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Comparative Studies of *Plumeria* Species for their Phytochemical and Antifungal Properties Against *Citrus sinensis* Pathogens

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ABSTRACT

Development of effective means with low risk to human health and environment is needed to control postharvest pathogens as fruits are vulnerable to attacks of various microorganisms upon harvest due to high moisture and nutrient content. Leaves and flowers of *Plumeria alba, P. acutifolia* and *P. rubra* were extracted with different solvents to compare the profile of phytochemicals. *C. sinensis* (sweet orange) was selected to determine the postharvest fungal pathogens of its fruits. Various methods were followed to isolate the fungal pathogens from oranges and investigated their control by using *Plumeria* species extracts. Phytochemical analysis has revealed the presence of alkaloids as the major phytoconstituent irrespective of plant species and parts used followed by flavonoids and glycosides. Terpenoids, tannins, phenols were the other major phytochemicals found in the extracts. Six fungi were isolated from sweet oranges with *Penicillium digitatum* as the predominant one followed by *Aspergillus* sp. and *Rhizopus arrhizus*. Antifungal assay revealed the potential fungistatic activity of petroleum ether leaf and flower extracts whereas chloroform, methanol and aqueous extracts were completely failed to control the growth of fungal isolates. Flower extracts of *P. rubra* had the best antifungal activity against all the isolates but with no activity of leaf extracts. However, a significant fungistatic activity was observed with both leaf and flower extracts of *P. acutifolia* and *P. alba*. The extracts exhibited significant activity against the blue green mold, *P. digitatum*. This study suggests the potential value of using crude flower and leaf extracts of *Plumeria* species to combat postharvest fungal pathogens of sweet oranges thereby extending their shelf life.

Key words: Antifungal, *Citrus sinensis, Penicillium digitatum,* phytochemical, *Plumeria*

INTRODUCTION

World orange production is estimated to be 69.41 million tonnes annually with Brazil as the highest producer (19.16 MT) and the total orange production in India is 6.26 MT (FAO, 2012). *Citrus sinensis* L. Osbeck (Rutaceae) commonly known as sweet orange is widely cultivated in India and fungal diseases are of major concern in postharvest decay of oranges, where sporulation from infected fruits affects the healthy ones during storage thereby reducing shelf life and market value. The major fungal pathogens of citrus fruits are *Penicillium digitatum, P. italicum* (Gardener *et al*., 1986), *Trichothecium roseum*, *Ceratocystis fimbriata* (Cheema *et al*., 1981; Singh and Chaudhary, 1974), *Alternaria, Aspergillus niger* and *Geotrichum candidum* (Snowdown, 1990). Many fungicides are applied during pre and post harvesting stages and the commonly used

chemicals are imazalil (IMZ), thiabendazole (TBZ), guazatine and carbendazim (Ladaniya and Singh, 2001; Brown and Miller, 1999). These fungicides are harmful to human health if the intake exceeds the specific quantity (Ladaniya, 2008). To retain the garden fresh nature of the fruits without the side effects of chemicals, postharvest technologies using fungistatic compounds from plant origin is needed to preserve the quality of fruits from farm to market and finally to consumer.

Plumeria (Apocynaceae) commonly known as frangipani found in tropical areas of the world has medicinal values. Various species of *Plumeria* have been reported to have antimicrobial, anticancer, antioxidant, anti-inflammatory, anti-mutagenic and antipyretic activities (Rasool *et al.*, 2008; Egwaikhide *et al.*, 2009; Radha and Sivakumar, 2009; Radha *et al.*, 2009; Merina *et al.*, 2010; Gupta *et al.*, 2006; Guevera *et al.*, 1996; Gupta *et al.*, 2007).

Extending the shelf life of citrus fruits and preserving the natural qualities of fruits are needed for the quality conscious people in and around the world. Development of effective and ecofriendly method for bio-control of postharvest diseases is necessary as postharvest decay losses of citrus fruits are high. This study was focused on biological methods to control postharvest diseases in sweet oranges (*Citrus sinensis*) by using various extracts of leaves and flowers of *Plumeria* sp.

MATERIALS AND METHODS

This study was conducted during January 2012 to June 2012 which focuses on screening phytochemical and antifungal properties of *Plumeria* species against fungal pathogens of sweet oranges.

