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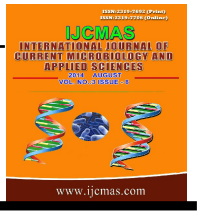


Original Research Article

Plant extract as natural food preservative against spoilage fungi from processed food

Reena Mohanka* and Priyanka

Plant pathology and Microbiology Laboratory, Department of Botany,
Patna University, Patna- 800005, India
**Corresponding author*

A B S T R A C T

In the present study, the emphasis was on employing different Citrus species extracts, to be efficiently used as an antifungal agent with an ultimate objective of developing replacements for the synthetic chemical additives in food products . Inspite of several related reports more scientific work is needed to support the claim. The purpose of this study is to examine the effectiveness of parts of few Citrus plants for preventing growth of some spoilage fungi viz. *Mucor sp.and Rhizopus* sp. isolated from processed food. *In vitro* antifungal screening of aqueous and ethanolic extracts of juice and peel of *Citrus limon* ,*Citrus sinensis* and *Citrus limetta* against the spoilage fungi with reference to commercially available synthetic preservative was attempted. It was observed that alcoholic extracts of all undertaken plants were more effective than aqueous extracts. Juice extracts were more effective than peel extracts. Among the investigated plants *C. sinensis* showed significant mycelial growth inhibition of followed by *C.limon* and *C.limetta* when compared with chemical preservative. The minimum inhibitory concentration of the extract ranged from 6.25 to 25 mg/ml. Among the plants tested *C.sinensis* ethanolic juice extract showed strong activity with best MIC value of 6.25 mg/ml against *Mucor* and *Rhizopus* . The results indicate that plant extracts possessing antifungal activity can be exploited as ideal food preservative.

Keywords

Antifungal activity, *Mucor sp.*, *Rhizopus sp.*, MIC, Citrus extracts.

Introduction

Globalization of food trade poses major challenges for food safety and quality. Food products are subjected to contamination by microorganisms causing undesirable changes including food borne illness. An internationally acceptable standard in food quality emphasized that food (processed or raw) should be wholesome and free of contaminants (FAO, 1992).

Food borne pathogens are the leading causes of illness and death in undeveloped countries, billing approximately 1.8 million people annually (Faruque, SM 2012). Fungi are the major cause of food deterioration and spoilage worldwide, ranking second to insects (Jarvis et al., 1983). To prevent spoilage is food several physical and chemical preservation techniques are

commonly employed. Nowadays, there is an increasing consumer awareness concerning the use of processed food having no chemical preservatives. The trend of today is the use of natural additives or preservatives instead of any chemical additives. Researchers have been studying antimicrobials derived from nature from many years but the interest is now stronger than ever. Currently permitted synthetic preservative are still being used safely and successfully, to prevent fungal spoilage of processed food. Increasing consumers demand for green food products with high safety and nutritional values, the present research focuses on using plant sources to meet this need.

Herbs have been used in foods since ancient times, not only as folk medicine, but also as flavoring agents and food preservatives. (Dillon, 1994; Cutler, 1995; Charalambous, 1994) due to their antimicrobial activity against certain pathogens (Tepe et al, 2004; Erasto et al. 2004); Fukai et al; 2002, Puupponen et al, 2001; Salie et al., 1996; Xu et al.,1998 Rauha et al, 2000; Ahmad and Beg, 2001 and wide array of medicinal values (Wood et al, 2001). The genus Citrus, belonging to the Rutaceae family comprises of about 140 genera and 1,300 species. *Citrus sinensis* (orange), *Citrus limon* (Lemon) and *citrus limetta* (sweet lime) are commonly used (Anwar et al 2008) .They do not exhibit toxicity at levels consumed and are considered as GRAS (Generally Recognized as safe) substance (*Souza et al, 2005, Pandit and Shelf, 1994*).

The present study was undertaken to determine and compare the potential of various extracts of *citrus limon*, *citrus sinensis* and *citrus limetta* as an antifungal agent against food borne microorganisms such as *Mucor* spp. and *Rhizopus* spp; isolated from processes food; an attempt to formulate as natural food preservatives.

