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Evaluation on Antifungal Property of Supercritical Carbon Dioxide Extract of *Prosopis juliflora* Leaves against Plant Pathogens

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A B S T R A C T

The aim of this study was to evaluate antifungal property of the *Prosopis juliflora* leaf extract as a potential natural source for synthesis of new drugs to avoid gaining of antifungal resistance. The *Prosopis juliflora* leaf extracts obtained by supercritical fluid extraction were assessed for their antifungal property against *Rhizoctonia bataticola, Alternaria alternata and Colletotrichum gloeosporioides* by food poisoned technique. *Prosopis juliflora* leaf extract obtained by SC-CO_2 (at 50 °C and 200 bar) showed strong antifungal activities against the fungal pathogens viz., *Rhizoctonia bataticola, Alternaria alternata and Colletotrichum gloeosporioides* with 94.07 per cent, 93.15 per cent and 92.43 per cent of inhibition, respectively which indicates that supercritical fluid extract shows best results having per cent of inhibition greater than that of the soxhlet extraction process (55.31, 75.63 and 45.13 per cent of inhibition, respectively). The results of investigation showed that all the extracts had inhibitory effect on the growth of all the isolates. It can be concluded that the *Prosopis juliflora* could be a potential source for antifungal agents, probably a novel inhibitory metabolite and is potential natural fungicide.

Introduction

Plants are known to be the source of effective and versatile therapeutic agents against various diseases. Records of indigenous knowledge from various parts of the world illustrate an age long tradition of plant being a major bio-resource base for health care (Wamburu *et al.*, 2015). About 70–80 per cent of the world populations, particularly in the developing countries, rely on non-conventional medicine in their primary health care as reported by the World Health Organization. The interest of finding natural bioactive components has increased due to the harmful effects of synthetic fungicides on environment and health. Natural plant products can be alternatives to currently used synthetic pesticides, since they provide unlimited opportunities for the discovery of new pesticides because of their rich bioactive chemical constituents. Scientific interest in

medicinal plant has burgeoned in recent times due to increased efficiency of new plant derived drugs and rising concerns about the side effects of modern medicine.

Prosopis juliflora is a shrub or small tree in the family Fabaceae. It has established itself as a weed, notably in Asia and Australia (Santos *et al.*, 2013). *Prosopis juliflora* DC., mesquite tree is one of the major invasive alien species of India and it has been used to treat eye problems, open wounds, dermatological ailments and digestive problems by the native tribes of many countries. It has soothing, astringent, antiseptic, antibacterial and antifungal properties. The extracts of *P. juliflora* seeds and leaves were well studied for several in vitro pharmacological effects such as antibacterial, antifungal and anti-inflammatory properties.

The plant has been reported to treat oral ailments like toothache. The leaves were used against asthma, bronchitis, conjunctivitis as well as against skin diseases, blood and venereal diseases and acts as an insecticide.

Prosopis juliflora has been used for its pesticidal properties. Extract from *P. juliflora* has shown its antifungal potential against *Aspergillus* isolated from sorghum, maize and paddy samples. For economic and eco-friendly disease management aqueous leaf extracts of *P. juliflora* and *L. esculentum* were used, which completely inhibited the *in vitro* germination of *P. personata* and *P. arachidis*.

There are a number of conventional extraction methods for *Prosopis juliflora*. Solvent extraction is being practiced for extraction from *Prosopis juliflora* leaves. However, this method has the major disadvantage of solvent residue in the extracts. In the field of natural products, the new technique of Supercritical Fluid Extraction (SFE) has gained increasing attention over the traditional techniques in the recovery of edible and essential oils, it does not have any of the negative effects related to traditional organic solvents, at optimal conditions (Casas *et al.*, 2009).

To date, any research article on supercritical fluid extraction of *Prosopis juliflora* leaves has not been reported. Keeping in view of these facts, the investigation on "Evaluation on antifungal property of supercritical carbon dioxide extract of *Prosopis juliflora* leaves against plant pathogens" was undertaken in the Department of Agricultural Microbiology, College of Agriculture, University of Agricultural Sciences, Raichur, Karnataka (India).

