Available online at www.ijpsdr.com
International Journal of Pharmaceutical Sciences and Drug Research 2014; 6(3): 229-234

Research Article ISSN 0975-248X

# Evaluation of Heavy Metal Distribution and Antibacterial Activities of Medicinal Plants *Tinospora cordifolia*, *Ocimum sanctum* and *Piper nigrum*

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**ABSTRACT**

To evaluate the inhibition and the *in vitro* interaction of the pathogens with the plant extracts, various solvent extracts of *Tinospora cordifolia*, *Ocimum sanctum*, *Piper nigrum* were screened for antimicrobial activity using agar well diffusion method. A synergistic inhibitory activity by the combination of aqueous extracts of the three plants against *Salmonella*, *Shigella* and *P. aeruginosa* was observed. The chloroform extracts in combination also showed maximum inhibition against *E. coli* but ethanolic extract in equimolar concentration showed maximum inhibition against *Staphylococcus aureus*. All these plant extracts were tested for heavy metal (HM) content as they are often contaminated during the growth, development and processing. *O. sanctum* and *P. nigrum* contained the maximum HM content while *T. cordifolia* contained less HM. The plants extracts in combination also showed low contamination with heavy metal which is a positive indication for their potential use in combating infections. These results indicate that plant extracts in combinations are less contaminated with heavy metals but they are very potent in fighting emerging pathogens.

**Keywords:** Antimicrobial, heavy metal, *Tinospora cordifolia*, *Ocimum sanctum*, *Piper nigrum*, Minimum inhibitory concentration, plant extract.

## INTRODUCTION

Medicinal plants are an attractive source to obtain a variety of drugs in developed countries. [1] According to World Health Organization, more than 80% of the world's population relies on traditional medicine for their primary healthcare needs. [2] Traditionally, medicinal plants were used in primary health due to their comparatively less side effects. [3] Based on the ethno pharmaceutical research, many new promises of potential high yielding new anti infective agents have emerged. [4] Medicinal plants are important source for the confirmation of pharmacological properties and can be natural composite sources that act as new agent against pathogens.

Many medicinal plants have been exploited for their useful constituents for screening antimicrobial properties. [5] *Ocimum sanctum* (family Lamiaceae), Holy basil, or tulasī, is an aromatic plant possessing medicinal properties against malaria, dengu fever, cough and cold [6] antibiotic and antimalarial activity [7], hyperglycemic, hypolipidemic and antioxidant property [8], hepatoprotective, renoprotective and neuroprotective activities. [9] The main chemical ingredients in this plant are eugenol, carvacrol, methyl eugenol and caryophyllene. [10] This plant is reported to exhibit antibacterial activity. Aqueous extract of fresh leaves of *O. sanctum* showed inhibition against *Escherichia coli, Proteus, Staphylococcus aureus, Staphylococcus cohni* and *Klebsiella pneumonia.* [11] Ethanolic extract of *O. sanctum* exhibited a wider inhibition zone against *Streptococcus mutans.* [12] Antibacterial activity of methanol and aqueous extract of *O. sanctum* showed zone of inhibition against *E.coli, P.mirabilis and S. aureus.* [10] The antibacterial activity of aqueous extract, chloroform extract, alcohol extract and oil obtained from leaves of *O. sanctum* reported activity against the selected bacteria like *E. coli, P. aeruginosa, S. typhimurium* and *S. aureus.* Among them, the chloroform extract was reported to exhibit the best activity. [13]

*Piper nigrum*, black pepper, is a flowering vine (family Piperaceae), cultivated for its fruit, which is usually dried and used as a spice and seasoning. A close relative of the same plant, *Piper longum*, contain piperine, piper longamine, volatile oil, resin, gums and fatty oil. [14] Black pepper is used to treat vertigo, asthma, chronic indigestion, colon toxins, obesity, sinusitis, congestion, fever, paralytic, arthritic disorders, diarrhea and cholera. [15] *P. longum* was reported as a strong antibacterial agent against *B. cereus* and *E. coli* [16], gram positive bacterial stains such as *Steptococcus faecalis, Steptococcus pyogens* and two gram negative

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bacteria such as *E. coli* and *Salmonella paratyphi* A. Hot ethyl acetate extract of *P. nigrum* was reported to inhibit *E. coli, B. subtilis* but found to be less active for *S. aureus.* [17] The acetone and dichloromethane extract of *P. nigrum* also inhibited growth of gram positive bacteria like *S. aureus, B. cereus, and S. feacalis.* [15] Ethyl acetate extract of *P. nigrum* showed inhibition against *S. aureus, P. aeruginosa* and *V. cholera.* [18] The ethanolic extract of *P. nigrum* showed maximum inhibition activity against a wide range of bacteria. [19]

*Tinospora cordifolia* (Family Menispermaceae) is known to produce diverse classes of pharmacologically active compounds. [20] In traditional medicine, it has been used in treatment of jaundice, rheumatism, urinary disorder, skin diseases, diabetes, anemia, inflammation, and allergic condition. [21] The pharmacological activity of *T. cordifolia* is related to several classes of secondary metabolites like alkaloids, glycosides, diterpenoid lactones, steroids, sesquiterpenoids, and aliphatic compounds specifically Cordifolioside A [22], Tinosporin, Columbin and Tinosporin acid. [23] The methanolic extract of *T. cordifolia* from the whole plant show significant dose dependent antibacterial activity against the gram negative microbes, especially *V. cholera, Shigella dysenteriae, E. coli* and gram positive bacteria like *Bacillus sp.* and *S. aureus.* [24] The aqueous extracts of *T. cordifolia* also inhibits both gram positive and gram negative bacteria like *Klebsiella pneumonia, E. coli, M. luteus, S. pneumoniae, S. aureus, B. cereus, L. acidophilus.* [25] Using disc diffusion method, the antibacterial activity of the aqueous, ethanol and chloroform extracts from the stems of *T. cordifolia* was reported to show inhibition studied against *E. coli, Proteus vulgaris, Enterobacter faecalis, S. typhi* (Gram-negative), *S. aureus* and *Serratia marcescens* (Gram-positive). The ethanolic extract was most active. [23]

