



Comparative analysis of the antibacterial efficacy and bioactive components of *Thuja occidentalis* obtained from four different geographical sites

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Abstract

The purpose of this study was to determine whether or not there were significant differences in the antibacterial potential of *Thuja occidentalis* collected from four distinct geographical sites, namely Chamba (Himachal Pradesh, India), Jalandhar (Punjab, India), Aurangabad (Bihar, India) and Kakching (Manipur, India). The plant extracts were prepared in three different solvents: ethanol, methanol, and acetone. The antibacterial potential of the plant extracts was tested against five different bacterial species using well diffusion test. The minimum inhibitory and bactericidal concentrations of the plant sample exhibiting maximum zone of inhibition against different bacterial strains were calculated. Further, the total phenols, flavonoids, and antioxidant efficacy (using DPPH assay) were also analysed biochemically. The activity of different antioxidant enzymes including SOD, CAT and APX were also recorded as these enzymes protect the cells from free radical damage. GC–MS analysis was also performed on all plant extracts to identify the bioactive components. The results showed that the *T. occidentalis* collected from the Kakching, Manipur, East side of India showed the highest zone of inhibition against all the bacterial strains, followed by Chamba, Jalandhar, and lastly Aurangabad. To analyse the impact of phytochemicals on the antibacterial efficacy, a correlation was drawn between the biochemical parameters and zone of inhibition using Karl Pearson's method. Most bacterial species demonstrated a positive correlation between antibacterial effectiveness (zone of inhibition) and biochemical markers. The GC–MS study revealed positive correlation between zone of inhibition and peak area percentages of α -Pinene, β -caryophyllene, Germacrene-D, and Humulene in all bacterial species indicating that these chemicals may play a key role in the bactericidal potential of *T. occidentalis*. Based on the results of this investigation, it is evident that the antibacterial effectiveness of *T. occidentalis* varies with its geographical location which may be attributed to the differences in the phytochemical makeup.

Keywords Antibacterial · Antioxidant · DPPH · GC–MS · Phenols · *Thuja occidentalis*

Introduction

Herbal remedies are becoming more popular across the world for several reasons. Majority of the population in Asian and African countries relies on plants and plant extracts to cure a wide range of illnesses [1]. We have depended on plant remedies to heal a broad variety of diseases since the Vedic era of India and China, much earlier. They are among the most effective treatments now available in terms of long-term cure, effectiveness, safety, and adverse effects [2]. To counter chemical medication side effects and gain substantial financial rewards, now western nations have grown their reliance on herbal medicine. Income from the sale of plants and plant by-products is difficult to put a dollar value on, although it is clear that the pharmaceutical

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industry grows by more than 4 per cent annually [3]. The usage of herbal medicines and phytonutrients, often known as nutraceuticals, continues to rise fast across the globe, including in the United States, where a growing number of individuals are turning to them for help with a wide range of medical issues [4]. In response to the substantial and increasing public interest in and use of complementary and alternative medicine (CAM) therapies in the United States, the National Center for Complementary and Alternative Medicine (NCCAM), along with 15 other NIH centres and institutes, and the Agency for Healthcare Research and Quality, asked the Institute of Medicine (IOM) to form a committee to investigate related scientific, policy, and practise questions [5]. The European Directive 2004/24/EC released in 2004 by the European Parliament and by the Council of Europe provides the basis for the use of herbal medicines in Europe going forward. The Directive establishes that herbal medicines released in the market need authorization by the national regulatory authorities of each European country and that these products must have a recognized level of safety and efficacy [6].

Since the rise in antibiotic use in response to an increase in antibiotic-resistant bacteria is a major cause for concern, and since the prevalence of new microbial diseases necessitates the use of antibiotics, the development of a pathogen-specific medication that is effective while causing minimal or no side effects is highly desirable. In this era, there is a plant present in the lap of Mother Nature that may provide medicines with little side effects, *Thuja occidentalis*.

Thuja trees are members of the order Pineales and the Cupressaceae family [7]. Native to eastern North America, the *T. occidentalis* tree is now widely grown for its decorative value in places like India, Brazil, and Europe [8, 9]. Arbour vitae and white cedar are two more names for the same plant that are often used [10]. In the United States, it is known by a variety of names, including Swamp Cedar, American Arborvitae, and Eastern Arborvitae. *Thuja* is known by several other names in other languages as well, including Thuya du Canada in French, Lebensbaum in German, Thuja and Tuya in Spanish, and Morpankhi in India [11]. In the sixteenth century, native Indians in Canada recognized this plant as a medicine for the first time and also found it effective in the treatment of scurvy-induced weakness [12]. Terpenoids, steroids, flavonoids, and polysaccharides are the compounds that have been found in *T. occidentalis* according to previous phytochemical reports [13]. Previous research has indicated that *T. occidentalis* essential oil may be used to effectively treat ovarian cysts [14]. When combined with other immunomodulatory herbs, *T. occidentalis* may also be used to treat acute and chronic upper respiratory tract infections [15]. Liver illness, bronchitis, psoriasis, amenorrhoea, cystitis, uterine cancer, diarrhoea, and rheumatism have all been treated with *T. occidentalis*

in traditional medicine [16]. Fungal infections, cancer, and intestinal worms have all been treated using essential leaf oil [17]. *T. occidentalis* have been found to be effective against *Saccharomyces cerevisiae*, *Macrophomina*, *Fusarium solani*, *Aspergillus niger*, *Aspergillus flavus*, *Aspergillus parasiticus*, and *Trichophyton rubrum*, among others [18]. It also contains antiviral chemicals that have a high in vitro therapeutic activity against herpes simplex virus, many of whose results are either old or published in German [19]. It was found to exhibit an emmenagogue action against blocked menses; these compounds are found to be effective in the treatment of gynaecological illnesses because they alleviate the stomach discomfort, cramps, nausea, and exhaustion that are associated with menstrual cycles. They regulate the menstrual cycle and maintain female reproductive organ health [14]. Due to widespread overuse of antibiotics, microorganisms have developed an unprecedented level of resistance to antibiotics. This marks the beginning of the "post-antibiotic period" during which some bacterial infections may no longer be treated, as they were in the past [20].

As a result of its varied topography, India has a wide range of climates, from tropical in the country's southernmost regions to temperate and alpine in the Himalayan highlands [21]. Changes in temperature and wind patterns are linked to climate change, and the plant's phytoconstituents may make a difference because of these oscillations. A multitude of environmental variables, including location, climate, soil type, sun exposure, and grazing stress have been shown to influence the phytochemical composition of plants in previous research [22]. The antimicrobial effectiveness may be affected by the variety in phytochemicals in the same plant obtained from distinct geographic sites. We found no previous research that compared the antibacterial activity of the same plant grown in areas with wildly diverse temperatures and environmental conditions. In light of the above knowledge, the present research work was done to compare the bactericidal potential of *T. occidentalis* from four different geographic locations (with varied climatic conditions) in India and correlate the same to the phytochemical composition of the plants. The selection of *T. occidentalis* was based on the plant's long history of usage in herbal medicine for the treatment of a broad spectrum of ailments, as stated above.