Materials and Methods

Raw materials

Clean and matured leaves of *Prosopis juliflora* were collected around the UAS campus, Raichur. Then the leaves were cut dried in dehumidified air dryer (make: Bry Air Asia; model: FSD-600) at 45 °C and 15% RH. The dried leaves were ground in laboratory hammer mill with LN_2 cooling to obtain fine powder (Sankalpa *et al.*, 2014). The solvents, chemicals and reagents (analytical grade) used throughout the experiment were procured from M/s. Sigma Aldrich Chemicals, Bangalore (Karnataka).

Fungal culture

Authentic pure culture of *Colletotrichum gloeosporioides* were obtained from Microbial Type Culture Collection Centre [MTCC] Institute of Microbial Technology [IMTECH] Chandigarh in lyophilized vial. Whereas *Alternaria alternata* and *Rhizoctonia bataticola* were collected from Department of Plant Pathology, UAS, Raichur. Procured cultures were maintained in appropriate media for further use.

Extraction of *Prosopis juliflora* leaf extract

The supercritical carbon dioxide extraction system (Thar; SFE 500 system) was used for extraction of *Prosopis juliflora* leaf powder. Deionized water (at 5 °C) was used for cooling different zones in the SC-CO_2 extraction system. The independent variables selected for the study were supercritical fluid (SC-CO_2) pressures of 100, 150 and 200 bar and temperatures of 40, 50 and 60 °C at constant dynamic extraction time of 90 min (Liza., 2010) Table 1.

Hundred grams of *Prosopis juliflora* leaf powder was placed into the extractor vessel. The flow rates of supercritical CO_2 and co-solvent (ethanol) were maintained at 20 and 2 g/min, respectively (Pradhan *et al.,* 2010). Static extraction process was performed for 30 min (Palafox *et al.,* 2012). After attaining desired pressure and temperature dynamic extraction time (90 min) was started by opening the exit valve of the SC-CO_2 extraction system.

The static extraction time allowed the sample to soak in the CO_2 and co-solvent in order to equilibrate the mixture at desired pressure and temperature. During the dynamic extraction time, CO_2 carrying the crude extract flowed out of the extraction vessel and then into a collection vessel, where the CO_2 was separated through the vent connected to the fume hood.

Prosopis juliflora leaves extraction was carried out by soxhlet extraction method using SOCS- PLUS apparatus (Make: Pelican Equipments; Model: SCS-08) with hexane as solvent. Accurately, 100 g of the *Prosopis juliflora* leaf powder was taken into the thimble and placed it in the sample compartment of the extractor. Sample compartment was attached to a 500 ml round bottom flask containing 300-350 ml hexane. SOCS- PLUS set-up was assembled and heated in a mantle. The SOCS- PLUS apparatus was run at 85 ^{o}C for 90 min. Hexane in the oil extract was distilled out by using a rotary flash vacuum evaporator (Superfit, Rotavap; PBU-6D) (Malapit, 2010). Extraction process of *Prosopis juliflora* leaf extract was standardized based on the extraction yield and extraction efficiency.

In vitro screening of antifungal activity SC-CO_2 extracted *Prosopis juliflora* leaf extract by food poison technique

Food Poison technique described by Nene and Thapliyal (1993) demonstrated by Prasad and Anamika (2015) was employed to test antifungal effect. Petri plates were washed, rinsed with sterile distilled water, dried, wrapped in tin foil and kept in autoclave at100 ^{o}C for 15 min to sterilize.

The potato dextrose media was prepared and sterilized. A volume of 0.5 ml of plant extract was aseptically poured into Petri plate followed by addition of 9.5 ml of melted PDA and was gently mixed. One inoculum disc of test fungus was aseptically inoculated upside down at the centre of the Petri plate and incubated at 25 $^{\circ}$C.

The media plate without extract set as a control. The average radial growth of the fungal mycelial was measured on the 7th day of incubation.

$$\text{Mycelial inhibition } (\%) = \frac{DC - DT}{DC} \times 100$$

Where,

DC - Average diameter of colony in control plate

DT - Average diameter of colony in treatment plate

Results and Discussion

Effect of SC-CO_2 temperature and pressure on extraction efficiency of *Prosopis juliflora* leaf extract

The effect of SC-CO_2 temperature and pressure on extraction efficiency of *Prosopis juliflora* leaf extract at three levels namely 40, 50 and 60 °C and 100, 150 and 200 bar are presented in Table 1. It is evident from the figure that as pressure increased from 100 to 200 bar, the extraction efficiency increased. This might be due to the fact that the increase in pressure increased the density of the CO_2 thereby increasing the solvent strength and solubility of the oil in CO_2 (Liza *et al.*, 2010). The highest extraction efficiency of 93.37 per cent were recorded at SC-CO_2 pressure of 200 bar, temperature of 50 °C which was considered as the optimum and best SC-CO_2 extraction condition for obtaining the highest extraction efficiency from *Prosopis juliflora* leaf powder.