However, the use of herbal medicines has come under scrutiny due to their perceived long term toxicity among other considerations. The causes of the toxicities, which could be attributed to the chemical and mineral contents of various plants. They are also linked to the source of the material. [26] Medicinal plants have been cited as a potential source of heavy metal toxicity to both humans and animals. [27] Because of the widespread presence of heavy metals in the environment, their residues also reach the plant and are assimilated into medicinal plants. The most common heavy metals related to human toxicity include lead, mercury, arsenic, and cadmium. Therefore, the world health organization recommends that medicinal plants, which form the raw materials for most herbal remedies, should be checked for the presence of heavy metals. Some metals like zinc, iron, copper, chromium and cobalt are toxic only at higher concentrations, while others like lead, mercury and cadmium are exclusively toxic. [28] Increasing use of traditional medicines is of special concern because they are not rigorously regulated. [29]

To date, synergistic study of plant extracts in combination is very limited and it should be conducted so that any synergistic activities may reverse the bacterial resistance. [30] The aim of the present study was to evaluate the antimicrobial properties of the plant extract singly and in combination and also characterize the impact of heavy metal contamination on the antimicrobial activity.

## MATERIALS & METHODS

**Collection of plant material**

The whole plant of *O. sanctum* & *T. cordifolia* were collected from Malaviya National Institute of Technology college campus, Jaipur during the month of February, 2014. *Piper nigrum* was collected from the local market. They were authenticated by Prof. Uma Kant of Rajasthan University, Jaipur. The herbariums are preserved in our laboratory for future reference.

**Extraction**

Green leaves of *O. sanctum* suitable for extraction were plucked. Leaves of *O. sanctum* & stem of *T. cordifolia* were washed under running tap water followed by sterilized distilled water wash. The plant materials were dried in shade for one week & then powdered with the help of mortar & pestle and preserved in air tight bottles for further use. 5 g of powdered plant material was thoroughly mixed with 200 ml of various solvents (distilled water, ethanol, methanol and chloroform) in separate beakers and extracted using soxhlet apparatus. The extracts were filtered using muslin cloth followed by filtration with Whatman No. 1 filter paper to obtain a clear filtrate. The filtrate was concentrated using rotary vacuum evaporator (IKA model RV 10). Filtrate was evaporated to near dryness to obtain a final concentration of 25 mg/ml. For further studies, the extract was reconstituted with 10 ml of solvent. Each solution was stored at 4°C in sterilized tubes until further use.

**Microorganism**

*E. coli (*MTCC-40), *Pseudomonas aeruginosa (*MTCC-424), *Salmonella sp. (*MTCC-3215), *Shigella flexneri (*MTCC-1457), *Staphylococcus aureus (*MTCC-3160) were purchased from Microbial Type Culture Collection (MTCC), Institute of Microbial Technology (IMTech), Chandigarh, India. The microorganisms were maintained in nutrient agar at 4°C until the assays were carried out. The cultures were checked for purity and biochemical tests were carried out. The cultures were grown in liquid medium at 37°C and maintained on agar slants at 2-8°C. The media, growth and incubation conditions are described in Table 1.

**Table 1: Media and incubation condition of standard reference bacterial strains**

| Bacterial Strain | Incubation Conditions | Microbiological Media Used | Reference method |
|---|---|---|---|
| *E. coli* | 37°C, 18-24 h; Aerobic | MacConkey Agar | IS: 5887 Pt1-1976 [31] |
| *Pseudomonas aeruginosa* | 37°C, 18-24 h; Aerobic | Milk Agar | IS:13428 2005[32] |
| *Salmonella sp.* | 37°C, 18-24 h; Aerobic | Deoxycholate Citrate Agar | IS: 5887 Pt.3-1999[33]. |
| *Shigella flexneri* | 37°C, 18-24 h; Aerobic | Deoxycholate Citrate Agar | IS: 5887 Pt.7 - 1999[34] |
| *Staphylococcus aureus* | 37°C, 18-24 h; Aerobic | Baird Parker Agar | IS: 5887 Pt2-1976[35] |

**Agar cup diffusion assay:** Petri Dishes containing 30 ml of cooled and molten agar media were seeded with 100µl inoculum of the respective microorganism. To each tube, approximately $3 \times 10^5$ CFU/ml of actively growing bacterial cultures in the log phase was inoculated and media was allowed to solidify. After solidification of the media, wells of 4 mm diameter were cut with the help of sterilized core borer. Wells were marked with the related solvent extract name (E= Ethanol; C= Chloroform; D= Distilled Water; X = Mixture of E + D + C). 100µl of each extract was poured in the respective well and the plates were incubated at 37°C overnight. For combinations, equal amount of 1 ml extracts were added, mixed well in a test tube and from that 100µl

was aliquoted to the respective wells. Negative control was prepared using solvents (Negative Control = Media + Solvent). Media containing microorganism were used as a positive control (Positive Control = Media + microorganism). The antibacterial activity for each of the extracts evaluated was expressed in terms of the average of the diameter of zone of inhibition (in mm.).

