

Original Article

PHYTOCHEMICAL ANALYSIS AND *IN VITRO* STUDIES ON ANTIBACTERIAL, ANTIOXIDANT AND ANTI-INFLAMMATORY ACTIVITIES USING *CASUARINA EQUISETIFOLIA* BARK EXTRACTS

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ABSTRACT

Objective: *Casuarina equisetifolia* is an important multipurpose exotic forest tree species widely cultivated in the coastal regions of Tamil Nadu that serves as a warehouse of essential secondary metabolites. Identification of these bioactive compounds in this forest tree species might lead to the discovery and development of a new drug to treat various diseases.

Methods: The present study was carried out with an objective to analyse the phytochemicals qualitatively and quantitatively. Gas Chromatography-Mass Spectrometry (GC-MS) analysis was performed to evaluate the presence of various volatile compounds. An *in vitro* antibacterial, antioxidant and anti-inflammatory properties of aqueous and organic solvents of *C. equisetifolia* bark was studied.

Results: The preliminary qualitative screening revealed the presence of alkaloids, glycosides, carbohydrates, proteins, flavonoids, phenols, terpenoids, and tannins. The quantitative analysis revealed the presence of maximum phenols (71.2±0.51 mg/g), flavonoids (35.12±0.34 mg/g), tannins (77.59±0.21) and terpenoids (6%) in methanolic root extract with respective standards. Several peaks were obtained in the GC-MS analysis which indicates the presence of different secondary metabolites. Antibacterial activity showed a maximum zone of inhibition against *Escherichia coli* (23±0.24 mm) and *Proteus vulgaris* (23±0.32 mm). The antioxidant potential of various extracts was compared with the standard ascorbic acid. Anti-inflammatory activity was compared with standard diclofenac sodium and the extract showed activities significantly in a dose-dependent manner.

Conclusion: From this study, it is revealed that *C. equisetifolia* bark extract possesses efficient antibacterial property, the potential in scavenging free radical, effective antioxidant, powerful anti-inflammatory source that can be employed in the development of a novel drug to treat various diseases.

Keywords: *Casuariana equisetifolia*, bark, Phytochemicals, Antioxidant, Antibacterial, Anti-inflammatory, GC-MS

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INTRODUCTION

Plants serve as a potential storage of various secondary metabolites (phytochemicals). These phytochemicals covers diverse and important biochemical components, that serves as a raw material for the development of pharmacologically active natural products. Plants still serve as the base for the existence and development of traditional medicine system for thousands of years in India. A study conducted by [1] reports that, the drugs that are developed for the commercial purpose were obtained from higher plants, of which 74% of drugs were developed with the help of ethnobotanical information. Plant-based drugs have greater scientific and economic significance. Phenols, flavonoids, tannins, terpenoids, alkaloids and steroids are a group of secondary metabolites obtained from plants that have drawn the attention of pharmaceutical industries in recent years [2].

Casuarina equisetifolia Linn. Belongs to the family Casuarinaceae. It is a non-leguminous, woody forest tree which is in symbiotic association with an actinomycete, *Frankia*. Thus, this association helps in nitrogen fixation and also makes the tree to adapt to varied soil types and extreme environmental stress conditions. *C. equisetifolia* is mainly cultivated in coastal regions to provide fuelwood, protection against shifting dunes, heavy wind blow and in addition it serves as a stable base for agriculture [3, 4]. In south India, *C. equisetifolia* is employed exclusively in agroforestry system. The pulp material of this tree crop is widely used in the paper industries. Other parts of the tree crop like root, bark and cladode which has potent phytochemical constituents are marked as waste material and are used for fuel purpose by local people. This study is an attempt to explore the potential phytoconstituents of one such resource namely bark and its pharmaceutical evaluation.

Presence of high amount of tannin in the bark of *C. equisetifolia* marks its action against toothache traditionally [5]. A study

conducted by [6] confirmed the presence of alkaloids, flavonoids, tannins and proteins, which could be responsible for the potent antibacterial and antioxidant property.

The objective of the current study is to evaluate the phytochemicals qualitatively and quantitatively, GC-MS analysis, antibacterial potential, antioxidant and anti-inflammatory ability of the bark extracts using aqueous and organic solvents.

MATERIALS AND METHODS

Collection of plant material

The bark samples of *Casuarina equisetifolia* Linn. Was collected from Nimilenchery, Pondicherry Union territory (Latitude: 12.10'; Longitude 79.9'), Tamil Nadu. The plant sample was taxonomically identified and authenticated as *Casuarina equisetifolia* Linn., Casuarinaceae, Ref No: BSI/SRC/5/23/2015/Tech/2012 Botanical Survey of India, Coimbatore.

Chemicals and reagents

Benzene, chloroform, methanol, ethanol, petroleum ether, folin-ciocalteu reagent, folin-denis reagent, sodium carbonate, gallic acid, sodium nitrite, aluminium chloride, sodium hydroxide, quercetin, 2,2-diphenyl-1-picrylhydrazyl, hydrogen peroxide, ascorbic acid, potassium ferric cyanide, sodium salicylate, sulphuric acid, sodium phosphate.