Material and methods

Sources of test bacterial strains

The Microbial Type Culture Collection and Gene Bank (MTCC) in Institute of Microbial Technology (IMTECH), Chandigarh, provided a total of five different bacterial cultures in lyophilized form for the present research work. They

Table 1 Temperature and incubation time required for the growth of bacterial cultures

| S. No | Bacterial culture | Temperature required | Incubation time |
|-------|---|----------------------|-----------------|
| 1 | MTCC-96 (<i>Staphylococcus aureus</i>) | 37°C | 48 h |
| 2 | MTCC-103 (<i>Pseudomonas fluorescens</i>) | 25°C | 48 h |
| 3 | MTCC-118 (<i>Escherichia coli</i>) | 37°C | 48 h |
| 4 | MTCC-741 (<i>Pseudomonas aeruginosa</i>) | 37°C | 12 h |
| 5 | MTCC-1272 (<i>Bacillus cereus</i>) | 30°C | 24 h |

Table 2 The description of geographical sites from which plant samples were collected [56]

| State | District | Geographical Site | Elevation above sea level | Latitude | Longitude | Average precipitation | Average temperature |
|------------------|------------|-------------------|---------------------------|------------|------------|-----------------------|---------------------|
| Himachal Pradesh | Chamba | GS-1 | 930.4 m | 32.5533633 | 76.1258083 | 30.1 mm | 19.46°C-30.54°C |
| Punjab | Jalandhar | GS-2 | 248.48 m | 31.3260152 | 75.5761829 | 85.21 mm | 20.65°C- 32.78°C |
| Bihar | Aurangabad | GS-3 | 108 m | 24.7033198 | 84.3542049 | 22.56 mm | 24.1°C- 33.66°C |
| Manipur | Kakching | GS-4 | 776 m | 24.496869 | 93.9830529 | 205.0 mm | 14.53°C-27.28°C |

were revived by incubating them overnight in nutrient broth at the temperatures indicated in Table 1. Using a standard of 0.5 McFarland, each strain was adjusted to a concentration of 10^8 CFU/ml in Mueller–Hinton Broth [23].

Sources of plant samples

The plant samples (leaves of *T. occidentalis*) were collected from four different geographical locations namely Chamba (Himachal Pradesh), Jalandhar (Punjab), Aurangabad (Bihar), and Kakching (Manipur) of India. These are referred to as GS-1, GS-2, GS-3, and GS-4, respectively. The sites were chosen because of the differences in climatic conditions. The details of the geographical locations are given in the Table 2 and Fig. 1. All the plant samples were validated by Dr Inam Mohammad, an experienced plant taxonomist at DAV University, Jalandhar, Punjab, India.

Preparation of plant extracts

All the plant leaves were washed properly and then shade dried for 20–25 days. 10 g of each plant material was dissolved in 100 ml of three different solvents namely ethanol, methanol, and acetone (referred to as S1, S2, and S3, respectively) and allowed to settle at a temperature of 37 °C for 24 h, 48 h, 72 h, and 96 h separately. In order to separate the liquid extracts from the solid residue, the liquid extracts were filtered using a Whatman No. 1 filter and then evaporated to dryness. Until additional analysis could be conducted, the crude extract was kept in a 4 °C refrigerator [24]. The extraction yield of plants collected from different sites was calculated using the following equation [25]:

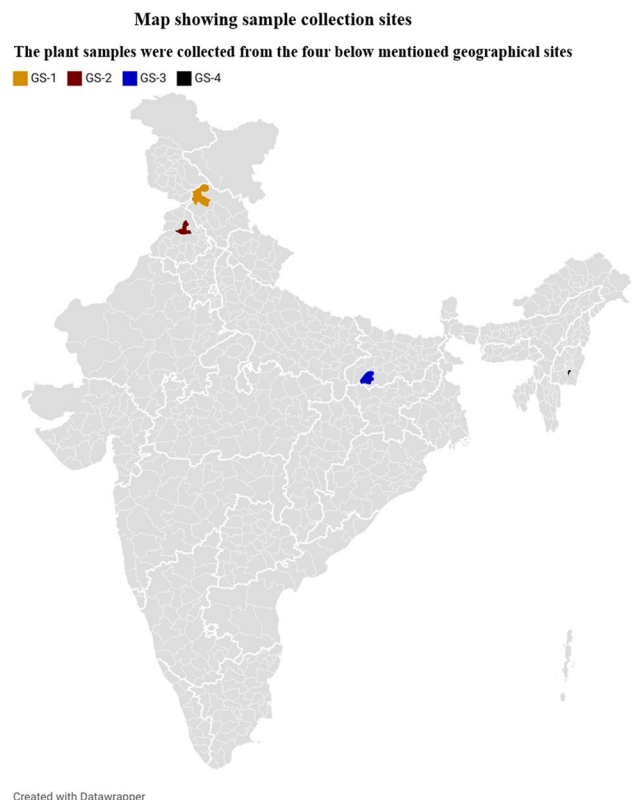


Fig. 1 Map showing the four different geographical sites from which *T. occidentalis* samples were collected for the present investigation. GS-1: Chamba, Himachal Pradesh, India. GS-2: Jalandhar, Punjab, India. GS-3: Aurangabad, Bihar, India. GS-4: Kakching, Manipur, India

$$\text{Yield (\%)} = D \times 100/d,$$

where D is the weight of the extract remaining after the solvent has been evaporated, and d is the dry weight of the plant powder before extraction.

Antibacterial efficacy

The antibacterial effectiveness of plant extracts was evaluated by conducting the well diffusion test. Nutrient agar was utilized for the cultivation of bacterial isolates. Spreading 100 μl of the microbial inoculum over the agar plate was the initial step in the process of inoculation. After solidification, a hole in the culture plate was made aseptically using a borer with a diameter of 6 mm. Each well received about 20 μl of the extracted plant material, which was then incubated for 24 h at 37 °C. Extracts' antibacterial activity was determined by measuring inhibition zones after they diffused into an agar medium; the zones were measured in millimetres using a transparent ruler [26]. In every experiment, ampicillin was utilized as a positive control, and all solvents (acetone, methanol, and ethanol) were employed separately as negative controls.

Estimation of minimum inhibitory concentration (MIC)

The MIC is the lowest dose of an antimicrobial compound that inhibits the growth of bacteria to a detectable level after an incubation time of 24 h. The MIC of plant extracts was measured using sterile 96-well plates. 0.5 ml of the test bacterial strain (prepared as mentioned above in Sources of Bacterial Strains) and 0.5 ml of plant extract was added into each well by serial dilution from 100% to 0.097% (100 mg/ml–0.097 mg/ml). The control well contained 0.5 ml of Mueller–Hinton broth only. The 96-well plates were then incubated for 24 h at 37°C. The MIC was observed by analysing the amount of visible turbidity [27].