**Analysis of metal ions using ICP-MS:** Fresh plant materials were collected in separate polythene bags. These plant samples were washed with running tap water followed by distilled water. The samples were accurately weighed, dried in oven at 60°C for 48-72 h and later powdered using mortar pestle. 500 mg of these samples were digested separately by pressurized digestion in a microwave heated system (SINEO microwave, China) with nitric acid, hydrogen peroxide and distilled water in the ratio 5:1:1 at 180°C. The heating programme was conducted in three steps of different time scale (130°C for 10 minutes, 150°C for 5 minutes and 180°C for 25 minutes with power of single vessel (W/w)-400 at pressure of 0.2 MPa; total time 40 minutes) and at constant power supply of 400 watts. The final extracts were filtered and made up to 50 ml with Milli Q water.

This digestion method is suitable for the determination of volatile elements since the process is completed in a doubly closed system. All samples were analyzed thrice for Al (Aluminium), Ni (Nickel), Se (Selenium), Ag (Silver), Hg (Mercury), Fe (Iron), As (Arsenic), B (Boron), Ba (Barium), Cd (Cadmium), Co (Cobalt), Cr (Chromium), Cu (Copper), Mn (Manganese), Pb (Lead) and Zn (Zinc) using ICP-MS (XSERIES 2, Thermo scientific, USA) with external calibration. The ICP-MS was operated at RF forward power of 1400 W, cool gas flow rate of 13 L/min, intermediate gas flow rate of 0.7 L/min, nebuliser gas flow rate of 0.98 L/min, and dwell time of 10 ms/peak. [36] Sample blanks were run after every five determinations.

The heavy metals content obtained from ICP MS analysis in mg/L were converted into mg/Kg using Temminghoff and Houba's formula. [37]

$$\frac{\{(a - b) \times v\}}{w}$$

Where, $a$ is the concentration of the heavy metal in the sample (mg/L); $b$ is the concentration of the heavy metal in the blank (mg/L); $v$ is the total volume of digest (mL) and $w$ is the weight of the plant material (g). Counts were recorded and analyte concentration was calculated with Plasma lab software.

**Data analysis:** The data from the antimicrobial activity and heavy metal contamination from the samples analyzed in replicate were evaluated for equality of their mean values ± standard deviation. All analyses were performed with statistical software SPSS version 20 by one way analysis of variance with Turkey's posthoc tests at $p \leq 0.05$. The $p$ values were considered to be statistically significant when $p < 0.05$.

## RESULT AND DISCUSSION

Antimicrobials of plant origin have enormous therapeutic potential. They are effective in the treatment of infectious diseases while simultaneously mitigating many of the side effects that are often associated with synthetic antimicrobials. [38] In the present study, three medicinal plants were evaluated for their antibacterial potential against gram positive and gram negative bacteria and also evaluated for their synergistic effects on the microorganisms by various combinations of extracts. Data from the crude extracts of in different solvents are presented in the Table 2.

In the present investigation, it was found that *T. cordifolia* used singly against microorganisms in various solvent and aqueous extracts, showed less activity than in combination except for *P. aeruginosa* with chloroform extract. Surprisingly, this trend was also common with the other two plants. They also did not show any inhibition when the extract was used in combination. On single use of the various extracts at 25 mg/ml concentration in different solvents, *Shigella* showed maximum inhibition by the ethanolic extract of *T. cordifolia* followed by chloroform extract of *P. nigrum* and ethanolic extract of *O. sanctum*. All the three herbs are known for their antimicrobial properties specially to combat fever. [10-13, 15, 17-19, 23-25]

Results showed that the most active solvent was distilled water since water extracts inhibited a total of five bacteria viz. *Salmonella*, *Shigella*, *E. coli*, *P. aeruginosa* and *S. aureus*. Ethanol and chloroform were found to be less effective and methanol was completely inactive against all the microorganisms. *P. aeruginosa* (Gram negative bacterium) was found to be the most resistant microorganism and showed the minimum inhibition against microorganisms in various solvent and in various combinations tested. Both *Salmonella* and *Shigella,* showed their highest susceptibility and maximum inhibition when all the three extracts (*T. cordifolia, P. nigrum* and *O. sanctum)* interacted to give a synergistic effect. *S. aureus* (gram positive bacterium) was the most susceptible bacterium and there was maximum inhibition in ethanolic extract of the mixture of all the three plants (*T. cordifolia, P. nigrum* and *O. sanctum). E. coli* (gram negative bacterium) showed maximum susceptibility and was inhibited by mixture of extracts (T. *cordifolia* and *O. sanctum*; *T. cordifolia* and *Piper nigrum* and *T. cordifolia*, *P. nigrum* and *O. sanctum)* in chloroform solvent. Contrary to our report, Bhatt *et al.*, 2012; [39] found methanolic extract of *Ocimum sanctum* highly potent against *E. coli*.