Preparation of bark extract

Dried bark samples of *C. equisetifolia* was surface sterilized, cut into small pieces and was finely powdered. Powdered bark samples were extracted using water and organic solvents (Benzene, Chloroform, Methanol and Ethanol) in the ratio 1:10 w/v, for 48 h, by cold percolation method. The extract was filtered, concentrated and dried and stored at 4 °C until further use.

Qualitative phytochemical screening

Preliminary phytochemicals screening of bark samples were carried out using different solvents (Aqueous, Benzene, Chloroform, Methanol and Ethanol). The qualitative phytochemical analysis was carried out to screen the presence of phenols, alkaloids, flavonoids, terpenoids, carbohydrates, saponins, proteins and amino acids, phlobatannins, glycosides and tannins using standard procedures.

Quantitative analysis

Estimation of total phenol content

The total phenolic content in aqueous, ethanol and methanol extracts of *C. equisetifolia* bark was determined by Folin-Ciocalteu reagent (FCR) [7]. To 1 ml of the various extracts, 1 ml of Folin-Ciocalteu reagent was added and the mixture was incubated for 5 min. To this, 10 ml of 7% Na₂CO₃ solution was added and incubated in dark at 23 °C for 90 min. The absorbance of the mixture was read at 765 nm against the blank using UV-visible spectrophotometer (UV 1650 Pc, Shimadzu). Gallic acid was used as a standard. The total phenolic content in the extracts was expressed in Gallic acid equivalent (GAE).

Estimation of total flavonoid content

The total flavonoid content was estimated using aluminium chloride colourimetric method [8]. 0.5 ml of various bark extracts were mixed with 2 ml of solvent and 0.30 ml of 5% sodium nitrite solution individually. To this solution mixture, 0.3 ml of 10% AlCl₃, 2 ml of 1M sodium hydroxide and 2.8 ml of distilled water was added and incubated at room temperature for 30 min. The absorbance was read at 510 nm using UV-visible spectrophotometer (UV 1650 Pc, Shimadzu). Quercetin was used as the standard for estimation. Total flavonoid concentration was expressed in terms of Quercetin equivalent (mg of QAE/g of extract).

Estimation of tannin

The total tannin content in aqueous, ethanol and methanol extracts of *C. equisetifolia* bark was estimated by Folin Denis method [9], with slight modifications. To 1 ml of bark extracts (aqueous, ethanol and methanol), 3 ml of water, 0.5 ml of Folin-Denis reagent and 1.0 ml of 1N sodium carbonate solution were added. This mixture was diluted with water. The solution was mixed thoroughly and the absorbance was read at 515 nm. Tannic acid was used as the standard. Total tannin content was expressed as milligrams of tannic acid equivalents per gram of dried sample (mg of TAE/g of extract).

Estimation of total terpenoids

Total terpenoid content was estimated [10] for ethanol, methanol and aqueous extract of *C. equisetifolia* bark. The crude extract was soaked in 20 ml of 95% ethanol for 24 h. The filtrate was extracted with petroleum ether (60°C - 80°C). The residue of the extract obtained from the petroleum ether extract was dried and weighed to estimate the total terpenoids.

$$\text{Terpenoid content (\%)} = \frac{\text{Weight of terpenoid extract (g)}}{\text{Weight of the sample (g)}} \times 100$$

GC-MS studies

The methanolic bark extract was subjected to GC-MS (Perkin Elmer, Sophisticated Instrumentation Facility, Vellore Institute of Technology University, Vellore, Tamilnadu) analysis to identify the bioactive compounds. The Clarus 680 GC was used in the analysis that employed a fused silica column, packed with Elite-5MS (5% biphenyl 95% dimethylpolysiloxane, 30 m × 0.25 mm ID × 250µm df). The components were separated using Helium as a carrier gas, at a constant flow of 1 ml/min. The injector temperature was set at 260 °C during the chromatographic run. 1µL of the bark extract was injected into the instrument. The oven temperature was as follows: 60 °C (2 min); followed by 300 °C at the rate of 10 °C min⁻¹; and 300 °C, where it was held for 6 min. The mass detector conditions were: transfer line temperature 240 °C; ion source temperature 240 °C; and ionization mode electron impact at 70 eV, a scan time 0.2 sec and scan interval of 0.1 sec. The fragments from 40 to 600 Da. The spectrum of the components were compared with the database of the spectrum of known components stored in the GC-MS NIST (2008) library.

Antibacterial activity

In vitro antibacterial assay using aqueous, ethanolic and methanolic bark extract of *C. equisetifolia* of various concentrations (25 µg, 50 µg, 75 µg, 100 µg) was determined by agar well diffusion method, using Muller Hinton agar medium. The antibacterial efficiency of bark extracts was tested against *Escherichia coli*, *Bacillus subtilis*, *Staphylococcus aureus*, *Proteus vulgaris* obtained from the Department of Microbiology, Ethiraj College for women, Chennai. Zone of inhibition was observed after incubating the plates at 37 °C for 24 h. Gentamycin (100µg) was used as positive control and the solvent was used as negative control for the bacterial assay. Triplicates were maintained.

