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RESEARCH ARTICLE
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In Vitro Biological Activity 0f Aqueous Extract of Cyanthillium Cinereum Against Oral Pathogens

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ABSTRACT

Background: Cyanthillium cinereum is used as a smoking cessation herb in Thailand as these leaves and flowers have a tiny amount of nicotine. Aim of the study is to investigate the antioxidant, anti-inflammatory, cytotoxicity and antimicrobial activity against common oral pathogens of Cyanthillium cinereum.

Methods: Aqueous extract was prepared and antioxidant activity assessed using DPPH Assay at 5 µL, 10 µL, 20 µL, 30 µL, 50 µL concentrations. Cytotoxic effect of extract was assessed using Brine Shrimp Assay at 5 µL, 10 µL, 20 µL, 40µL and 80µL concentrations, anti-inflammatory activity using Bovine Serum Albumin (BSA) assay and antimicrobial activity against oral pathogens was assessed using well diffusion method. Independent t test and ANOVA were used for data analysis.

Results: Antimicrobial activity against S. mutans was more in 100 µL (ZOI – 15.23±0.25; p value < 0.001); but there was no antifungal activity against Candida albicans; antioxidant activity of aqueous extract of Vernonia cinerea was more when compared with the standard ascorbic acid (p value < 0.05); Antiinflammatory activity was more in 50 µL of aqueous extract when compared with Diclofenac (94.16±0.76 vs 83.86±0.51). Cytotoxicity increases with increased concentration, 50% death at 5 µL and 90% death at 50 µL.

Conclusion: Cyanthillium cinereum showed good antimicrobial activity against S mutans. Cyanthillium cinereum have very less cytotoxic effects and also has a potential to serve as a good anti-inflammatory and antioxidant agent.

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INTRODUCTION

Indigenous medicine is widely used by the majority of the people in developing countries. Researches been conducted to assess the medicinal properties of plants and the constituents which are responsible for their medicinal value.1

Medicinal properties of the plants are observed to be due to the presence of bioactive compounds such as flavonoids, alkaloids, tannins, steroids, glycosides, terpenoids, coumarin, phlobatannin etc. Researches to probe the therapeutic role and the action of bioactive compounds involved in the medicinal properties are given importance due to its inevitable role in the primary health care system. 2, 3

Vernonia cinerea is a perennial plant belonging to the family Asteraceae and is also known as little iron weed. It is found in China, India, Bangladesh, Sri Lanka, Myanmar, Malay Island, Africa, Philippines, Australia, New Zealand, Asia and Vietnam. In malayalam it is called as poovamkurunnila, in Bengali it is known as kushkim, in Tamil as Puvamkuruntal and as sahadevi in sanskrit and hindi.1

The plant occurs mostly in sunny or slightly shaded habitats, in wasteland, roadsides, cultivated land and other anthropogenic habitats. V. cinerea reproduces and spreads by seeds which are adapted to wind dispersal.2 It is been reported that Cyanthillium cinereum plant is consumable and can be used as a medicine.1 Phytochemical research of Cyanthillium explored the presence of chemicals such as flavonoids, steroids, triterpenoids, tannins and sesquiterpenes. Different parts of the plant possess different therapeutic values and have been used in different traditional medicines of the world. Cyanthillium plant is used for treating malarial fever, infections, worms, pain, diuresis, cancer, abortion, and various gastro intestinal disorders. 3

The flowers of the plant are used to treat conjunctivitis, fever, and rheumatism; leaf extracts of the plant are reported to be diuretic and antidiuretic 4, anti-inflammatory 5, analgesic, and antipyretic 6, methanol extract of V. cinerea exhibited a good antibacterial and anti yeast activity 7.

