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RESEARCH ARTICLE

In Vitro Efficacy of *Piper betle* Leaf Extract against *Rhizoctonia solani* Causing Damping off Disease of Chilli

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ABSTRACT

In this study, antifungal activity against the pathogen *Rhizoctonia solani*, Preliminary phytochemical studies and GC-MS analysis of the leaf extracts of *Piper betle* were carried out. The pathogen was isolated by tissue segment method and identified. Phytochemical analysis of the plant extracts was undertaken. The ethanolic leaf extracts were subjected to GC-MS studies to identify the bioactive compounds. *In vitro* efficacy of *Piper betle* leaf extract against pathogen. Antifungal activity of crude extract of *P. betle* leaves was evaluated against the fungal pathogen *R. solani*. Hence the bioactive compounds were separated by Gas Chromatography Mass Spectrometry (GC-MS) analysis which revealed the presence of 25 compounds. All concentration of the leaf extract were found to be inhibitory to growth and the rate of inhibition increased generally by increasing the concentration, 100% concentration was the most effective concentration. This inhibition may be of different bioactive compounds such as Squalene, Dibutyl phthalate, Heptaxyloxane, 9-Octadecenamide, Cyclononasiloxane etc., from *P. betle* having antifungal properties. This result support an interesting direction of research, the use of plant extracts in controlling disease and is eco-friendly.

KEYWORDS

Rhizoctonia solani, GC-MS, Crude extract, Fungitoxicity, Squalene, Dibutyl phthalate

INTRODUCTION

Chilli (*Capsicum annum*)is one of the most important spice crop in the world having nutritive value especially rich in vitamin C. Chilli is known to suffer from as many as 83 different diseases, of which more than 40 are caused by fungi[1]. *Rhizoctonia solani* is very important soil borne pathogen. Common symptom produced by *Rhizoctonia solani* is damping off and root rot[2]. Chemical fungicides are commonly used successfully for control of *Rhizoctonia*[3].

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However, their field application may not always be desirable. The persistence, injudicious use of chemicals was discouraged owing to their toxic effects[4]. Keeping in view the drawback of chemical control of plant diseases, the use of plant extracts in the control of plant diseases is gaining importance. Various plant products were shown to exert biological activity *in vitro* and *in vivo* and are used as bio-fungicidal compounds[5-7]. They may have a minimum adverse effect on physiological processes of plants and less environmental hazards compared to their synthetic alternatives, being plant products are easily convertible into a common organic material (eco-friend)[8]. *Piper betle* Linn. (betel vine) is a tropical plant closely related to the common pepper and belongs to the family

Piperaceae[9]. The leaves of *Piper betle* have been used in Indian system of medicine for its antifungal, antibacterial and antioxidant properties[10]. In the present study, the antifungal activity of crude extract of *P. betle* leaves was evaluated against the fungal pathogen *R.solani*.

MATERIALS AND METHOD

Isolation and Identification of Test Pathogen

The pathogen was isolated from chilli seeds were sown thickly in pots under green house conditions. After sowing, the pots were kept under shade and watered daily to favour the incidence of damping-off diseases. After 14 days, seedlings showing damping-off symptom were collected and the pathogen was isolated by tissue segment method[11] on potato dextrose agar medium (PDA) under laboratory conditions. It was purified by single hyphal tip method and maintained in potato dextrose agar slants. The pathogenic fungi was identified by using standard manuals such as Manual of soil fungi[12].

Plant Materials Collection

Fresh leaves of *Piper betle* were collected from Nanjikkottai, Thanjavur Dt., Tamilnadu. The leaves were washed in clean water and air dried in room temperature. The dried plant materials were milled to a fine powder using grinder and stored in the dark at room temperature in airtight containers.

Preparation of Plant Extract

The powdered sample was successively extracted with ethanol by hot continuous percolation method in Soxhlet apparatus[13] for 24 hrs. The extract was concentrated on Rota vapour under reduced pressure.

Screening of Phytochemicals

Phytochemical analysis of the plant extracts were undertaken by[14].

Gas Chromatography Mass Spectrometry (GC-MS) Analysis of *Piper betle* Leaf Extract

2µl of the *Piper betle* leaf extract was employed for GC-MS analysis. The GC-MS analysis was carried out using a Clarus 500 Perkin- Elmer (Auto System XL) Gas Chromatograph equipped and coupled to a mass detector Turbo mass gold – Perking Elmer Turbomas 5.2 spectrometer with an Elite-1 (100% Dimethyl ply siloxane), 300 m x 0.25 mm x 1 µm df capillary column. The instrument was set to an initial temperature of 110°C, and maintained at this temperature for 2 min. At the end of this period, the oven temperature was raised upto 280°C, at the rate of an increase of 5°C/min, and maintained for 9 min. Injection port temperature was ensured as 250°C and Helium flow rate as 1 ml/min. The ionization voltage was 70 eV. The samples were injected in split mode as 10:1. Mass Spectral scan range was set at 45-450 (mhz). The chemical constituents were identified by GC-MS. The fragmentation patterns of mass spectra were compared with those stored in the spectrometer database using National Institute of Standards and Technology Mass Spectral database (NIST-MS). The percentage of each component was calculated from relative peak area of each component in the chromatogram.