Antioxidant assay

2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay

The free radical scavenging activity of aqueous, ethanol and methanol bark extract of *C. equisetifolia* was tested using DPPH radical scavenging assay [11]. Four different concentration of bark extracts were prepared (20 µg/ml, 40 µg/ml, 60 µg/ml, 80 µg/ml). 2 ml aliquot of DPPH solution was added to 0.5 ml of each concentration of various bark extracts. The reaction mixture was incubated at room temperature for 30 min. The absorbance was determined at 517 nm using UV-visible spectrophotometer (UV 1650 Pc, Shimadzu). Ascorbic acid was used as a standard. Percent inhibition and Inhibitory concentration (IC₅₀) value were calculated. Experiments were performed in triplicates and the average was calculated for each concentration.

$$\text{DPPH scavenging effect (\%)} \text{ or Percent inhibition} \\ = A_0 - A_1/A_0 \times 100$$

Where A₀ was the absorbance of the control (blank, without extract) and A₁ was the absorbance in the presence of the extract.

Hydrogen peroxide scavenging assay

The ability of bark extracts to scavenge hydrogen peroxide was determined spectrometrically [12]. Different concentrations (20 µg/ml, 40 µg/ml, 60 µg/ml, 80 µg/ml) of various bark extracts were added to 0.6 ml of 2 mmol/l of hydrogen peroxide solution prepared in phosphate buffer (pH=7.4). Absorbance was measured at 230 nm against blank solution (phosphate buffer) using UV-visible spectrophotometer (UV 1650 Pc, Shimadzu) and was compared with the standard Ascorbic acid. Experiments were performed in triplicates.

$$\text{Hydrogen peroxide scavenging effect (\%)} = A_c - A_o/A_c \times 100$$

Where A₀ was the absorbance of the control (blank, without extract) and A₁ was the absorbance in the presence of the extract.

Reducing power assay

The reducing power of aqueous, ethanol and methanol extract of *C. equisetifolia* bark were determined [13]. Various concentrations (20 µg/ml, 40 µg/ml, 60 µg/ml, 80 µg/ml) of corresponding solvent extracts of *C. equisetifolia* bark, were mixed with 0.2M phosphate buffer (2.5 ml) and 1% potassium ferricyanide (2.5 ml). The reaction mixture was incubated at 50 °C for 20 min. After cooling, 2.5 ml of 10% trichloroacetic acid was added to the reaction mixture and centrifuged at 3000rpm for 10 min. The supernatant (2.5 ml) was collected and mixed with freshly prepared 0.1% FeCl₃ (0.5 ml).

The absorbance was measured at 700 nm using UV-visible spectrophotometer (UV 1650 Pc, Shimadzu). Ascorbic acid was used as a reference compound. Experiments were performed in triplicates.

$$\text{Reducing power assay (\%)} = \frac{\text{Control OD} - \text{Test OD}}{\text{Control OD}} \times 100$$

Hydroxyl radical scavenging assay

The hydroxyl radical scavenging assay experiment was carried out using standard method [14]. The reaction mixture was prepared using 1 ml FeSO₄ (1.5 mmol), 0.7 ml hydrogen peroxide (6 mmol), 0.3 ml sodium salicylate (20 mmol). Different concentrations (20

µg/ml, 40 µg/ml, 60 µg/ml, 80 µg/ml) of various bark extracts were added to 3 ml of reaction mixture. After incubation for 1h at 37°C, the absorbance of the hydroxylated salicylate complex was measured at 562 nm using UV-visible spectrophotometer (UV 1650 Pc, Shimadzu). Ascorbic acid was used as the standard. Experiments were performed in triplicates.

$$\text{Per cent inhibition} = A_0 - A_1/A_0 \times 100$$

Where A_0 was the absorbance of the control (blank, without extract) and A_1 was the absorbance in the presence of the extract.

Total antioxidant capacity assay

The total antioxidant capacity assay of aqueous extract, ethanol extract and methanol extracts of *C. equisetifolia* bark were determined by a standard method [15]. Different concentrations of *C. equisetifolia* bark extracts (20 µg/ml, 40 µg/ml, 60 µg/ml, 80 µg/ml) were added to 1.0 ml of the reagent solution (0.6 M sulphuric acid, 28 mmol sodium phosphate and 4 mmol ammonium molybdate). The reaction mixture was incubated at 95 °C for 90 min. After cooling, the absorbance was measured at 695 nm against a blank using UV-visible spectrophotometer (UV 1650 Pc, Shimadzu). Ascorbic acid was used as a standard. Experiments were performed in triplicates.

$$\text{Per cent inhibition} = A_0 - A_1/A_0 \times 100$$

Where A_0 was the absorbance of the control (blank, without extract) and A_1 was the absorbance in the presence of the extract.

In vitro anti-inflammatory activity

Protein denaturation ability of various solvents extracts of *C. equisetifolia* was studied using inhibition of albumin denaturation technique [16]. Various concentration of test solution (20 µg, 40 µg, 60 µg, 80 µg) was prepared using different solvents. The reaction mixture consists of 100 µl of different concentrations and 500 µl of 1% of aqueous bovine albumin fraction.

The reaction mixture was incubated at 37 °C for 10 min and then heated at 51 °C for 20 min. The turbidity was measured at 660 nm using UV-visible spectrophotometer (UV 1650 Pc, Shimadzu) after cooling and was compared with the reference compound Diclofenac sodium. Experiments were performed in triplicates.

