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Research Article
Anti-fungal Potential and Brine Shrimp Lethality Assay of *in vitro* Raised Clones of *Celastrus paniculatus*

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Abstract

Background and Objective: *Celastrus panicultus* Willd (Celastraceae) a climbing shrub is well known for its ability to improve memory. Because of its high therapeutic application, it has been over exploited and is now considered as endangered species. The present investigation was focused the anti-fungal potential and brine shrimp lethality assay of leaf extracts of *in vitro* raised clones with that of their mother plant. **Materials and Methods:** The powdered leaves were extracted sequentially using a soxhlet apparatus with solvents such as petroleum ether, chloroform and methanol for 24 h. The extracts of both mother plant and *in vitro* raised clones were tested for anti-fungal assay and brine shrimp lethality assay. **Results:** The results showed that the methanolic extract of both mother plant and *in vitro* raised clones were equally and effectively inhibited *Phytophthora capsici* and *Rhizoctonia solani* completely. The chloroform and methanol extract showed maximum mortality against *Artemia salina.* **Conclusion:** The study revealed the anti-fungal activity and non-toxic nature of the leaf extracts of both mother plant and *in vitro* raised clones of *C. paniculatus*.

Key words: *Celastrus paniculatus*, anti-fungal assay, *Phytophthora capsici*, *Rhizoctonia solani*, *Artemia salina*

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Medicinal plants are not only a major resources base for traditional medicine and herbal industry but also provide livelihood and health security to a large segment of Indian population[1]. Most of these phytochemicals have beneficial role in the primary health care system as well as in various herbal drug formulation. These phytochemicals are of two types. Primary metabolites such as sugars and fats which is commonly found in all plants and secondary metabolites such as alkaloids, terpenoids, phenolics and tannins, these are not directly involved in the normal growth, development and reproduction. They often play a major role in plant defense mechanism. Human beings use secondary metabolites as medicines, flavorings and recreational drugs. The drug discovery based on the natural products provides a golden opportunity in the new clinical application of plant secondary metabolites and their derivatives[2].

The versatile properties associated with the medicinal plants were over exploited for various applications as a result most of these plants are endangered. Hence an alternative method must be devised to cultivate these plants to meet the large scale requirements. Plant biotechnology has an impact on agricultural crop productivity, food quality and human nutrition and health. The application of biotechnological tools to the regeneration of medicinal plants and production of plant derived compounds turn out to be a mainstay to conserve the plants and to achieve the aims in a limited time frame without distressing the natural sources. *In vitro* propagation provides superb opportunity to exploit our heritage of medicinal and aromatic plants without harm to natural bio resourses. Micropropagation studies on medicinal plants progressed well with several avenues during the last decades. The explant used for the *in vitro* propagation, ranges from large seedlings and organs to small single cells and protoplast[3,4].

Celastrus paniculatus Willd belongs to the family Celastraceae, is an endangered medicinal plant[5] which is large, woody, climbing shrub, distributed almost all over India is known for its ability to improve memory[6]. It is a climbing or scrambling shrub, with erect branches, the young shoots and branches are pendulous. Leaves are glabrous, broadly ovate or obovate, acuminate or acute. Flowers are unisexual, yellowish-green, borne in terminal, pendulous panicles (flowering throughout the year). This plant has much more importance in Indian system of medicines such as Ayurveda, Folk, Siddha and Unani to cure many diseases such as leprosy, leucoderma, skin diseases, paralysis, depression, arthritis, asthma and cancer[7]. Many pharmacological studies proved that the anti-oxidant, anti-inflammatory, anti-fungal, anti-malarial and anti-cancerous activity of *Celastrus paniculatus*[8-10]. The broad range of biological activities of this particular genus is mainly due to the presence of various phytoconstituents and it was proved that most of them are sesquiterpenes (β-agarofurans), diterpenes, triterpenes, alkaloids, phenolics and flavonoids[11].

