

POTENT BIOACTIVE METABOLITES OF *CASUARINA JUNGHUHNIANA* MIQ. ROOTS–A THERAPEUTIC APPROACH

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ABSTRACT

Objective: *Casuarina junghuhniana* Miq. is a multipurpose tree, belongs to the family Casuarinaceae. It has a symbiotic association with actinomycete *Frankia* which makes the tree to grow vigorously on varied environmental conditions. Not much work has been documented on secondary metabolites and therapeutic approach of this species in India. Therefore, the objective of the present study was to explore the potential phytochemicals in the root extracts and its therapeutic values.

Methods: *Casuarina junghuhniana* root samples were collected from the State Forest Research Institute, Kolappakam, Chennai. The qualitative and quantitative phytochemicals screening were carried out using standard procedures. Antibacterial assay was tested against different pathogens using well diffusion method. Antioxidant activity was carried out using reducing power, hydrogen peroxide scavenging and 1, 1-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging methods. The anti-inflammatory assay was tested using protein denaturation method. Further, the phytochemicals in the root extract were evaluated using GC-MS studies. The functional role of the phytoconstituent was confirmed by docking against the target using mcule software.

Results: The qualitative screening revealed the presence of various phytoconstituents. The quantitative analysis revealed the presence of maximum phenols (68.95 ± 0.46 mg/g), flavonoids (34.15 ± 0.33 mg/g) and terpenoids (3.9%) in methanolic root extract with respective standards. Antibacterial activity showed a maximum zone of inhibition against *Bacillus subtilis* (19 ± 0.2 mm) followed by *Staphylococcus sp* (14 ± 0.3 mm). The antioxidant and anti-inflammatory activity was compared with standard ascorbic acid and the extract showed activities in a dose-dependent manner. Several peaks were obtained in the GC-MS analysis which indicates the presence of different secondary metabolites. The specific phytocompound showed prominent binding affinity against the target enzyme in docking studies.

Conclusion: The presence of potent phytochemicals in the *Casuarina junghuhniana* root with antioxidant and anti-inflammatory activities could be used as a promising source for developing novel plant-based therapeutic agents.

Keywords: *C. junghuhniana*, Root, Phytochemicals, Antioxidant, GC-MS, *In silico*

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INTRODUCTION

Plants are the rich source of bioactive secondary metabolites that has been used as a potential source of many therapeutic agents in the treatment of a wide spectrum of human ailments. The Plant based therapeutic agents have scientific, social and commercial significance. The medicinal property of the plant lies in the bioactive compound synthesised in their secondary metabolism. The most important secondary metabolites include phenols, flavonoids, tannins, alkaloids, steroids, etc.[1] There is a continuous and urgent need to discover new plant-based therapeutic compounds with the diverse chemical structure for new and re-emerging infectious diseases [2].

Casuarina junghuhniana Miq. is one of the most important members of nitrogen fixing, woody, a non-leguminous tree belonging to family Casuarinaceae. It has a symbiotic association with actinomycete *Frankia* which makes the tree to grow vigorously on varied soil types and in extreme environmental conditions.

C. junghuhniana is an exotic species originated in Indonesia. In India, within two decades of its systematic introduction in 1996, *C. junghuhniana* proved to be more preferred species than widely cultivated *C. equisetifolia* [3]. In recent years, *C. junghuhniana* has gained popularity among farmers in South India due to its various desirable characters such as growth rate, cultivation practices and marketability [4]. It is a fast growing, drought resistant with the good coppicing ability and has not affected by blister bark disease-host specific as well as free from pests and pathogens. The tree has various end uses such as pulpwood, fuel wood, and poles in India. It is also widely used for sand dune stabilisation, shelterbelts, and windbreak in agroforestry systems.

However, the emphasis is being laid over on the improvement of yield and quality of species; not much work has been documented on secondary metabolites and therapeutic approach of this species in India.

Casuarina junghuhniana tree has recorded faster growth which is widely grown in Tamil Nadu with 3 to 4 y of crop rotation period and hence is a desirable tree crop to the farmers in the field of agroforestry. Since the plant root and bark are the waste materials during pulping in the paper industry, the plant root has been taken for the present study to evaluate the potential phytoconstituents and its therapeutic values.

Therefore the objective of the present work was to screen the qualitative and quantitative phytochemicals, antimicrobial, antioxidant and anti-inflammatory potential of root using different solvents. The GC-MS analysis was carried out to characterize the presence of bioactive compounds in the root extract. The functional role of selected potent phytochemical was further studied using *in silico* work.

MATERIALS AND METHODS

Collection of plant material

The plus trees of *Casuarina junghuhniana* were identified from State Forest Research Institute, Kolappakam which is located at latitude ($12^{\circ} 8' 13''$ N) and longitude ($80^{\circ} 10' 18''$ E) of Chennai City, Tamil Nadu, India. Healthy root samples were collected from 4 y old plantation site. Identification (authentication) of the plant sample was confirmed at Botanical Survey of India (BSI), Coimbatore, Tamil Nadu. (BSI/SRC/5/23/2015/Tech/2153).