The lowest of 55.62 per cent were recorded at SC-CO_2 pressure of 100 bar, temperature of 40 °C. The extraction efficiency decreased with the rise of temperature at low pressures, due to the reduced density of CO_2 with increased temperature (Zhao and Zhang, 2013).

In vitro screening of SC-CO_2 extracted *Prosopis juliflora* leaf extract for antifungal activity

The antifungal activity of SC-CO_2 extracted *Prosopis juliflora* leaf extract at different temperature and pressure combinations against the plant pathogens are presented in Table 2. Among the different treatments of SC-CO_2 extracted *Prosopis juliflora* leaf extract, all the treatments showed significant antifungal activity against all the three fungal pathogens tested.

From the Table 3, it is observed that the percent of inhibition of extract were in the range of 55.31 per cent to 94.07 per cent for *Rhizoctonia bataticola*, 75.63 per cent to 93.15 per cent for *Alternaria alternata*, and 46.40 per cent to 92.43 per cent for *Colletotrichum gloeosporioides*.

The highest zone of inhibition for *Rhizoctonia bataticola* of 94.07 per cent was found at 200 bar and 50 °C treatment and lowest of 55.31 per cent was found in soxhlet extraction. The highest zone of inhibition for *Alternaria alternata* of 93.15 per cent was found at 200 bar and 50 °C treatment and lowest of 75.63 per cent was found in soxhlet extraction.

The highest zone of inhibition for *Colletotrichum gloeosporioides* of 92.43 per cent was found at 200 bar and 50 °C treatment and lowest of 55.67 per cent was found in soxhlet extraction. Effect of *Prosopis juliflora* leaf extract by supercritical fluid extraction against *Rhizoctonia bataticola*, *Alternaria alternata*, and *Colletotrichum gloeosporioides* was shown in Plate 1, Plate 2 and Plate 3, respectively.

Results of present investigation were in agreement with Raghavendra *et al.*, (2009) that the activity of aqueous extract of *Prosopis juliflora* against *Alternaria alternata showed* 71.59 per cent inhibition of mycelial growth. Ikram and Dawar (2013) studied on *Prosopis juliflora* leaf, stem and flower powder as a soil amendment carried to control of *Rhizactonia solani* in cowpea and mung bean. Deressa *et al.*, (2015) used methanol, acetone and aqueous extract of *Prosopis juliflora* leaves against *Colletotrichum gloeosporioides* the results showed radial growth inhibition of 100 per cent, 100 per cent, 79.60 per cent, respectively. Bazie *et al.*, (2014) reported the activity of methanolic extract of *Prosopis juliflora* against *Colletotrichum musae*, which showed 30.70 mm zone of inhibition.

Table.1 Treatment combinations for supercritical fluid extraction of *Prosopis juliflora* leaf extract

Treatment	Temperature °C	Pressure(bar)
T_1	40	100
T_2	40	150
T_3	40	200
T_4	50	100
T_5	50	150
T_6	50	200
T_7	60	100
T_8	60	150
T_9	60	200
T_{10}	Soxhlet Extraction (Conventional method)	

T_{10} = Control - Soxhlet extraction carried out at 85 ^{o}C for 90 minutes

Table.2 Effect of SC-CO_2 temperature and pressure on extraction yield and extraction efficiency of *Prosopis juliflora* leaf extract

Treatments	Extraction yield (g/100g)	Extraction efficiency (%)
T_1	8.40^{f}	55.62 ^{f}
T_2	9.15^{e}	60.59 ^{e}
T_3	12.00^{c}	79.46 ^{c}
T_4	9.80^{d}	64.89 ^{d}
T_5	12.60 ^{b}	83.44 ^{b}
T_6	14.10^{a}	93.37 ^{a}
T_7	8.60^{f}	57.17^{f}
T_8	9.74^{d}	64.67 ^{d}
T_9	12.70^{b}	84.10^{b}
T_{10}	9.25^{e}	61.25 ^{e}
Mean	**10.64**	**70.49**
SEm±	**0.070**	**0.440**
CD (1%)	**0.210**	**1.318**