Based on the growth inhibition zone diameter obtained by 25 mg/ml plant extract concentration, bacterial strains were divided into three categories, resistant (>3), intermediate (>5), and susceptible (>10). When these extracts were used in combination, their antimicrobial activity increased. Similar reports of synergistic role of microbial inhibition on combined usage of plant extracts were also reported by many earlier workers. [40-42] It was noted that when *T. cordifolia* was present in the mixture, the effect of inhibition was more pronounced. In case of *Salmonella* and *Shigella*, the maximum inhibition was shown by the aqueous mixture of all the three extracts (8±0.57; 8±1.29). Salmonellae are a group of Gram-negative food borne pathogenic bacilli that can cause a wide spectrum of diseases both in animals as well as humans. [43] Shigellosis caused by *Shigella* is an invasive disease of the human colon which is particularly prevalent among children of the developing world. [44] This aqueous mixture of three plant extracts can be focus for further study to design a novel drug against these two gram negative bacteria. The chloroform extract of the various combinations containing *T. cordifolia* showed the maximum inhibition against *E. coli* (7±1.73 in *T. cordifolia* and *O. sanctum*; 7±1.25 in *T. cordifolia* and *P. nigrum*; 7±0.81 in *T. cordifolia, P. nigrum* and *O. sanctum*). Lunavath *et al.*, (2012) [45] also reported that *T. cordifolia* showed high activity across *E. coli, P. aeruginosa* and *S. aureus* and

suggested that the aqueous and chloroform extracts showed moderate antibacterial activity. *E. coli* strains colonizing the small intestines produce enterotoxins, the major cause of diarrheal disease in humans and animals. [46] The ethanolic extract of *T. cordifolia*, *P. nigrum* and *O. sanctum* exhibited the maximum inhibition (6 ± 0.01) against *S. aureus*. *Staphylococcus aureus* causes a spectrum of human infection [47] from minor skin infections, such as pimples, impetigo, boils (furuncles), cellulitis folliculitis, carbuncles, scalded skin syndrome, and abscesses, to life-threatening diseases such as pneumonia, meningitis, osteomyelitis, endocarditis, toxic shock syndrome (TSS), bacteremia, and sepsis. Piperine from *P. nigrum* is reported to have pharmacological and antimicrobial effects. [48] Essential oil from *Ocimum* sp. containing eugenol, carvacrol, methyl eugenol, caryophyllene, is responsible for various antimicrobial properties. [49] Preliminary phytochemical screening of *T. cordifolia* revealed the presence of alkaloids, carbohydrates, glycosides, saponins and flavonoids responsible for the antimicrobial activity. The arabinogalactan present in aqueous extract of *T. cordifolia* stem has also been shown to produce immunological activity. [50] Probably all these secondary metabolites from the three plants contribute to provide a synergistic effect and greater inhibition for the microbes under investigation. It was further noticed that those extract combinations with *T. cordifolia* showed better inhibition and susceptibility to various pathogens. This study supports the traditional use of *T. cordifolia* and indicated that it contains some major bioactive compounds inhibiting the growth of microorganisms thereby proving very effective source of derived drugs.

It is clear from the above results that different extracts show varying degree of antimicrobial activity against gram positive and gram negative bacteria. These plants in combination have great antimicrobial properties and can act as antimicrobial agents. Since these extracts inhibit disease causing microorganisms, they can be used for treatment of infectious diseases caused by resistant microorganisms. Such screening can lead to successful prediction of the active compounds responsible for the antimicrobial activity. Further work is needed to isolate the active component from the plant extracts to carry out pharmaceutical studies.

In the second part of the study, the concentration of the metals were evaluated from the digested extracts of *T. cordifolia*, *Ocimum sanctum* and *Piper nigrum* singly and also in various combinations. According to WHO recommendations (1998) [51], medicinal plants which form the raw materials for the finished products may be checked for the presence of heavy metals and the regulated limit of toxic metals like lead (Pb), Cadmium (Cd), and arsenic (As) amounts to 10.00 mg/kg, 0.03 mg/kg and 1.0 mg/kg respectively.

The amount of heavy metals in the plants was analyzed to show the potential threat of their effects to the animals and human beings who consume them as such or their derived products. This study reports the investigation on the presence of fifteen (15) elements Al, Cr, Mn, Fe, Co, Ni, Cu, Zn, As, Se, Ag, Cd, Ba and Pb (Table 3) in the plant extracts.

