**In vitro antimicrobial and antioxidant activities of the essential oil of Craniotome furcata**

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**Abstract:** The aim of present study was, to investigate the essential oil of Craniotome furcata for its antimicrobial and antioxidant activities. The essential oil was obtained by steam distillation of the flowering aerial parts of *C. furcata*. The oil was tested for its antimicrobial activity *in vitro* against four Gram-positive, four Gram-negative bacteria and three fungi, by using disk diffusion and tube dilution methods. The antioxidant potential of the essential oil was investigated by two different models DPPH free radical and ABTS free radical cation scavenging. The essential oil was effective against all the tested microorganisms, with minimum inhibitory concentrations (MICs) ranged from 4.11±1.44 to 0.30±0.10 mg/ml. The oil showed antioxidant activity in DPPH free radical and ABTS free radical cation scavenging model. The results of this study revealed that the essential oil of *C. furcata* possesses antimicrobial and antioxidant activities.

**Keywords:** Craniotome furcata, Antimicrobial activity, MIC, Antioxidant activity, DPPH, ABTS

**INTRODUCTION**

Various medicinal plants have been used for years in day to day life for treating diseases all over the world. The use of traditional medicine and medicinal plants in developing countries, to meet some of their primary health care needs, has been widely documented (WHO, 2007). Several essential oils and their isolates have been found to exhibit strong antibacterial and antifungal activities. The essential oils are supposed to interfere with intermediary metabolism of microorganisms by changing the rate of an enzyme reaction influencing nutrient uptake from the medium affecting enzyme synthesis at nuclear or ribosomal level or changing membrane structures and kill the microorganisms (Delaquis, et al., 2002; Burt and Reinders, 2003). Plants have an almost limitless ability to synthesize aromatic substances, most of which are phenols or their oxygen-substituted derivatives (Geissman, 1963). These compounds protect the plant from microbial infection and deterioration (Cowan, 1999). Some of these phytochemicals can significantly reduce the risk of cancer due to polyphenol antioxidants and anti-inflammatory effects. Some preclinical studies suggested that phytochemicals can prevent colorectal cancer and other cancers (Michaud, et al., 2000; Greenberg, et al., 1994; Birt, et al., 2001). *Craniotome furcata* Link. O. Kuntze belongs to family Lamiaceae, the only species of the genus *Craniotome*, is widely distributed in temperate areas from Simla to Bhutan at a height of 1500-2300 m (Gupta, 1968). The phytochemicals present in *C. furcata* are cranioside A and B, mussaeniside, ningpogenin (Yue, et al., 2001) triterpene saponins, cranosaponin A and buddelesaponins Ia and I (Fan, et al., 2002) from the ethyl acetate and n-butanol extracts respectively, while germacrene D, germacrene D-4-ol, epi-α-cadinol and 10-epi-α-eudesmol have been reported as the main constituents of the essential oil of flowering aerial parts of this herb (Joshi and Pande, 2008). The leaves of *C. furcata* have been traditionally used for treatment of cuts and wounds (Manandhar, 1992). The present study was carried out to evaluate the antimicrobial effects and antioxidant potential of the essential oil of *C. furcata*. To the best of my knowledge, this is the first report on the antimicrobial and antioxidant activities of the essential oil of flowering aerial parts of this plant.

**MATERIALS AND METHODS**

**Test material:** The plant was collected from Nainital, India. The botanical identification was done at Botany Department, Kumaun University, Nainital, which was further confirmed by the Forest Research Institute, Dehradun, India (herbarium specimen No-34806).

**Extraction of essential oil:** Fresh flowering aerial parts (1 kg) of *C. furcata* were steam distilled using copper still fitted with spiral glass condensers for 3 hours and water distillate was extracted with n-hexane and dichloromethane. The organic phase was dried over anhydrous sodium sulphate. The solvent was removed by distillation using a thin film rotary vacuum evaporator.
The yield of essential oil was 0.25% (v/w). Composition of essential oil: The compounds studied in this assay were reported as 3-octanone (0.2%), α-cubebene (0.6%), α-caryophyllene (1.6%), germacrene D (49.2%), epi-cubeol (0.6%), trans-α-γuaiene (1.9%), germacrene A (3.2%), cubebol (2.1%), α-cadinene (1.7%), selina-3,7(11)-diene (1.9%), germacrene B (0.3%), germacrene D-4-ol (8.8%), α-biotol (0.4%), 10-epi-α-eudesmol (4.2%), epi-α-cadinol (5.9%), α-cadinol (2.3%), khusinol (1.4%) and oplopanone (1.5%) from the essential oil of flowering aerial parts of C. furcata (Joshi and Pande, 2008).