C. cinereum exhibited protective activity as it contains antioxidant compounds such as tannins, catechins, and flavonoids in ex vivo study8. A previous study reported that CC extract significantly inhibited the secretion of IFN- in a dose-dependent fashion, while IL-10 anti-inflammatory cytokine increased.9

Saponins present in flower extracts and flavonoids present in leaf and flower extracts are known to have anti- microbial activity. Hexane and crude extracts of flower show maximum inhibition against B. cereus, E. aerogenus and S. aureus whereas leaf extracts showed activity against B. cereus and E. aerogenus but not against S. aureus. Ether extracts also showed antibacterial against B. cereus, E. aerogenes and S. aureus. Antibacterial activity of various extracts of the plant against both gram positive and gram-negative bacteria exhibited different effects with a maximum antibacterial activity in case of methanolic extract than hexane extract10. The crude methanolic extract showed significant antioxidant activity by the DPPH free radical scavenging method.11

Few studies reported the use of Cyanthillium in tobacco cessation.12- 14 Behaviour counselling, nicotine replacement therapy, pharmacotherapies such as bupropion and varenicline are the common methods used to treat tobacco addiction. Role of herbals in smoking cessation has been researched as an inexpensive and safer alternative to pharmacotherapies. Hence as an initiative step to explore the herbals in smoking cessation in India, the present in vitro study was done to investigate the in vitro activities of Cyanthillium in some of the oral pathogens.

To the best of the knowledge, only few studies were conducted to evaluate the antioxidant activity, anti-inflammatory activity, cytotoxicity of aqueous extract of Cyanthillium cinereum; hardly few or no studies to evaluate the

antimicrobial activity of aqueous extract of Cyanthillium cinereum against S aureus, E faecalis, S mutans, Candida albicans. Hence an in vitro study was done to evaluate the antioxidant, anti-inflammatory, cytotoxicity and antibacterial activity of Cyanthillium cinereum plant.

MATERIALS AND METHODS

Collection of Plant

Whole plant of Cyanthillium cinereum including the root was collected from Erode district in Tamilnadu. Healthy plants were thoroughly screened, washed to remove the dust and soil. Washed plants were shade dried for about two weeks (Figure 1) and were grinded to powder. Aqueous extract of the whole plant was prepared and assessed for anti-inflammatory, anti-oxidant, antimicrobial and cytotoxic properties.

Aqueous extract preparation

Cyanthillium cinereum aqueous solution was prepared by dissolving 1 g Cyanthillium cinereum powder in 100ml of distilled water, followed by boiling for 5–10 min at 60–70°C, the aqueous extract was filtered and stored in 4°C.

Antimicrobial activity

Antimicrobial activity against Streptococcus mutans, Staphylococcus aureus, Candida albicans and E. faecalis were determined.

The pure bacterial cultures were obtained from Saveetha Institute of Medical and Technical Sciences, Chennai and maintained on nutrient agar medium and fungal culture on potato dextrose agar (PDA) medium, which was further maintained by regular subculturing on the same medium and stored at 4 degree celsius. Antimicrobial activities of different concentrations of aqueous extracts of Cyanthillium cinereum were evaluated by agar well diffusion method.

Rose Bengal Agar and nutrient agar plates were swabbed with broth culture of respective bacteria and fungi. Sterile cork borers were used to create wells in each of these plates. Wells were 10mm diameter and about 2 cm apart. Stock solutions of Cyanthillium cinereum were prepared at a concentration of 1 mg/ml in Cyanthillium cinereum extract of 25µl, 50µl and 100 µl concentrations. Solutions were added to the wells and allowed to diffuse at room temperature for 2 hrs. The plates were incubated at 37°C for 18-24 h for Streptococcus mutans, Staphylococcus aureus and E. faecalis, zone of inhibition was recorded and compared with the activity of the Ampicillin (1.0 mg/disc). The plates were incubated at 28°C for 48 hours for Candida albicans, a zone of inhibition was recorded and compared with the Fluconazole (1.0 mg/disc. The experiment was done thrice and the average values were recorded.