Evaluation of minimum bactericidal concentration (MBC)

After the minimum inhibitory concentration (MIC) of the plant extract was determined, 50 μl solution from each of the wells that did not exhibit any obvious bacterial growth was spread onto Mueller Hinton agar plates. The plates were then placed in an incubator at 37 °C for 24 h. The plates were then checked for bacterial growth. MBC endpoint refers to the point at which 99.9% of a bacterial population may be eradicated by an antimicrobial agent at the lowest effective concentration of the agent [28].

Estimation of total phenols

The total phenols of *T. occidentalis* extracts obtained from different geographical sites prepared in different solvents were determined using the previously described method [29, 30]. The extract was incubated at room temperature for 40 min with 0.25 ml of 1N Folin-Ciocalteu reagent and 1.25 ml 20% sodium carbonate. The absorbance was read at 725 nm using a UV–Vis spectrophotometer.

Estimation of total flavonoids

The flavonoids from *T. occidentalis* extracts prepared in three different solvents obtained from four different geographical sites were estimated by Chang et al. [31]. 0.1 ml of 10% aluminium chloride and 1 M potassium acetate were added to the plant extracts followed by an incubation period of 30 min at room temperature. The absorbance was measured at 415 nm.

Estimation of free radical scavenging activity

The free radical scavenging experiment of *T. occidentalis* extracts obtained from four different geographical sites was carried out using 2,2-diphenyl-1-picrylhydrazyl (DPPH), as specified by Guleria and Yadav [32] and Sharma et al. [30]. 50 μl of plant extracts were mixed with 1.95 ml of 0.1 mM DPPH solution. DPPH per cent inhibition was calculated by measuring absorbance at 517 nm after 30 min of room temperature incubation. The per cent inhibition was calculated by using following formula:

$$\text{Percentage inhibition (\%)} = A_0 - A_1/A_0 \times 100,$$

where A_0 refers to the absorbance of control and A_1 refers to the absorbance of sample.

Enzyme assays

Superoxide dismutase (SOD)

The technique developed by Giannopolitis and Reis for measuring SOD enzyme activity was used [33]. The fresh leaves of four different *T. occidentalis* plants (obtained from different geographic locations) were quickly ground up and placed into an ice-cold extraction solution containing 100 mM potassium phosphate and 0.1 mM EDTA. The supernatant from the homogenate was collected and centrifuged. The reaction mix was prepared by adding 50 mM phosphate buffer, 0.1 mM EDTA, 13 mM methionine, 75 mM nitroblue tetrazolium, and 2 mM riboflavin. After

adding 50 µl of crude enzyme extract to the reaction mixture, 15 min of exposure to 20 W of fluorescent light was allowed to pass. The absorbance was read at 560 nm.

Catalase (CAT)

Enzyme activity was determined for catalase by using the method outlined by Sharma et al. [30]. The crude extract was prepared by pulverizing fresh *T. occidentalis* leaves collected from four distinct locations with 100 mM sodium phosphate extraction buffer and then centrifuging the resulting supernatant. The reaction mixture included 20 mM of hydrogen peroxide, 0.1 M of EDTA, and 100 mM of potassium phosphate buffer. 50 µl more of crude enzyme extracts were added to the mixture. Hydrogen peroxide levels were measured by their decrease in optical density at 240 nm, which indicates enzyme activity. A 40 mM⁻¹ cm⁻¹ extinction coefficient was used to quantify the enzyme's activity.

Ascorbate peroxidase (APX)

Ascorbate peroxidase enzyme activity was analysed and calculated according to the previously described procedure [30, 34]. An extract of fresh leaf samples was made in 50 mM phosphate buffer, and the crude enzyme extract was obtained by centrifuging the supernatant. 50 mM phosphate buffer, 0.2 mM EDTA, 0.5 mM ascorbic acid, and 0.25 mM hydrogen peroxide were all components of this solution. The addition of the crude extract at 37°C initiated the process. The absorbance of the reaction mixture was measured at 290 nm at the start of the reaction and again at the end of the reaction. A molar extinction coefficient of 2.8 mM⁻¹ cm⁻¹ was selected.

GC–MS analysis

The methanolic extracts of *T. occidentalis* collected from the four different geographical sites were used for GC–MS analysis of the bioactive components. The analysis was carried out using Shimadzu's GCMS-TQ8040 NX instrument with a capillary column (30 m × 0.25 mm). Helium gas was used as solvent. The parameters used to perform the analysis are summarized in Table 3. The obtained spectrum was analysed using NIST online library as reference.

Correlation of Biochemical Parameters and bioactive components obtained from GC–MS analysis with the antimicrobial activity

To analyse the correlation of the antimicrobial activity with biochemical parameters or the bioactive components, the correlation matrices for all the bacterial species were prepared using zone of inhibition, biochemical parameters, and

the area percentage of the bioactive components as obtained after GC–MS analysis of *T. occidentalis* plant extracts. Coefficient of correlation was calculated using Karl Pearson's method [35]. Plots for correlation matrices were created using `corrplot` and `ggplot2` in R studio.

Statistical analysis

All the results are expressed as mean ± standard deviation of 10 individual experiments, and statistical analysis was done by student's t test and two-way ANOVA.

Results

Extraction yield

The extraction yield of *T. occidentalis* was analysed and the results showed a considerable difference in the extraction yields across the three solvents. The methanol extract had the greatest yield which was found to be 10.5%, followed by ethanol (7.75%) and acetone (3.5%), this would indicate that highly polar solvents are optimal for extraction.

Antibacterial activity

In this research, the antibacterial potential of *T. occidentalis* obtained from a variety of different places was evaluated. According to the findings, all the extracts from the various locations were able to inhibit the development of the different pathogens that were tested, to various levels. The GS-4 (Kakching, Manipur), out of all the geographical locations, demonstrated the most promising antibacterial activity in each of the three solvents against all the pathogenic microorganisms. When compared methanol to ethanol and acetone, it was observed that methanol was the suitable solvent to be used since it displayed the maximum level of efficacy after the extract had been allowed to rest for 48 h. The 48 h methanolic extract of site 4 plant samples had the greatest level of inhibition against *S. aureus*, which was measured to be 27.5 mm in diameter followed by ethanolic extract (23 mm) and acetonic extract (20 mm). At the same time frame and solvent, geographical site 1 demonstrated the second most promising antibacterial activity, with a 25 mm zone against *S. aureus*. The least promising action of the plant's methanolic extract against *S. aureus* was discovered at sites 2 and 3, with diameters of 16 mm and 12 mm, respectively.