**Table 2: Antimicrobial activity of plant extract in different solvents**

| Solvent | Microorganism | *T. cordifolia* (A) | *Piper nigrum* (B) | *Ocimum sanctum* (C) | A + C | B + C | A + B | A+B+C | Streptomycin (25mcg/disc) |
|---|---|---|---|---|---|---|---|---|---|
| Distilled water | *Salmonella* | 1 ± 0.5 | 3 ± 0.5 | 1 ± 0. 1 | 7 ± 1.25 | 5 ± 0.96 | 5 ± 0.81 | 8 ± 0.57 | 11 ± 1.52 |
| | *Shigella* | 1 ± 1.15 | 1 ± 0.58 | 1 ± 1.15 | 6 ± 0.95 | 5 ± 0.81 | 8 ± 1.73 | 8 ± 1.29 | 10 ± 0.57 |
| | *E. coli* | 2 ± 1.5 | 3 ± 1.29 | 4 ± 3.1 | 6 ± 0.57 | 3 ± 1.9 | 5 ± 1.41 | 6 ± 1.25 | 10 ± 1.0 |
| | *P. aeruginosa* | 1 ± 0.5 | 2 ± 0.5 | 2 ± 1.70 | 3 ± 0.5 | 3 ± 0.57 | 3 ± 0.50 | 3 ± 0.57 | 11 ± 1.73 |
| | *S. aureus* | 2 ± 1.25 | 3 ±1.5 | 5 ± 2.62 | 4 ± 0.57 | 3 ± 0.57 | 4 ± 0.95 | 2 ± 0.95 | 10 ± 0.57 |
| Ethanol | *Salmonella* | 1 ± 0.57 | 3 ± 0.81 | 3 ± 3.36 | 3 ± 1.73 | 3 ± 0.81 | 3 ± 0.57 | 4 ± 0.57 | 11 ± 0.57 |
| | *Shigella* | 5 ± 0.89 | 5 ± 0.70 | 7 ± 0.54 | 4 ± 1.82 | 4 ± 0.81 | 6 ± 1.50 | 6 ± 1.50 | 10 ± 1 |
| | *E. coli* | 3.2 ± 0.44 | 4 ± 1.30 | 3 ± 1.94 | 5 ± 0.50 | 6 ± 0.95 | 4 ± 0.95 | 4 ± 0.81 | 10 ± 0.57 |
| | *P. aeruginosa* | 3 ± 1.78 | 4 ± 1.51 | 4 ± 0.89 | 3 ± 0.57 | 3 ± 0.81 | 2 ± 0.5 | 3 ± 0.95 | 10 ± 1.52 |
| | *S. aureus* | 3 ± 1.51 | 4 ± 2.64 | 5 ± 1.51 | 4 ± 0.50 | 5 ± 1.00 | 5 ± 0.95 | 6 ± 0.01 | 11 ± 1.15 |
| Chloroform | *Salmonella* | 2 ± 0.70 | 4 ± 1.30 | 3 ± 0.70 | 4 ± 0.57 | 3 ± 0.57 | 4 ± 0.95 | 4 ± 0.95 | 11 ± 1.52 |
| | *Shigella* | 5 ± 1.14 | 6 ± 1.64 | 6 ± 1.00 | 5 ± 0.50 | 2 ± 0.5 | 2 ± 0.81 | 3 ± 1.00 | 12 ± 0.57 |
| | *E. coli* | 4 ± 0.89 | 4 ± 1.51 | 5 ± 0.83 | 7 ± 1.73 | 6 ± 0.81 | 7 ± 1.25 | 7 ± 0.81 | 12 ± 057 |
| | *P. aeruginosa* | 4 ± 0.70 | 1 ± 1.41 | 3 ± 0.70 | NI | NI | NI | NI | 12 ± 1.15 |
| | *S. aureus* | 3 ±1.09 | 3 ±1.14 | 5 ± 0.83 | 3 ± 0.50 | 3 ± 0.95 | 3 ± 0.50 | 3 ± 0.57 | 11 ± 2.51 |
| Methanol | *Salmonella* | NI | NI | NI | NI | NI | NI | NI | NI |
| | *Shigella* | NI | NI | NI | NI | NI | NI | NI | NI |
| | *E. coli* | NI | NI | NI | NI | NI | NI | NI | NI |
| | *P. aeruginosa* | NI | NI | NI | NI | NI | NI | NI | NI |
| | *S. aureus* | NI | NI | NI | NI | NI | NI | NI | NI |

*Mean of three values ±Standard deviation; NI= No inhibition was observed

**Table 3: Metal contents in medicinal plants used for the antimicrobial activity**

| Metals | *T. cordifolia* (A) | *P. nigrum* (B) | *O. sanctum* (C) | A+C | B+C | A+B | A+B+C |
|---|---|---|---|---|---|---|---|
| **Al** | 10.39 ± 0.50 | 32.14 ± 0.64 | 419.92 ± 1.74 | 108.54 ± 1.61 | 204.69 ± 1.3 | 26.87 ± 1.37 | 67.20 ± 0.34 |
| **Cr** | 0.22 ± 0.02 | 0.38 ± 0.09 | 0.88 ± 0.13 | 0.55 ± 0.23 | 0.53 ± 0.07 | 0.36 ± 0.02 | 0.42 ± 0.09 |
| **Mn** | 5.64 ± 0.26 | 74.29 ± 1.97 | 20.39 ± 1.48 | 12.41 ± 0.64 | 32.42 ± 0.83 | 32.58 ± 0.81 | 22.49 ± 1.62 |
| **Fe** | 11.97 ± 1.72 | 43.63 ± 1.91 | 354.49 ± 1.55 | 38.08 ± 1.80 | 81.45 ± 1.44 | 52.17 ± 1.67 | 50.72 ± 1.85 |
| **Co** | 0.02 ± 0.00 | 0.04 ± 0.03 | 0.20 ± 0.08 | 0.10 ± 0.00 | 0.12 ± 0.03 | 0.03 ± 0.00 | 0.08 ± 0.00 |
| **Ni** | 0.16 ± 0.02 | 1.36 ± 0.08 | 1.16 ± 0.12 | 0.67 ± 0.07 | 1.19 ± 0.02 | 0.66 ± 0.03 | 0.80 ± 0.14 |
| **Cu** | 5.53 ± 0.21 | 11.97 ± 1.47 | 6.64 ± 0.66 | 6.31 ± 0.24 | 9.70 ± 0.81 | 8.70 ± 0.26 | 7.34 ± 0.27 |
| **Zn** | 10.63 ± 0.10 | 6.08 ± 1.21 | 15.01 ± 0.66 | 12.54 ± 0.35 | 9.55 ± 1.19 | 8.37 ± 0.10 | 11.09 ± 1.35 |
| **As** | 0.01 ± 0.00 | 0.03 ± 0.02 | 0.07 ± 0.06 | 0.04 ± 0.00 | 0.04 ± 0.01 | 0.02 ± 0.00 | 0.04 ± 0.02 |
| **Se** | 0.02 ± 0.002 | 0.08 ± 0.031 | 0.02 ± 0.021 | 0.02 ± 0.005 | 0.05 ± 0.03 | 0.06 ± 0.01 | 0.04 ± 0.04 |
| **Ag** | 0.08 ± 0.05 | 0.05 ± 0.02 | 0.09 ± 0.05 | 0.15 ± 0.05 | 0.18 ±0.03 | 0.07 ± 0.02 | 0.35 ± 0.47 |
| **Cd** | 0.01 ± 0.00 | 0.005 ± 0.00 | 0.01 ± 0.00 | 0.01 ± 0.00 | 0.01 ± 0.00 | 0.01 ± 0.00 | 0.01 ± 0.00 |
| **Ba** | 8.70 ± 0.13 | 51.75 ± 1.19 | 61.82 ± 1.97 | 43.01 ± 0.38 | 54.99 ± 1.64 | 24.43 ± 0.90 | 37.71 ± 0.50 |
| **Pb** | 0.15 ± 0.05 | 0.07 ± 0.01 | 0.60 ± 0.05 | 0.39 ± 0.13 | 0.28 ± 0.13 | 0.08 ± 0.04 | 0.20 ± 0.003 |