In plants majority of defense mechanism against pathogen attack are induced after recognition. Whereas a minor case may be non-specific and constitutive in nature. Such responses, include localized cell death[12] and followed by synthesis of Pathogenesis Related (PR) proteins and thereby inducing systemic acquired resistance[13]. Such resistance activated a broad range of response first locally and then to distal tissues from the site of attack. Widely diverse organisms such as fungi, bacteria and viruses provide similar resistance mechanisms[14]. Studies proved that the anti-fungal properties of natural products from many indigenous medicinal plants are being increasingly reported from different parts of the world. The World Health Organization estimated, plant extracts or their active constituents to be used as folk medicine in traditional therapies of 80% of the world's population[15].

Brine shrimp lethality assay is considered as a useful tool for preliminary assessment of toxicity and it has been used for the detection of fungal toxins, plant extract toxicity, heavy metals, pesticides and cytotoxicity testing of dental materials and it is based on the ability to kill laboratory cultured brine shrimp (*Artemia nauplii*)[16-21].

The objective of the study was:

- To determine the anti-fungal potential and toxicity of the organic leaf extracts from both mother plant and *in vitro* raised clones of *Celastrus paniculatus*

MATERIALS AND METHODS

The experiment was done at the laboratory of Department of Biotechnology, University of Calicut on 2016 June-December. The chemicals used for the experiments were procured from Himedia, Mumbai; Merck Mumbai; SRL Mumbai. The chemicals used were of analytical grade.

Preparation of solvent extract: Fresh leaves were collected, washed with distilled water, shade dried for 2-3 weeks and ground into course powder. The extract was prepared by

single solvent extraction method. About 100 g of powdered leaves were extracted sequentially using a soxhlet apparatus with solvents such as petroleum ether, chloroform and methanol based on the increasing order of polarity for 24 h for each solvent. The suspension was filtered through Whatman's filter paper and collected in large Petri plates. These were allowed to dry completely in water bath set at 40±0.2°C for 30 min.

Anti-fungal activity: Three different organic extracts of leaves were tested for anti-fungal activity against some plant pathogens such as *Phytophthora capsici* and *Rhizoctonia solani* were used in the present experiment. Test fungi were isolated on potato dextrose agar medium (PDA). Periodic observations were made after every 24, 48 and 72 h for the growth of the fungus and the growth was recorded in percentage. All the experiments were conducted in triplicate.

Brine shrimp lethality assay: Brine shrimp lethality bioassay was carried out to investigate the cytotoxicity of organic extracts of leaves. Brine shrimps (*Artemia salina*) were hatched using brine shrimp eggs maintained in sterile artificial seawater (prepared using sea salt 38 g L^{-1} and adjusted to pH 8.5 using 1 N NaOH) under constant aeration for 48 h. After hatching, active nauplii were collected from brighter portion of the hatching chamber and used for the assay. Ten nauplii were drawn through a glass capillary and placed in each vial containing 4.5 mL of brine solution. In each experiment 0.5 mL of the plant extract was added to 4.5 mL of brine solution and maintained at room temperature for 24 h under the light and surviving larvae were counted[22].

Data analysis: Brine shrimp lethality assay determined the toxicity by counting the survived nauplii after 24 h of exposure to the tested sample and survivors have been counted by magnifying glass or a microscope. After 24 h of exposure, the median lethal concentration (LC_{50}) of the test samples has been obtained by a plot of percentage of the shrimps killed against the logarithm of the sample concentration[23-25]. The experiment has been repeated twice to get statistically reproducible data[26-28].

Toxicity testing criteria: The toxicity of extracts was expressed as LC_{50} values, either by comparing to Meyer's or to Clarkson's toxicity index.

According to Meyer's toxicity index, extracts with LC^{50}<1000 μg mL^{-1} are considered as toxic, while extracts with LC^{50}>1000 μg mL^{-1} are considered as non-toxic[23].