The plant's ethanol extract (at 48 h' time frame) obtained from GS-4 was most effective against *B. cereus* (25 mm) followed by *S. aureus* (23 mm), *P. aeruginosa* (21.5 mm), *P. fluorescens* (21 mm), and *E. coli* (20.5 mm). The acetonic extract of the GS-4 was the most efficient against *B. cereus* (24 mm) after a time frame of 48 h. This

was followed by *P. fluorescens* (22 mm), *E. coli* (22 mm), *S. aureus* (20.5 mm), and *P. aeruginosa* (20 mm). The antibacterial activity of the plant extracts revealed the same pattern of findings across all the geographical regions that were investigated. Figure 2a to e depicts the detailed results of antibacterial activity shown by different geographical sites against five different bacterial isolates at four different extraction time frames of three solvents.

Since, the plant extract of *T. occidentalis* obtained from GS-4 after a time frame of 48 h showed the most antagonistic effects against all the bacterial strains, it was chosen to determine the minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC) of the plant extract. The minimum inhibitory concentration (MIC) of methanolic extracts against *B. cereus* and *P. aeruginosa* was determined to be 0.78 mg/ml, whereas the MIC for

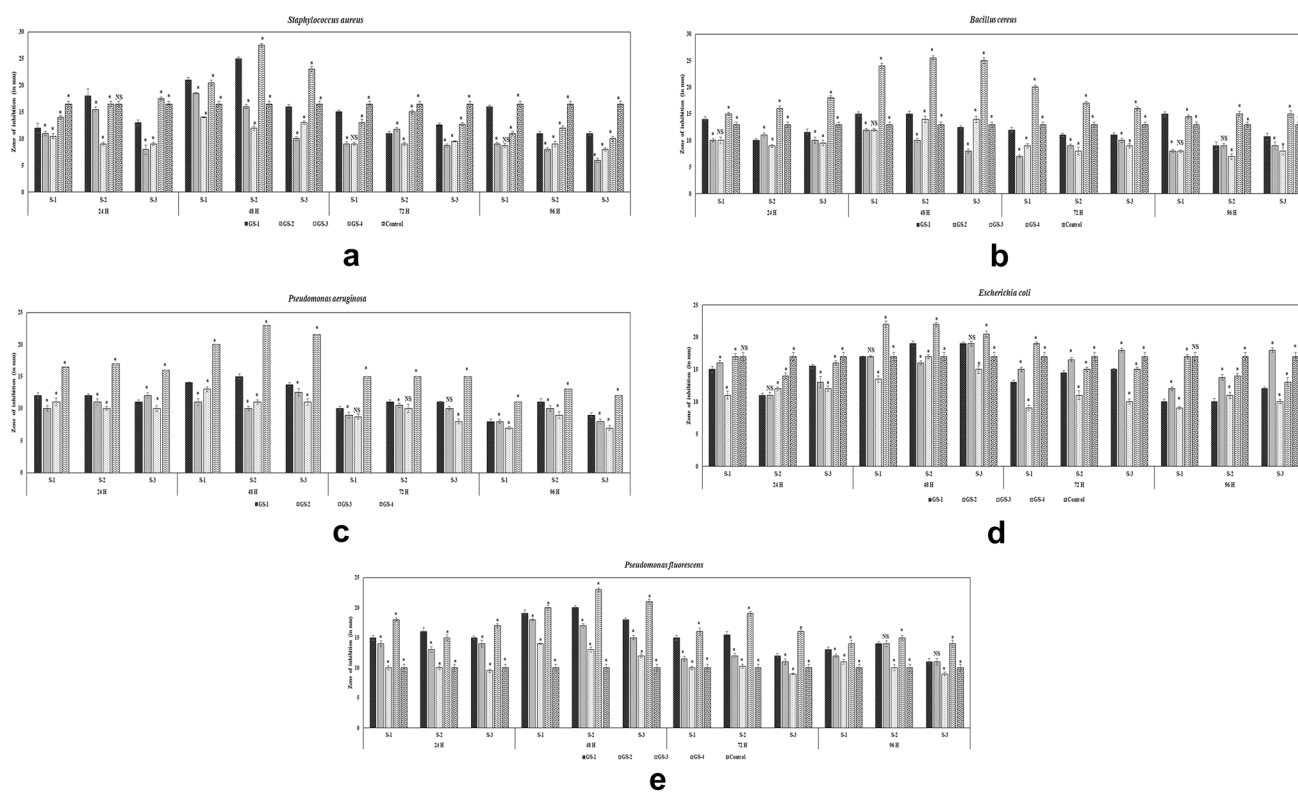


Fig. 2 **a** Graph showing zone of inhibition against *Staphylococcus aureus* recorded in *T. occidentalis* samples collected from four different geographic sites (GS-1, GS-2, GS-3, GS-4). The plant extracts were prepared in three different solvents (S1, S2, S3) and four different time intervals (24H, 48H, 72H, 96H). GS-1: Chamba, Himachal Pradesh, India. GS-2: Jalandhar, Punjab, India. GS-3: Aurangabad, Bihar, India. GS-4: Kakching, Manipur, India. S1: Acetone, S2: Methanol, S3: Ethanol. P value: GS-1 vs GS-2 vs GS-3 vs GS-4. * represents $P < 0.05$, NS represents Non-Significant ($P > 0.05$). **b** Graph showing zone of inhibition against *Bacillus cereus* recorded in *T. occidentalis* samples collected from four different geographic sites (GS-1, GS-2, GS-3, GS-4). The plant extracts were prepared in three different solvents (S1, S2, S3) and four different time intervals (24H, 48H, 72H, 96H). GS-1: Chamba, Himachal Pradesh, India. GS-2: Jalandhar, Punjab, India. GS-3: Aurangabad, Bihar, India. GS-4: Kakching, Manipur, India. S1: Acetone, S2: Methanol, S3: Ethanol. P value: GS-1 vs GS-2 vs GS-3 vs GS-4. * represents $P < 0.05$, NS represents Non-Significant ($P > 0.05$). **c** Graph showing zone of inhibition against *Pseudomonas aeruginosa* recorded in *T. occidentalis* samples collected from four different geographic sites (GS-1, GS-2, GS-3, GS-4). The plant extracts were prepared in three different solvents (S1, S2, S3) and four different time intervals (24H, 48H, 72H,