Identification of Compounds

Interpretation of mass spectrum of GC-MS was conducted using the database of National Institute Standard and Technology (NIST) having more than 62,000 patterns. The spectrum of the known component was compared with the spectrum of the known components stored in the NIST library. The name, molecular weight and structure of the components of the test materials were ascertained.

In vitro Efficacy of *Piper betle* Leaf Extract against Pathogen

In vitro efficacy of *Piper betle* leaf extract was done by poisoned food technique[15]. Leaves of *Piper betle* were collected from Nanjikkottai, Thanjavur Dt. Tamilnadu. 20g of leaves were taken and surface sterilized with 70% alcohol and finally sterilized with distilled water. Then they were crushed by pestle and mortar and extracted with 20 ml of sterilized distilled water and filtered aseptically through double layered cheese cloth. The extracts were poured in the flasks plugged with cotton to avoid contamination. The extracts were further diluted to different concentrations (25, 50, 75, 100%) by adding

distilled sterile water for further use in the experiment. Then 10ml aqueous extract were mixed with 10ml of Potato Dextrose Agar medium in a pre sterilized petriplates separately and swirled properly. In control set the medium was supplemented with the same amount of sterilized distilled water. A mycelial disc (4 mm diameter) cut from the periphery of 7 days old culture of Rhizoctonia *solani* was aseptically inoculated in the centre of the medium in each petriplate of treatment and control. Three replicates were used for each treatment and control. All petriplates were incubated at 28ºC for six days.

RESULTS

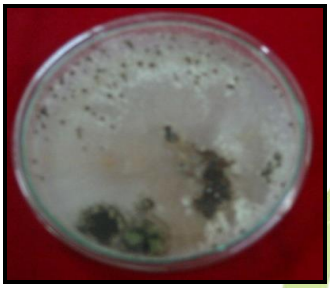

Figure 1: Colony of *R. solani*

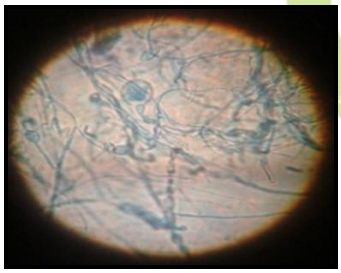

Figure 2: Fungal hyphae

Table 1: Qualitative Phytochemical Screening of *P.betle*

Phytochemicals	***P. betle***	**Phytochemicals**	***P. betle***
Alkaloids	+	Saponins	+
Flavonoids	+	Tannins	+
Carbohydrates	+	Phytosterols	+
Protein	+	Terpenoids	+
Phenols	+	Phlobatannins	-

+ indicates present**;** **-** indicates absent

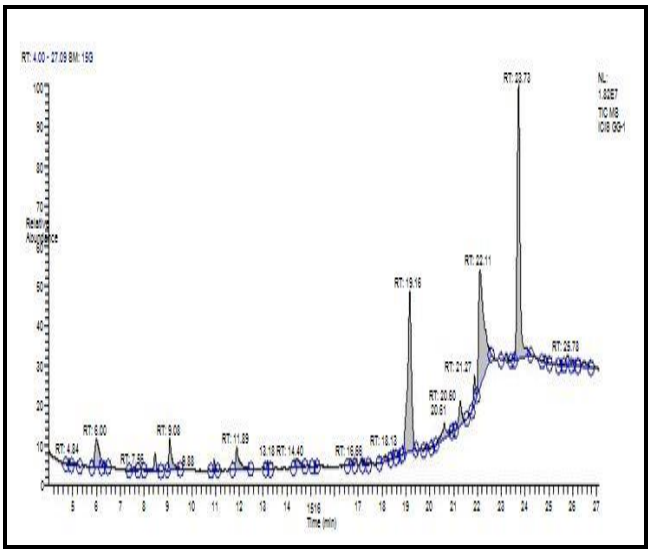

Figure 3: GC-MS Chromatogram of *P.betle*

DISCUSSION

Rhizoctonia solani is characterized by runner hyphae usually wider than 7mm, mycelium buff-colored to dark brown, sclerotia irregular in shape, light to dark brown (Figure 1 and 2). The mycelium consists of hyphae partitioned into individual cells by a septum. The hyphae often branch at a 90º angles and usually possess more than three nuclei per hyphal cell. The width of hyphae ranges from 4.3-8.0 µm[16] and 4.75-13.5 µm[17]. Sclerotial colour ranged from brown, light/dark brown, black brown, chocolate brown, salmon and dark salmon[18].