Percent inhibition of protein denaturation was calculated using the formula:

$$\text{Per cent Inhibition} = \frac{\text{Control OD} - \text{Test OD}}{\text{Control OD}} \times 100$$

Statistical analysis

The experiments were performed in triplicates. Data was represented as mean±SD.

RESULTS AND DISCUSSION

Qualitative phytochemical screening

Preliminary qualitative phytochemical screening of aqueous and organic solvent extracts of *Casuarina equisetifolia* bark showed the presence of varied phytochemicals (table 1). All the extracts showed the presence of proteins and phenols. Aqueous, ethanol and methanol extracts showed the presence of alkaloids, glycosides, carbohydrates, flavonoids and tannins. Benzene and chloroform extracts showed the presence of proteins, phenols and terpenoids. Ethanolic and methanolic extract revealed the presence of all the phytochemicals. The observations from the present study can be rationalized in terms of the polarity of the compounds being extracted by each solvent. The presence of phytochemicals such as alkaloids, flavonoids, carbohydrates, and tannins from the aqueous and methanolic extracts of *Casuarina equisetifolia* bark has been reported by [17].

Phytochemicals are plant derivatives that protect the plants from varied stress conditions and pathogens. Though these phytochemicals are not highlighted as essential nutrients, it has become a key constituent in the pharmaceutical industry for the development of novel drugs that has a minimum or no side effects. Plant phenolic compounds are responsible for antimicrobial activity and antioxidant activity since it reduces lipid peroxidation [18]. Flavonoids possess a functional hydroxyl group that mediates the antioxidant effect by scavenging free radical or by chelating the metal ions [19] and it is also responsible for the modification of topography of microbial cell wall, thus functioning as an antimicrobial agent [20]. Tannin is a polyphenolic compound and it has been evaluated that polyphenolic compounds acts as a potent source of antimicrobial, antioxidant [21], anti-inflammatory [22] and analgesic agent. It has been documented that the plants with antimicrobial and free radical scavenging properties accelerate wound healing by enhancing the process of re-epithelialization and contraction of the wound [23]. The medicinal value of a plant is also dependent on other phytochemicals like, alkaloids, carbohydrates, glycosides and proteins which produces definite physiological actions on the human body.

In the qualitative phytochemical screening, maximum solubility of phytochemicals was observed in aqueous, ethanol and methanol bark extracts. Thus, these three extracts were carried on for further quantification and *in vitro* studies.

Table: 1 Phytochemical analysis of different extracts of *C. equisetifolia* bark

S. No.	Phytochemicals	Aqueous	Benzene	Chloroform	Ethanol	Methanol
1	Alkaloids	+	-	-	+	+
2	Glycosides	+	-	-	+	+
3	Carbohydrates	+	-	-	+	+
4	Protiens	+	+	+	+	+
5	Phenols	+	+	+	+	+
6	Flavonoids	+	+	-	+	+
7	Tannins	+	+	-	+	+
8	Terpenoids	+	+	+	+	+

+: Presence.-: Absence

Quantitative phytochemical analysis

The total phenolic content in aqueous, ethanol and methanolic extracts of *Casuarina equisetifolia* bark was determined by Folin-cioaltea method and was expressed in Gallic acid equivalent (Standard Curve equation $y = 0.175x - 0.118$, $R^2 = 0.995$). Among the three extracts, methanol extract contained maximum phenol content (71.2±0.12 mgGAE/g of extract), followed by ethanol extract (66.8±0.17 mg GAE/g of extract) and aqueous extract (43.2±0.21 mg GAE/g of extract). (fig. 1a) The total flavonoid content in aqueous,

ethanol and methanolic extracts of *Casuarina equisetifolia* bark was determined by the aluminium chloride colourimetric method and was expressed in Quercetin equivalent (Standard Curve equation $y = 0.013x - 0.161$, $R^2 = 0.990$). Among the three extracts, methanol extract contained maximum flavonoids content (35.12±0.18 mg of QE/g of extract), followed by ethanol extract (32.4±0.31 mg of QE/g of extract) and aqueous extract (27.86±0.23 mg of QE/g of extract). (fig. 1a). The total tannin content was calculated using standard curve of Tannic acid ($y = 0.003x - 0.007$, $R^2 = 0.994$) and expressed in Tannic acid equivalents/g of extract.

Among the various solvents used methanolic extract revealed the highest tannin content of 77.59 ± 0.27 mg TAE/g of extract followed by ethanol and aqueous extracts with 70.45 ± 0.26 and 22.29 ± 0.19 mg TAE/g of extract respectively (fig. 1a).

The methanol root extracts showed maximum terpenoid content (6%) followed by ethanol extract (5.1%) and aqueous extract (2.9%) (fig. 1b).

Phenolic compounds are the most abundant secondary metabolite group which is very important for the defence mechanism of plants. Phytochemical compounds like phenol, flavonoids and tannins possess efficient antibacterial property [24]. Epidemiological studies have proved positive effect of plant phenolic compounds on human health that are capable of inhibiting free radicals and hence can retard the aging process [25]. Polyphenolic compounds like tannins and flavonoids inhibit the initial process of inflammation and thus acts as anti-inflammatory agents [26].

