

## Analysis of chemical composition of *Cupressus torulosa* (D. Don) essential oil and bioautography guided evaluation of its antimicrobial fraction

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*Cupressus torulosa* D. Don, known as the Himalayan or Bhutan cypress, is one of the medicinal plants commonly used in the Indian System of Medicine for various ailments. The present study evaluates the chemical composition and inhibitory potential of the essential oil and three different extracts (chloroform, methanol and aqueous) of aerial parts of *C. torulosa*. Chemical composition of essential oil was determined by GC-MS that showed the presence of four major components viz.,  $\alpha$ -pinene (45.44%), 3-carene (38.34%), terpinolene (5.36%) and aromadendrene (6.32%). Essential oil showed significant inhibitory activity against *Bacillus subtilis*, *Pseudomonas alcaligenes*, *Micrococcus luteus* and *Bacillus cereus* in comparison to the different extracts. Essential oil also showed good antifungal activity against the three fungal pathogens viz., *Alternaria alternata*, *Curvularia lunata* and *Bipolaris specifera*. TLC-bioautography was used to screen the antibacterial components of the essential oil. Analysis showed the presence of four zones of inhibition on bioautography plate at  $R_f$  values 0.80, 0.70, 0.61 and 0.46. This study has demonstrated the presence of four potential antibacterial compounds in the essential oil of *Cupressus torulosa*.

**Keywords:** *Alternaria alternata*, Antimicrobial, Antifungal, *Bipolaris specifera*, *Curvularia lunata*, 3-Carene, *Cupressus*, Essential oil, Fungal pathogens, GC-MS, Himalayan cypress,  $\alpha$ -Pinene

Plants have been used as therapeutics over thousands of years in the traditional system of medicine and still hold importance as sources of natural medicine. In recent investigations, several medicinal plants have been found to possess interesting biological activities like antibacterial, antifungal, anticancer, antioxidant etc. that have proved to be thrust area of interest around the world.

Of late, there has been a growing interest in research concerning the possible use of natural products for preventing the growth of microbial pathogens. In agriculture, pesticides are generally used in the control of plant pathogens. However, there is a serious problem in the effective use of these chemicals due to the development of resistance. To overcome this problem, higher concentrations of these chemicals are used but this increases the risk of high level of toxic residues in the products. Also, antibiotics for preventing human disease causing microbes that reached market in the last 20 years clearly shows that there is paucity of new classes of antibiotics<sup>1</sup>. Due to risk of adverse effects encountered with the use of synthetic antibiotics, medicinal plants may offer an

alternative source for antimicrobial compounds. In addition, a number of antibiotics have lost their effectiveness due to the development of resistant strains, mostly through the expression of resistance genes. Medicinal plants could be a potential source of integrative antimicrobial system as they are known to possess more than one antimicrobial compounds<sup>2</sup>. Therefore, the present focus has shifted onto the botanicals for their possible use in inhibiting the growth of human and plant disease causing pathogens. Therefore, their characterization based on chemical profile may be of great importance.

*Cupressus torulosa* D. Don, commonly called the Himalayan cypress, is a tall evergreen tree, widely found throughout India, Nepal, Tibet, Pakistan and Bhutan at elevation of 1800-3300 m on limestone substrates. It is an evergreen tree that grows up to 35 m tall. Essential oil obtained from the leaves of *Cupressus torulosa* is used to treat rheumatism and whooping cough, as an astringent and also to protect stored grains from insect infestation<sup>3,4</sup>. Some researchers also reported remarkable activity of *Cupressus sempervirens* leaf extracts in enhancing liver and kidney functions<sup>5</sup>. It is expected that *C. torulosa* possesses some active components which are responsible for its biological activity. In view to

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the above facts, we studied the phytochemistry and inhibitory potential of the essential oil and extracts of *C. torulosa* leaves growing in Jammu and Kashmir.

## Material and Methods

### Plant material and Isolation of essential oil

Plant material was collected locally from University of Jammu, Jammu and Kashmir and identified by a Dr. Harish Dutt, Assistant Professor and Taxonomist, Department of Botany, University of Jammu. *Cupressus torulosa* (arial parts) were cleaned and subjected to hydro-distillation for 4 h in a Clevenger type apparatus for isolation of essential oil. The extracted oil was dried over anhydrous sodium sulphate and stored at low temperature.