Plants have a rich source of secondary metabolites with interesting biological activities. In general, these secondary metabolites are an important source with a variety of structural arrangements and properties[19]. Phytochemical analysis conducted on the plant extracts revealed the presence of constituents which are known to exhibit medicinal as well as physiological activities[20]. In the present study reveals that ethanolic leaf extract of *P. betle* exhibited the presence of Alkaloids, Flavonoids, Carbohydrates, Protein, Phenols, Saponins, Tannins, Phytosterols and Terpenoids (Table 1). GC-MS is one of the best techniques to identify the constituents of volatile matter, branched chain hydrocarbons, alcohols, acids, esters etc.

Table 2: Components detected in ethanol extract of leaves of *Piper betle*

S. No	Compound Name	Molecular Formula	RT	Area %	Compound Nature
1	2,7Diphenyl1,6dioxopyridazino[4,5:2', 3']pyrrolo[4',5'd]pyridazine	$C_{20}H_{13}N_5O_2$	4.84	0.3	Alkaloid compound
2	Cyclotetrasiloxane, octamethyl	$C_8H_{24}O_4Si4$	6	4.12	Organo Silicone compound
3	3Carene	$C_{10}H_{16}$	6.3	0.48	Monoterpene
4	Gibberellic acid	$C_{19}H_{22}O_6$	7.56	0.32	Diterpenoid
5	Cyclopentasiloxane, decamethyl	$C_{10}H_{30}O_5Si_5$	8.46	1.36	Cyclic dimethyl siloxanes
6	Cyclopropane, 1methyl2octyl	$C_{12}H_{24}$	9.08	3.17	Gaseous Hydrocarbons
7	Cyclohexasiloxane, dodecamethyl	$C_{12}H_{36}O_6Si_6$	10.95	0.56	Silicone group
8	1Hexadecanol	$C_{16}H_{34}O$	11.89	2.86	Fatty alcohol
9	Cycloheptasiloxane, tetradecamethyl	$C_{14}H_{42}O_7Si_7$	13.18	0.47	Organo Silicone compound
10	2-Nonadecanone 2,4-dinitrophenylhydrazine	$C_{25}H_{42}N_4O_4$	17.22	0.34	Hydrocarbons
11	Squalene	$C_{30}H_{50}$	18.13	0.9	Hydrocarbon and triterpene
12	Dibutyl phthalate	$C_{16}H_{22}O_4$	18.46	0.57	Plastilizer compound
13	Hexadecanoic acid, ethyl ester	$C_{18}H_{36}O_2$	18.68	0.37	Palmitic acid
14	Cyclononasiloxane, octadecamethyl-	$C_{18}H_{54}O_9Si_9$	19.16	21.43	Organo Silicone compound
15	1Monolinoleoylglycerol trimethylsilyl ether	$C_{27}H_{54}O_4Si_2$	19.86	0.52	Steroid compound
16	9,12,15Octadecatrienoic acid, 2,3bis [(trimethylsilyl)oxy]propyl ester, (Z,Z,Z)	$C_{27}H_{54}O_4Si_2$	21.88	1.8	Linolenic acid ester
17	9-Octadecenamide, (Z)	$C_{18}H_{35}NO$	22.11	20.72	Nitrogen compound
18	Heptasiloxane, hexadecamethyl	$C_{16}H_{48}O_6Si_7$	23.73	27.62	Organo Silicone compound
19	Hexasiloxane, 1,1,3,3,5,5,7,7,9,9,11,11 dodecamethyl	$C_{12}H_{38}O_5Si_6$	25.5	0.33	Organo Silicone compound
20	Hexasiloxane, 1,1,3,3,5,5,7,7,9,9,11,11 dodecamethyl	$C_{12}H_{38}O_5Si_6$	26.48	0.94	Organo Silicone compound

Table 3: List of important bioactive compounds and its biological activity obtained through the GCMS study of the leaves of *Piper betle*