Clarkson's toxicity criterion for the toxicity assessment of plant extracts classifies extracts in the following order: Extracts with LC_{50} above 1000 μg mL^{-1} are non-toxic, LC_{50} of 500-1000 μg mL^{-1} are low toxic, extracts with LC_{50} of 100-500 μg mL^{-1} are medium toxic, while extracts with LC_{50} of 0-100 μg mL^{-1} are highly toxic[29].

RESULTS

Anti-fungal assay: The leaf extracts of both mother plant and *in vitro* raised clones inhibited *Phytophthora capsici* and *Rhizoctonia solani* considerably when treated at a concentration of 0.1 mg mL^{-1}. Among the extracts methanol and chloroform-methanol extract showed maximum anti-fungal activity in case of *Phytophthora capsici* (Table 1). Whereas in case of *Rhizoctonia solani*, both methanol and chloroform extracts equally and effectively inhibited.

Effect on *Phytophthora capsici*: The plant fungus *Phytophthora capsici* exhibited a zonal diameter of 25, 53 and 90 mm, respectively for 24, 48 and 72 h. Whereas the methanolic extract of both mother and *in vitro* propagated plant inhibited *Phytophthora capsici* completely with a 100% of inhibition. On the other hand chloroform extracts of mother plant exhibited lesser activity but the *in vitro* propagated plant exhibited 40% of inhibition at 24 h (Table 2). So from the above results it was concluded that methanolic extract of both mother and *in vitro* raised plants are effectively inhibited *Phytophthora capsici* (Fig. 1).

Effect on *Rhizoctonia solani*: The plant fungus *Rhizoctonia solani* exhibited a zonal diameter of 30, 55 and 90 mm, respectively for 24, 48 and 72 h (Table 3). The methanolic

Table 1: Fungal growth diameters of *Phytophthora capsici*

Samples	24 h	48 h	72 h
1 mg mL^{-1}	0.1 mg mL^{-1}	0.1mg mL^{-1}	0.1 mg mL^{-1}
Control	25 mm	53 mm	90 mm
Protein extract	0 mm	0 mm	0 mm

Table 2: Percentage of inhibition of methanol and chloroform leaf extract on *Phytophthora capsici*

Samples	24 h	48 h	72 h
1 mg mL^{-1}	0.1 mg mL^{-1}	0.1 mg mL^{-1}	0.1 mg mL^{-1}
Methanolic extract of mother plant	100%	100%	100%
Methanolic extract of *in vitro* plant	100%	100%	100%
Chloroform extract of mother plant	8%	0%	0%
Chloroform extract of *in vitro* plant	40%	1.8%	0%