Preparation of plant extracts

The collected roots were thoroughly washed, shade dried and finely powdered. Powdered root materials were extracted with different solvents such as Acetone, Chloroform, Ethanol, Methanol and Aqueous using cold percolation method. The extracts were then concentrated and dried under reduced pressure and preserved at 5 °C until further use.

Qualitative phytochemical screening

The different root extracts (Acetone (RA), Chloroform (RC), Ethanol (RE), Methanol (RM) and Aqueous extracts (RW)) were checked for the presence of secondary metabolites such as alkaloids, phenols, tannins, flavonoids, terpenoids, proteins and amino acids, carbohydrates, steroids, phlobatanin saponin, Gum and mucilage using standard procedures [5, 6].

Quantitative estimation

Estimation of total phenol content

Total phenolic content of ethanol, methanol and aqueous extract of *C. junghuhniana* root was determined by Folin-ciocalteu reagent method with modification. To the crude extract (1 mg), 5 ml distilled water and 0.5 ml Folin-ciocalteu reagent was added and incubated for 5 min. Later 20% Na₂CO₃ (1.5 ml) was added to the solution and volume was made up to 10 ml with distilled water. Finally, the test solutions were kept in the dark for 2 h. After incubation, the absorbance of blue color was read at 750 nm using UV Spectrophotometer (UV 1650PC Shimadzu). Total phenolic content was calculated as Gallic acid equivalents (mg of GAE/g of extract) [7].

Estimation of total flavonoid content

Total flavonoids content of ethanol, methanol and aqueous extract of *C. junghuhniana* root was determined by aluminium chloride colorimetric assay. To 1 ml of the test sample, 4 ml of distilled water and 0.3 ml of 5% sodium nitrite solution was added and incubated for 5 min. After incubation, 0.3 ml of 10% aluminium chloride and 2 ml of 1M sodium hydroxide was added. Finally, the volume was made up to 10 ml with distilled water. Orange yellowish colour was developed. The absorbance was measured at 510 nm using UV Spectrophotometer (UV 1650PC Shimadzu). Total flavonoids content was calculated as Quercetin equivalents (mg of QAE/g of extract) [7].

Estimation of total terpenoids

Total terpenoid content of ethanol, methanol and aqueous extract of *C. junghuhniana* root was determined by Ferguson's method. The crude extract was soaked in 20 ml alcohol (95% ethanol) for 24 h. The filtrate was extracted with petroleum ether (60 °C-80 °C) and the ether extract was treated as total terpenoids. The residue obtained was dried and weighed [8].

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

Antibacterial activity

The different root extracts (ethanol, methanol and aqueous extracts) of 50µg and 100µg were assayed for its antibacterial activity by well diffusion method using Muller Hinton Media. The extracts were tested against *Bacillus subtilis*, *Escherichia coli*, *Proteus vulgaris*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus*. Gentamycin (100µg) was used as positive control. The plates were incubated at 37 °C. Zone of inhibition around the well was observed after 24 h for antibacterial assay. Triplicates were maintained.

Antioxidant activity

Hydrogen peroxide scavenging assay

The ability of root extracts to scavenge hydrogen peroxide was determined according to the method of Ruch *et al.* 1989 [9]. The extracts of 0.1 ml of different concentrations (100, 200, 300, 400, 500µg), were dissolved in 3.4 ml of 0.1M phosphate buffer (pH-7.4) and mixed well with 0.6 ml of 43 mmol hydrogen peroxide solution. The absorbance of the reaction mixture was read at 230 nm using a

UV Spectrophotometer. Ascorbic acid was used as a standard. The hydrogen peroxide scavenging activity was calculated using the formula:

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

Where A_c is the absorbance of control, A_o is the absorbance of sample

Reducing power assay

The reducing power assay of the root extracts was analyzed using standard method [10]. To different concentrations of the samples (100, 200, 300, 400, 500µg), 2.5 ml of phosphate buffer (pH-6.6) and 2.5 ml of 1% potassium ferricyanide solution was added and mixed well. This mixture was kept at 50 °C in a water bath for 20 min. After cooling the test tubes, 2.5 ml of 10% trichloroacetic acid was added and centrifuged at 3000 rpm for 10 min. 2.5 ml of the supernatant was mixed with 2.5 ml of distilled water and 0.5 ml of 0.1% ferric chloride solution and the mixture was kept undisturbed for 10 min. The absorbance of the resulting solution was measured at 700 nm using UV Spectrophotometer. The increase in absorbance of the reaction mixture indicates increased reducing power. Ascorbic acid was used as a standard. The reducing power assay was calculated using the formula.