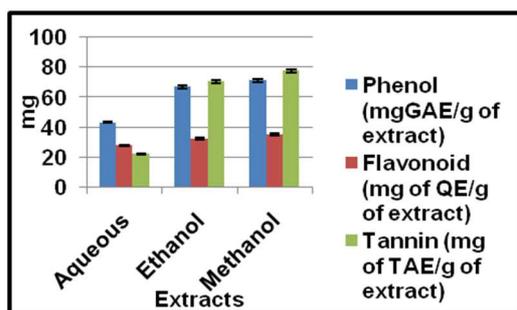


Fig. 1(a): Quantitative analysis (total phenol, flavonoid and tannin) data presented are the means of three replicates

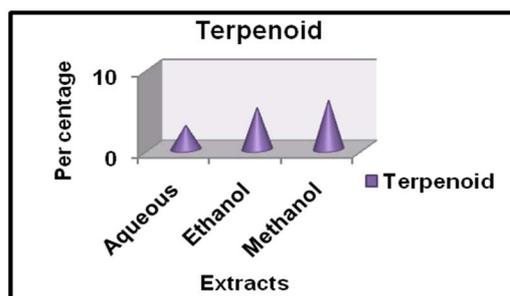


Fig. 1(b): Quantitative analysis–Total Terpenoid Values are expressed as mean of three replicates

GC-MS analysis

In the present study, methanolic bark extract of *Casuarina equisetifolia* showed significant bioactivities, hence Gas chromatography-mass spectroscopy analysis (fig. 2) was carried out in methanolic crude extracts of the bark. The peaks in the chromatogram were compared with the NIST database GC-MS library to identify the bioactive compounds.

The chromatogram revealed the presence of trihydroxy benzoic acid, isosorbide dinitrate, 1,2 benzenedicarboxylic acid, mono(2-ethylhexyl) ester, di-n-decylsulfone, sulfurous acid, decyl 2-propyl ester, silane, 1,4-phenylenebis, octadecane, 9-ethyl-9-heptyl, cyclotrisiloxane, hexamethyl.

Among the identified compounds, trihydroxybenzoic acid is a phenolic compound (gallic acid), which is a potent radical scavenger. This compound has the therapeutic effect to treat diseases like cancer, neurodegenerative disorder and aging [27-28] which is caused due to oxidative stress. Isosorbide dinitrate comes under nitrate group, which helps to treat congestive heart failure, suppress inflammation and oesophageal spasm [29]. 1,2 benzene dicarboxylic acid, mono(2-ethylhexyl) ester is a phthalates group, which is an effective source to suppress the oxidative stress of the cell [30].

Octadecane, 9-ethyl-9-heptyl is a lipid compound that degrades hydrocarbons of the cell wall and helps in antimicrobial activity, thus also serves as a potential antioxidant. Cyclotrisiloxane hexamethyl is a cyclic phenolic compound that plays a significant role in free radical scavenging. The better antioxidant property of the compound is mainly due to the high degree of unsaturation of the cyclic compound than the $-\text{COH}=\text{COH}-\text{enediol}$ group present in the standard ascorbic acid [31].

Antibacterial activity

The results indicated that the bark extracts of *C. equisetifolia* showed varied degrees of antibacterial activity at different concentration against different bacterial pathogens. The maximum zone of inhibition was seen at a $100\mu\text{g}$ concentration of methanolic extract against *Escherichia coli* (23 ± 0.24 mm) and ethanolic extract against *Proteus vulgaris* (23 ± 0.32 mm). Aqueous bark extract showed a maximum zone of inhibition against *Escherichia coli* with 20.47 ± 0.3 mm. (fig. 3).

The study conducted by [32] recorded that the methanolic bark extract of *Casuarina equisetifolia* showed a maximum zone of inhibition at 10 mg/disc against gram-negative bacteria, *E. coli* (35 ± 0.22 mm). Variation in the zone of inhibition may be correlated to the polarity of the solvent and concentration of the extracts. The synergistic effect of phytochemicals also plays a major role in the antimicrobial activity.

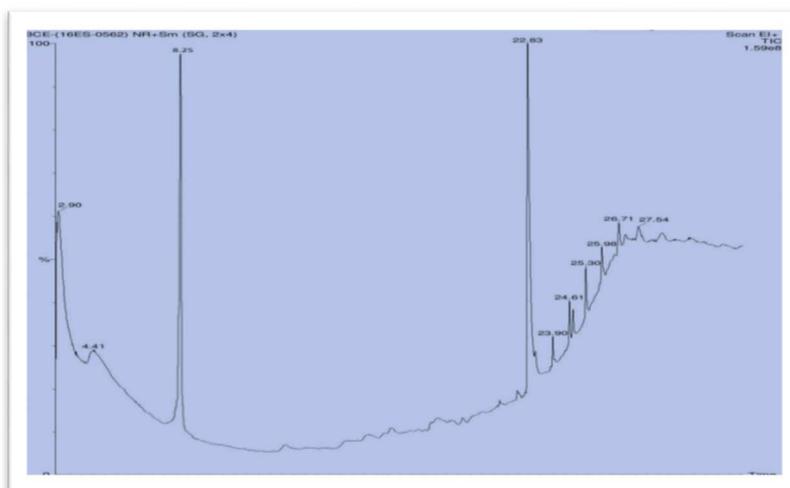


Fig. 2: GC-MS chromatogram of a methanolic extract of *C. equisetifolia*

The antimicrobial ability of phenolic compounds are based on its potential to penetrate the cell membrane by altering the cell permeability thereby inactivating the cellular enzymes [33]. Flavonoids are synthesized by the plants in response to the microbial infection which could be the reason for potential *in vitro* antibacterial assay. Flavonoids are found to form a complex with a soluble extracellular protein of bacterial cell wall, thereby destructing the topography leading to the death of the cell [34].