Note: Mean values followed by same superscript letters are not significantly different

Table.3 Antifungal activity of *Prosopis juliflora* leaf extract obtained by supercritical fluid extraction Method against fungal test organisms

Treatments	*Rhizoctonia bataticola*		*Alternaria alternata*		*Colletotrichum gloeosporioides*	
	Mycelia growth (mm)	**Inhibition (%)**	**Mycelia growth (mm)**	**Inhibition (%)**	**Mycelia growth (mm)**	**Inhibition (%)**
T_1	21.40	76.04^{de}	21.50	76.65^{i}	32.10	61.74^{g}
T_2	20.50	75.24^{e}	18.50	80.05^{gh}	30.10	64.15^{f}
T_3	19.50	78.06^{cd}	10.60	87.87^{d}	24.70	70.72^{d}
T_4	25.30	71.66^{f}	19.20	78.82^{h}	28.43	66.18^{e}
T_5	17.30	80.96^{b}	13.20	89.40^{c}	14.13	83.36^{c}
T_6	5.30	94.07^{a}	6.10	93.15^{b}	6.37	92.43^{b}
T_7	35.20	61.00^{g}	16.50	81.12^{g}	37.20	55.67^{i}
T_8	27.20	69.70^{f}	15.20	82.98^{f}	34.30	59.26^{h}
T_9	18.10	79.58^{bc}	13.30	85.07^{e}	28.13	66.57^{e}
T_{10}	40.50	55.31^{h}	22.50	75.63^{i}	45.13	46.40^{j}
Negative control	90	Nil	90	Nil	84	Nil
Positive control	5	94.6^{a}	4.9	94.57^{a}	5.1	93.77^{a}
Mean*	**26.69**	**69.69**	**20.55**	**77.11**	**30.38**	**63.35**
Sem	**0.115**	**0.555**	**0.097**	**0.283**	**0.187**	**0.157**
CD1%	**0.457**	**2.195**	**0.384**	**1.119**	**0.739**	**0.620**

Note: Mean values followed by same superscript letters are not significantly different.

Plate.1 Effect of *Prosopis juliflora* leaf extract obtained by supercritical fluid extraction method against *Rhizoctonia bataticola*

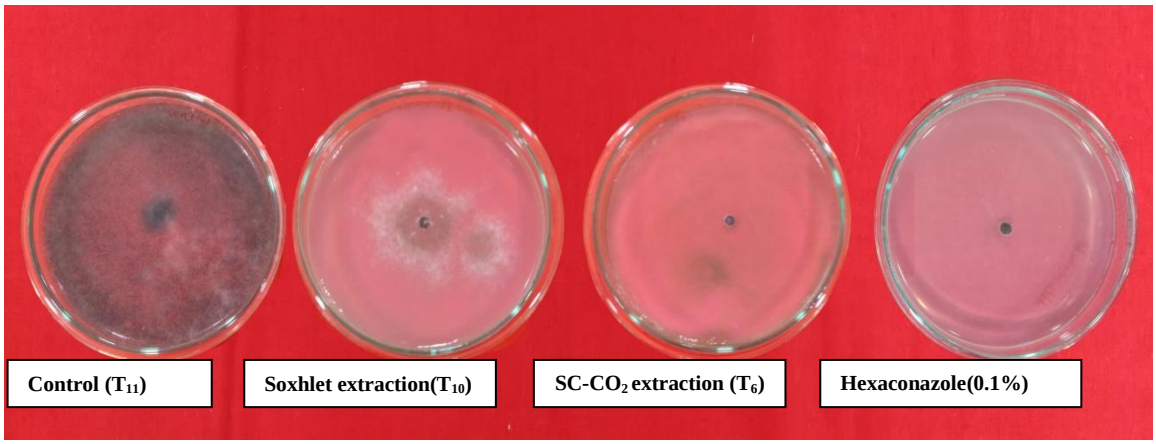


Plate.2 Effect of *Prosopis juliflora* leaf extract obtained by supercritical fluid extraction method against *Alternaria alternate*