All the extracts were found to contain very high amount of detectable levels of Al and Fe. *O. sanctum* leaf digest showed 419.92 mg/kg of Al, whereas the mixture of all the three plant digests showed only 67.20 mg/kg Al.

There is no known physiological role for aluminum within the body and hence this metal may produce adverse physiological effects. The impact of aluminum on neural tissues is well reported. [52] From the Table 3 it is clear that the micronutrients present in the extracts could serve as a good dietary source for essential micronutrients but they probably do not pose any serious hazards. [53]

Chromium was highest in *O. sanctum* (0.88 mg/kg) and least in *T. cordifolia* (0.22 mg/kg). But all the samples were within the maximum safe limit of 2 mg/kg for chromium. *O. sanctum* also contained highest amount of arsenic (0.07 mg/kg) but it did not cross the WHO limit of 0.3 mg/kg. [51] Supraoptimal levels of arsenic are often fatal leading to toxic symptoms include peripheral polyneuropathy, liver cirrhosis, visual disturbance, and blindness. [54]

Lead is considered very harmful for plants, animals, and particularly for microorganisms. [55] It has no physiologic role. The highest level of lead occurred in *Ocimum sanctum* digest (0.39 mg/kg), but did not exceeded the WHO standard of 10 mg/kg for lead in raw materials for herbal medicines. Levels of lead beyond the permissible values or long term use of these plants could lead to toxicity characterized by colic, anemia, headache, convulsions and chronic nephritis of the kidneys, brain damage, and central nervous system disorders. [56]

The highest levels of mercury (0.007 mg/kg) were found in combination of the entire three plant digest but were less than the permissible value of 5 mg/kg. Mercury levels in humans were above the allowable values and were associated with male infertility, inhibition of endogenous antioxidant enzymes, and brain damage among others. [57]

The level of cadmium in all the plant samples was less than the level of WHO recommended value of 0.3 mg/kg. Cadmium levels above permissible values may result in irreversible kidney damage. [58] The mineral and elements $Fe^{2+}$, $K^{+}$, $Mn^{2+}$, $Zn^{2+}$ and $Cu^{3+}$ has been classified as essential elements. This investigation confirmed that all the plant extracts were rich in iron, manganese, copper, zinc and barium (Table 3). Thus, human activities in the areas from the places of plant collection did not lead to the accumulation of arsenic and mercury in the soils. A correlation between the antimicrobial activity and the heavy metal content was also investigated. The study clearly shows that both *T. cordifolia* and the mixture of the three plants contained fewer amounts of heavy metals. *Ocimum sanctum* and *P. nigrum* contained maximum contamination with heavy metals and showed least antimicrobial activity. This mixture shows least contamination of heavy metals and probably this may be the reason for its highest intensity of inhibition for antimicrobial activity. Our findings support the view that the medicinal plants should be collected from areas not contaminated with heavy metals. It is therefore advised that, the metal content in medicinal plants should be checked for purity considering the levels of heavy metals before their use for pharmaceutical purposes.

From the above result, it can be concluded that different plant extracts in different solvents showed varying degree of antimicrobial activity against bacteria. The extracts in combination contained heavy metals below the permissible limits and hence can be used as potential candidates for screening against pathogens. The results also justify the synergistic use of *Tinospora cordifolia*, *Ocimum sanctum* and *Piper nigrum* plant extracts as a potential antimicrobial agents for the therapy of infectious diseases caused by pathogens. This mixture can be the probable candidate for further screening for novel drug.

**ACKNOWLEDGEMENT**

The authors are grateful for the kind help and cooperation from the Management, CEG test house and research centre for providing all support for this work.