Tannin serves as a potential antibacterial agent because of its ability to bind with proline-rich protein that interferes with the protein production [35].

The lipophilic nature of terpenoids disturbs the membrane-bound protein of bacterial cell wall, increase the permeability of the bacterial cell, which in turn influences the ion transport process and also inhibit respiration that ultimately results in cell death.

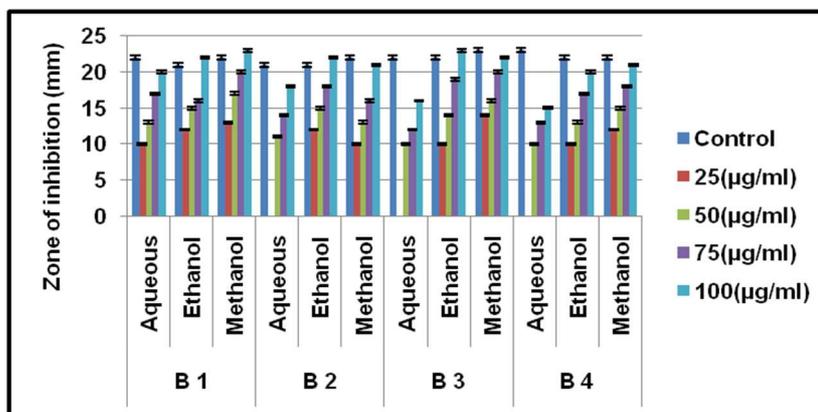


Fig. 3: Antibacterial activity of *Casuarina equisetifolia* bark extracts against different bacterial pathogens. Data presented are the means of three replicates. Values are expressed as mean \pm SD of three replicates, B1-*Escherichia coli* B2-*Bacillus subtilis*, B3-*Proteus vulgaris* B4-*Staphylococcus aureus*

Antioxidant activity

DPPH radical scavenging assay

DPPH radical scavenging assay estimates the ability of the extract to donate Hydrogen or to scavenge the free radical. The methanol extract showed percent maximum inhibition at 80 μ g concentration (91.82 \pm 0.93) followed by ethanol extract (89.55 \pm 0.75) and aqueous extracts (88.19 \pm 0.63) with IC 50 value of 49.15 \pm 0.64 μ g/ml, 51.33 \pm 0.86 μ g/ml and 54.01 \pm 0.28 μ g/ml respectively.

The DPPH activity of standard ascorbic acid showed maximum percent inhibition of 96.34 \pm 0.82 at 80 μ g concentration with IC₅₀ value of 39.22 μ g/ml. Lower IC₅₀ value indicates greater antioxidant activity (fig. 4).

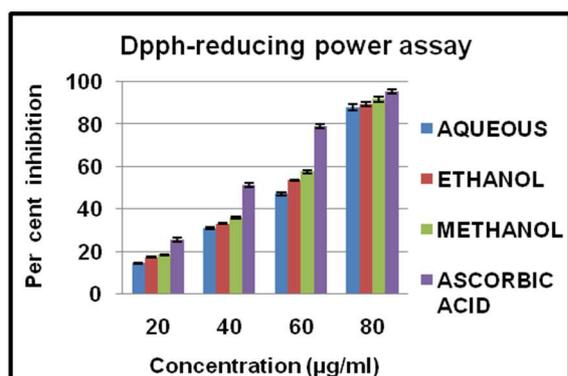


Fig. 4: 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging activity of *C. equisetifolia* bark extracts. Data presented are the means of three replicates, values are expressed as mean \pm SD of three replicates

Hydrogen peroxide scavenging assay

Hydrogen peroxide has the ability to degrade hemeprotein and release Fe ions thus, supporting the scavenging activity. The methanol bark extract showed percent maximum inhibition at 80 μ g

concentration (62.90 \pm 0.73) followed by ethanol extract (57.19 \pm 0.68) and aqueous extracts (50.47 \pm 0.23), with IC 50 value of 58.47 \pm 0.63 μ g/ml, 65.34 \pm 0.47 μ g/ml and 75.02 \pm 0.85 μ g/ml respectively. Maximum percent inhibition and IC₅₀ value indicate, methanolic bark extract as an efficient scavenger. The IC₅₀ value of Standard Ascorbic acid was 44.56 \pm 0.15 μ g/ml with percent inhibition of 89.2 \pm 0.76 (fig. 5).