96H). GS-1: Chamba, Himachal Pradesh, India, GS-2: Jalandhar, Punjab, India, GS-3: Aurangabad, Bihar, India, GS-4: Kakching, Manipur, India. S1: Acetone, S2: Methanol, S3: Ethanol. P value: GS-1 vs GS-2 vs GS-3 vs GS-4. * represents $P < 0.05$, NS represents Non-Significant ($P > 0.05$). **d** Graph showing zone of inhibition against *Escherichia coli* recorded in *T. occidentalis* samples collected from four different geographic sites (GS-1, GS-2, GS-3, GS-4). The plant extracts were prepared in three different solvents (S1, S2, S3) and four different time intervals (24H, 48H, 72H, 96H). GS-1: Chamba, Himachal Pradesh, India, GS-2: Jalandhar, Punjab, India, GS-3: Aurangabad, Bihar, India, GS-4: Kakching, Manipur, India. S1: Acetone, S2: Methanol, S3: Ethanol. P value: GS-1 vs GS-2 vs GS-3 vs GS-4. * represents $P < 0.05$, NS represents Non-Significant ($P > 0.05$). **e** Graph showing zone of inhibition against *Pseudomonas fluorescens* recorded in *T. occidentalis* samples collected from four different geographic sites (GS-1, GS-2, GS-3, GS-4). The plant extracts were prepared in three different solvents (S1, S2, S3) and four different time intervals (24H, 48H, 72H, 96H). GS-1: Chamba, Himachal Pradesh, India. GS-2: Jalandhar, Punjab, India. GS-3: Aurangabad, Bihar, India. GS-4: Kakching, Manipur, India. S1: Acetone, S2: Methanol, S3: Ethanol. P value: GS-1 vs GS-2 vs GS-3 vs GS-4. * represents $P < 0.05$, NS represents Non-Significant ($P > 0.05$)

S. aureus, *P. fluorescens*, and *E. coli* was 0.39 mg/ml and the MBC of *B. cereus* and *P. aeruginosa* was found to be 0.39 mg/ml, followed by *S. aureus*, *P. fluorescens*, *E. coli* which was 0.19 mg/ml. In contrast, the ethanolic and acetonic extracts showed MIC at 0.39 mg/ml and MBC at 0.19 mg/ml against all the abovementioned bacterial strains.

Biochemical screening results

TFC and TPC

The total flavonoid content (TFC) and total phenolic content (TPC) of methanolic extract of *T. occidentalis* obtained from four different geographical sites were assessed and are presented in Fig. 3a and 3b, respectively. It was observed that both TPC and TFC values of the *T. occidentalis* extracts were found to be different at different geographical sites. The geographical site4 (GS-4) showed highest TPC(365.53 $\mu\text{g/g}$ FW) followed by GS-1 (336.30 $\mu\text{g/g}$ FW), GS-3 (301.84 $\mu\text{g/g}$ FW), and GS-2 (259.38 $\mu\text{g/g}$ FW). The TFC results were also found in the same order, GS-4 showed the highest levels (136.49 mg/g FW) followed by GS-1 (128.71 mg/g FW), GS-3 (126.76 mg/g FW), and GS-2 (117.85 mg/g FW). Hence, the GS-4 showed highest TPC and TFC contents in contrast to the other geographical sites.

Antioxidant activity

Plants that are abundant in secondary metabolites, such as phenolics, flavonoids, and carotenoids have been shown to possess antioxidant properties. The antioxidant capacity of the extracts was analysed, and the results were compared among the four different geographical sites (Fig. 3c). When compared to the results of other sites, the methanolic extract of GS-4 revealed significantly higher levels of antioxidant activity. The levels of DPPH scavenging activity identified in GS-4, GS-3, GS-2, and GS-1 were 44%, 36%, 25%, and 11%, respectively.

Enzymes

Free radicals may be neutralized by oxidoreductases like catalase and superoxide dismutase (SOD). The evaluation of these enzymes showed that GS-4 had the highest SOD, CAT, and APX activity. The results of these are depicted in Fig. 3d–f.

Correlation of biochemical parameters with the antimicrobial activity

A positive correlation was observed between antimicrobial efficacy (zone of inhibition) and biochemical parameters in all bacterial species except for *P. fluorescens* and *S. aureus*

which showed no correlation between DPPH activity and zone of inhibition. In addition to this, the biochemical parameters showed positive correlation with each other also in all the bacterial species. The results are summarized in Fig. 4.

GC–MS analysis

The GC–MS analysis revealed 54 different components in plant extracts collected from GS-1 while the GS-2 sample showed 56 compounds. Similarly, the plant extracts from GS-3 showed 58 components and those from GS-4 showed 54 components. All these components were scanned for their antimicrobial and antioxidant potential by analysing the previous literature. Out of all these, six bioactive components were selected and their peak area percentages for the samples collected from all the locations were compared because the peak area is directly proportional to the concentration of the component. These compounds were selected on the basis of their occurrence in all the four plant samples and their antimicrobial or antioxidant potential. The details of these compounds are summarized in Table 4.

Correlation of the peak area percentages of the bioactive components with the antimicrobial activity

A positive correlation was observed in all bacterial species between zone of inhibition and peak area percentages of α -Pinene, β -caryophyllene, Germacrene-D, and Humulene. Thunbergol showed negative correlation with the zone of inhibition in all bacterial species. Neophytadiene showed either an insignificant positive correlation or negative correlation with the zone of inhibition in different bacterial species. The results are depicted in Fig. 5.

Discussion

The selected plant species (*T. occidentalis*) commonly known to be used as ornamental was evaluated for its antibacterial efficacy in vitro against five bacterial species: *S. aureus*, *P. aeruginosa*, *P. fluorescens*, *B. cereus*, and *E. coli*. The different zones of inhibition were recorded for each bacterium. The extracts were made using three different solvents: methanol, ethanol, and acetone, and were kept for four different time frames for comparative analysis. The present study first time documents the variations in the antibacterial efficacies of the same plant extract obtained from four different geographical sites of a country. The maximum zone of inhibition was recorded by GS-4 plant extract (27.5 mm) against *S. aureus* from methanol extract at 48 h time frame. Methanol extract

