

Full Length Research Paper

## Antibacterial, antioxidant and phytochemical investigation of *Thuja orientalis* leaves

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In the present study, leaves of *Thuja orientalis* were powdered and extracted by soxhlet extractor in two solvent systems that is, (E<sub>1</sub>) ethyl acetate: chloroform: ethanol (40: 30: 30) and (E<sub>2</sub>) methanol: distilled water (70:30). This study conferred the screening of phytochemical constituents, antioxidant activity and antibacterial activity of crude E<sub>1</sub> and E<sub>2</sub> extract and its fractions. Antioxidant activity was carried out by 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay. The results indicate that E<sub>2</sub> extract (70% methanolic extract) had the highest antioxidant effect (85.25% inhibition) at 100 µg/ml concentration and the crude extracts (E<sub>1</sub> and E<sub>2</sub> extract) showed significant ( $P \leq 0.05$ ) inhibitory activity against both gram positive and gram negative organisms. It was active against *Staphylococcus aureus*, *Bacillus subtilis*, *Escherichia coli*, and *Agrobacterium tumefaciens*. The minimum inhibitory concentrations (MICs) of E<sub>1</sub> extract ranged from 0.40 to 0.85 mg/ml and E<sub>2</sub> extract 0.55 to 1.15 mg/ml. The highest antibacterial potentiality was exhibited by E<sub>2</sub> extract. The fractions also exhibited antimicrobial activity against all the selected microorganisms. The study revealed that *T. orientalis* is a promising phytomedicine for antioxidant and antibacterial activity.

**Key words:** *Thuja orientalis*, 2,2-diphenyl-1-picrylhydrazyl (DPPH), extracts, fractions, minimum inhibitory concentrations (MICs).

### INTRODUCTION

Plant derived bioactive substances are good source of medicines that play a significant role for human health and also used against different types of microbial disease (Ateş et al., 2003; Şengül et al., 2005; Nair, 2005; Dülger et al., 2005; Kumar et al., 2006; Mathabe et al., 2006). Plants have great medicinal relevance; infections have increased to a great extent and resistant against antibiotics become an ever increasing therapeutic problem (Venkatesan and Karrunakaran, 2010). Moreover, in recent years, plant extract and their phytochemicals are

getting more importance as they have the great potential sources for microbial and viral inhibitors. A number of researchers have focused their interest to investigate phytochemical constituents of plant for human health (Jasuja et al., 2012a). The bioactive constituents of plants such as tannins, flavonoids (Mandalari et al., 2007), saponins (Avato et al., 2006), terpenoids (Funatogawa et al., 2004) and alkaloids (Navarro and Delgado, 1999) have great antimicrobial activity.

Nowadays, research has gained a renewed focus to

develop herbal antioxidant formulation (Jasuja et al., 2012b; Bairwa et al., 2011). Antioxidants from plant extract are compounds that demonstrated biological activity which can protect the body from damage caused by free radical-induced oxidative stress (Sulaiman et al., 2011). Various synthetic antioxidants such as butylated hydroxytoluene (BHT), L-ascorbic acid, and butylated hydroxyanisole (BHA) were restricted by legislative rules because of doubts over their carcinogenic and toxic effects. Therefore, a considerable interest in the food industries and pharma industries has developed to find natural antioxidants to replace the synthetic ones (Dubey and Batra, 2009; Jasuja et al., 2012a).

*Thuja orientalis* (commonly- morpankhi, family- Cupressaceae) is an evergreen and monoecious tree or shrub has been used in the different activity that is, antipyretic, antitussive, astringent, diuretic, refrigerant and stomachic (Yeung, 1985). The phytoconstituents of *T. orientalis* such as flavonoids and terpenoids showed the biological activities (Hassanzadeh et al., 2001). Various phytochemical compounds had been isolated by the different parts of *Platyclusus orientalis* such as flavonoids from leaves, mono and sesquiterpenoids in essential oils of different parts of the plant (Yan-hua et al., 2006), some labdane and isopimarane diterpenoids from pericarpes and leaves (Kuo et al., 1990; Koo et al., 2002), two monogol derivatives from pollens (Ohmoto et al., 1988). The most beneficial chemical compounds of *T. orientalis* oil are camphor, fenchone, isothujone and thujone (Asili et al., 2007).