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

DPPH radical scavenging assay

Free radical scavenging activity of the root extracts was measured by 1, 1-diphenyl-2-picrylhydrazyl (DPPH). 0.1 mmol solution of DPPH in methanol was prepared. 1 ml of DPPH was added to 3 ml of different concentrations of the sample (100, 200, 300, 400, 500µg). The above mixture was shaken vigorously and allowed to stand at room temperature for 30 min. The absorbance was measured at 517 nm in UV Spectrophotometer. The experiment was carried out using ascorbic acid as standard. The DPPH scavenging effect was calculated using the following equation:

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

Where A_0 is the absorbance of control, and A_1 is the absorbance of the test sample [10].

All the above assays were performed in triplicates.

In vitro anti-inflammatory assay (Inhibition of the albumin denaturation)

To 100 µl of different concentrations (100, 200, 300, 400, 500µg) sample, 500 µl (1%) Bovine Serum Albumin was added. The sample extracts were incubated at 37°C for 10 min and then heated to 51°C for 20 min. After cooling the samples, the turbidity was measured using a UV Spectrophotometer at 660 nm. Diclofenac sodium was used as a standard drug. The experiment was performed in triplicates [10]. Percent inhibition of protein denaturation was calculated using the formula.

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

GC MS studies

The methanol root extract (extracted using HPLC grade Methanol) was subjected to GC MS analysis to identify the bioactive compounds. GC-MS analysis was performed at the SAIF, IIT-Madras, Chennai, Tamil Nadu. The sample was subjected to GC and MS JEOL GC mate equipped with a secondary electron multiplier. JEOL GCMATE II GC-MS (Agilent Technologies 6890N Network GC system for gas chromatography). The column (HP5) was fused silica 50 m X 0.25 mm I.D. The experimental conditions were 20 min at 100 °C, column temperature: 235 °C for 3 min; injector temperature: 240 °C; carrier gas: helium; and split ratio: 5:4. 1µl of the sample was evaporated in a splitless injector at 300 °C and the run time was 40 min. The active phytochemical components were identified by gas chromatography coupled with mass spectrometry. The spectrum of GC-MS was analyzed using the database of National Institute Standard and Technology (NIST) having more than 62,000 patterns.

In silico analysis (Docking studies)

In silico analysis through molecular docking plays a significant role in studying the functional role of the specific bioactive compound. The compounds identified by GC-MS analysis in the *Casuarina junghuhniana* were screened against the target protein (sortase) in order to study the anti-microbial property. The target molecule was retrieved from (PDB) Protein Data Bank (<http://www.rcsb.org/pdb/>). The bioactive compound details were retrieved from the Pubchem database. The bioactive compounds were docked against the target protein using bioinformatics tools. Docking results in interactions between the target and ligand molecules. Docking studies were carried out to confirm the anti-microbial property of the phyto compound. Streptomycin was used as a standard anti-microbial compound.

Statistical analysis

For each experiment, data presented are the means of three replicates. Values are expressed as mean±SD of three replicates.

RESULTS AND DISCUSSION

Casuarina junghuhniana roots were collected from State Forest Research Institute, Kolapakkam. The roots were dried and powdered. (fig. 1a and 1b).

Qualitative phytochemical screening

The results of preliminary qualitative phytochemical screening of different extracts of root of *Casuarina junghuhniana* showed the presence of varied phytochemicals. (table 1). The ethanol, methanol and aqueous extract of the root alone showed the presence of phenol, flavonoids, tannin and terpenoids. Aqueous root extract alone showed the presence of alkaloids and glycosides. Except for

chloroform root extract, all other root extracts showed the presence of carbohydrates and proteins.



Fig. 1a: *Casuarina junghuhniana* plantation



Fig. 1b: *Casuarina junghuhniana* root

Table 1: Phytochemical Analysis of different extracts of root of *C. junghuhniana*

S. No.	Phytochemical test	RA	RC	RE	RM	RW
1	Alkaloid	-	-	-	-	+
2	Glycoside	-	-	-	-	+
3	Carbohydrate	+	-	+	+	+
4	Proteins	+	-	+	+	+
5	Phenol	-	-	+	+	+
6	Flavonoid	-	-	+	+	+
7	Tannin	-	-	+	+	+
8	Terpenoid	-	-	+	+	+

+ : Presence.-: Absence, RA-Acetone root extract, RC-Chloroform root extract, RE-Ethanol root extract, RM-Methanol root extract, RW-Aqueous root extract of *Casuarina junghuhniana*.