**REFERENCES**

1. Borde VU, Pawar DP, Shelar SR, Apturkar RM. Antimicrobial activity of some medicinal plants. Sci. Res. Rep. 2013; 3(1):33-37.
2. Issazedeh K, Massiha A, Pahlaviani MRMK. Minimum inhibitory concentration (MIC) of Myrtus communis extract and nystatin on clinical isolated and standard strains of *Candida albicans*. J. Appl. Environ. Biol. Sci.2012; 2(9):466-468.
3. Biswas P. evaluation of antibacterial activities of leaf extract of two medicinal plants *Ocimum canum sims* and *Ocimum tenuiflorum* Linn. J. Microbiol Biotech Res. 2013; 3(3):20-23.
4. Chandra R, Dwivedi V, Shivam K, Jha AK. Detection of antimicrobial activity of *Ocimum sanctum* (Tulsi) and *Trigonella foenum graecum* (Methi) against selected bacterial and fungal strains. Research J Pharma., Bio.& Chem. Sci. 2011; 2(4): 809-813.
5. Debnath M. Clonal propagation and antimicrobial activity of an endemic medicinal plant *Stevia rebaudiana*. J Medl Plts Res 2008; 2(2): 45-51.
6. Mohan L, Amberkar MV, Kumari M. *Ocimum sanctum linn (*Tulsi)- an overview. Int. J. Pharma. Sci. Rev. & Res. 2011; 7(1): 51-53.
7. Rajeshwari S. *Ocimum sanctum* , the Indian home remedy, In Current medicinal scnene, Rajeshwari Foundation Limited Bombay. 1992.
8. Hussain AI, Anwar F, Sherazi STH , Przybylski R. Chemical composition. Antioxidant and antimicrobial activities of basil (*Ocimum basilicum*) essential oils depends on seasonal variations. Food Chem. 2008; 108: 986-995.
9. Chattopadhyay RR, Sarkar SK, Ganguly S, Medda C, Basu TK. Hepatoprotective activity of *Ocimum sanctum* leaf extract against paracetamol induced hepatic damage in rats. Ind. J. Pharmacol. 1992; 24: 163-165.
10. Singh AR, Bajaj VK, Shekhawat VS, Singh K. Phytochemical estimation and Antimicrobial activity of Aqueous and Methanolic extract of *Ocimum sanctum* L. J. Nat. Prod. Plant Resour. 2013; 3 (1):51-58.
11. Ali H, Dixit S. *In-vitro* antimicrobial activity of flavanoids of *Ocimum sanctum* with synergistic effect of their combined form. Asian Pacific J of Trop. Dis. 2012; S396-S398.
12. Agarwal P, Murlikrishnan NL. Evaluation of the antimicrobial activity of various concentrations of Tulsi (*Ocimum sanctum*) extract against Streptococcus mutans: an *in-vitro* study. Indian J Dent Res. 2010; 21(3):357-9.
13. Mishra P, Mishra S. Study of Antibacterial Activity of *Ocimum sanctum* Extract Against Gram Positive and Gram Negative Bacteria. Amer J of Food Tech, 2011; 6: 336-341.
14. Sindhu S, Manorama S, Alfamol PM. Preliminary phytochemical analysis and antimicrobial activity of *Piper longum* L. (Piperaceae) Mintage. J of Pharma & Med Sci. 2013; 2(1): 21-23.
15. Karsha PV, Lakshmi OB. Antibacterial activity of black pepper (*Piper longum* Linn.) with special reference to its mode of action on bacteria. Ind J of Nat Prod & Res. 2010;1(2): 213-215.
16. Williamson EM. Major herbs of Ayurveda Churchill living stone, Elsevier Science Ltd, London, 2002.
17. Joy B, Sandhya CP, Remitha KR. Comparison and bioevaluation of *Piper longum* fruit extracts. J. Chem. Pharm. Res. 2010; 2(4):696-706.
18. Singh C, Rai NP. *In-vitro* Antibacterial Activity of *Piper longum* L. Fruit. Int. J. Pharm. Sci. Rev. Res. 2013; 18(2): 89-91.
19. Khan M, Siddique. Antimicrobial activity of piper fruits. Natural products radiance 2007; 6(2): 111-113.
20. Molla AH, Zakaria MG , Molla MTH, Alam MT, Ahsan MS. Chemical investigations and microbial activity of *Tinospora cordifolia* miers. J. Bio. Sci. 2012; 29:153-160.