The present work deals with the antioxidant and antibacterial bioefficacy of *T. orientalis* (Figure 1) leaves extract and various fractions.

## MATERIALS AND METHODS

### Chemicals

All chemicals and reagents used in this study were of analytical grade and obtained from Merck Company, Germany.

### Plant

*T. orientalis* were collected from the National Institute of Ayurveda, Jaipur. Further, plant was identified and registered (Reg. No. RUBL21110) by Herbarium, Department of Botany, University of Rajasthan, Jaipur, India.

### Preparation of extracts

The plant material was dried under shade at room temperature for about 10 days. The dried plant samples were powdered by mechanical grinder and sieved to give particle size 50 to 150 mm. The powder was stored in polythene bags at room temperature before extraction. Powder (34 g) was filled in the thimble and extracted successively with 70% methanol (methanol: water; 70: 30) and ethyl acetate: chloroform: ethyl alcohol (40: 30: 30) solvents in soxhlet extractor for 48 h. The extracts were concentrated to dryness using rotary evaporator and crude extracts were tested



Figure 1. *Thuja orientalis*.

for antibacterial activity.

### Fractionation of the total extract

The crude ethyl acetate: chloroform: ethanol (E<sub>1</sub>) extract and 70% methanolic (E<sub>2</sub>) extract of the leaves of *T. orientalis* were subjected to thin layer chromatography using 0.25 mm thick silica gel/UV<sub>254</sub> pre-coated plate. After ascertaining the major spots on the chromatographic plate, a column chromatography (silica gel mesh 60 grade) was run using 1 g of the extract. Six fractions were obtained from each extract (E<sub>1</sub> and E<sub>2</sub>) by the column chromatography using different mobile solvent systems that is, petroleum ether-ethyl acetate (9:1, 8:2, 7:3, 6:4, 5:5, 4:6) used for E<sub>1</sub> extract whereas dichloromethane - methanol (9:1, 8:2, 7:3, 6:4, 5:5, 4:6) used for E<sub>2</sub> extract. Fractions obtained from E<sub>1</sub> and E<sub>2</sub> extracts were designated as E<sub>1</sub>F<sub>1</sub>, E<sub>1</sub>F<sub>2</sub>, E<sub>1</sub>F<sub>3</sub>, E<sub>1</sub>F<sub>4</sub>, E<sub>1</sub>F<sub>5</sub>, and E<sub>1</sub>F<sub>6</sub> for E<sub>1</sub> extract and E<sub>2</sub>F<sub>1</sub>, E<sub>2</sub>F<sub>2</sub>, E<sub>2</sub>F<sub>3</sub>, E<sub>2</sub>F<sub>4</sub>, E<sub>2</sub>F<sub>5</sub>, and E<sub>2</sub>F<sub>6</sub> for E<sub>2</sub> extract thus separated, concentrated, dried and tested for antibacterial activity.

### Phytochemical analysis

The extracts of *T. orientalis* leaves was tested for the presence of various phytoconstituents such as carbohydrate, alkaloids, glycoside, phenolic compound and tannins, saponins, flavonoids, fixed oils and fat test. All phytochemical tests were done as per the procedure given in the standard book (Practical Pharmacognosy by C. K. Kokate).

### Total phenolics

Phenolics are most important plant constituents for their scavenging ability due to their hydroxyl groups. So, phenolic compound are directly involved for anti oxidative action. Total phenolic content was determined by Folin Ciocalteu method (Akhlaghi et al., 2011) with slight modification. Dried extracts (10 mg) was dissolved in 10 ml of 70% methanol. Extract solution (1000 µl) was added followed by 0.5 ml of 10% Folin Ciocalteu reagent and after 10 min were mixed with 0.4 ml of 7.5% aqueous sodium bicarbonate. Then the

mixtures were allowed to stand for 30 min and the amount of total phenols was determined by colorimetry at 730 nm using a spectrophotometer. The results are expressed as mg of Gallic acid equivalents/g of extract (GAEs).