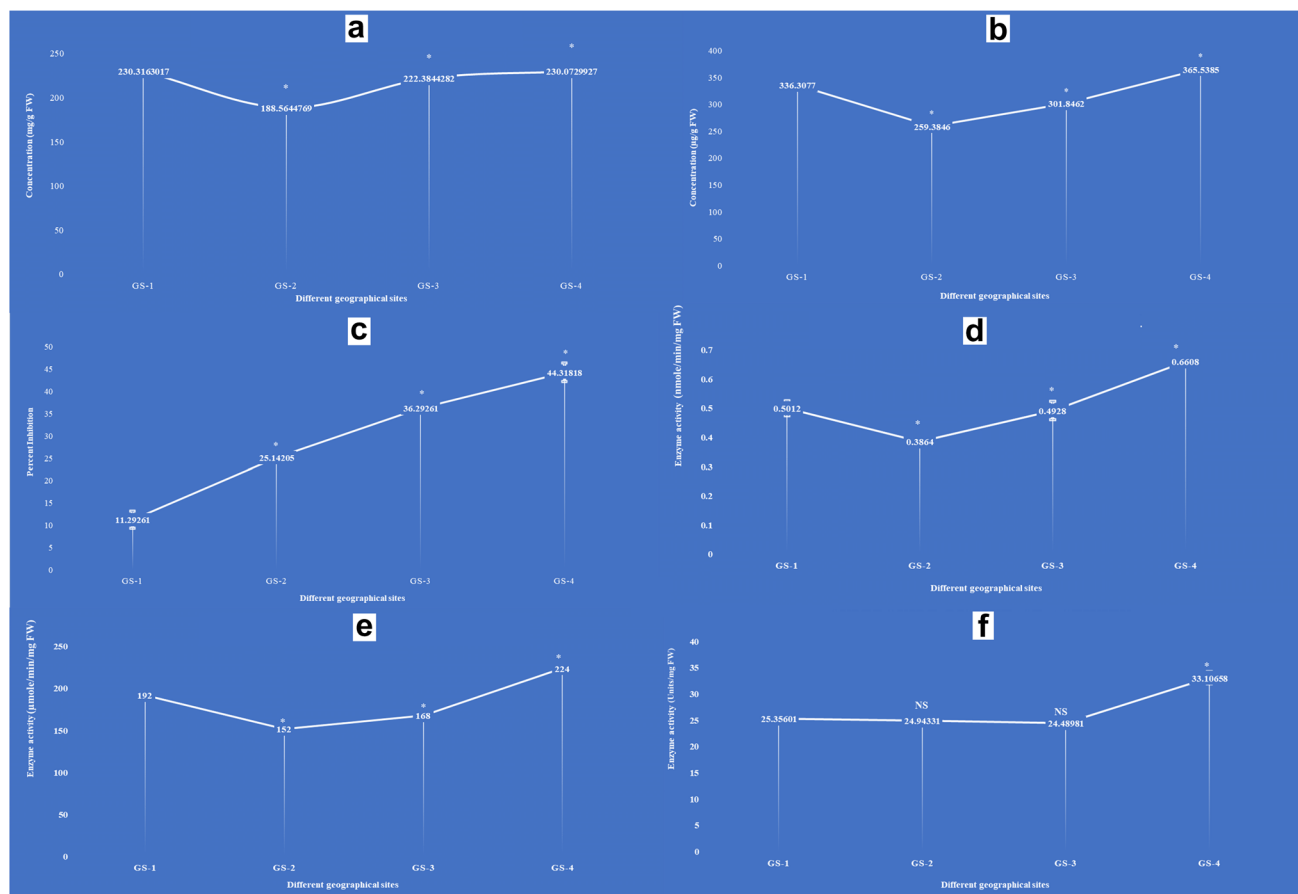


Fig. 3 **a** Graph showing comparison of total flavonoid content in *T. occidentalis* samples collected from four different geographic sites. The biochemical test was done on the plant extracts prepared in methanol for 48 h. **b** Graph showing comparison of total phenolic content in *T. occidentalis* samples collected from four different geographic sites. The biochemical test was done on the plant extracts prepared in methanol for 48 h. **c** Graph showing comparison of free radical scavenging potential in *T. occidentalis* samples collected from four different geographic sites. The biochemical test was done on the plant extracts prepared in methanol for 48 h. **d** Graph showing comparison of ascorbate peroxidase activity in *T. occidentalis* samples collected from four different geographic sites. The biochemical test was done

on the plant extracts prepared in methanol for 48 h. **e** Graph showing comparison of catalase activity in *T. occidentalis* samples collected from four different geographic sites. The biochemical test was done on the plant extracts prepared in methanol for 48 h. **f** Graph showing comparison of SOD (superoxide dismutase) activity in *T. occidentalis* samples collected from four different geographic sites. The biochemical test was done on the plant extracts prepared in methanol for 48 h. GS-1: Chamba, Himachal Pradesh, India, GS-2: Jalandhar, Punjab, India, GS-3: Aurangabad, Bihar, India, GS-4: Kakching, Manipur, India. P value: GS-1 vs GS-2 vs GS-3 vs GS-4. * represents P < 0.05, NS represents Non-Significant (P > 0.05)

showed more antagonistic effects against all the bacterial species at 48 h time frame as compared to ethanol and acetone extract. The results prove that methanol the best solvent for extraction purposes as compared to ethanol and acetone. Some previous studies also confirmed the similar results [36, 37]. The GS-4 methanolic extract at 48 h' time frame showed highest zone of inhibition against *P. aeruginosa* (23 mm) and *B. cereus* (25.5 mm) in comparison with the previous studies which was found to be 7.75 mm and 6.75 mm, respectively [38]. The antibacterial activity of *T. occidentalis* ethanol and acetone plant extract was previously verified for all the bacterial isolates evaluated in the present study except *P. fluorescens*; however, the zone of inhibition assessed in the current investigation was

less than in the previous studies [39–41]. The possible causes for the discrepancy in zones of inhibition might include variations in extraction procedure and sample concentration.

The minimum inhibitory concentration and minimum bactericidal concentration values of the plant extract also validates the effectiveness of *T. occidentalis* to be used as an alternative to antibiotics that are resistant to the aforementioned bacterial species. To this day, no studies have been conducted to verify whether or not there is a difference in the antibacterial activity of any plant sample that was taken from a variety of different geographical sites of different elevation, and temperature. The present study validates the variations in the antibacterial activity of *T.*

Table 3 Parameters for GC–MS analysis

| | |
|------------------------|-------------|
| Oven Temperature | 50.0 °C |
| Injection Temperature | 250.00 °C |
| Injection Mode | Splitless |
| Sampling Time | 0.50 min |
| Total Flow | 14.3 mL/min |
| Column Flow | 1.02 mL/min |
| Linear Velocity | 36.7 cm/sec |
| Purge Flow | 3.0 mL/min |
| Split Ratio | 10.0 |
| Ion Source Temperature | 240.00 °C |
| Interface Temperature | 310.00 °C |
| Solvent Cut Time | 4.00 min |
| Start m/z | 45.00 |
| End m/z | 800.00 |

occidentalis obtained from different geographical sites with different elevation, temperature, and average rainfall.

To analyse the differences in phytochemical composition, different biochemical tests were performed. The methanol

extract of *T. occidentalis* obtained from GS-4 has showed the maximum total phenol and total flavonoid content. Phenolic compounds are powerful hydrogen donors, which contribute to the fact that they are excellent antioxidants [42]. Because of the presence of hydroxyl groups, phenols have the potential to scavenge free radicals, making them a very vital component of plants. It is possible that the phenolic component makes a direct contribution to the anti-oxidative activity [43]. As suggested previously that the phenolic and flavonoid chemicals found in the extract might be responsible for the antibacterial activity [44, 45]. The substantial phenolic and flavonoid content of these crude extracts may be responsible for the antibacterial and antioxidant action that they possess.