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, phenols, tannins and Terpenoids from the root of related *Casuarina* species (*Casuarina equisetifolia*) has been reported [11]. The chemical constituents in the plants or in crude extracts are known to be biologically active metabolites. Some chemical constituents are considered as secondary metabolites [12]. The primary function of phytochemical is to protect the plants from pathogens that bring damage to the plant. Besides that, it is also a source of colour, aroma and flavor for specific parts of the plant. Moreover, phytochemicals are also useful in plant protection over environmental hazards such as pollution, UV exposure and stress drought [13]. The secondary metabolites in plants are directly responsible for various activities such as antimicrobial, antioxidant and anti-cancer activities [14]. The presence of phytochemicals within root can be a potential source for medicinal use and it is also an indicator that they can be a promising source for developing novel plant-based therapeutic agents.

Since, ethanol, methanol and aqueous root extracts alone revealed positive results in qualitative phytochemical analysis, further work was carried out only with these three root extracts.

Quantitative phytochemical analysis

The total phenolic content in 3 different extracts of *Casuarina junghuhniana* root determined by Folin-ciocalteu method was

reported as Gallic acid equivalent (Standard Curve equation $Y=0.027x+0.176$, $R^2=0.994$). Among the three extracts, methanol extract contained maximum phenol content (68.5 ± 0.2 mg/g), followed by ethanol extract (63.4 ± 0.3 mg/g) and aqueous extract (43.5 ± 0.3 mg/g). (fig. 2)

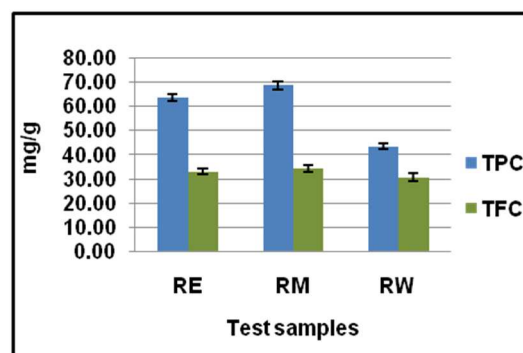


Fig. 2: Total phenol content (TPC) and total flavonoids content (TFC) in *Casuarina junghuhniana* root. Data presented are the means of three replicates. Values are expressed as mean±SD of three replicates

The total flavonoid content in 3 different extracts of *Casuarina junghuhniana* root determined by aluminium chloride colorimetric method was reported as Quercetin equivalent (Standard Curve equation $Y=0.009x+0.136$, $R^2=0.997$). Among the three extracts, methanol extract contained maximum flavonoids content (34.3 ± 0.4 mg/g), followed by ethanol extract (32.9 ± 0.2 mg/g) and aqueous extract (30.5 ± 0.3 mg/g). (fig. 2)

The methanol root extracts showed maximum terpenoid content (3.9%) followed by ethanol extract (3%) and aqueous extract (2.5%) (fig. 3)

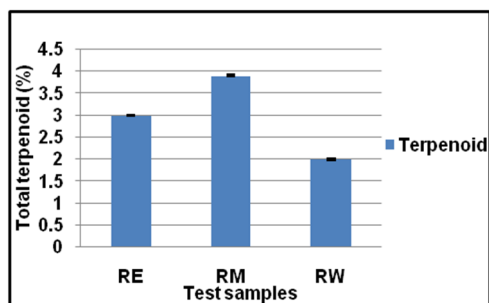


Fig. 3: Total Terpenoid content in *Casuarina junghuhniana* root. Data presented are the means of three replicates. Values are expressed as mean \pm SD of three replicates

In the present study, the quantitative analysis revealed that methanol root extract has maximum phenols, flavonoids and terpenoids. The biological activity of plant matter are greatly influenced by the nature of the extracting solvent owing to the presence of different bioactive compounds of different chemical properties and polarities that may or may not be soluble in a particular solvent [15]. Determination of total phenolic content of aqueous extract of root bark of *Casuarina littorea* was found to be 6.68 ± 0.03 mg/g GAE [16].

Phenolic compounds are broadly distributed in the plant kingdom and are most abundant secondary metabolites of the plant. They are generally involved in defence against UV radiations, aggression by pest and pathogens and also contribute to plant colour [17]. These phenolics compounds are a class of antioxidant agents which act as free radical terminators [18] and exhibit strong antioxidant properties due to their redox potential, which allow them to act as reducing agents, hydrogen donors, metal chelators and singlet quencher [19].

Flavonoids are one of the most diverse and largest classes of plant phenolics performing various functions in plant system including pigmentation and defence [20]. Several flavonoids have been reported to quench active oxygen species and inhibit *in vitro* oxidation of low-density lipoproteins [21, 22]. Flavonoids have been reported to possess various properties such as antimicrobial, antioxidant, anti-inflammatory, estrogenic, enzyme inhibition, antiallergic, vascular and cytotoxic antitumor activities [23]. The antioxidant properties of flavonoids are due to the presence of a number of phenolic hydroxyl groups attached to ring structures [24].

Terpenoids are also known as isoprenoids, constituting the largest class of natural products. They are widely used in the field of medicine and in industrial sectors as flavors, fragrance, spice, in the preparation of perfumes and cosmetics [25]. Terpenes react with free radicals by partitioning themselves into fatty membranes by virtue of their long carbon side chain and thus exhibiting unique antioxidant activity [26].