21. Singh SS, Pandey SC, Srivastava S, Gupta VS, Patro B, Ghosh AC. Chemistry and medicinal properties of *Tinospora cordifolia* (Guduchi). Indian J. Pharmacol. 2003; 35: 83-91.
22. Alam P, Ali M, Singh R, Madhurima, Ahmad S, Shakeel F. A validated HPLC method for estimation of cordifolioside A in *Tinospora cordifolia*, Miers and marketed formulations. J Chromatogr Sci. 2009; 47(10):910-3.
23. Jeyachandran R, Xavier TF, Anand SF. Antibacterial activity of stem extracts of *Tinospora cordifolia* (Willd) Hook. f & Thomson. Anc Sci Life. 2003; 23(1): 40-43.
24. Gahlawat D, Mazumdar A. *In-vitro* antibacterial activity of the whole plant of *Tinospora cordifolia*. Int J of Health and Pharma Sci.2013; 2(3): 1-6.
25. Upadhay RK, Tripathi R, Ahmad S. Antimicrobial activity of two Indian medicinal plants *Tinospora cordifolia* (family Menispermaceae) and Cassia fistula (Family: Caesalpinaceae) against human pathogenic bacteria. J of Phar Res. 2011; 4(1):167-170.
26. Lekouch N, Sedki A, Nejmeddine A, Gamon S. Lead and traditional Moroccan pharmacopoeia. Sci Total Environ. 2001; 280:39-43.
27. Dwivedi SK, Dey S. Medicinal herbs: A potential source of toxic metal exposure for man and animals in India. Arch Environ Health. 2002; 57:229-31.
28. Gajalakshmi S, Iswarya V, Ashwini R, Divya G, Mythili S, Sathiavelu A. Evaluation of heavy metals in medicinal plants growing in Vellore District. Eur J of Exp Biol. 2012; 2(5):1457-1461.
29. Dzomba P, Chayamiti T, Togarepi E. Heavy Metal Content of Selected Raw Medicinal Plant Materials: Implication for Patient Health. Bull. Environ. Pharmacol. Life Sci. 2012; 1(10): 28-33.
30. Sivananthan M .Antibacterial activity of 50 medicinal plants used in folk medicine International Journal of Biosciences 2013; 3(4):104-121.
31. IS 5887 (Part 1). Isolation, Identification and Enumeration of *E. coli*.1976 (Reaffirmed 2005).
32. IS 14843. Meat and meat products – Enumeration of *Pseudomonas* spp. 2000.
33. IS 5887 (Part 3). General Guidance on Methods for the Detection of *Salmonella*. 1999 (Reaffirmed 2005).
34. IS 5887 (Part 7).General Guidance on Methods for the Isolation and Identification of *Shigella*. 1999 (Reaffirmed 2005).
35. IS 5887(Part 2). Isolation, Identification and Enumeration of *S. aureus* and *F. streptococci.* 1976 (Reaffirmed 2005).
36. Debnath M, Chejara V, Vijaya BK, Bika D, Sajal K, Dixit A, Jain R, Jain V. Evaluation of quality and impact of untreated wastewater for irrigation. Amer J Res Com. 2014: 2(4): 200-231.
37. Temminghoff EJM, Houba VJG. Plant analysis procedures. 2nd ed. Dordrecht: Kluwer Academic Publishers. 2004.
38. Joshi B, Lekhak S, Sharma A. Antibacterial Property of Different Medicinal Plants: *Ocimum sanctum*, *Cinnamomum zeylanicum*, *Xanthoxylum armatum* and *Origanum majorana*. Kat Uni J of Sci Eng and Tech. 2009; 5(1): 143- 150.
39. Bhatt MK, Shankar MB, Saluja AK, Dholwani KK, Captain AD. Evaluation of antimicrobial activity of *Ocimum sanctum* methanolic extract. J Pharm Sci Innov. 2012; 1(4): 39-41.
40. Abeysinghe PD, Wanigatunge RP. Evaluation of antibacterial activity of different mangrove plant extracts. Ruh J of Sci. 2006; 1:104-112.
41. Kamegam N, Karuppusamy S, Prakash M, Jayakumar M, Rajasekar K. Antibacterial potency and synergistic effects of certain plant extracts against food borne diarrheagenic bacteria. Int J of biomed and pharma sci. 2008:2(2): 88-93.
42. Rivera SEV, Escobar-Saucedo MA, Morales D, Aguilar CN, Herrera RR. Synergistic effects of ethanolic plant extract mixtures against food-borne pathogen bacteria. Afr J of Biotech.2014; 13(5):699-704.
43. Sahu M, Sujatha S, Chaya DR, Parija SC. Pericardial effusion–an unusual manifestation of salmonellosis: a case report. Cases J. 2008; 1:375.
44. Fontaine A, Arondel J, Sansonetti PJ. Construction and evaluation of live attenuated vaccine strains of *Shigella flexneri* and *Shigella dysenteriae*. Res Microbiol.1990; 141(7-8):907-12.
45. Lunavath V, Porika R, Priya S , Mamidala E Preliminary phytochemical analysis and anti bacterial activity of *Tinospora cordifolia* leaf extracts. Inter J of Health and Pharma Sci. 2012; 1(4):18-21.
46. Zhang W, Robertson DC, Zhang C, Bai W, Zhao M, Francis DH. *Escherichia coli* constructs expressing human or porcine enterotoxins induce identical diarrheal diseases in a piglet infection model. Appl. Environ. Microbiol.2008; 74:5832-5837.
47. Ahn SH, Tsalik EL, Cyr DD, Zhang Y, van Velkinburgh JC, Langley RJ, Glickman SW, Cairns CB, Zaas AK, Rivers EP, Otero RM, Veldman T, Kingsmore SF, Lucas J, Woods CW, Ginsburg GS, Fowler VG Jr. Gene expression-based classifiers identify *Staphylococcus aureus* infection in mice and humans. PLoS One. 2013; 8(1):e48979.
48. Shamina A, Sarma YR. Secondary metabolites in black pepper (*Piper nigrum*) and their effect on the foot-rot pathogen Phytophthora capsici. J of Plantation Crops, 2001; 29 (2): 22-26.
49. Singh V, Amdekar S, Verma O. *Ocimum sanctum* (tulsi): Bio-pharmacological Activities. Pharmacol. 2010; 1(10):WMC001046.
50. Mishra A, Kumar S, Pandey AK. Scientific Validation of the Medicinal Efficacy of *Tinospora cordifolia*. Scientific World J. 2013(2013); Article 292934.8 pages.
51. WHO: Geneva Switzerland; 1998. Quality control methods for medicinal plant materials. available at http://whqlibdoc.who.int/publications/1998/9241545100.pdf .
52. Nayak P. Aluminum: impacts and disease. Environ Res. 2002; 89(2):101-15.
53. Abdurrahman FI, Tijjani MA, Osuji UO. Proximate content and chemical composition of *Ocimum viridis* leaf and *Ocimum gratissum* leaf. Internat Res J of Phar 2012; 3(4): 153-156.
54. Ling LJ, Clark RF, Erickson TB, Trestrail JH. philadelphia: Hanley and Belfus Inc; 2001. Toxicology secrets, 2001, pp. 153-69.
55. Khan MA, Ahmad I, Rahman I. Effect of environmental pollution on heavy metals content of *Withania somnifera*. J of the Chinese Chem Soc. 2007; 54:339-43.
57. Annan K, Dickson, Amponsah IK, Nooni IK. The heavy metal contents of selected plants sampled from different locations. Pharmacog Res. 2013; 5(2): 103-108
58. Choy CMY, Lam CW, Cheung LT, Briton-Jones CM, Cheung LP, Haines CJ. Infertility, blood mercury concentrations and dietary seafood consumption: A case-control study. BJOG. 2002;109:1121-5.