It is a well-known process for anti-oxidation that free radicals may be scavenged by the donation of hydrogen. The methanol extract of GS-4 findings of the present investigation indicated that the extracts derived from *T. occidentalis* had the maximum ability to scavenge free radicals by donating hydrogen to the DPPH radical. The previous studies also confirmed the DPPH activities of the plant extract at different concentrations [46]. DPPH

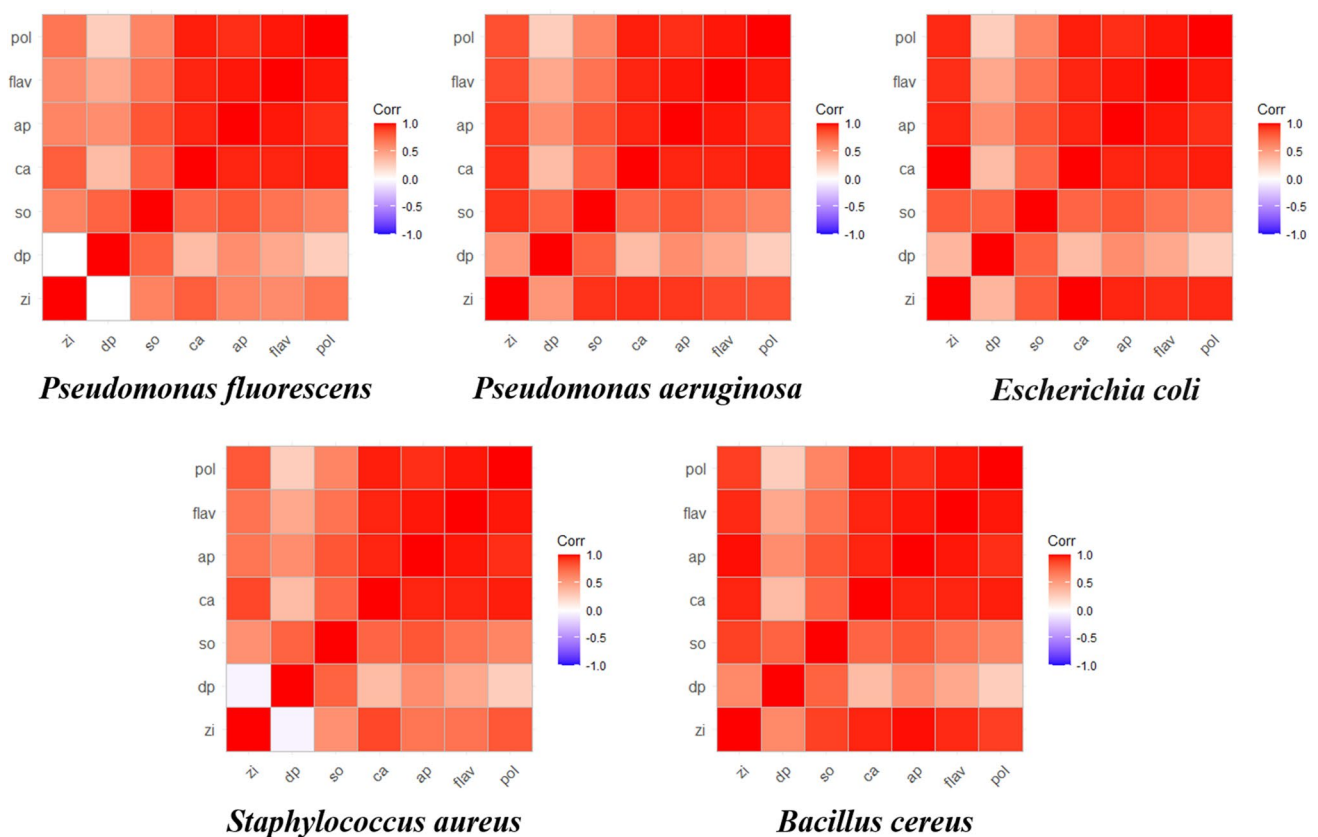
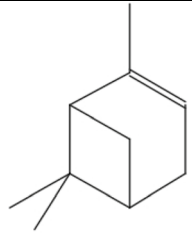
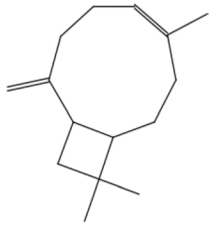
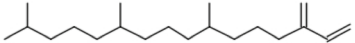
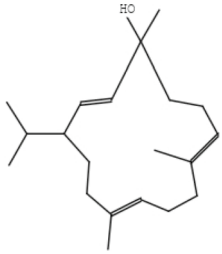
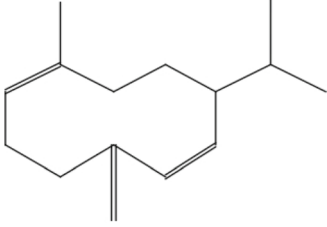
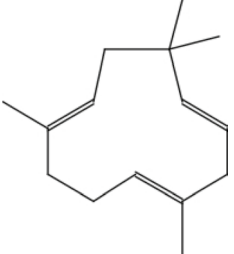


Fig. 4 Plots showing the correlation between the various biochemical parameters of *T. occidentalis* and its antibacterial potential (measured by zone of inhibition) in different bacterial strains. zi: zone of inhibition, dp: free radical scavenging activity, so: superoxide dismutase

activity, ca: catalase activity, ap: ascorbate peroxidase activity, flav: total flavonoid content, pol: total phenolic content. White colour represents no correlation, Shades of red represent positive correlation, and Shades of blue represent negative correlation

Table 4 Details of *Thuja occidentalis* bioactive compounds obtained from GC–MS analysis of the plant samples collected from four different geographical sites

| S. No | Name of the compound | Property | Formula | Structure |
|-------|------------------------|---|-----------------|---|
| a) | α -Pinene | Anti-inflammatory, likely antimicrobial | $C_{10}H_{16}$ |  |
| b) | β -Caryophyllene | Antimicrobial, Antioxidant, anti-inflammatory | $C_{15}H_{24}$ |  |
| c) | Neophytadiene | Antioxidant, antimicrobial | $C_{20}H_{38}$ |  |
| d) | Thunbergol | Antimicrobial | $C_{20}H_{34}O$ |  |
| e) | Germacrene D | Antimicrobial | $C_{15}H_{24}$ |  |
| f) | Humulene | Antimicrobial | $C_{15}H_{24}$ |  |