### Preparation of extracts

Plant material (leaves) was shade dried, powdered in an electronic grinder and extracted with three different solvents. The grounded powder (100 g) of plant material was extracted with 500 ml of three different solvents *viz.*, chloroform, methanol and aqueous. Methanol and chloroform extracts were prepared by continuous stirring at room temperature (37°C) for 6 h. Aqueous extract was prepared at 60°C overnight. This procedure was repeated thrice and extracts were filtered, pooled and evaporated using rotary vacuum evaporator, and finally lyophilized to dried powder<sup>6</sup>.

### GC-MS analysis of essential oil

Analysis of the *C. torulosa* leaf essential oil was carried out at Indian Institute of Integrative Medicine (CSIR, India), Canal Road, Jammu, India. System used for analysis was GC-MS 4000 (Varian, USA) with a varian CP-SIL 8CB column (30 mm×0.32 mm i.d., 1 µm film thickness). Injector temperature was 230°C. Oven temperature program used was holding at 60°C for 5min, heating to 250°C at 3°C/min and keeping the temperature constant at 250°C for 10 min. Helium was used as a carrier gas at a constant flow of 1 mL/min and an injection volume of 0.20 µL was employed. The MS scan parameters included electron impact ionization voltage of 70 eV, a mass range of 40-500 m/z. The identification of the essential oil components was based on comparison of their mass spectra with those of NIST05 (version 2.0) library<sup>7</sup>.

### Antibacterial assay

Qualitative screening of the essential oil and extracts for antibacterial activity was carried out by agar well diffusion assay against ten different

Gram positive and Gram negative strains<sup>8</sup>. Bacterial strains used were *Bacillus subtilis*, *Bacillus cereus*, *Pseudomonas aeruginosa*, *Pseudomonas alcaligenes*, *Campylobacter coli*, *Micrococcus luteus*, *Escherichia coli*, *Enterococcus faecalis*, *Alcaligenes denitrificans* and *Pseudomonas florescens* (Purchased from MTCC Chandigarh, India). Autoclaved nutrient agar (20 mL) was inoculated with 100 µL test bacterial suspension (10<sup>8</sup> CFU/mL) and poured into a sterilized petriplates. Plates were allowed to solidify and a well of 6 mm was aseptically bored into the agar plate by using a cork borer. 20 µL of Essential oil (E.oil: DMSO; 1:1) or extract (50 mg/mL) was added into each well. Finally, the plates were kept for incubation at 37°C for 24 h. Chloramphenicol (10 µg) was used as positive reference and DMSO was used as negative control.

### Antifungal assay

The antifungal activity of essential oil and extracts was determined by Poisoned food technique against three pathogenic fungal strains *viz.*, *Alternaria alternata*, *Curvularia lunata*, and *Bipolaris specifera*. Test essential oil or extract was added to the sterilized potato dextrose agar in a petri plate. Plates containing different concentration of essential oil or extract were inoculated with test fungal culture (5 mm bit). Inoculated plates were incubated at 26°C. Hyphal growth was measured at every 24 h interval till the growth of test fungus in the control plate reached the edge of the plate. Percentage inhibition was calculated by using the equation mentioned below and IC<sub>50</sub> (50% inhibition concentration) value was calculated by plotting a graph between the different test concentrations and their respective percentage growth inhibition. Amphotericin B was used as positive control. The experiment was conducted in triplicates and the results were expressed as average<sup>9</sup>.

$$\text{Antifungal activity (\%)} = [(D_a - D_b) / D_a] \times 100$$

D<sub>a</sub>= Diameter of Growth in control; D<sub>b</sub>= Diameter of growth in test

### Evaluation of antibacterial activity by TLC-Bioautography method

Components of the essential oil were separated on TLC plate (silica gel 60 F<sub>254</sub>) using hexane:ethyl acetate (8:2) solvent system and separation of analytes was checked by visualization under UV light (365 and 254 nm) or by spraying with vanillin/sulphuric acid solution.