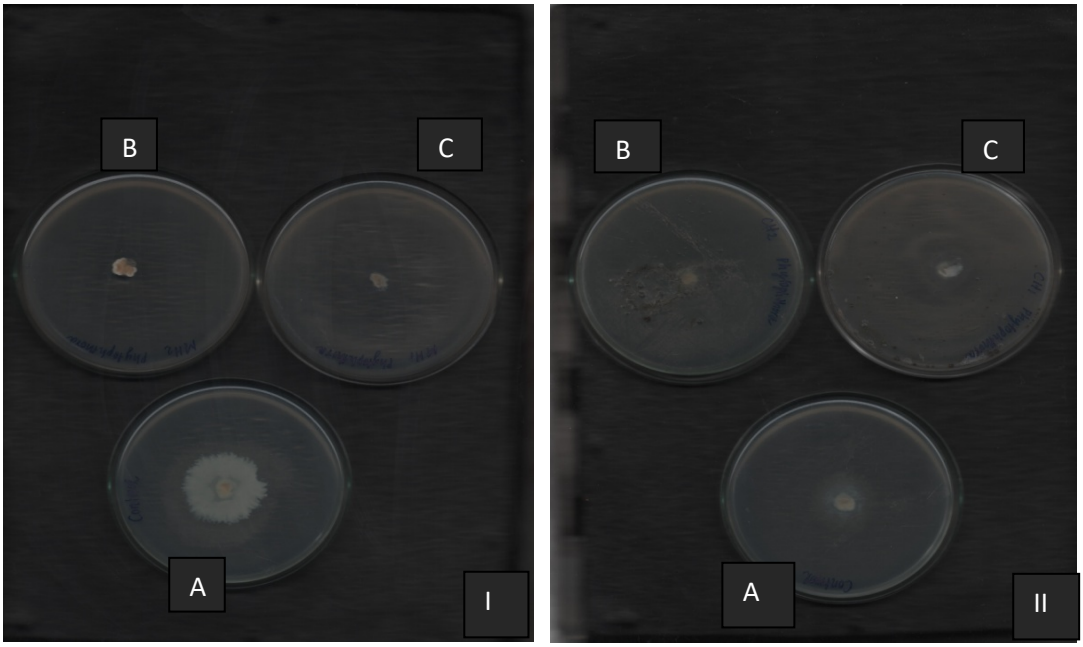

Fig. 1: Anti-fungal activity of leaf extracts on *Phytophthora capsici*
A: Control, B: *In vitro* propagated plant, C: Mother plant, I: Methanol extract, II: Chloroform extract

Table 3: Fungal growth diameters of *Rhizoctonia solani*

Samples	24 h	48 h	72 h
1 mg mL^{-1}	0.1 mg mL^{-1}	0.1 mg mL^{-1}	0.1 mg mL^{-1}
Control	30	55	90
Protein extract	10	12	13

Table 4: Percentage of inhibition of methanol and chloroform leaf extract on *Rhizoctonia solani*

Samples	24 h	48 h	72 h
1 mg mL^{-1}	0.1 mg mL^{-1}	0.1 mg mL^{-1}	0.1 mg mL^{-1}
Methanolic extract of mother plant	66.66%	78.18%	85.55%
Methanolic extract of *in vitro* plant	73.33%	80%	85.55%
Chloroform extract of mother plant	66.66%	72.72%	77.77%
Chloroform extract of *in vitro* plant	70%	81.81%	86.66%

extract of both mother and *in vitro* raised plant inhibited *Rhizoctonia solani* at a range of 85.55% at 72 h (Table 4). Whereas a variation exhibited in the chloroform extract of mother and *in vitro* plant being 77.77 and 86.66%, respectively. Hence forth it can be considered as methanolic and chloroform extracts of both mother and *in vitro* raised plants are equally and effectively inhibited *Rhizoctonia solani* (Fig. 2).

Brine shrimp lethality assays: The organic leaf extract of *Celatrus paniculatus* showed brine shrimp lethality at higher concentrations. The median lethality concentration (LC_{50}) of petroleum ether, chloroform and methanol extracts was 1000, 2500 and 1000 ppm (μg mL^{-1}), respectively for mother plant. Whereas the *in vitro* raised plants exhibited LC_{50} values of 1000 ppm (μg mL^{-1}) for petroleum ether extract and 2500 ppm (μg mL^{-1}) for both chloroform and methanol extracts. The degree of lethality and concentration of the extracts are directly proportional to each other. The maximum mortality was observed (100%) at a concentration of 2500 ppm (μg mL^{-1}) for chloroform extract of mother plant, chloroform and methanol extract of *in vitro* plant. The least mortality rate was observed at a concentration of 1000 ppm (μg mL^{-1}) for petroleum ether of both mother plant and *in vitro* raised clones and methanolic extract of mother plant as shown in (Table 5). So from the above results it was concluded that the organic leaf extracts of mother plant and *in vitro* raised clones of *Celastrus paniculatus* are non-toxic in nature.