### Total flavonoids

The amount of total flavonoids content was determined using aluminum chloride colorimetric method with slight modifications (Quettier-Deleu et al., 2000). Dried extracts (10 mg) was dissolved in 10 ml of 70% methanol. Extract solution (1000  $\mu$ l) was mixed with 1 ml of 2% aluminum chloride and 6 ml of 5% potassium acetate. Then the mixtures were allowed to stand for incubation at room temperature for 40 min and the absorbance of the reaction mixture was measured at 415 nm versus the prepared blank using spectrophotometer. Total flavonoids were expressed in mg of quercetin equivalent per gram of the dry plant extract (mg QE/g).

### Antioxidant activity determination by DPPH free radical scavenging assay

The free radical scavenging activity of plant extract was observed with stable 1, 1-diphenyl – 2-picryl hydrazyl radical (DPPH) (Braca et al., 2002). DPPH solution was prepared in solvent. The mixture was incubated for 20 min in the dark at room temperature. Test solution of plant extract ( $E_1$  and  $E_2$  extract) was prepared into two different concentrations (50 and 100  $\mu$ g/ml). Free radical scavenging activity was determined spectrophotometrically by monitoring the decrease of the absorbance at 517 nm. Lower absorbance of the test sample indicates a higher free radical scavenging activity. Ascorbic acid was used as control and the blank reaction only containing the solvent which was used for the extraction. The percent DPPH radical scavenging activity was calculated in terms of percentage inhibition using the following equation:

$$\% \text{ inhibition} = [100 \times (A_c - A_s)] / A_c$$

Where  $A_c$  is the absorbance of the control and  $A_s$  is the absorbance of sample. Tests were carried out in triplicate.

### Micro-organisms

The bacterial strains *Agarobacterium tumefaciens* and *Bacillus*

*subtilis* were collected from University of Rajasthan and *Staphylococcus aureus* and *Escherichia coli* were isolated from soil sample of Jharna village in the laboratory of Jayoti Vidyapeeth Women's University, Jaipur. All the strains were confirmed by cultural and biochemical studies (Tambekar and Dahikar, 2011) and maintained in yeast extract mannitol and nutrient agar media slants at 4°C for further use.

### Preparation of the Inoculum

A loopful of the test organism was taken from their respective agar slants and sub-cultured into test tubes containing nutrient broth and yeast extract mannitol broth for bacteria. The test tubes were incubated for 24 h at 37°C. The obtained bacteria in the broth were standardized using normal saline to obtain a population density of  $10^8$  cfu/ml (Kim et al., 2012).

### Determination of antimicrobial activity

The disk diffusion method was used for evaluation of antimicrobial activity (Rios et al., 1988; Kim et al., 2012). Standard size Whatman No. 1 filter paper discs, 5.0 mm in diameter, sterilized by moist heat at 121 lb in an autoclave for 15 min were used to determine antibacterial activity. Muller hinton agar (MHA) medium for *E. coli*, *B. subtilis*, *S. aureus* and yeast extract mannitol (YEM) medium for *A. tumefaciens* were prepared for disc diffusion test. After sterilization, it was poured into sterilized petri plates and allowed to solidify. Then, one day old fresh culture of bacteria will be used for inoculums preparation. A suspension that was just turbid (~0.5 McFarland standard) by visual inspection was prepared by suspending bacteria in 0.9% NaCl solution and the homogeneous suspension was used for inoculation. Using a sterile cotton swab, bacterial cultures were swabbed on the surface of sterile agar plates. The dried plant extracts were re-suspended to 20 mg/ml in Dimethyl sulfoxide (DMSO) and sonicated to dissolve and sterilize the extracts. Sterile 5 mm discs were impregnated with 50  $\mu$ l of extract and placed on the surface of agar plates inoculated with a microbial culture. Each extract was tested in triplicate. Streptomycin sulphate (40 mg/disc) served as a control. The plates were incubated at 37°C for 24 h. The diameter of the inhibition zones was measured in millimeter. Three replicates were kept in each case and average values were calculated. The activity of extract was measured by the following formula:

$$AI \text{ (Activity index)} = \frac{\text{Zone of inhibition of the extract}}{\text{Zone of inhibition obtained for standard antibiotic drug}}$$