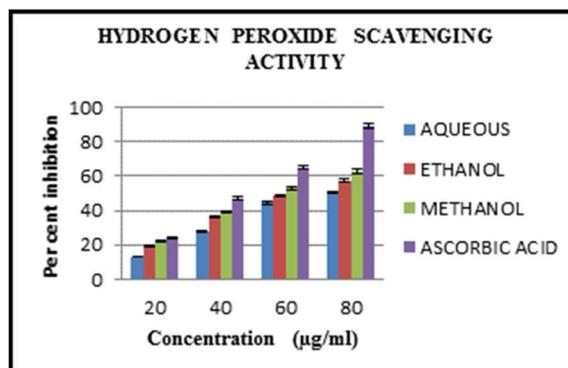


Fig. 5: Hydrogen peroxide scavenging activity of *C. equisetifolia* bark extracts. Data presented are the means of three replicates. Values are expressed as mean \pm SD of three replicates

Reducing power assay

In the reducing power assay, Fe³⁺-complex (Ferric cyanide) is reduced to ferrous, with the help of reductones present in the extract. There was an increase in reducing power with an increase in the concentration of aqueous, ethanol and methanolic bark extracts of *C. equisetifolia*. (fig. 6). The methanol extract showed percent maximum inhibition at 80 μ g concentration (79.81 \pm 0.74) followed by ethanol extract (75.58 \pm 0.54) and aqueous extracts (69.95 \pm 0.62) respectively, with IC 50 value of 49.09 \pm 0.85 μ g/ml, 52.78 \pm 0.42 μ g/ml and 59.13 \pm 0.19 μ g/ml respectively. The reducing power of standard ascorbic acid showed maximum percent inhibition of 82.16 \pm 0.15 (IC 50 value of 47.27 \pm 0.31 μ g/ml) at 80 μ g concentration.

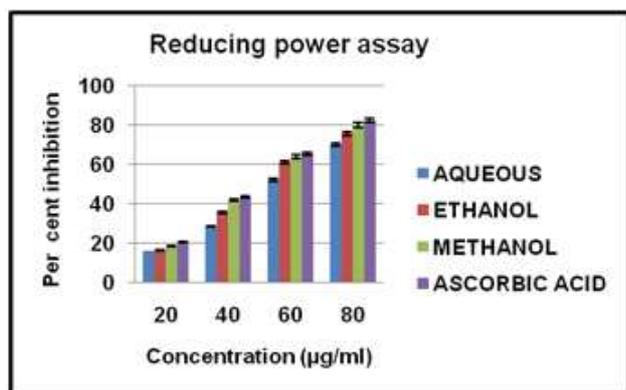


Fig. 6: Reducing power assay of *C. equisetifolia* bark extracts. Data presented are the means of three replicates. Values are expressed as mean±SD of three replicates

Hydroxyl radical scavenging assay

Hydroxyl radical is the most reactive oxygen centered species and causes severe damage to adjacent biomolecule. The methanol extract showed percent maximum reducing ability at 80µg concentration (78.75±0.29) followed by ethanol extract (72.08±0.06) and aqueous extract (60.83±0.94) with IC 50 value of 50.01±0.37µg/ml, 55.51±0.15µg/ml and 63.91±0.68µg/ml respectively. The reducing power activity of standard ascorbic acid showed maximum percent inhibition of (82.08±0.82) at 80µg concentration with IC₅₀ value of 47.54±0.82µg/ml (fig. 7).

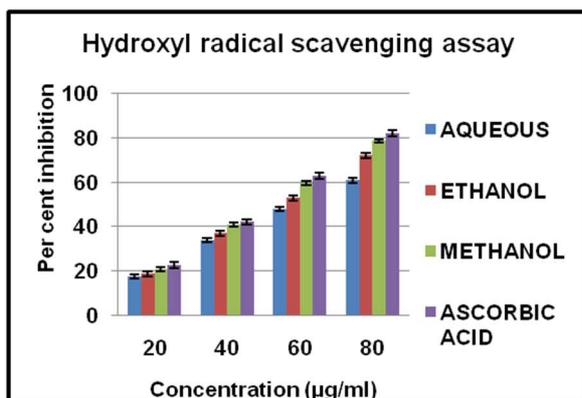


Fig. 7: Hydroxyl radical scavenging assay of *C. equisetifolia* bark extracts. Data presented are the means of three replicates. Values are expressed as mean±SD of three replicates

Total antioxidant capacity assay

The methanol extract showed percent maximum reducing ability at 80µg concentration (83.43±0.47) followed by ethanol extract (77.18±0.32) and aqueous extract (69.37±0.28) with IC 50 value of 47.89±0.36µg/ml, 51.61±0.09 µg/ml and 57.22±0.13µg/ml respectively. The reducing power activity of standard ascorbic acid showed maximum percent inhibition of 86.87±0.83 at an 80µg concentration with IC₅₀ value of 45.71±0.59µg/ml (fig. 8).

The study of [36], reported that the stem bark of *C. equisetifolia* exhibited potent radical scavenging and reducing power effect. But in the study conducted by [37] reported lower DPPH scavenging activity by catechin (compound isolated from bark).

Antioxidants are substances that significantly delay or prevent the oxidation of an oxidizable substrate when present in low concentrations compared to the substrate [38]. The results obtained

in this investigation revealed that all the extracts are free radical scavengers. However, the methanolic extract showed the highest scavenging activity which could be due to the high phenol content.