S. No	Compound Name	***Activity
1	2,7Diphenyl1,6dioxopyridazino[4,5:2',3'] pyrrolo[4',5'd]pyridazine	Antimalarial, Antiasthma, Anticancer, Antiarrhythmatic, Analgesic and Antibacterial
2	Cyclotetrasiloxane, octamethyl	Hair conditioning agent, Skin conditioning agent, Emollient, Solvent, Antimicrobial and Antiseptic
3	3Carene	Allergenic, Fungicide, Irritant and Pesticide
4	Gibberellic acid	Insecticide
5	Cyclopentasiloxane, decamethyl	Antistatic, emollient, conditioner, delivery agent, lubricant, solvent and humactant
6	Cyclopropane, 1-methyl 2-octyl	Anesthetic agent
7	Cyclohexasiloxane, dodecamethyl	Antifungal, health related products, cosmetics, paints, varnishes, surface treatments and cookware
8	1Hexadecanol	Flavour
9	Cycloheptasiloxane, tetradecamethyl	Not reported
10	Hexadecen-1-ol, trans 9-	Measure of the detonation of diesel fuel
11	Squalene	Antimicrobial, Antioxidant, Antitumour, Cancer preventive and Pesticide
12	Dibutyl phthalate	Antimicrobial, Solvent, Plastilixer, Pesticide and Repellent
13	Hexadecanoic acid, ethyl ester	Antioxidant, Nematicide and Pesticide
14	Cyclononasiloxane, octadecamethyl-	Not reported
15	1Monolinoleoylglycerol trimethylsilyl ether	Antimicrobial, Antioxidant, Anti-inflammatory, Antiarthritic, Antiasthma and Diuretic
16	8,9Dihydrocyclopenta[def]phenanthrene	Not reported
17	9,12,15Octadecatrienoic acid, 2,3bis [(trimethylsilyl)oxy]propyl ester, (Z,Z,Z)	Analgesic, Antipyretic, Anticonvulsant and Antiseptic
18	9-Octadecenamide, (Z)	Rubber additive, Antistatic agent, Dispersion aid, mold release agent
19	Heptasiloxane, hexadecamethyl	Antimicrobial, Antifungal, health related products, cosmetics, paints, varnishes, surface treatments and cookware

***Source: Dr. Duke's phytochemical and ethno botanical databases [online database]

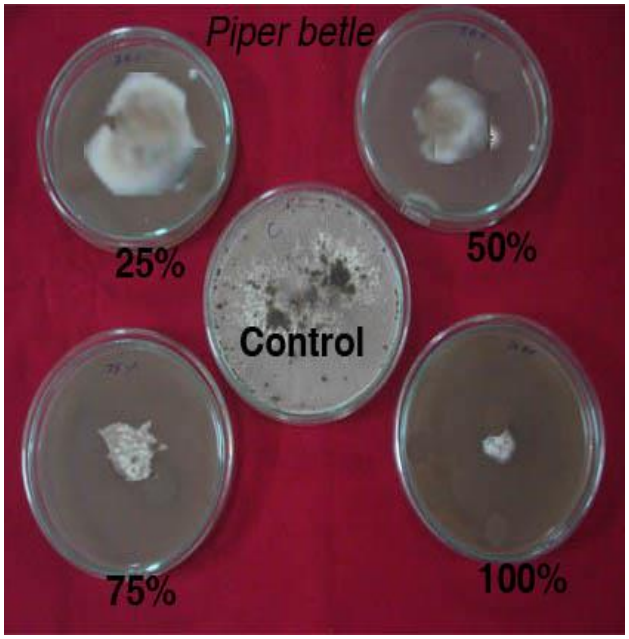

Figure 4: *In vitro* evaluation of plant extract against pathogen by food poison technique

The present study, GC-MS analysis of ethanolic leaf extract of *P. betle* showed 20 major peaks and listed out the important bioactive compounds and its biological activity obtained through Dr. Duke's phytochemical and ethno botanical databases (Table 2 and 3). They possess biological properties such as Antifungal, Antimicrobial, Antioxidant, Anti-inflammatory, Antiarthritic, Antiasthma, Diuretic, Analgesic, Antipyretic, Anticonvulsant and Antiseptic activities.

In vitro efficacy of Piper *betle plant* extract against the pathogen *Rhizoctonia solani* proved at different concentrations significantly reduced the percentage of the disease incidence. All concentration of the leaf extract were found to be inhibitory to both growth and the rate of inhibition increased generally by increasing the concentration, 100% concentration was the most effective concentration (Figure 4). This inhibition may have originated from the release of different phytocompounds such as Squalene, Dibutyl phthalate, 1-Monolinoleoylglycerol trimethylsilyl ether, Cyclohexasiloxane, dodecamethyl etc., from the plant having antifungal properties. It has been well documented by several workers that the antifungal compounds present in plant's extracts have inhibitory effect on the growth of pathogen.

The investigation concluded that ethanolic leaves extraction of *Piper betle* produce enormous number of phytochemical constituents which are responsible for many biological activities. So it can be utilized for the development of novel medicines and further investigations need to elute new bio active compounds from the these three experimental medicinal plants and it may create a new path to treat many incurable diseases. This study would benefit the farmers who wish to lessen the impact of *Rizoctonia* root rot on chilli production.

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