### Minimum inhibitory concentration (MIC) of extract

The minimum inhibitory concentration (MIC) value of extracts was determined by serial dilution method (Ibekwe et al., 2001; Sung et al., 2006). The extracts were diluted to make different concentrations. Serial dilutions of each extract were individually placed in tubes labeled 1 to 6. Tube 1 was filled with 2 ml of Muller Hinton broth including extract. 1 ml of extract from tube 1 was transferred to tube 2 and diluted with Muller Hinton broth. This procedure was repeated from tube 2 to 6 and each tube was filled with 1 ml Muller Hinton broth including bacterial suspension. The resulting mixture was incubated at  $37 \pm 0.1^\circ\text{C}$  for 24 h. Turbidity was

taken as an indication of growth and the lowest concentration which remained clear was recorded as the relative minimum inhibitory concentration. This test was repeated in triplicates.

### Statistical analysis

Statistical analysis was carried out by statistical package for social sciences (SPSS) version 16.0 software for antimicrobial activity of plant extract ( $E_1$  and  $E_2$  extract). The result express as arithmetic mean  $\pm$  standard deviation (SD) and analysis was performed using student T-test and analysis of variance (ANOVA).

**Table 1.** Preliminary phytochemical test of extract of *Thuja orientalis*.

S/No.	Phytochemical tests		Results
	Chemical constituents	Test	
1	Carbohydrate	Fehling's test	Positive
2	Alkaloids	Mayer's test, Wagner's test, Hager's test	Positive
3	Glycoside	Molish' test, Keller-Killani test	Positive
		With con. H <sub>2</sub> SO <sub>4</sub>	Negative
4	Phenolic compound and Tannins	With ferrous sulphate and sodium potassium Tartarate, lead acetate test, ferric chloride test	Positive
5	Saponins	Foam test	Positive
6	Flavonoids	With NaOH, with lead acetate test	Positive
		With H <sub>2</sub> SO <sub>4</sub> , zinc test	Negative
7	fixed oils and fat test	Spot test	Positive

## RESULTS AND DISCUSSION

The preliminary phytochemical analysis of the plant extract of *T. orientalis* showed the presence of carbohydrates, alkaloids, glycoside, phenolic compound and tannins, saponins, flavonoids, fixed oils and fat test (Table 1) (Practical Pharmacognosy by C. K. Kokate). The total contents of phenolic and flavonoids are shown in Table 2. The average total phenols content (mg GAE/g crude extract) and total flavonoids content (mg QE/g crude extract) of E<sub>2</sub> extract was significantly high (95 and 10 mg/g) than E<sub>1</sub> extract (70 and 8 mg/g) (Quettier-Deleu et al., 2000; Akhlaghi et al., 2011). Determination of antioxidant activity of *T. orientalis* leaves extract using stable 2, 2'-diphenyl- 1- picryl hydrazyl (DPPH) assay spectrophotometrically. The DPPH free radical is a very fast method for determination of antioxidant activity (Dubey and Batra, 2009). Data presented in Table 3 show the inhibition percentage of DPPH radical generation by 50 and 100 µg/ml of *T. orientalis* plant extract comparable to the same dose of ascorbic acid. However, results of antioxidant activity showed that the E<sub>2</sub> extract (70% methanolic extract) of *T. orientalis* had higher inhibition percentage (70.45 and 85.25%) than control (ascorbic acid). The presence of antibacterial substances in the medicinal plants are well established as they have provided a source of inspiration for novel drug compounds as herbal medicine have made significant contribution towards human health (Srivastava et al., 1996).