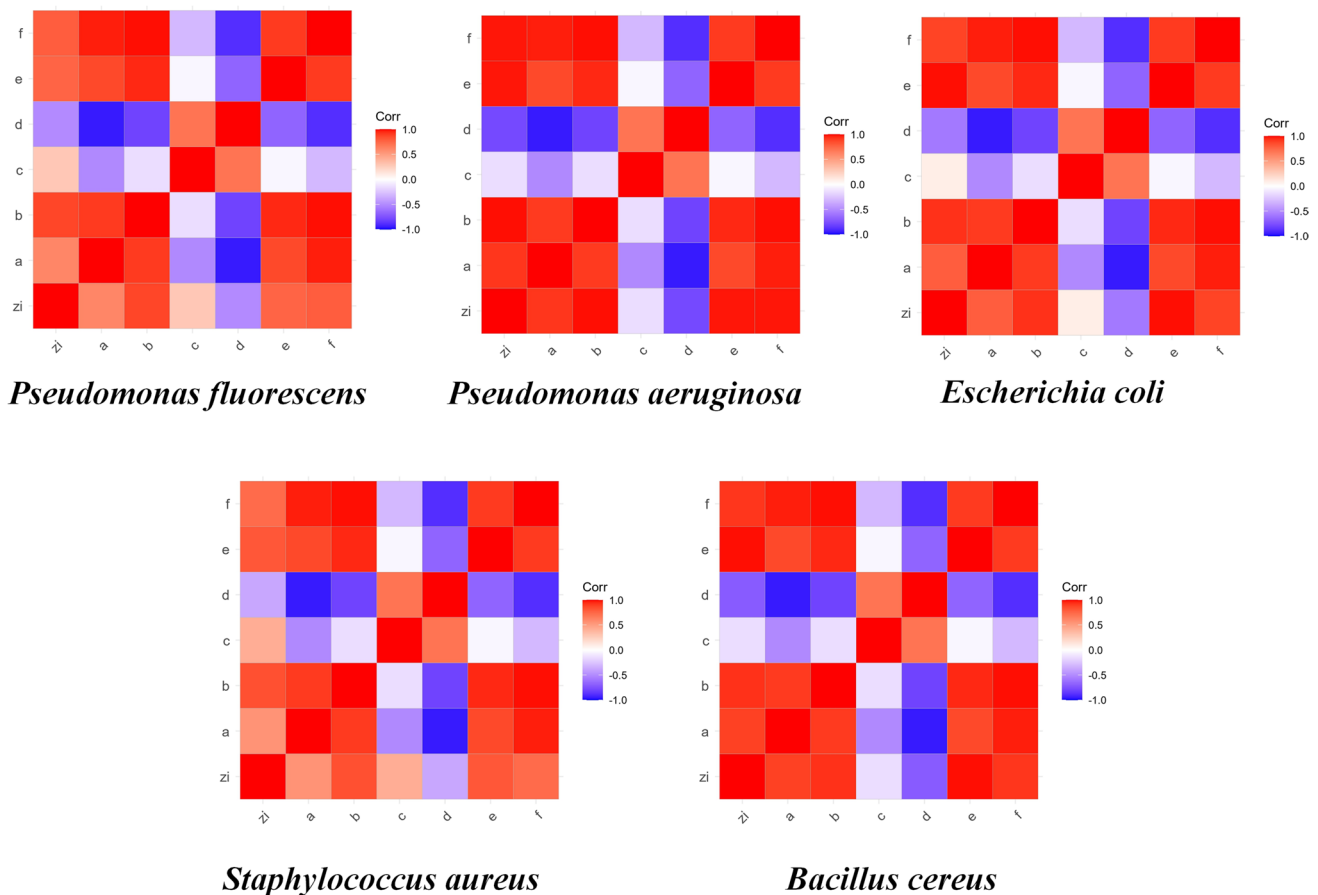


Fig. 5 Plots showing the correlation between the various bioactive components of *T. occidentalis* and its antibacterial potential (measured by zone of inhibition) in different bacterial strains. zi: zone of inhibition, a: α -Pinene, b: β -caryophyllene, c: Neophytadiene, d:

Thunbergol, e: Germacrene-D, f: Humulene. White colour represents no correlation, Shades of red represent positive correlation, and Shades of blue represent negative correlation

radical scavenging activity of the plant extract showed it might be a suitable option in the quest for natural, effective compounds with antioxidant activity.

In plant cytoplasm, flavonoids further interact with and improve the activity of enzymes that regulate free radicals. The cells have defence mechanisms against free radical damage, including the enzymes catalase and superoxide dismutase (SOD) [47]. Our study showed the plant extracts collected from GS-4 had the maximum flavonoid content and SOD, APX, and CAT activity. As suggested previously these enzymes might be responsible for the more antagonistic effects against different bacterial species [48].

The biochemical parameters showed positive correlation with the zone of inhibition suggesting that they might play a role in the bactericidal potential of the plant. Variation in the phytochemical composition with change in geographic location might be the reason for the different antibacterial efficacies of *T. occidentalis* samples collected from different sites.

The GC–MS analysis showed that a positive correlation was observed in all bacterial species between zone of inhibition and peak area percentages of α -Pinene, β -caryophyllene, Germacrene-D, and Humulene suggesting that the significant role of these compounds in the bactericidal potential of *T. occidentalis*. These results are in coherence with previous studies. For example, Borges et al. [49] compiled work of many scientists and confirmed the antimicrobial potential of α -pinene. Danham et al. [50] researched essential oil of *Aquilaria crassna* for analysing the anticancerous, antimicrobial and antioxidant potential. The study showed positive results and β -caryophyllene was reported to be the bioactive principle behind the anticancerous and antimicrobial potential of the plant. Germacrene-D is a sesquiterpenoid germacrene. Germacrene A, B, C, and E are another four isomers of germacrene. Out of all the four isomers, Germacrene-D has been reported to have bactericidal potential [51]. The antimicrobial efficacy of α -humulene has been previously reported by many workers [52, 53]. In the present study,

Neophytadiene showed either an insignificant positive correlation or negative correlation with the zone of inhibition in different bacterial species. This contrasts with a previous study done by Ceyhan-Güvensen and Keskin [54] that reported the antimicrobial potential of *Mentha pulegium* and attributed the same to neophytadiene. Similarly, thunbergol showed negative correlation with the zone of inhibition in all bacterial species. A previous study done by Mitic et al., [55] stated that thunbergol can be a potential antimicrobial if used alone or in combination with other compounds.

Conclusion

One of the most significant challenges has been the emergence of novel infections that are resistant to many drugs. In spite of the availability of large quantities of chemical drugs for the treatment of various diseases, the use of herbs as the natural drugs that were traditionally used has continued to remain the alternative treatment option for deformities made in the normal physiological system by foreign organisms or by any malfunctioning of the body. *T. occidentalis* extracts are rich in phenolics, flavonoids, and other free radical scavengers that may minimize lipid oxidation and possess great antibacterial activity against number of bacterial species. α -Pinene, β -caryophyllene, Germacrene-D, and Humulene are suggested to be the bioactive components behind the bactericidal potential of the plant. The present study validates novel information of variations in the antibacterial activity of plant extract obtained from different geographical sites. It should be looked up to gain more knowledge and further research is required to confirm the findings. The present study concludes with the quote that location must be taken into consideration while choosing plant for the research purposes.

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Author contributions Mr. Manish Thakur prepared the material, collected the data, carried out the analysis, and wrote the manuscript. Ms. Ayushi Gautam carried out the biochemical analysis. The work was supervised by Dr. Tejinder Kaur and Dr. Praveen Guleria. The manuscript was edited by Dr. Tejinder Kaur and Dr. Ranbir Chander Sobti.

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Data availability All data generated or analysed during this study are included in this manuscript.

Declarations

Conflict of interest The authors have no financial or non-financial interests to disclose.

Ethical approval Not applicable.

Consent to participate Not applicable.

Consent to publish The authors give consent to Molecular and Cellular Biochemistry journal to publish the present research work.

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