Plant material collection and extraction: Fresh plant materials (leaves and flowers) of *Plumeria alba, P. acutifolia* and *P. rubra* were collected, air dried, ground into powder and sieved (60 mesh). About 100 g of dry leaf and flower powder were extracted with solvents of increasing polarity (petroleum ether, chloroform, methanol and aqueous solution) at room temperature for 48 h. The extracts were filtered using Whatman No.1 filter paper and concentrated to dryness under reduced pressure in a rotary evaporator and stored in sterile vials at 4°C until used.

Phytochemical analysis: Phytochemical analysis of the extracts was done by following the methods described by Trease and Evans (1989), Harborne (1998) and Edeoga *et al.* (2005).

Test for alkaloids (Hager's test): To the 0.5 mL of the extract, few drops of 0.1% picric acid were added. Formation of yellow color indicates the presence of the alkaloids.

Test for anthraquinones: Two milliliter of the Chloroform was added to 1 mL of the extract and the resulting mixture was shaken for 5 min using vortex mixer followed by filtration. The filtrate was shaken with equal volume of 10% ammonia. The bright pink color in the aqueous layer indicates the positive result.

Test for flavonoids (ammonia test): One milliliter of the extract was taken in the test tube and ammonia solution was added (1:5) followed by the addition of conc. sulphuric acid. Appearance of yellow color and its disappearance on standing indicates the positive test for flavonoids.

Test for glycosides (Keller Kiliani test): Five milliliter of each extract was added with 2 mL of glacial acetic acid which was followed by the addition of 1 drop of ferric chloride solution and 1 mL of conc. sulphuric acid. Formation of brown ring at interface confirms the presence of glycosides.

Test for phenols (ferric chloride test): The 0.5 mL of the extract was added with few drops of neutral ferric chloride (0.5%) solution. Formation of dark green color indicates the presence of phenolic compounds.

Test for phlobatannins: One percent of HCl was added to the extract (1 mL) and boiled in hot water bath. Formation of red precipitate indicates the presence of phlobatannins.

Test for saponins (Froth test): One milliliter of the extract was taken in a test tube and distilled water (2 mL) was added to it. The test tube was then kept in boiling water bath for boiling and was shaken vigorously. Existence of froth formation persisted for next one hour confirms the presence of saponins.

Test for steroids: Two milliliter of acetic anhydride was added to 0.5 mL of the extract and then added 2 mL of H_2SO_4. Change of colour from violet to blue or green indicates the presence of steroids.

Test for tannins (ferric chloride test): One milliliter of the extract was added with 5 mL of distilled water and kept for boiling in hot water bath. After boiling, sample was cooled down and to this 0.1% ferric chloride solution was added. Appearance of brownish green or blue black coloration confirms the presence of tannins.

Test for terpenoids: Five milliliter of extract was taken in a test tube and 2 mL of chloroform was added to it followed by the addition of 3 mL of conc. sulfuric acid. Formation of reddish brown layer at the junction of two solutions confirms the presence of terpenoids.

Isolation of fungal pathogens: Different methods were carried out for fungal isolation from *C. sinensis* by collecting infected fruits from local market; injuring the fruit surface; cut open the fruits; incubating the whole fruits at room temperature. The fungus was inoculated into PDA plates with chloramphenicol and incubated at 28±1°C for 5-7 days. The colonies were identified (Alexopoulos *et al.*, 2007; Naqvi, 2004) and pure culture was done by streaking on the surface of PDA slants and the developed colonies were stored at 4°C until use.

Determination of *Plumeria* extracts effect on citrus fungi: Agar well diffusion method was followed to determine the effect of *Plumeria* extracts against the fungal isolates by using PDA. Spore suspensions of seven day old fungal cultures were prepared in 0.5% tween 80 in sterile distilled water and adjusted to give a spore count of 10^6 spores mL^{-1} using haemocytometer. One hundred microliter of the spore suspension was swabbed on the surface of PDA plates and 5 mm well was created on the agar. Fifty microliter of the different extracts were added to the well and the plates were kept undisturbed for 30 min for the pre diffusion of the extracts. The plates were incubated at 28±1°C for 3-5 days and the zone of inhibition was measured using HiMedia scale.

Statistical analysis: Each antifungal test was carried out in 3 replications. MS Excel was used to calculate means and standard deviations. Significant differences between values were determined by Duncan's multiple range test ($p<0.05$), following one way ANOVA.

RESULTS

Phytochemical screening of four extracts (petroleum ether, chloroform, methanol and aqueous) of both leaves and flowers has been summarized in Table 1. Alkaloids was found to be universally occurring in all extracts irrespective of species, plant part and solvent used followed by flavonoids and glycosides. However, terpenoids and tannins were also found in most of the extracts. Anthraquinones were present only in the aqueous extract of *P. acutifolia* leaf extract. In general, more number of phytochemicals was present in *P. alba* and *P. acutifolia*.