This study also revealed that the *T. orientalis* leaves extract may be useful as a broad spectrum antibacterial agent following extensive investigation (Figures 2 and 3). The inhibitory effects of E<sub>1</sub> and E<sub>2</sub> extract of *T. orientalis* against different test microorganisms are shown in Table 4. The E<sub>1</sub> and E<sub>2</sub> extract indicated significant ( $P \leq 0.05$ ) antibacterial activity (ZOI 40 and 38 mm) against *A. tumefaciens* and *S. aureus* (ZOI 35 mm of E<sub>1</sub> extract) than that of the standard drug solution (ZOI 30 mm). Moreover, Figure 2 showed that E<sub>1</sub> extract may contain

**Table 2.** Total phenolics and flavonoids content in E<sub>1</sub> and E<sub>2</sub> extract of *Thuja orientalis*.

Plant extract	Phenolic (mg/g)	Flavonoids (mg/g)
E <sub>1</sub> extract	70	8
E <sub>2</sub> extract	95	10

**Table 3.** DPPH radical scavenging activity of E<sub>1</sub> and E<sub>2</sub> extract of *Thuja orientalis* at different concentration.

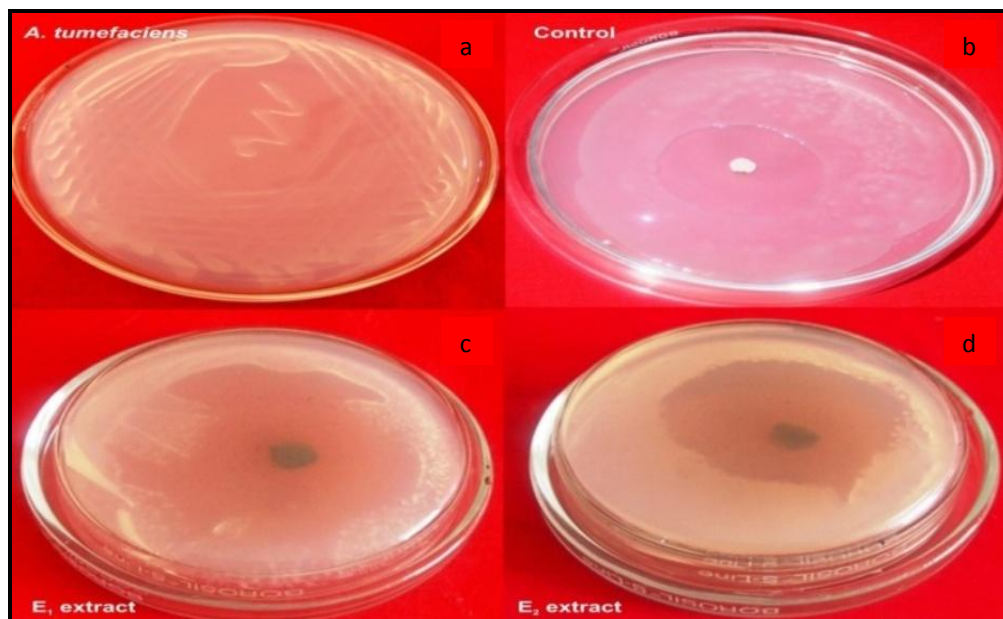
Test compound	Antioxidant activity	
	Inhibition percentage (%)	
	50 µg/ml	100 µg/ml
E <sub>1</sub> extract	50.25	74.27
E <sub>2</sub> extract	70.45	85.25
Ascorbic acid	55.15	75.25

more antimicrobial compounds as compared to E<sub>2</sub> extract which better inhibit the growth of *A. tumefaciens*. The results of antimicrobial activity of extracts of *T. orientalis* were also conferring with previous studies.