To screen the antibacterial activity of the essential oil, direct bioautography was performed<sup>10</sup>. The

developed plate was dried completely so that residual solvent would not inhibit the growth of bacteria. *Bacillus subtilis* as a test organism was then allowed to grow on the TLC plate and incubated at 37°C for 24 h in humid conditions. After incubation, the plate was sprayed with 2 mg/mL solution of iodo-nitro-tetrazolium (INT). One of the replication of plate was developed with vanillin/sulphuric acid spray reagent as control. Clear zones on chromatogram showed inhibition of bacterial growth.

#### Phytochemical analysis of extracts

The extracts of *Cupressus torulosa* were investigated for the presence of various secondary metabolites like terpenoids, coumarins, glycosides, quinones, saponins, tannins, anthraquinones, alkaloids, phenols, and flavonoids by different qualitative methods<sup>11,12</sup>.

Further, total phenols in the extracts were determined by Folin-Ciocalteu method<sup>13</sup>. In Brief, 0.5 mL of extract solution was mixed with 0.5 mL of 1 N Folin-Ciocalteu reagent. The mixture was kept for 5 min, followed by the addition of 1 mL of 20% Na<sub>2</sub>CO<sub>3</sub>. After 10 min of incubation at room temperature, the absorbance was measured at 730 nm using UV-VIS spectrophotometer. The concentration of phenolic compounds was calculated according to the equation obtained from the Gallic acid as standard:

$$\text{Absorbance} = 0.0364 \text{ Gallic acid } (\mu\text{g}) + 0.009$$

#### Results and Discussion

Fresh leaves of *C. torulosa* were subjected to Hydro-distillation and the dark yellow coloured essential oil was obtained with yield of 0.7%. The main chemical constituents of the essential oil were determined by using gas chromatography and mass

spectrometry. GC-MS analysis showed the presence of four major components accounting for 96% of the total components of the essential oil eluted between 10-50 minutes. The main components of the essential oil are  $\alpha$ -pinene (45.44%), 3-carene (38.34%), terpinolene (5.36%) and (+)-aromadendrene (6.32%). The oil is rich in monoterpenes and  $\alpha$ -pinene was the major component identified by mass spectrometry. Lohani and coworkers also investigated the composition of the *C. torulosa* essential oil from Uttarakhand Himalaya and also found  $\alpha$ -pinene (34.25%) and 3-carene (18.67%) as a major constituent of the oil<sup>14</sup>. Sellappan and coworkers reported the chemical composition of *C. torulosa* essential oil growing in Nilgiri, India<sup>15</sup>. An interesting evidence of spatial variation was observed in the report as terpiene-4-ol (25.91) as the major constituent of the essential oil and  $\alpha$ -pinene was minor one (2.89). Essential oils of different *Cupressus* species mainly contain monoterpene hydrocarbons (namely  $\alpha$ -pinene,  $\beta$ -phellandrene, sabinene, p-cymene, limonene,  $\alpha$ -thujene, myrcene and 3-carene) and oxygenated monoterpenes ( $\alpha$ -terpinyl acetate, terpinen-4-ol, umbellulone and bornyl acetate). In addition to these, sesquiterpenoids ( $\alpha$ -cedrene,  $\beta$ -cedrene, cis-thujopsene, cuparene, cedrol, germacrene D, caryophyllene and  $\alpha$ -murolool) and diterpenoids also constitute major proportion of essential oil composition in various *Cupressus* species<sup>16</sup>.

Yield of the three different extracts of leaves of *C. torulosa* viz., Methanol, aqueous and chloroform extracts were 17.5%, 13.4% and 7.3% respectively. Essential oil and the three extracts were subjected to *in vitro* analysis to determine their phytochemicals and biological activities.