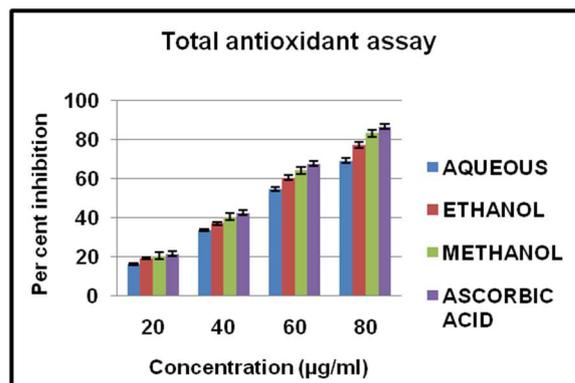


Fig. 8: Total antioxidant activity of *C. equisetifolia* bark extracts, data presented are the means of three replicates. Values are expressed as mean±SD of three replicates

Phenols acts as an efficient antioxidant or free radical terminators by donating hydrogen to radicals and breaks the lipid oxidation reaction at the initiation step [39]. Flavonoid compounds have been considered as potential antioxidants because of its ability to terminate radical free species and by upregulating or protecting antioxidant defence [40]. Flavonoids also inhibit LDL (Low-Density Lipoprotein) cholesterol oxidation which in turn scavenges free radicals [41].

Tannin are high molecular weight phenolic compounds that function as both primary and secondary antioxidants [42]. The ability of tannin to chelate metal ion [43] retards the oxidation process. The antioxidant property is attributed to tannin because of its potency to inhibit cyclooxygenase that leads to lipid peroxidation [44].

Terpenoids have the ability to quench the singlet oxygen and to transfer hydrogen or electron that proves it to be a potential antioxidant [45].

Anti-inflammatory assay

The anti-inflammatory assay was carried out using different concentrations (20, 40, 60, 80 µg/ml) of *Casuarina equisetifolia* bark extracts. Inhibition of thermally induced protein (albumin) denaturation was based on the concentration of different extracts. The methanol extract showed maximum percent inhibition (87.05±0.42) when compared to ethanol extract (83.52±0.62) and aqueous extract (70.29±0.14) with IC 50 value of 42.99±0.53µg/ml, 45.94±0.42µg/ml and 54.48±0.31µg/ml respectively. The anti-inflammatory activity of standard diclofenac sodium showed maximum percent inhibition of 92.35±0.7 at 80µg concentration with IC₅₀ value of 40.80±0.47 µg/ml (fig. 9).

A study conducted by [46] in evaluating the clinical efficacy of *Casuarina equisetifolia* bark extract in comparison with the standard, benzoyl peroxide, proved to cure papules, pustules and nodulocystic acne caused by acute inflammation.

The result clearly indicates that the phytoconstituents of extracts have the ability to reduce the synthesis of mediators that are responsible for the development of inflammation. The preliminary phytochemical screening showed the presence of phenols, flavonoids, tannins and terpenoids. This compound has greater potential to inhibit serotonin, leukocyte migration and histamine [47].

Flavonoids are observed to act as a potential anti-inflammatory material in both proliferative phase and exudative phase of inflammation, which may be attributed due to its ability to inhibit histamine, cytokine, and prostaglandin and leukotriene release [26, 48] and proves that phenolic compounds, flavonoids can be used in the treatment against various inflammatory disorders [49,50].

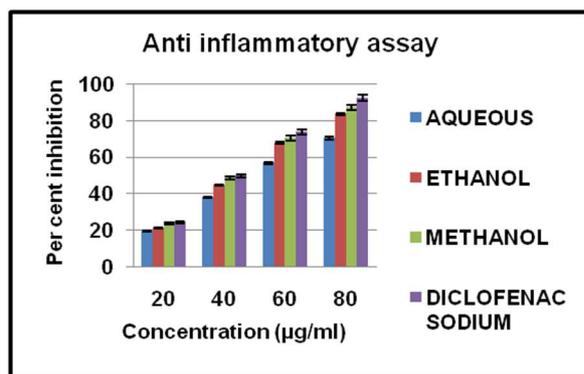


Fig. 9: Anti-inflammatory assay of *C. equisetifolia* bark extracts. Data presented are the means of three replicates. Values are expressed as mean±SD of three replicates

CONCLUSION

The current research work concludes that *C. equisetifolia* bark has a wide pharmacological spectrum, as the plant shows the presence of several secondary metabolites like phenols, flavonoids, tannins and terpenoids in crude extracts, which are responsible for the varied medicinal property. The bioactivity of the secondary metabolites was based on the polarity of the solvents used in the study. The methanolic extract of bark was found to be very efficient antibacterial, antioxidant and anti-inflammatory agent, which may be due to the consortium effect of these secondary metabolites along with the volatile compounds of GC-MS analysis. Therefore more evaluation on *C. equisetifolia* bark is being carried out to formulate the chemical constituents that could be employed in drug discovery leading to the application in the biomedical field.

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AUTHORS CONTRIBUTIONS

All the authors contributed equally

CONFLICT OF INTERESTS

The authors declare that they have no conflict of interest in the publication

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