A total of six fungal strains were isolated from *C. sinensis* with *Aspergillus* (*A. flavus, A. fumigatus, A. niger, A. terreus*) as the major pathogen followed by *Penicillium digitatum* and *Rhizopus arrhizus*. (Table 2).

Results of antifungal activity of plant extracts of *Plumeria* sp. against *C. sinensis* fungal pathogens are shown in Fig. 1. It was observed that none of the extracts except petroleum ether was able to control the growth of fungal isolates throughout the study. Inhibitory action of petroleum ether extract of leaf and flower were seen with *P. acutifolia* whereas, petroleum ether extract of only flowers of *P. alba* and *P. rubra* were observed. Among the *Aspergillus* isolates, *A. terreus* and *A. flavus* were significantly controlled by most of the extracts. However, a moderate activity was observed against *A. fumigatus* and *A. niger*.

Table 1: Phytochemical analysis various extracts of *Plumeria* species

			Phytochemicals									
Plant	Parts	Extract	Alk	Ant	Fla	Gly	Phe	Phl	Sap	Ste	Tan	Ter
P. alba	Leaves	Aq	+	-	+	-	+	-	-	-	+	+
		Met	+	-	+	+	+	-	-	-	+	-
		Chl	+	-	-	+	+	-	-	-	-	+
		Pet	+	-	+	+	-	-	-	-	+	+
	Flowers	Aq	+	-	+	+	+	+	+	-	+	+
		Met	+	-	+	+	+	-	+	-	+	+
		Chl	+	-	+	+	-	+	+	-	-	+
		Pet	+	-	+	+	+	+	+	-	+	+
P. acutifolia	Leaves	Aq	+	+	+	+	+	-	-	-	+	-
		Met	+	-	+	-	-	-	-	-	-	+
		Chl	+	-	+	+	-	-	+	+	-	-
		Pet	+	-	+	+	-	-	-	+	-	-
	Flowers	Aq	+	-	+	+	+	-	-	-	+	+
		Met	+	-	+	+	+	+	-	-	+	+
		Chl	+	-	+	+	-	-	-	-	-	+
		Pet	+	-	-	+	-	-	-	-	-	+
P. rubra	Leaves	Aq	+	-	+	-	-	-	-	-	-	-
		Met	+	-	+	+	-	-	-	+	-	-
		Chl	+	-	+	+	-	-	-	-	-	+
		Pet	+	-	+	+	-	-	-	-	-	-
	Flowers	Aq	+	-	+	-	+	-	-	-	+	+
		Met	+	-	+	+	+	-	-	-	+	+
		Chl	+	-	+	+	-	-	-	-	-	+
		Pet	+	-	-	+	-	-	-	-	-	+

Aq: Aqueous, Met: Methanol, Chl: Chloroform, Pet: Petroleum ether, +: Presence and -: Absence of respective phytochemical, Alk: Alkaloids, Ant: Anthraquinones, Fla: Flavonoids, Gly: Glycosides, Phe: Phenols, Phl: Phlobatannins, Sap: Saponins, Ste: Steroids, Tan: Tannins, Ter: Terpenoids

Table 2: Morphological characteristics of the isolates

	Characteristics	
Organism	Microscopic	Macroscopic
Aspergillus niger	Rough, globose and brownish black conidia with globose vesicles	Black
Aspergillus flavus	Radiate conidia, rough, colourless conidiophores with metula and globose vesicles	lemon green
Aspergillus fumigatus	Columnar conidia, smooth, colourless conidiophores with dome shaped vesicles	Greyish green
Aspergillus terreus	Globose conidia with phialides and metulae, smooth walled conidiophores with globose vesicles	Yellowish brown
Penicillium digitatum	Elliptical conidia with metula and phialides	Olive green
Rhizopus arrhizus	Irregularly branched hyphae, globose sporangia with collapsed columellae	Grey colonies with small black dots

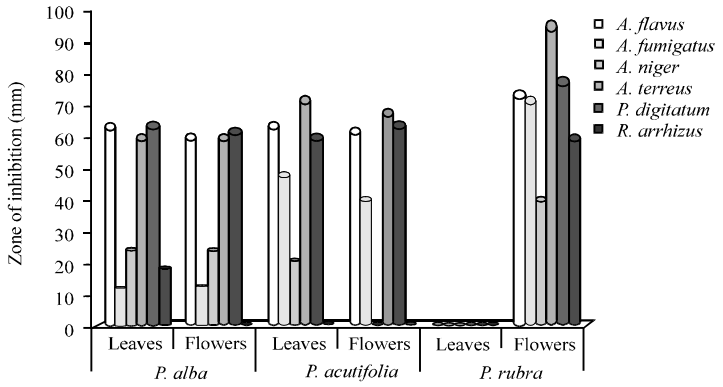

Fig. 1: Antifungal activity of petroleum ether extract of *Plumeria* sp.