The results of antibacterial activity of the essential oils and extracts are depicted in Table 1. Both

Table 1 — Antibacterial activity of essential oil and extracts of leaves of *Cupressus torulosa* by agar well diffusion assay  
Mean zone of inhibition excluding well (mm)

Bacterial strains	Essential oil	Methanol	Aqueous	Chloroform	Cmp
<i>Micrococcus luteus</i>	12±0.4	9±0.3	5±0.23	-	26±1.05
<i>Staphylococcus aureus</i>	5±0.2	9±0.4	-	-	18±0.6
<i>Escherichia coli</i>	-	-	-	-	15±0.5
<i>Enterococcus fecalis</i>	-	-	-	-	18±0.43
<i>Campylobacter coli</i>	-	6±0.23	-	-	28±0.9
<i>Bacillus subtilis</i>	21±0.7	-	-	-	15±0.55
<i>Bacillus cereus</i>	10±0.5	-	-	-	8±0.25
<i>Alcaligenes denitrificans</i>	7±0.3	11±0.32	-	8±0.35	21±0.75
<i>Pseudomonas aeruginosa</i>	9±0.42	-	-	-	20±0.0.9
<i>Pseudomonas alcaligenes</i>	13±0.7	-	-	-	15±0.6

[Values are expressed as mean ± SD of triplicate experiments, Essential oil: 20  $\mu$ L (Essential oil:DMSO; 1:1); Extract: 20  $\mu$ L (50 mg/mL); Cmp+ = Chloramphenicol (standard antibiotic, 10  $\mu$ g)]

essential oil and extracts were examined by agar well diffusion assay against various bacterial strains and inhibitory activity was determined by zone of inhibition. Essential oil of *C. torulosa* showed potential inhibitory activity against *B. subtilis* ( $21 \pm 0.7$  mm), *P. alcaligenes* ( $13 \pm 0.7$  mm), *M. luteus* ( $12 \pm 0.4$  mm) and *B. cereus* ( $10 \pm 0.5$  mm), and moderate activity against *P. aeruginosa* ( $9 \pm 0.42$  mm), *A. denitrificans* ( $7 \pm 0.3$  mm) and *S. aureus* ( $5 \pm 0.2$  mm). *E. coli*, *E. fecalis* and *C. coli* were resistant to the effect of essential oil. Methanol extract showed antimicrobial activity only against *A. denitrificans* ( $11 \pm 0.32$  mm), *S. aureus* ( $9 \pm 0.4$  mm), *M. luteus* ( $9 \pm 0.3$  mm) and *C. coli* ( $6 \pm 0.23$  mm) whereas, aqueous and chloroform extracts did not show any inhibitory effect. Essential oil of *C. torulosa* exhibited significant inhibitory effect on test bacterial strains in comparison to its extracts.

Antifungal activity of the essential oil and extracts of *C. torulosa* was evaluated by poisoned food technique against three fungal plant pathogens *viz.*, *A. alternata*, *C. lunata* and *B. specifera*. The results of the antifungal activity are shown in Table 2. Essential oil of *C. torulosa* demonstrated significant antifungal activity against *B. specifera*, *A. alternata* and *C. lunata* with  $IC_{50}$  values 0.19%, 0.32% and 0.96% (v/v) respectively. Chloroform, methanol and aqueous extracts of *C. torulosa* showed weak antifungal activity in comparison to the essential oil.

Bioautography technique can be used to evaluate the antibacterial activity of plant components. The inhibitory potential of the components of the essential oil was also determined by TLC-bioautography method. Analytes of the essential oil were separated on TLC plate and *Bacillus subtilis* was used as test organism. Application of the *Cupressus torulosa* essential oil in direct bioautography for observing antibacterial activity showed four inhibition zones at  $R_f$  values 0.80, 0.70, 0.61 and 0.46 as shown in Fig. 1. Four zones of inhibition indicated the presence of four

active antibacterial compounds present in essential oil separated on TLC plate. (Fig. 1)

Essential oils are a mixture of volatile compounds containing monoterpenes which are known to possess antimicrobial activity<sup>17</sup>. GC-MS analysis of *C. torulosa* essential oil showed the presence of four major compounds (monoterpene) accounting for more than 96% of the total composition of the essential oil. Antimicrobial activities of different chemical constituents of the essential oil have also been investigated by many researchers. Various reports on essential oil where major constituents are terpenoids are known to possess significant fungitoxic, antimicrobial and free radical scavenging activity<sup>18,19</sup>.