Anti-bacterial activity

The results indicated that the root extracts showed antibacterial activities at variable degrees against different bacterial pathogens. The methanol extracts of root showed remarkable antibacterial activity compared to ethanol and aqueous extracts against the test pathogens. The maximum zone of inhibition was seen at 100 μ g

concentration against *Bacillus subtilis* (19 ± 0.2 mm), followed by *Staphylococcus aureus* (14 ± 0.2 mm). (fig. 4). Ethanol root extract and aqueous root extract showed a maximum zone of inhibition against *Staphylococcus aureus* with 11 ± 0.3 mm and 10 ± 0.4 mm respectively. The control gentamycin at 100 μ g concentration showed (21 ± 0.7 mm) against the tested bacterial pathogens.

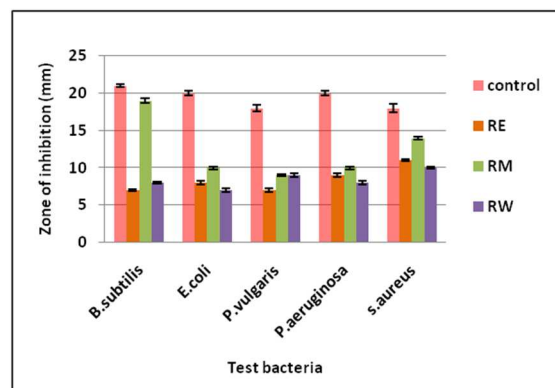


Fig. 4: Antibacterial activity of *Casuarina junghuhniana* root extract against different bacterial pathogens at 100 μ g concentration. Data presented are the means of three replicates. Values are expressed as mean \pm SD of three replicates

In this study, Methanol extracts of root had a higher solubility for more phytoconstituents resulting the highest antibacterial activity. The presence of phytochemicals such as higher phenol and flavonoids in root extract may be responsible for the antimicrobial activity. There is an increasing rate of resistance to existing antibiotics, and this problem extends beyond the clinical application of antimicrobial drugs. Plant-based antimicrobials have wide therapeutic potentials as they serve with lesser side effects [27]. Flavonoids are grouped under phenolics, and it acts as an antimicrobial agent by inhibiting the nucleic acid synthesis, cytoplasmic membrane function and energy metabolism [28].

Anti-oxidant activity

The antioxidant assay was carried out at different concentrations (100, 200, 300, 400, 500 μ g) of ethanol, methanol and aqueous extracts of *Casuarina junghuhniana* root and was compared with standard ascorbic acid. The antioxidant potential of different extracts was determined based on their IC_{50} value. The IC_{50} value is the measure of the extract concentration that is required for 50% inhibition. Lesser IC_{50} value denotes the higher antioxidant potential of the sample.

Hydrogen peroxide scavenging assay

H_2O_2 inactivate few enzymes directly, usually through oxidation of essential thiol (-SH) groups. H_2O_2 react with Fe^{2+} and possibly Cu^{2+} ions resulting in the formation of hydroxyl radical which may be the origin of oxidative damage. In the present study, the percent inhibition at various concentrations (100-500 μ g) was evaluated. The methanol extract showed per cent maximum inhibition at 500 μ g concentration (83 ± 0.6) followed by ethanol extract (79 ± 0.4) and aqueous extract (74 ± 0.5) with IC_{50} value of 165.45 μ g/ml, 224.56 μ g/ml and 247.27 μ g/ml respectively. The hydrogen peroxide scavenging activity of standard ascorbic acid showed maximum percent inhibition of (91 ± 0.8) at 500 μ g concentration with IC_{50} value of 105.08 μ g/ml. (fig. 5)

Reducing power assay

The reducing power has a significant correlation with the antioxidant activity. The concentration of Fe^{2+} formed by the reduction of the Fe^{3+} /ferricyanide complex was monitored by measuring the formation of Prussian blue at 700 nm [29]. In the present study, the reducing power activity of different extracts of

Casuarina junghuhniana root was evaluated. The methanol extract showed per cent maximum reducing ability at 500 μ g concentration (87 \pm 0.3) followed by ethanol extract (83 \pm 0.2) and aqueous extract (78 \pm 0.3) with IC₅₀ value of 147.86 μ g/ml, 175.67 μ g/ml and 236.84 μ g/ml respectively. The reducing power activity of standard ascorbic acid showed maximum percent inhibition of (91 \pm 0.8) at 500 μ g concentration with IC₅₀ value of 105.08 μ g/ml. (fig. 6)