Bissa et al. (2008) reported that petroleum ether extract of *T. orientalis* stem exhibited maximum antibacterial activity against *A. tumefaciens*. In the similar way, Chen et al. (1989) reported the antibacterial activity of *T. orientalis* against *Streptococcus mutans*. Further, Hafez and Abdel- Salam (2004) also examined the chemical composition and antimicrobial activity of the volatile constituents of *T. occidentalis*. Studies on pharmaceutical, pharmacological and clinical properties of plants were reported by Naser et al. (2005). However, in the present study, E<sub>1</sub> (ethyl acetate: chloroform: ethanol) extract of *T. orientalis* leaves showed the highest antibacterial activity than E<sub>2</sub> extract (methanol: distilled water). Figure 3 showed that 10% fraction of E<sub>2</sub> extract (DCM: methanol; 9:1) exhibited the maximum antibacterial activity against *E. coli* as compared to all other

**Table 4.** Minimum inhibitory concentration (MIC), and Active index results of E<sub>1</sub> & E<sub>2</sub> extracts of *Thuja orientalis* Linn. tested against the four microorganisms.

Bacteria used	MIC (mg/ml)		Active index	
	E <sub>1</sub> extract	E <sub>2</sub> extract	E <sub>1</sub> extract	E <sub>2</sub> extract
<i>Escheriachia coli</i>	0.85	1.15	0.825	0.45
<i>Bacillus subtilis</i>	0.50	0.97	0.9090	0.6363
<i>Staphylococcus aureus</i>	0.40	0.87	1.166	0.833
<i>Agarobacterium tumefaciens</i>	0.45	0.55	1.142	1.08



**Figure 2.** Antibacterial activity of E<sub>1</sub> and E<sub>2</sub> extract of *Thuja orientalis* against *Agarobacterium tumefaciens*.