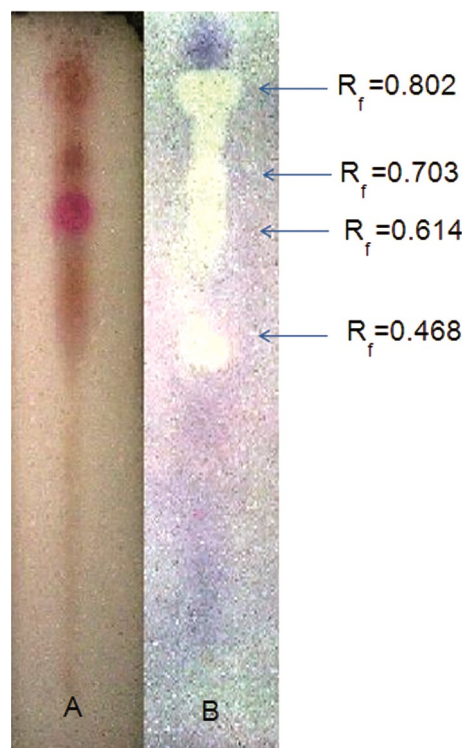


Fig. 1 — Screening of antibacterial compounds in *Cupressus torulosa* essential oil using bioautography (a) Visualization of essential oil compounds by vanillin/sulphuric acid reagent. (b) Bioautography with *B. subtilis* for screening of antibacterial compounds in essential oil.

Table 2 — Antifungal activity of essential oil and extracts of leaves of *Cupressus torulosa* by poisoned food technique

Fungal strains	Inhibition of fungal growth, $IC_{50}$ (% v/v)				
	Essential oil (mg/mL)	Methanol (mg/mL)	Aqueous (mg/mL)	Chloroform (mg/mL)	Amphotericin B ( $\mu$ g/mL)
<i>Alternaria alternata</i>	0.32	2.07	1.91	2.15	9.5
<i>Curvularia lunata</i>	0.96	2.25	1.51	1.39	12.1
<i>Bipolaris specifera</i>	0.19	4.99	2.25	2.78	5.7

[Amphotericin B: positive control;  $IC_{50}$  values are expressed as mean  $\pm$  SD of triplicate experiments]

Table 3—Preliminary phytochemical analysis of three extracts of *Cupressus torulosa*

Phytochemicals	Chloroform extract	Methanol extract	Aqueous extract
Terpenoids	++	++	-
Coumarins	-	++	+
Quinones+	++	+	
Saponins-	+	+	
Glycosides	-	+	-
Tannins-	+	+	
Alkaloids	+	+	-
Anthraquinones	-	-	-
Phenols-	++	-	
Flavonoids	-	-	-

[++ indicates good amount; + indicates low amount; - indicates absence]

The phytochemical analysis showed that the methanol extract contains significant amounts of terpenoids, coumarins, quinones and phenols, whereas, glycosides, alkaloids, and tannins were found in low quantity (Table 3). Trace amounts of coumarins, quinines, tannins and saponins were found in aqueous extract and chloroform extract was rich in terpenoids. Polyphenols are known to have biological activities like antimicrobial, antioxidants, etc. These activities are related to their molecular structures; by their hydroxyl groups or by phenolic ring, phenolic compounds have capacity to link with proteins and bacterial membrane to form complexes<sup>20-22</sup>. Total phenol content in the extracts of *Cupressus torulosa* was evaluated by Folin-Ciocalteu method. Qualitative and quantitative analysis were found in agreement as it showed that the methanol extract contained noteworthy amounts of phenolic compounds (123.56 mg GAE/g dry wt.) and negligible traces were observed in chloroform and aqueous extracts. It was interesting to observe that among the three, only methanol extract showed antimicrobial activity which could be due to the presence of wide range of phytochemicals in the extract.

### Conclusion

The present study demonstrates that the essential oil of *Cupressus torulosa* bears good inhibitory activity against different bacterial and fungal strains tested as compared to the different extracts. TLC-bioautography of essential oil confirmed the presence of four compounds which are responsible for its antibacterial activity. GC-MS analysis of *Cupressus torulosa* essential oil showed the presence of high amounts of 3-carene and  $\alpha$ -pinene, which are known antibacterial

agents. Therefore, it can be concluded that the antibacterial activity of *C. torulosa* essential oil may be attributed to the presence of these compounds.

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