/kg) | *Ballota pseudodictamnus* (L.) Benth parts | | | Standard Deviations |
|---|---|---|---|---|---|
| | | Root | Stem | Leaves | |
| 1 | Mn | 12.61±1.23 | 9.54±0.06 | 4.29±0.14 | 4.207 |
| 2 | Cr | 5.34±0.65 | 3.71±0.59 | 0.84±1.03 | 2.278 |
| 3 | Ni | 4.12±0.89 | 1.04±0.79 | 0.67±0.09 | 1.894 |
| 4 | Fe | 17.17±1.22 | 13.86±0.79 | 9.37±0.96 | 3.915 |
| 5 | Cd | 3.39±0.13 | 3.05±0.10 | 2.79±0.08 | 0.301 |
| 6 | Zn | 25.57±0.36 | 23.38±0.11 | 17.32±0.18 | 4.274 |
| 7 | Pb | 5.37±0.016 | 6.53±0.013 | 8.25±0.026 | 1.449 |

Values are mean ± standard error

***Antifungal activities***

*Ballota pseudodictamnus* extracts antifungal activities were performed in comparison of Clotrimazole (2µg/µL). Leaves, stem and roots antifungal activities are given in tables 4, 5 and 6. All plant roots extracts were active except water and *n*-butanol, where as all fractions except *n*-hexane, ethyl acetate were active in the stem and leaves against *Aspergillus niger*. Roots water, *n*-hexane and *n*-butanol fractions were inactive, while the remaining were active against *A. fumigates.* Stem crude extract, while leaves all extracts except aqueous and ethyl acetate showed activities *A. fumigates.* Roots crude, *n*-hexane and butanol fractions showed significant activity against *Aspergillus flavus*. Stem all fractions except *n*-hexane, while leaves chloroform and *n*-hexane extracts revealed activities against *Aspergillus flavus*. Roots all fractions with the exception of aqueous and ethyl acetate, stem fractions except crude and leaves all extractions except aqueous divulged significant activities against *F. solani.*

***Antileishmanial bioassay***

Crude extracts and five fractions of different parts of both the plants were analyzed for identification of active fractions against leishmanial parasite. Stock solutions of extracts and different fractions were made in DMSO at a concentration of 25µg/mL and 50µg/mL and were tested against *leishmanial* parasite (table 7). In RPMI 1640 media Amastigote from vertebrate (Human) blood grows to another life stage of Promastigote in the 5$^{th}$ day of refreshing. Promastigote then divides very rapidly in 7$^{th}$ day of refreshing in th media. The antileishmanial results of Ballotapseudodictamnus are shown in the following tables. The results indicate that crude extract, chloroform and ethyl acetate fractions in root of *Ballota pseudodictamnus* have antileishmanial activity (table 8). The crude extract, *n*-butanol and ethyl acetate fractions in stem of *Ballota pseudodictamnus* show inhibition of amastigote (table 9). Crude extract, chloroform and *n*-butanol fractions in leaves of *Ballota pseudodictamnus* showed inhibition of leishmanial parasite (table 10). Ethyl acetate fraction was only an active fraction in leaves and shows good inhibition. The *n*-hexane fraction in all parts of *Ballota pseudodictamnus* does not show any promising inhibitory activity. The *n*-hexane fraction shows inhibition after 7$^{th}$ day. It starts inhibition when an amastigote form of leishmanial parasite was start converting into promastigote form. Aqueous fraction was completely inactive in all parts of the *Ballota pseudodictamnus.*

***Heavy metals analysis***

The selected medicinal plant *Ballota pseudodictamnus* was analyzed for heavy metals concentration. The concentration levels of Mn, Cr, Ni, Fe, Cd, Zn and Pb have been determined in units of mg/kg. The results are shown in table 11. From the results it was concluded that the heavy metals concentration in *Ballota pseudodictamnus* root were seen in decreasing order of Zn> Fe >Mn>Pb> Cr> Ni > Cd in general. The concentration level of heavy metals in *Ballota pseudodictamnus* stem was found to decrease in the order of Zn> Fe >Mn>Pb> Cd> Ni >Cr. The concentration level of heavy metals in *Ballota pseudodictamnus* leaves were present in decreasing order of Zn>Fe>Pb>Mn>Cd>Cr> Ni.

## DISCUSSION

*Ballota pseudodictamnus* different extracts exhibited significant antimicrobial and antiprotozoal bioassays. Crude ethanol, ethyl acetate and *n*-butanol extracts revealed noteworthy antibacterial activities against different bacterial species and were highly active (21-23mm) against *Enterococcus faecalis*. Researchers in Turkey executed antimicrobial assays of sixteen Ballota species (Saltan *et al*., 2003). Different Gram negative and Gram positive bacterial species were reviewed against crude ethanolic extract. Fungal strains were also assessed against crude extracts. *Ballota* species results revealed prominent antibacterial and antifungal activities (Basaran and Alper, 2010). Previously, plants were investigated for antileishmanial activities, but studies were carried out on whole plant. In this study different parts of plants (root, stem and leaves) were selected to execute the correlative antileishmanial study. The investigated plants show good inhibition of leishmanial parasite (Hamid *et al*., 2012). Ballota specie (*Ballot nigra*) various extracts were examined formerly against different leishmanial parasites. Crude, chloroform and ethyl acetate extracts were found active and showed significant leishmanicidal activity (Ullah *et al*., 2014). Heavy metals concentration in medicinal plants above WHO limit is an issue of great worry for the people worldwide. This is a more serious problem in Pakistan, because medicinal plants don't not checked for heavy metals and used blindly by the people or advised by local Hakims for different curing purposes (Ali *et al*., 2013). Chromium was above permissible value in all parts except *Ballota pseudodictamnus Leaves* where as the maximum permissible limit of chromium is 1.5mg/kg (Fazli *et al*., 2012). Nickel was above the WHO limit in *B. pseudodictamnus* root and the WHO Permissible value of nickel is 1.5mg/kg (Hashmi *et al*., 2007). Cadmium was above permissible value in all parts of the plant and its MPL value is 0.3mg/kg (Fazli *et al*., 2012). Lead was above permissible value in the leaves of the plant where as WHO MPL value is 10mg/kg (Ejaz *et al*., 2007).

## CONCLUSION

The results of antimicrobial (antibacterial and antifungal) study of different parts of *Ballota pseudodictamnus* indicates that crude extract; ethyl acetate and chloroform fractions were the most active fractions. The antiprotozoal

(antileishmanial) activity of *Ballota pseudodictamnus* was evaluated for the first time. The crude extract; ethyl acetate and chloroform fractions of *Ballota pseudodictamnus* show good leishmanicidal properties. But different parts have different inhibition properties. So each part of plant should be checked for antimicrobial and antiprotozoal assay. It is clear from the results that plant *Ballota pseudodictamnus*a ccumulates different phytochemicals in different parts with different concentrations. These chemicals are responsible for various biological activities by disturbing the life cycle of different microbes and kill them. The results revealed that the plants accumulate different metals in its different parts (root, stem and leaves) with diverse concentration. Only few metals (Cr, Ni, Fe, Cd, Pb) were observed above WHO limits. So each medicinal plant should be analyzed for contaminant load or heavy metals before processing it for further pharmaceutical purposes or for local human consumption.

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