Anti-inflammatory Activity

Anti-Inflammatory Activity was measured using Bovine serum albumin (BSA) assay. 2 ml of 1% bovine albumin fraction was mixed with 400 µl of Cyanthillium cinereum plant crude extract in different concentration (500–100 µg/mL), and 1N HCl was used to adjust the pH of reaction mixture to 6.8. Then it was incubated at room temperature for 20 min and then heated for 20 min at 55°C in a water bath. Absorbance value was recorded at 660 nm and Diclofenac sodium in different concentrations was used as standards. Aqueous extract of Cyanthillium cinereum extract with concentrations of 10 µL, 20 µL, 30 µL, 40 µL and 50 µL were taken in 5 test tubes and to each test tube 2 ml of 1% Bovine Serum Albumin (BSA) was added. 2 mL of Dimethyl Sulphoxide (DMSO) added to 2 mL of BSA solution served as control.

% Inhibition was calculated using the following formula:

Percentage of inhibition = [Control Optical Density – (Sample Optical Density/ Control Optical Density] / 100

Cytotoxicity

The cytotoxicity of aqueous extract of Cyanthillium cinereum plant extract was assessed using Brine shrimp assay. 10 nauplii was added to each well of 12 well ELISA plates which contains 6-8 ml of saltwater. Five different concentrations (5 µL, 10 µL, 20 µL, 40 µL, 80 µL) of aqueous extract of Cyanthillium cinereum extract were added to each well and were then incubated for 24 h. After 24 h, the total

number of live and dead nauplii was counted and the mortality rate was checked by the formula

% death = [Number of dead nauplii / (Number of dead nauplii − number of live nauplii)] × 100

Antioxidant Activity

Antioxidant potential of aqueous extract of plant was measured by 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay. Control group used 1 mL of DPPH was added to 2 mL of methanol solution and ascorbic acid was used as standard. The test tubes were incubated in a dark cupboard for around 20 minutes. Absorbance was measured at 517 nm in the UV Spectrophotometer.

% Inhibition was calculated using the following formula:

% of inhibition = (Control Absorbance − Sample Absorbance% Control Absorbance) × 100

Statistical analysis

All experiments were performed in triplicate and the results are expressed as mean ± standard deviation. Independent t test analyzed the mean difference in the zone of inhibition between the control group and different concentrations of aqueous extract of Cyanthillium cinereum. Mean differences within various groups were analysed using repeated measures ANOVA, followed by multiple comparisons using Tukey's method.

RESULTS

Anti-inflammatory, antioxidant, cytotoxic and antimicrobial activity of Cyanthillium cinereum against Streptococcus mutans, Staphylococcus aureus and Candida albicans was assessed in vitro. (Figure 2) There was an increase in the Antioxidant activity of aqueous extract of Cyanthillium plant with increase in the concentration with the exception of 30µl concentration in which there was a slight decrease when compared with 20 µl. Also, antioxidant activity of Cyanthillium extract was more when compared with the standard ascorbic acid at all concentrations other than 30µl concentration. This difference was found to be statistically significant at 10 µl, 30 µl, 40 µl and 50 µl concentrations. (Table 1)

Antibacterial activity of Cyanthillium cinereum extract showed significant activity against Streptococcus mutans at 100µl with zone of inhibition 15.23± 0.25. Antibacterial activity of Cyanthillium cinereum extract showed significant activity against Staphylococcus aureus at 25 µl with zone of inhibition 9.20 ± 0.20. Though there was statistically significant difference in the antifungal activity against Candida albicans, there is no antifungal activity. (Table 2)

There was an increase in the anti-inflammatory activity of aqueous extract of Cyanthillium plant with increase in the concentration. Also, anti-inflammatory activity of Cyanthillium extract was more when compared with the standard Diclofenac at all concentrations. This difference was found to be statistically significant at 10 µl, 20 µl, 30 µl, 40 µl and 50 µl concentrations. (Table 3)

Cytotoxic activity of Cyanthillium cinereum aqueous extract showed that there is increase in the cytotoxicity with increase in the aqueous extract concentration. (Table 4)