DISCUSSION

Aspergillus rot is an important postharvest disease of sweet orange (Srivastava and Tandon, 1969) and a total of four *Aspergillus* species were isolated in this study. Effectiveness of *P. alba* and *P. rubra* against *R. arrhizus* was observed in the first two days of incubation but was not very persistent in subsequent days. This could be due to the fungistatic effect of volatile compounds present in the extract which was evaporated or nullified during prolonged incubation. Volatile compounds from alcoholic extract of flowers from *P. rubra* were identified by Pino *et al.* (1994). Leaf extracts of *P. rubra* was observed with no fungistatic activity which may be due to the absence of phytochemicals which are present in other *Plumeria* spp. Appreciable fungistatic activity against most of the fungal isolates was noted in *P. acutifolia* and *P. alba* extracts.

Highest inhibitory action against *P. digitatum* was observed with *P. rubra* flower extract which was followed by *P. acutifolia*. *P. digitatum* causes significant commercial loses worldwide during citrus fruit production (Holmes and Eckert, 1995). Fungistatic activity of food additives and low toxicity compounds against *P. digitatum* and *P. italicum* on citrus fruit were studied (Palou *et al.*, 2002). Biocontrol of *P. digitatum* in oranges using yeasts and essential oils were reported by previous studies (Sallam *et al.*, 2012; Badawy *et al.*, 2011; Yigit *et al.*, 2000; Soylu *et al.*, 2005). In the present study, potential antifungal effect of *Plumeria* species against *P. digitatum* was identified and suggests the use of these extracts to control the blue green mold infection in oranges.

The presence of *Plumeria* extracts prevented or reduced the growth of most of the isolates thereby revealing its potential activity against the *C. sinensis* pathogenic fungi. The order of antifungal activity were *P. acutifolia*>*P. alba*>*P. rubra*. Antimicrobial activity of ethanolic extract

of *P. acutifolia* and *P. rubra* was reported by previous studies (Rasool *et al*., 2008; Egwaikhide *et al*., 2009). Antifungal and antibacterial activities of *P. rubra* have been reported by previous studies (Gaitan *et al*., 2011; Dey *et al*., 2011).

Plant extracts (Babu and Reddy, 1986; Dixit *et al*., 1995; Godara and Pathak, 1995) and oils (Badawy *et al*., 2011; Camele *et al*., 2010; Arras and Usai, 2001) were widely used to control the growth of fungi in citrus fruits. Differences in the antifungal activity of the extracts could be due to the solubility of bioactive compounds in various solvents. Phytochemical analysis suggests the presence of biologically active compounds in the petroleum ether extract of the sample could be correlated to the antifungal activities. Screening of plant extracts has led to the discovery of many antimicrobial compounds. In the present work, various extracts of *Plumeria* sp. have been determined for their antifungal properties for controlling postharvest diseases in *C. sinensis*. The present study was able to highlight the phytochemicals present in various species of the genus *Plumeria* and preliminary antifungal screening has indicated petroleum ether extracts of *P. acutifolia* to be effective against the isolated pathogens.

Though, the hazardous impacts of fungicides which include public health, environmental pollution and toxic effects are well known, its application is essential for the control of plant diseases (Gullino *et al*., 2000). Due to the predominant fungicide resistant strains and increasing customer concern over pesticides, an effective means with less risk to human health and environment is needed to control postharvest diseases. Screening of plant species having antimicrobial compounds for plant protection is one of the biological methods of disease control in *C. sinensis*.

CONCLUSION

From the three species of *Plumeria* investigated in this work, petroleum ether extract of *P. acutifolia* and *P. alba* have potential antifungal activities against the fungal isolates from *C. sinensis*. Identification of the active constituents and their molecular structure from the extracts responsible for antifungal activity is underway to develop an effective fungicide compound for the biological control of postharvest diseases in sweet oranges.

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