DISCUSSION

The anti-fungal activity of chloroform and methanol leaf extracts of both mother and *in vitro* raised plants were tested against phytopathogenic fungus such as *Phytophthora capsici* and *Rhizoctonia solani.* The anti-fungal activity of methanolic crude extracts *of C. paniculatus* against *Alternaria solani*, *Curvularia lunata*, *Fusarium* sp. *Bipolaris* sp. and

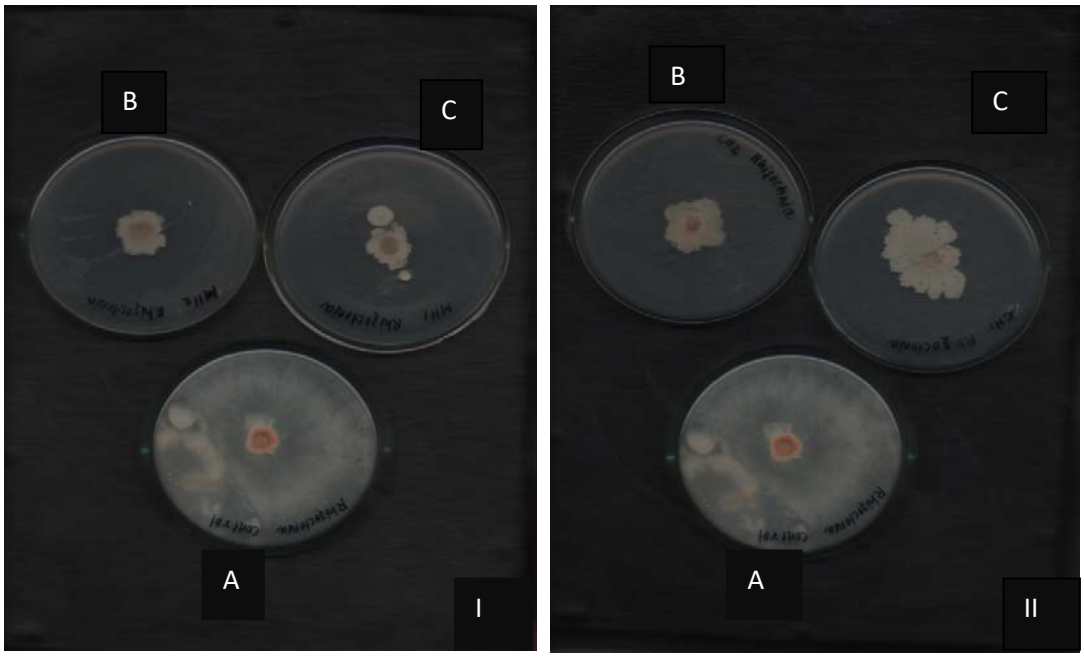

Fig. 2: Anti-fungal activity of leaf extracts on *Rhizoctonia solani*

A: Control, B: *In vitro* propagated plant, C: Mother plant, I: Methanol extract, II: Chloroform extract

Table 5: Brine shrimp lethality assay of leaf extracts of *Celastrus paniculatus*

Sample concentration (mg/100 mL)	Number of survival of brine shrimp/10	Percentage
Petroleum ether extract of mother plant		
Control	10	100
500	0	0
250	0	0
100	5	50
Petroleum ether extract of *in vitro* plant		
Control	10	100
500	0	0
250	1	10
100	5	50
Chloroform extract of mother plant		
Control	10	100
500	0	0
250	5	50
100	9	90
Chloroform extract of *in vitro* plant		
Control	10	100
500	0	0
250	5	50
100	9	90
Methanol extract of mother plant		
Control	10	100
500	0	0
250	4	40
100	5	50
Methanol extract of *in vitro* plant		
Control	10	100
500	0	0
250	5	50
100	8	80

Helminthosporium sp. at different concentrations (1000, 2000, 3000, 4000 and 5000 µg mL^{-1}). Better anti-fungal activity was noted against *A. solani* and *Helminthosporium* at a concentration of 5000 µg mL^{-1} and showed least activity against *C. lunata* and *Fusarium* sp[30]. The present study revealed that out of the two extracts, methanolic extract of both mother and *in vitro* raised plants are effectively inhibiting *Phytophthora capsici* at different time intervals. Whereas in the case of *Rhizoctonia solani* methanol and chloroform extracts of both mother and *in vitro* raised plants are effectively inhibiting. The methanol leaf extract was found to be most effective and evidenced excellent inhibitory activity against *Phytophthora capsici* and *Rhizoctonia solani.* Studies reported that *Celastrus paniculatus* exhibited anti-fungal activity against six species of fungi *Trichophyton mentagrophytes*, *T. rubrum*, *T. soudanense*, *Candida albicans*, *Torulopsis glabrata* and *C. krusei*[31].