TABLE 1: Antioxidant activity of Cyanthillium cinereum aqueous extract

Conc. µg/ml	Absorbance (Mean ± SD)		P value
	Cyanthillium cinereum aqueous extract	Ascorbic acid (Standard)	
10	41.26±0.64	34.60±0.40	0.000***
20	45.33±1.17	42.13±1.80	0.06
30	44.73±1.61	47.50±0.45	0.04*
40	55.56±1.83	48.73±0.55	0.003**
50	61.30±0.81	53.26±0.90	0.000***
p value	0.001**	0.004**	

TABLE 2: Antimicrobial activity of Cyanthillium cinereum aqueous extract against oral pathogens

	Zone of inhibition of Cyanthillium cinereum extract (Mean± Std. Deviation)				
	25µl	50µl	100µl	Control	P value
S mutans	9.20 ± 0.20	13.30± 0.26	15.23± 0. 25	35.43±0.51	0.000***
S aureus	9.20 ± 0.20	9.16 ± 0.15	9.03 ± 0.05	45.16 ± 0.15	
Candida	4.01± 0.31	4.95± 0.51	4.21± 0.41	25.01± 0.37	

TABLE 3: Anti-inflammatory activity of Cyanthillium cinereum aqueous extract

Conc. µg/ml	Absorbance (Mean ± SD)		P value
	Cyanthillium cinereum aqueous extract	Diclofenac (Standard)	
10	73.53±2.04	47.53±0.55	0.000***
20	85.60±1.15	61.80±1.50	0.01*
30	91.36±1.25	70.56±0.85	0.04*
40	93.20±0.20	77.26±0.55	0.001**
50	94.16±0.76	83.86±0.51	0.000***
p value	0.001**	0.000***	

TABLE 4: Cytotoxic activity of Cyanthillium cinereum aqueous extract

Aqueous extract of Cyanthillium cinereum - Conc in µl	Viable nauplii (Percentage of death)
5µl	10 (0%)
10µl	9(10%)
20µl	9(10%)
40µl	7(30%)
80µl	6(40%)

FIGURE 1: showing the Cyanthilium cinereum plant

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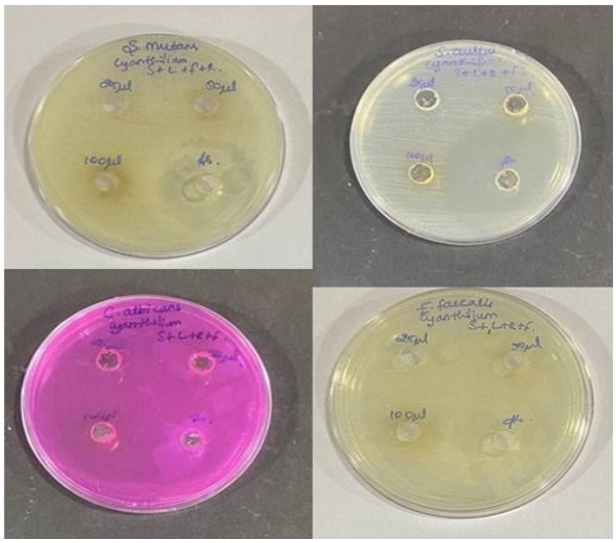

FIGURE 2: showing zone of inhibition of Cyanthillium cinereum aqueous extract against oral pathogens

DISCUSSION

Plants have been used in synthesis of antibiotics to treat infectious diseases and antimicrobials produced from plants are found to have less side effects compared with synthetic antibiotics. Secondary metabolites produced by plants act as chemotherapeutic, bactericidal, and bacteriostatic agents. Researches done on the metabolic compounds in plants to analyse the medicinal properties of plants provides an evidence-based approach to use plants in therapeutics. 3

Aqueous and methanol plant extracts of leaves of guava, leaves of neem, seeds of black cumin, root of chaff-flower, buds of cloves showed significant antimicrobial activity against S. Mutans with Minimal Inhibitory Concentration value range from 0.2 to 5.0 mg mL− 1. It was reported that aqueous extracts of all plants were less active than methanol extracts.15