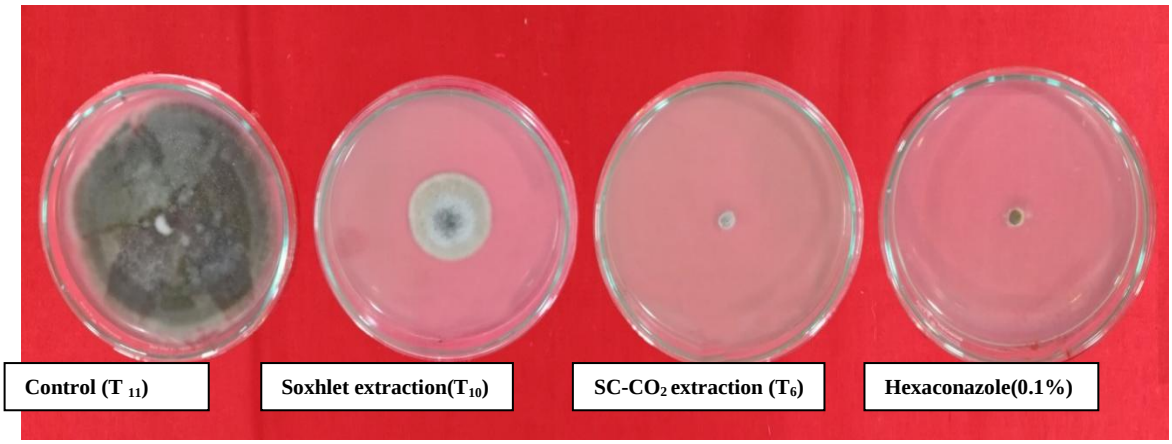
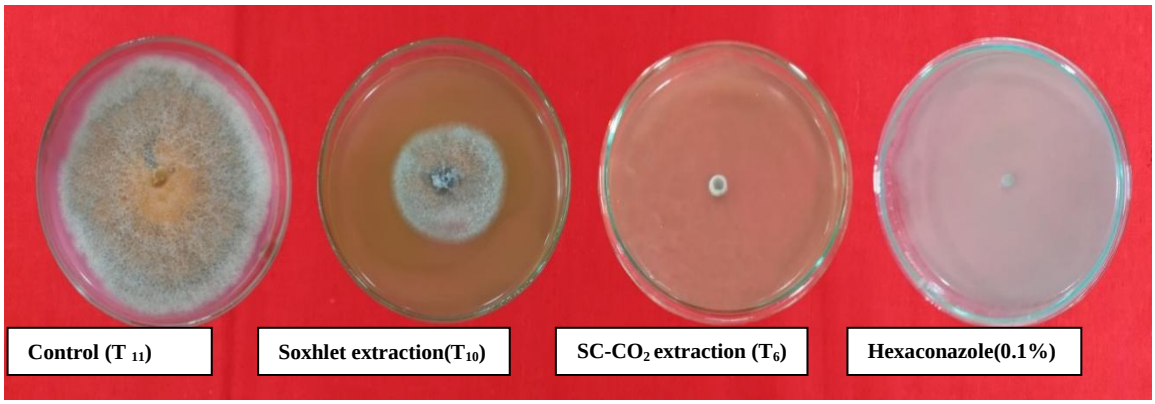


Plate.3 Effect of *Prosopis juliflora* leaf extract obtained by supercritical fluid extraction method against *Colletotrichum gloeosporioides*



Prosopis juliflora showed notable antifungal activity and it can be used to discover bioactive natural products that may serve as leads for the development of new pharmaceuticals that address hither unmet therapeutic needs. The results support the idea that *Prosopis juliflora* plant extracts could be used as a promising source of potential antifungal agents and can be used in medicinal applications. The results obtained from the above conducted study provide evidence for the presence of antimicrobial bio active compounds in *Prosopis juliflora* leaf extract. These compounds can degrade the cell wall, disrupt the cytoplasmic membrane, damage membrane proteins and interference with active transport of metabolic enzymes, or dissipation of cellular energy in ATP form (Davidson, 2001), which may eventually lead to cell death. More elaborative study in this plant with its pure compounds may lead to the development of natural antioxidant and alternative antifungal agents of against plant pathogens.

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