Antimicrobial activity: Microbial strains: Bacillus subtilis (NCIM 2063), Micrococcus flavus (NCIM 2379), Staphylococcus aureus (NCIM 2079), Staphylococcus faecalis (NCIM 2080) (Gram-positive bacteria), Escherichia coli (NCIM 2574), Klebsiella pneumoniae (NCIM 2957), Pseudomonas aeruginosa (NCIM 5029), Salmonella typhimurium (NCIM 2501) (Gram-negative bacteria) and Aspergillus niger (NCIM 620), Aspergillus fumigatus (NCIM 902) and Penicillium chrysogenum (NCIM 733) (Fungi) were obtained from the National Collection of Industrial Microorganisms (NCIM), National Chemical Laboratory, Pune, India.

Preparation of test sample: The essential oil was dissolved in 10% dimethyl sulfoxide (DMSO) which is reported to be non toxic to microorganisms at this percentage (Pujol et al., 1990) with Tween 80 (1.0% v/v for easy diffusion) to a concentration range 3.0 to 0.005 mg/ml for bacteria and 5.0 to 0.009 mg/ml for fungi. Negative controls were prepared using discs impregnated with 10% aqueous DMSO with Tween 80 (1.0% v/v) as solvent control and virgin control (blank control). Streptomycin (Nicholas Piramal India Limited, Gujarat, India) and fluconazole (Shwarde Pharmaceuticals (I) Pvt. Ltd., Goa, India) were used as positive reference standards for bacterial and fungal strains, respectively.

Preparation of inoculums: The inocula of microbial strains were prepared from 18 hours old culture and suspensions were adjusted to 0.5 McFarland standard turbidity (~10^6 colony forming unit (CFU) per milliliter (McFarland, 1987).

Disk diffusion assay: Petri plates were prepared by pouring 20 ml of nutrient agar (NA) (HI MEDIA, India) for bacterial strains and potato dextrose agar (PDA) (HI MEDIA, India) for fungal strains and allowed to solidify for half an hour. The inoculum (0.05 ml) was spread on the top of the solidified media and allowed to dry for 10 min. The sterile discs (6 mm diameter) were impregnated with 20 µl of different concentrations (3.0 to 0.005 mg/ml for bacteria and 5.0 to 0.009 mg/ml for fungi) of essential oil. The plates were left for 30 min at room temperature to allow the diffusion of essential oil. The plates were then incubated for 24 h and 48 h at 37 °C for bacteria and fungi, respectively. The diameters of inhibition zones were measured in millimeters (Joshi et al., 2009).

Tube dilution assay (minimum inhibitory concentration): The minimum inhibitory concentration (MIC) was determined using a microdilution method in 96-well microtiter plates (microdilution test system – Thermo Fisher Scientific, India) and read using an ELISA plate reader (Synergy 2, Bio-Tek, USA).

Table 1. Antibacterial activity of the essential oil of Craniothome furcata.

| Microbial strains | Essential oil (mg/ml) (Zone of inhibition in mm) | RA* (mg/ml) | Minimum inhibitory concentration (MIC) |
|-------------------|-----------------------------------------------|--------------|----------------------------------------|
|                   | Essential oil (mg/ml) | 3.0 | 1.5 | 0.75 | 0.37 | 0.1 | Essential oil (mg/ml) | RA (mg/ml) |
| Gram-positive      |                               |               |    |     |       |     |                           |             |
| Bacillus subtilis  | 13                             | 12            | 11 |    - |       | 18  | 0.62 ± 0.21               | 0.003 ± 0.001 |
| Micrococcus flavus | 12                             | 11            | 10 | 8   |       | 13  | 0.62 ± 0.21               | 0.002 ± 0.001 |
| Staphylococcus aureus | 14                           | 13            | 11 | 10  |       | 15  | 0.30 ± 0.10               | 0.003 ± 0.001 |
| Staphylococcus faecalis | 14                        | 12            | 11 | 9   |       | 20  | 0.49 ± 0.21               | 0.002 ± 0.001 |
| Gram-negative      |                               |               |    |     |       |     |                           |             |
| Escherichia coli   | 14                             | 12            | 9  |    - |       | 16  | 0.62 ± 0.21               | 0.002 ± 0.001 |
| Klebsiella pneumonia | 13                           | 12            | 10 |    - |       | 20  | 1.0 ± 0.43                | 0.002 ± 0.001 |
| Pseudomonas aeruginosa | 13                        | 12            | 10 | 8   |       | 13  | 0.43 ± 0.29               | 0.003 ± 0.001 |
| Salmonella typhimurium | 12                          | 11            | 9  |    - |       | 14  | 0.87 ± 0.57               | 0.003 ± 0.001 |