DPPH radical scavenging assay

DPPH is a stable free radical at room temperature, purple in colour. The degree of discolouration of DPPH by its reduction indicated the radical scavenging activity. In our present study, DPPH radical scavenging activity was recorded in terms of percent inhibition. The methanol extract showed per cent maximum inhibition at 500 μ g concentration (84 \pm 0.7) followed by ethanol extract (71 \pm 0.4) and aqueous extracts (70 \pm 0.6), with IC₅₀ value of 170.08 μ g/ml, 265.71 μ g/ml and 279.79 μ g/ml respectively. The DPPH activity of standard ascorbic acid showed maximum percent inhibition of (91 \pm 0.8) at 500 μ g concentration with IC₅₀ value of 105.08 μ g/ml (fig. 7)

In our present findings, among the three extracts, the methanol root extract showed maximum antioxidant activity. Plant extracts are widely used as a natural antioxidant because of the presence of polyphenols [30]. Compounds with reducing power indicated that they are electron donors and can reduce the oxidised intermediates of lipid peroxidation process. The reduction of potassium ferricyanide by the various extracts of *C. junghuhniana* root showed their hydrogen donating ability.

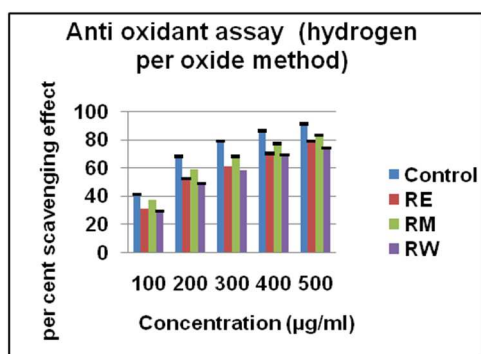


Fig. 5: Hydrogen peroxide scavenging activity of *C. junghuhniana* root. Data presented are the means of three replicates. Values are expressed as mean \pm SD of three replicates

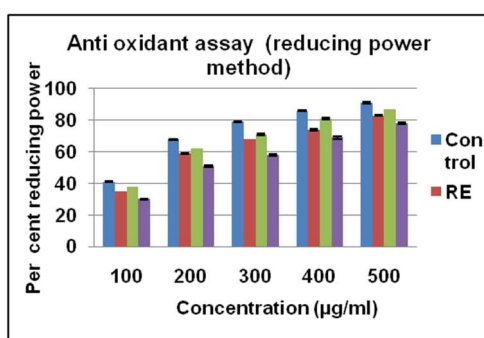


Fig. 6: Reducing power activity of *C. junghuhniana* root. Data presented are the means of three replicates. Values are expressed as mean \pm SD of three replicates

The antioxidant activity of a phenolic compound is mainly due to the redox properties, which play an important role in adsorbing and neutralising free radical, quenching singlet and triplet oxygen, or decomposing peroxides [31]. Plant polyphenols are produced either from phenylalanine or its precursor shikmic acid, are important dietary antioxidants because they possess an ideal structural chemistry for free radical scavenging activity. Phenolic compounds showed higher

antioxidant activity than antioxidant vitamins and carotenoids [32]. Thus, the antioxidant activity of *Casuarina junghuhniana* may be attributed to the presence of polyphenols in the extracts.

Similar observations were reported in *Casuarina equisetifolia* using condensed tannins extract from the fine root sample showing the IC₅₀ value of 89.32 μ g/ml [33].

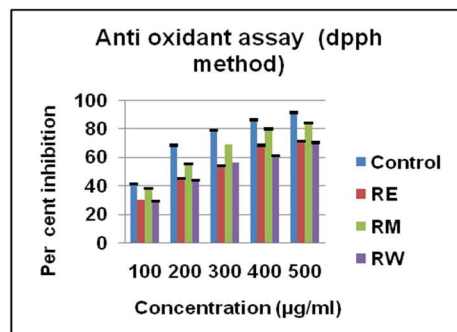


Fig. 7: DPPH free radical scavenging activity of *C. junghuhniana* root. Data presented are the means of three replicates. Values are expressed as mean \pm SD of three replicates

In vitro Anti-inflammatory assay (Inhibition of the albumin denaturation)

The anti-inflammatory assay was carried out at different concentrations (100, 200, 300, 400, 500 μ g) of *Casuarina junghuhniana* root extracts. Results revealed inhibition of thermally-induced protein (albumin) denaturation in a dose-dependent manner. The methanol extract showed maximum percent inhibition (85 \pm 0.5) when compared to ethanol extract (62 \pm 0.4) and aqueous extract (60 \pm 0.2) with IC₅₀ value of 228.57 μ g/ml, 367.27 μ g/ml and 404.34 μ g/ml respectively. The anti-inflammatory activity of standard diclofenac sodium showed per cent maximum inhibition 90 \pm 1.5 at 500 μ g concentration with IC₅₀ value of 181.11 μ g/ml. (fig. 8).