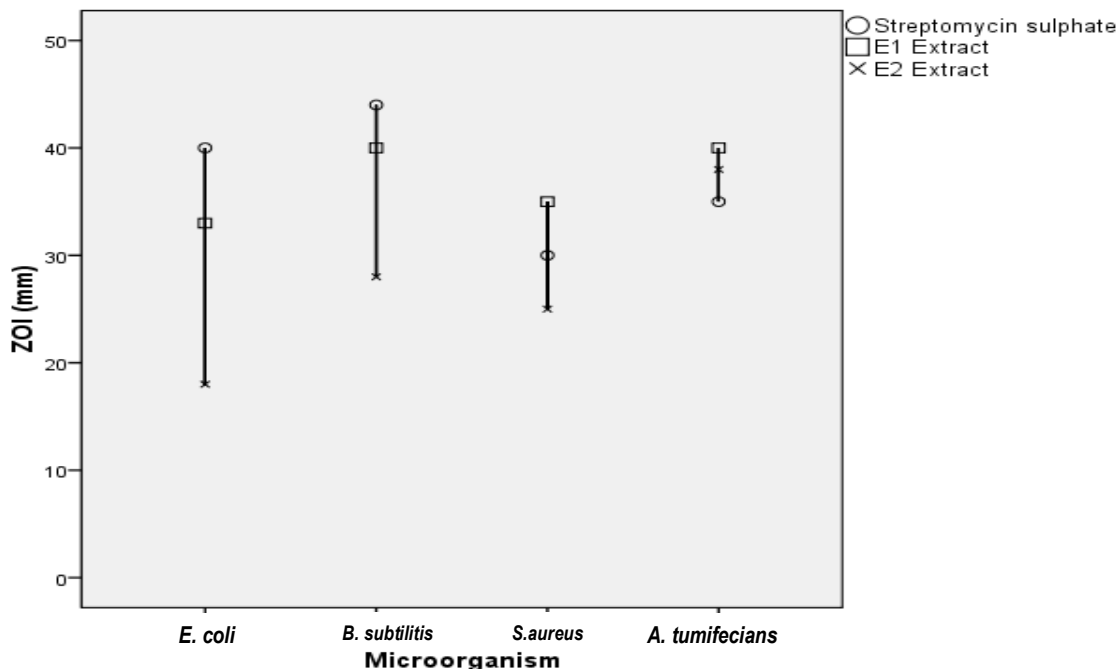


**Figure 3.** Antibacterial activity of *Thuja orientalis* fraction of E<sub>2</sub> extract against *E. coli*.

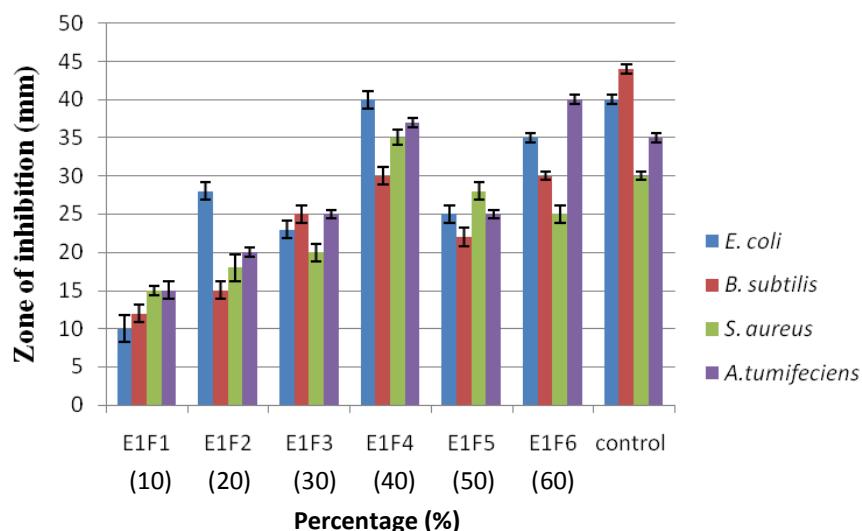
fractions. Analysis of variance (ANOVA) result revealed that E<sub>2</sub> extract had shown F value 4.027 whereas E<sub>1</sub> had shown F value 1.668. Both extract had shown significant inhibition against *A. tumefaciens* and *S. aureus* which was greater than control value.

In ANOVA study, R squared value was observed as 0.98. E<sub>1</sub> extract had shown better inhibitory activity as

compared to control (streptomycin sulphate) on *S. aureus* and *A. tumefaciens* (Figure 4). E<sub>1</sub> extract was observed as the significant minimum inhibitory concentration (MIC) against *S. aureus* (MIC 0.40 mg/ml and active index 1.16 mm) and *A. tumefaciens* (0.45 mg/ml and active index 1.142 mm). Almost similar inhibitory effect was observed when disc of E<sub>1</sub> extract was applied on *B. subtilis* (ZOI 40



**Figure 4.** Antibacterial activities (ZOI) of E<sub>1</sub> and E<sub>2</sub> extract of *Thuja orientalis* leaves. Each point represents the mean ± SEM value (n=3). R Squared = 0.986 (Adjusted R Squared = 0.973)

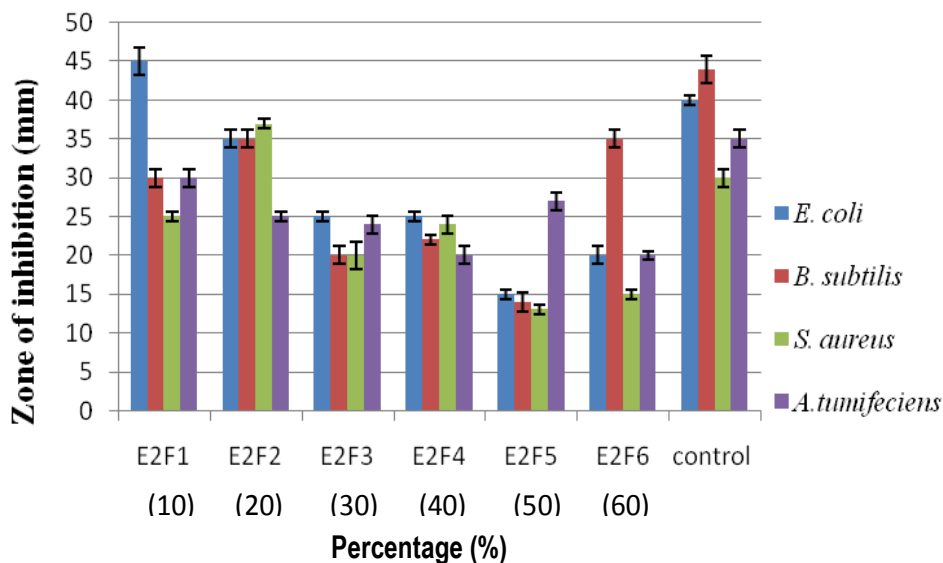


**Figure 5.** Antibacterial activity of fractions of petroleum ether: ethyl acetate (9:1-4:6) of E<sub>1</sub> Extract. Data are mean ± SD (Standard deviation); n=3.