Bioactive phytocomponents and also the solvent systems determine the medicinal value of plants. 16In a study done in Indonesia to explore the antifungal activity of ethanol extracts from different plant species used in traditional medicine, the leaf extract of cat's whiskers plant and Japanese wisteria plant exhibited a significant anti-candidal effect.17

Willow leaves were used to treat pain and inflammation in the earliest period by Celsius in 30 AD. 18, 19 Based on the use of herbs in relieving pain, acetyl salicyclic acid has been used in treating inflammation as an important component in the most commonly used anti inflammatory drug, aspirin. It was reported that more than 115 plant species are used as anti-inflammatory medicine in Soh Africa. 20, 21

Cyanthillium cinereum containing various chemical constituents like luteolin 7 mono beta D glucopyranoside, which possess antioxidant activity and along with triterpene compounds like beta amyrin acetate and lupeol acetate that has anti-inflammatory properties.22 Aqueous extract of Cyanthillium cinereum possesses excellent antioxidant activity which is higher than the antioxidant activity of standard ascorbic acid. Compounds including quinones, alkaloids, phenols, saponins, coumarin, cardiac glycosides and phlobatannins are present in C. cinereum leaf extract.3 In a study which investigated methanolic, aqueous, hexane and chloroform extracts of the Cyanthillium cinereum whole plant, it was reported that aqueous extract have protective role against oxidative breakdown. 23

Significant antioxidant activity was reported to be present in the leaves and bark of the stem extract of Cyanthillium cinereum. The antioxidant activity is generally due to the presence of phenolic compounds.14 Whereas it was shown that the stem of Cyanthillium had

lesser scavenging activity against DPPH radicals when compared with leaves and flower extracts.11 Inflammation is an intricate phenomenon, in which inflammatory mediators mediate humoral and cellular reactions. Inflammatory reactions are upregulated by proinflammatory cytokines such as IL-6, TNF-α and IL-1β. Anti-inflammatory agents are used to reduce the inflammatory responses and various natural products are nowadays used as potential anti-inflammatory agents.

Cyanthillium contains Vernolides, sesquiterpene lactones which have cytotoxic properties against human tumour cell lines. Studies supported the anti-inflammatory activity of Vernonia cinerea and there was inhibition in the elevated inflammatory cytokine. 6, 24, 25 Aqueous extracts of Cyanthillium cinereum is found to have excellent antiinflammatory activity, which is twice that of Diclofenac control. It was reported in a study that Interferon gamma cytokine secretion is inhibited by Cyanthillium cinereum extract. 26 The results on brine shrimp assay showed that CC extract had an LC50 value more than 1.0mg/mL, which is the desirable cut-off point for finding cytotoxic activity. 7

Different viral, bacterial and fungal species are reported in the oral cavity of humans. 27 S. mutans and C. albicans are the primary pathogens responsible for dental caries and candidal stomatitis.28 Aqueous extract of CC showed significant results for anti-inflammatory action and antimicrobial action on S. mutans and S. aureus; however, there is only minimal antifungal activity against C. albicans. Antimicrobial activity against S. aureus is reported in another study.26 Supportive evidences encourage the use of Cyanthillium cinereum in treating general ailments such as common cold, fever, diarrhea. 25

Present study has the following strength including standard tests and assays were used to assess the activities, antimicrobial activity against oral pathogens was assessed. Limitations of the present study were the following, extract of the parts of the plant have to be studied separately, cell line studies to be conducted to assess the toxic effects and further clinical and animal trials are needed to support the invitro results.

CONCLUSION

Cyanthillium cinereum showed good antimicrobial activity against S mutans and the antibacterial activity increased with increase in concentration of Cyanthillium cinereum aqueous extract; mild antimicrobial activity against S aureus and E faecalis. Cyanthillium cinereum have very less cytotoxic effects and also has a potential to serve as a good anti-inflammatory and antioxidant agent.

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