Harmful stimuli, pathogens, irritants characterised by redness, warmth, swelling and pain are the causes of inflammation of vascular tissue which is a complex biological process. The prolonged inflammation leads to various diseases such as rheumatoid arthritis, atherosclerosis, hay fever, ischemic heart disease [34]. The cause of inflammation is due to protein denaturation which has been well documented [35]. Agents that can prevent protein denaturation, therefore, would be worthwhile for anti-inflammatory drug development. Protein denaturation is a process in which proteins lose their tertiary structure and secondary structure by application of external stress or compound, such as strong acid or base, a concentrated inorganic salt and organic solvent or heat. Most biological proteins lose their biological function when denatured [10]. In the present study, methanol extract showed maximum anti-inflammatory activity. The presence of polyphenols and flavonoids in the extracts may be responsible for the anti-inflammatory activity.

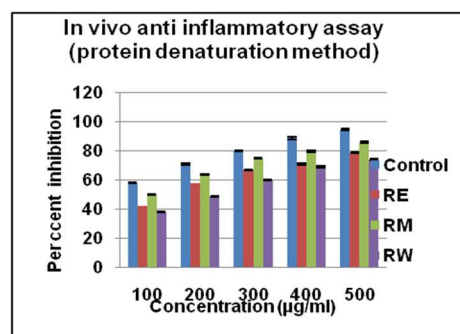


Fig. 8: Anti-inflammatory activity of *C. junghuhniana* root. Data presented are the means of three replicates. Values are expressed as mean \pm SD of three replicates

GC-MS study

In the present study, methanolic root extract showed maximum bioactivities; hence GC-MS analysis was carried out only in methanol root extract.

Several peaks were obtained in the GC-MS analysis of methanol extract of *C. junghuhniana* root indicating the presence of different secondary metabolites (fig. 9). These bioactive compounds were identified using NIST database on comparison with actual mass spectral obtained.

The chromatogram revealed the presence of Phenol 3,5 bis (1,1 dimethyl ethyl), 4,5,7-trihydroxy isoflavone, 4H-1-Benzopyran 4-one 2-(3,4-dimethoxy phenyl)7-hydroxy, Oleic acid, Ethanol 2 (9 octadecenyloxy) z, Palmitic acid, 3a,9 dimethyl dodecahydrocyclo hepta(d) inden-3-one, Phytol, 4methoxy 5,7 dihydroxyisoflavone, flavone. Among the compounds identified, phenol 3,5 bis (1,1 dimethyl ethyl), 4,5,7-Trihydroxy isoflavone, phytol, 4methoxy 5,7 dihydroxyflavone, flavone belongs to phenolic and flavonoid group which possess antibacterial, anti-inflammatory and antioxidant activities [36].

GC-MS is one of the technique to identify the bioactive constituents of long chain branched chain hydrocarbons, alcohols, acids, esters,

etc [37]. The results pertaining to GC-MS analysis led to the identification of a number of compounds from the GC fraction of the methanol extract of *C. junghuhniana* root. These compounds were identified through mass spectrometry attached with GC. Most of the compounds in the GC-MS spectrum are grouped under phenolic group. This reveals that *C. junghuhniana* is a rich source of phenolic compounds. Phenolic compounds are most widely occurring groups of phytochemicals and derivatives of the pentose phosphate, shikimate and phenylpropanoid pathway in plants. These compounds are secondary metabolites which have an important role in reproduction, growth, protection against pest and pathogens.

The present study strongly supports the presence of therapeutic values such as anti-microbial, anti-oxidant, anti-inflammatory activities which may be due to the presence of a wider range of phenolic phytoconstituents reported [38].

Phytol is one among the identified phytochemicals in the GC-MS analysis. Phytol was observed to have antibacterial activities against *Staphylococcus aureus* by causing damage to cell membranes. As a result, there is a leakage of potassium ions from bacterial cells [39]. Phytol is a key acyclic diterpene alcohol that is a precursor for Vitamins E and K1. Vitamin E is the main lipid, a soluble antioxidant in the body [40].