mm, MIC 0.50 mg/ml and active index 0.9090 mm) when compared to control (ZOI 44 mm). E<sub>2</sub> extract exhibited maximum antibacterial activity against *A. tumefaciens* (ZOI 38 mm, MIC 0.55 mg/ml, active index 1.08 mm) when compared to control (ZOI 35 mm) whereas E<sub>2</sub> extract was found less effective against *E. coli* (ZOI 18 mm, MIC 1.15 mg/ml and active index 0.45 mm). All bacteria showed the growth inhibition diameter ranging

from 30 to 44 mm against the control (streptomycin sulphate 1 mg/10 ml).

Results showed that different fraction of petroleum ether: ethyl acetate (E<sub>1</sub>F<sub>1</sub>; 9:1, E<sub>1</sub>F<sub>2</sub>; 8:2, E<sub>1</sub>F<sub>3</sub>; 7:3, E<sub>1</sub>F<sub>4</sub>; 6:4, E<sub>1</sub>F<sub>5</sub>; 5:5, E<sub>1</sub>F<sub>6</sub>; 4:6) of E<sub>1</sub> extract and dichloromethane: methanol (E<sub>2</sub>F<sub>1</sub>; 9:1, E<sub>2</sub>F<sub>2</sub>; 8:2, E<sub>2</sub>F<sub>3</sub>; 7:3, E<sub>2</sub>F<sub>4</sub>; 6:4, E<sub>2</sub>F<sub>5</sub>; 5:5, E<sub>2</sub>F<sub>6</sub>; 4:6) of E<sub>2</sub> extract displayed reasonable activity against selective bacteria (Figures 5



**Figure 6.** Antibacterial activity of fraction of dichloromethane: methanol (9:1 to 4:6) of  $E_2$  extract.

and 6).  $E_1F_1$  fraction of  $E_1$  extract showed the poor activity against all selected bacteria whereas  $E_1F_4$  showed the good activity against *E. coli* (ZOI 40 mm), *S. aureus* (ZOI 35 mm), *A. tumefaciens* (ZOI 37 mm).  $E_1F_6$  fraction of  $E_1$  showed the good activity against *A. tumefaciens* (ZOI 40 mm) better than control drug streptomycin sulphate (ZOI 35 mm). In case of DCM: methanol fraction,  $E_2F_1$  showed the highest zone of inhibition against *E. coli* (ZOI 45 mm) whereas  $E_2F_5$  and  $E_2F_6$  fraction of  $E_2$  extract showed poor activity against *S. aureus* and  $E_2F_2$  fraction showed the best antibacterial activity against *S. aureus* (ZOI 37 mm).

The antibacterial drug preparations were used as alternative medicines for synthetic drugs. It is suggested that plant extracts are economic and effective herbal drugs may be prepared for microbial infections. The finding of this study indicates the effective *in vitro* activity of the crude extracts of *T. orientalis* against selective bacterial species. This study demonstrates the potential of traditional medicine system.  $E_1F_4$  and  $E_1F_6$  fractionation of  $E_1$  extract and  $E_2F_1$ ,  $E_2F_2$  and  $E_2F_6$ , fractionation of  $E_2$  extract found to be most effective against selected microorganisms.

## Conclusion

Moreover, *T. orientalis* can be considered as good sources of natural compounds with significant antioxidant and antimicrobial activity, and this can be attributed to the high percentage of polyphenolic compounds content. Therefore, with attention to results obtained, the present study provides a basis for the isolation and identification of compounds of biological interest from *T. orientalis* for its potent activity.

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