* RA= Reference antibiotic (Streptozotocin); MIC values are mean ± SD of three experiments in triplicate.
Table 2. Antifungal activity of the essential oil of Craniotome furcata.

| Microbial strains   | Essential oil (mg/ml) (Zone of inhibition in mm) | RA* (mg/ml) | Minimum inhibitory concentration (MIC) |
|---------------------|-----------------------------------------------|-------------|--------------------------------------|
|                     | 5.0 | 2.5 | 1.2 | 0.62 | 0.1 | Essential oil (mg/ml) | RA (mg/ml) |
| **Fungi**           |     |     |     |     |     |                      |            |
| Aspergillus niger   | 9   | -   | -   | 13  | 4.11 ± 1.44 | 0.006 ± 0.002 |
| Aspergillus fumigatus | 8   | -   | -   | 12  | 2.91 ± 1.90 | 0.005 ± 0.002 |
| Penicillium chryogenum | 8   | -   | -   | 14  | 3.75 ± 2.16 | 0.006 ± 0.002 |

RA= Reference antibiotic (Fluconazole); MIC values are mean ± SD of three experiments in triplicate.

ABTS radical decolourization assay: 2, 2-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) diammonium salt radical cation decolourization test was performed using spectrophotometric method of Pellegrini et al., 1999.

RESULTS AND DISCUSSION

Antimicrobial activity: Table 1 and 2 summarizes the qualitative (diameters of inhibition zones) and quantitative results (minimum inhibitory concentrations) of the in vitro antimicrobial effect of the essential oil of C. furcata on the eleven microbial strains. The diameters of inhibitory zones recorded in millimeter. It was observed that the oil was effective against all tested microbial strains. The size of all inhibition zones were between 8 to 14 mm. Average size of inhibition zones was around 11 mm. The effective dilution range of essential oil was determined using tube-dilution technique. The essential oil was dissolved in 10% dimethyl sulfoxide (DMSO) with Tween 80 (1.0% v/v for easy diffusion). The final concentration of essential oil was 3.0 mg/ml for bacterial strains while 5.0 mg/ml for fungal strains, respectively. Serial (two-fold) dilution was prepared from the stock solution to give concentration ranging from 3.0-0.005 mg/ml of the oil for bacterial strains. The concentration range screened for fungal strains was 5.0-0.009 mg/ml of the oil. Streptomycin and fluconazole were dissolved in sterile distilled water and two-fold dilutions were prepared (1.02 mg/ml to 0.001 mg/ml). One ml of each concentration was mixed with 1.0 ml of sterile peptone water (10⁶ CFU/ml for bacteria and 10⁵ CFU/ml for fungal concentration, obtained from a McFarland turbidity standard (no. 0.5). Solvent control was prepared with DMSO (10%) with Tween 80 (1.0% v/v) and blank control was prepared by virgin media. Tubes were incubated for 24 h and 48 h at 37°C for bacteria and fungi, respectively. Assay was repeated in triplicate. The MIC values are expressed as mean ± standard deviation. The lowest concentration that inhibits the visible microbial growth was determined as MIC (Murthy et al., 2006 and Kuta, 2008).

Antioxidant activity:

DPPH radical scavenging activity: The antioxidant activity of the essential oil was determined using 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical according to the method of Hou et al., 2001, with slight modification. The principle of the reduction of DPPH free-radical assay is that the antioxidants react with the stable DPPH free radical and convert it into 1, 1-diphenyl-2-picrylhydrazine. The ability to scavenge the stable DPPH free radical is measured by a decrease in the absorbance. The DPPH free stable radical scavenger, which was dissolved in ethanol to give a 0.05 mM solution. Aliquots containing various concentrations (10-100 µg) of antioxidant reference (ascorbic acid) in the final volume of 1.0 ml were mixed with 1.0 ml of ethanolic DPPH solution. The oil was tested using the same method. The ethanolic solution of DPPH (1.0 ml) with equal amount of ethanol served as control. The reaction mixtures were incubated at 37°C for 20 min and the absorbance of reaction mixtures was recorded at 517 nm.

The reaction mixtures were incubated at room temperature. The absorbance was measured at 734 nm after initial mixing of the reactants within 1-3 minutes. The change in the absorbance of the reaction mixture in DPPH and ABTS assay were measured using a double beam UV-Vis spectrophotometer (ECIL) and the percent scavenging or inhibition was calculated according to the following formula.