The azaron or 1, 2, 4-trimethoxy-5-(1-propenyl) Benzene, isolated from the rhizome extract of *Acorus calamus* showed strong antifungal activity against three phytopathogenic fungi viz., *Macrophomina phaseolina*, *C. lunata* and *Alternaria alternata*[32] at 400 µg mL^{-1}. The methanol extract of *A. calamus* containing Asarone as a major component showed high anti-fungal activity against *M. gypseum, T. rubrum* and *P. marneffei* and had moderated activity against *C. albicans* and *C. neoformans*[33]. A constitutively

expressing protein was purified from leaves of *Acorus calamus* which exhibits anti-fungal activity against phytopathogens such as *M. phaseolina*, *Fusarium moniliforme* and *Trichosporium vesiculosum*[34]. The anti-fungal potential of *T. cordifolia* against 8 important species of *Aspergillus* such as *A. candidus*, *A. columnaris*, *A. flavipes*, *A. flavus*, *A. fumigatus*, *A. niger*, *A. ochraceus* and *A. tamari*[35].

The lethality of various leaf organic extracts of both mother plant and *in vitro* propagated plant of *C. paniculatus* was tested in the shrimp (*Artemia salina*) was utilized in the Brine Shrimp Cytotoxicity Test (BSCT). It is very simple, inexpensive, practical and economic method for determination of bioactivities of synthetic compound as well as plant products[36-38]. It has been well utilized to screen and fractionate physiologically active plant extracts and correlate reasonably well with cytotoxic and other biological properties[39]. The significant correlation between the Brine shrimp assay and *in vitro* growth inhibition of human solid tumor cell lines is significant because it shows the value of this bioassay as a pre-screening tool for anti-tumor drug research[40]. In toxicity evaluation of plant extracts by Brine shrimp lethality bioassay LC_{50} values lower than 1000 μg mL^{-1} are considered as toxic. According to this the present study revealed that different organic leaf extracts of both mother plant and *in vitro* raised clones of *C. paniculatus* are non-toxic. This lethality bioassay also indicates antifungal effects, pesticidal effects, teratogenic effects, toxicity to environment. The degree of lethality was found to be directly proportional to the concentration of the extract ranges from the lowest concentration to the highest concentration.

CONCLUSION

The anti-fungal activity was evaluated against two plant fungus such as *Phytophthora capsici* and *Rhizoctonia solani* using paper disc method. Results concluded that methanolic extract of both mother and *in vitro* raised plants are effectively inhibited *Phytophthora capsici*. But methanol and chloroform extracts of both mother and *in vitro* raised plants are equally and effectively inhibited *Rhizoctonia solani.* The Brine Shrimp lethality assay proved that the organic leaf extracts of mother plant and *in vitro* raised plant of *C. paniculatus* are non toxic in nature.

SIGNIFICANCE STATEMENT

The study authenticated the anti-fungal potential and non-toxic nature of various organic extracts of both mother plant and *in vitro* raised clones of *Celastrus paniculatus* Willd. The methanol leaf extract completely inhibit both the fungi *Phytophthora capsici* and *Rhizoctonia solani*, whereas chloroform extract showed inhibition on only one of the fungi, *Rhizoctonia solani.* The Brine Shrimp lethality assay proved that the organic leaf extracts of mother plant and *in vitro* raised plant of *C. paniculatus* are non toxic in nature.

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