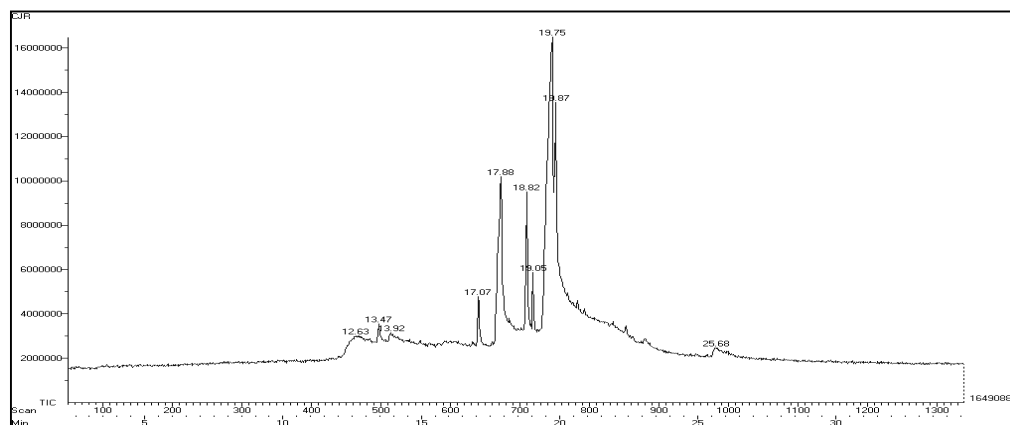


Fig. 9: GC-MS spectrum of *C. junghuhniana* root

Docking studies

The qualitative and quantitative phytochemical analysis revealed the presence of potent phytochemicals in the root extracts of *Casuarina junghuhniana*. Further, GC-MS studies confirmed the presence of various phytoconstituents. Thus, from the GC-MS analysis, screening was carried out for the therapeutic compounds related to the anti-microbial property.

Docking studies were carried out to confirm the functional role of the bioactive compound. Among the compounds screened, 4 methoxy 5,7 dihydroxyisoflavone from root extract was found to be related to the present study having anti-microbial activity is found to be grouped under flavonoids. Flavonoids are reported to have various biological activities such as antibacterial, antiviral and effective antioxidant potential.

The bioactive compound docked against the target protein Sortase using bioinformatics tool showed the anti-microbial property of the compound through docking scores. More negative values are an indication of higher binding affinity which clearly indicates the strong anti-microbial property. Docking analysis of 4 methoxy 5, 7 dihydroxy-isoflavone showed docking scores of -6.5, -6.2, -6.1 and -6.0. Docking analysis of standard Streptomycin showed docking scores of -6.7, -6.6, -6.3 and -6.2 (fig. 10 and 11).

The gram-positive bacterium *Staphylococcus aureus* colonises the human skin and causes invasive diseases such as skin and soft tissue infections, osteomyelitis, pneumonia, bacteremia, sepsis and

endocarditis [41]. Methicillin-resistant *S. aureus* (MRSA) acquired resistance against many different drugs including β -lactam, cephalosporin, fluoroquinolone, aminoglycoside, tetracycline, macrolide, trimethoprim-sulfamethoxazole, and vancomycin antibiotics [42].

The infections caused by *S. aureus* and other pathogens can be treated by using small molecules that effectively strip the bacteria of their surface proteins, which frequently function as virulence factors [43]. *Staphylococcus aureus* and many other gram-positive pathogens use sortase enzymes to anchor surface proteins to their cell walls that play a key role in the infection process by promoting nutrients acquisition from the host, bacterial adhesion and immune evasion [44-47]. Enzyme Sortase involves in the pathogenesis of a variety of bacterial infections, including respiratory tract, bloodstream, skin and tissue infection.

Sortase has been attracted great interest as potential drug targets since decades. The inhibition of *Sortase* activity results in the separation of *S. aureus* from the host cells and ultimately alleviation of the infection [48].

In the present study, we used the compound from GC-MS analysis to identify their binding affinity by performing molecular docking studies against the target enzyme Sortase. The compound 4 methoxy 5,7 dihydroxyflavone, which is present in the *C. junghuhniana* root showed prominent binding affinity against *Sortase enzyme* on par with the standard drug compound Streptomycin. Thus, the docking results are clear evidence for *Casuarina junghuhniana* root extracts

possessing anti-microbial properties. The effective potential phyto-constituents can be further purified and taken for future studies.

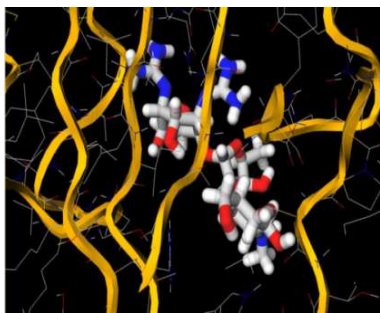


Fig. 10: Illustration of Streptomycin docked against target Sortase



Fig. 11: Illustration of 4 Methoxy 5,7 dihydroxyisoflavone compound from *C. junghuhniana* root (Methanol extract) docked with target Sortase

CONCLUSION

Casuarina junghuhniana root revealed a broad spectrum of potential phytochemicals, possessing antibacterial, antioxidant and anti-inflammatory activities. GC-MS analysis revealed the specific phytoconstituents present in root. Further, docking studies confirmed the compound 4 methoxy 5,7 dihydroxyflavone showing a strong specific antibiotic against the target Sortase. Thus, the study confirms the presence of rich phenol and flavonoid content in *Casuarina junghuhniana* root extracts. The present work ascertains the potent secondary metabolites of the plant, which could be used in the development of novel drugs. The screened bioactive compounds from the plant will be further purified and studied for its pharmaceutical efficiency and efficacy.

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CONFLICT OF INTERESTS

Declared none

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