Percent scavenging or inhibition = (Absorbance of control – Absorbance of test / Absorbance of control) x 100. All the experiments were performed three times and the values were presented as Mean ± SEM.
mg/ml of essential oil was effective against fungal strains, which were comparatively resistant than the bacterial strains. Microorganisms most susceptible at a concentration of 3.0 mg/ml of the essential oil were S. aureus, S. faecalis, E. coli (inhibition zones (IZ) 14 mm), B. subtilis, K. pneumoniae, P. aeruginosa (IZ 13 mm) and M. flavus (IZ 12), while 5.0 mg/ml was observed with zone of inhibition 9 mm for A. niger and 8 mm for A. fumigates and P. chrysogenum, respectively.  The MICs of the oil demonstrated good activity against tested microorganisms. The organisms like S. aureus was found to be most susceptible with MIC value of 0.30 ± 0.10 mg/ml, while P. aeruginosa and S. faecalis were found moderately susceptible with MIC values 0.43 ± 0.29 and 0.43 ± 0.21 mg/ml. The microorganisms B. subtilis, M. flavus and E. coli were less susceptible at a concentration of 0.62 ± 0.21 mg/ml. The organisms like K. pneumonia and S. typhimurium were comparatively less sensitive with a concentration of 1.0 ± 0.43 and 0.87 ± 0.57 mg/ml respectively. The MIC value was found 2.91 ± 1.90 for A. niger while, the organisms P. chrysogenum and A. niger were comparatively less susceptible at a concentration of 3.75 ± 2.16 and 4.16 ± 1.44 mg/ml. The essential oil was effective against bacterial strains than fungal strains.

Antimicrobial activity: Due to its complexity more than one test must be carried out when evaluating the antioxidant activity of pure compounds or extracts (Aruoma, 2003). In this study, two models were selected to evaluate the antioxidant potential of the essential oil of C. furcata. Antioxidant activities of the essential oils from aromatic plants are mainly attributed to the active compounds present in them. This can be due to the high percentage of the main constituents, but also to the presence of other constituents in small quantities or to synergy among them (Abdalla et al., 1999). The chemical complexity of the essential oil, often a mixture of dozens of compounds with different functional groups, polarity and chemical properties, makes it difficult to predict its overall activity. A notable finding in this study is the high antioxidant activity of C. furcata, which may be attributed to the presence of certain phenolic compounds and other classes of secondary metabolites.

Table 3. Antioxidant potential of essential oil of DPPH and ABTS free radicals.

| Material      | DPPH (µg/ml) | ABTS* (µg/ml) |
|---------------|--------------|---------------|
| Essential oil | 30.33 ± 0.66 | 48.33 ± 0.66  |
| Ascorbic acid | 8.33 ± 0.57  | 7.33 ± 0.88   |

Values expressed as mean ± SEM; *Expressed by IC50 values (µg/ml).

Fig. 1. Antioxidant activity of the essential oil of Craniotome furcata.
behavior, the antioxidant activity can occur through several mechanisms. For determination of in vitro antioxidant activity of the essential oil of C. furcata, DPPH free radical and ABTS free radical cation scavenging models were used. DPPH and ABTS are stable free radicals that can accept an electron or hydrogen radical to become stable diamagnetic molecule. The DPPH radical is widely used as a model system to investigate the free-radical scavenging activities of several plant extracts. Fig. 1 shows the concentration of essential oil on DPPH radical and ABTS radical cation scavenging activity. The DPPH and ABTS scavenging activities were compared with standard ascorbic acid (Table 3). The essential oil scavenged the DPPH radicals in a concentration-dependent manner with the maximum scavenging activity of 80% at 50 μg/ml did not attained saturation with further increase in the essential oil concentration. The IC50 value was found to be 30.33 ± 0.66 μg/ml. Similarly essential oil demonstrated ABTS radical scavenging activity in a concentration dependent manner with 76% at 100 μg/ml essential oil concentration attaining saturation with higher concentrations. The IC50 value was observed 48.33 ± 0.66 μg/ml. (Fig. 1). The results presented in this study revealed that the C. furcata essential oil possesses antioxidant capacity to scavenge free radicals, which can be ascribed the content of the phenolic compounds in this oil. In fact, various studies reported the correlation between the phenolic content and the antioxidant capacity of plant extracts (Gulcin, et al., 2004; Chun et al., 2005). Gemaacrene-D4-ol, 10-epi-α-eudesmol, epip-α-cadinol, α-cadinol, and khusinol were the main compounds of this oil, can be responsible for antioxidant activity due to the presence of hydroxyl groups in their chemical structure.

Conclusion
The Lamiaceae plants have many medicinal uses especially due to their high content of essential oils. The results in this study revealed that, the essential oil of C. furcata has antibacterial property while the oil was less effective against the tested fungal strains. The antioxidant activity of the essential oil of C. furcata may be an alternative to more toxic synthetic antioxidants as additives in food, pharmaceutical and cosmetic preparations. In conclusion the essential oil of C. furcata has antimicrobial and antioxidant properties.

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