Materials and Methods

For the present study, Citrus fruits were purchased from markets in the city of Patna. Peel and juicy parts of *C.limon, C.sinensis and C. limetta* were separated and kept for further aqueous and ethanolic extraction by grinding each part.

Spoilage Fungi Isolation and Identification

A survey concerning processed foods like buns(type of bread) with fungal contamination was done and collected. A number of spoilage fungi were detected but dominant spoilage fungi viz. *Mucor spp.* and *Rhizopus* spp. were chosen as the test organism. The spoiled portion of processed food sample was transferred with sterile forceps into Petri plates containing sabourauds dextrose agar (SDA) as the growth medium for the test fungi. Three replicates were made and the plates were incubated at 25 $\pm$ 2^{o}C for 5 days. Further, a pure culture of each colony type was obtained and maintained. The maintenance was done by sub- culturing each of obtained different colonies into SDA plates (sub cultured) and incubated (*Jha, 1995*) Technique of James and Natalie (2001) was adopted for identification of the unknown isolated fungi. Cultures growing on SDA were identified based on macro and micro morphology, reverse and surface coloration of colonies and slide culture technique (Ellis, 1971; Ellis,1976; Samson and van Reenen- Hoekstra, 1988; Domsch et al., 1993; Cheesbrough (2000) Pitt and Hocking, 1994; Abbey, 2007).

Extract Preparation

Aqueous and ethanolic extracts of peel and

juice of *C.limon, C. sinensis* and *C.limetta* were prepared by steeping 20g in 100 ml sterile distilled water and ethanol in sterile flasks separately. The flasks were kept for 48h at room temperature with occasional shaking. The plant extracts were filtered through Whatman filter paper No.1 and kept for use.

Antifungal screening

For screening of antifungal activity of aforesaid plants and their parts, Poisoned food technique was followed (*Sinha et al 1993*) SDA medium was supplemented with different fruit peels and juice extracts. 20 ml of this medium was poured into each Petri plate and allowed to solidify. *Mucor sp* and *Rhizopus sp*. were placed at the centre of the each petriplate and incubated at 25 $\pm$ 2^{o}C for ten days. Experiment was performed in triplicate. The medium without any plant extract in Petri plates served as negative control and medium with sodium benzoate served as positive control. Radial growth of the mycelium in each plate was recorded as the average of two diameters measured at right angles to one another at 24 hours interval, till the control grew to cover the entire plate. The percent inhibition of growth was calculated according to following formula

% inhibition – dc – dt / dc x 100

Where dc = Average increase in mycelia growth in control,

dt = Average increase in mycelia growth in treatment (*Singh and Tripathi 1999*).

Determination of Minimum Fungicidal Concentration (MIC) using agar dilution

The minimum inhibitory concentrations of the plant extracts were determined according to the National Committee for Clinical Laboratory Standards (NCCL) (now known as the CLSI [Clinical and Laboratory Standards Institute] guidelines by doubling dilution method. To 4 ml of sterile SD broth in test tubes were added 4 ml of extract. Doubling dilution was done to have extract concentrations of 50, 25, 12.5, 6.25 and 3.13 mg/ml. Afterwards, the isolated food spoilage molds viz. Mucor and Rhizopus sp. were inoculated into the test tubes and incubated at 25±^{o}C for 72 h. SDB. without extract served as negative control and with sodium benzoate as positive control.. From the test tubes used in the determination of MIC, the tube that showed no visible growth was sub cultured onto freshly prepared SDA medium. MIC was regarded as the lowest concentration of that extract that prevented growth of mold colony after at least 10 -12 days of incubation compared to positive and negative control

Results and Discussion

There has been an increased interest in the study of Citrus plants in the past few years because of the presence of secondary metabolites in different parts of the plants (*Piccinelli et al, 2008*). The current study was focused on the biological activity of fruit peel and juice extracts from *C.limon, C. sinensis* and *C. aurantifolia* for possible use as antimicrobial agents.

Influence of plant extracts on mycelia growth in percentage

The following results as per inhibition was thus obtained. All the undertaken plant samples viz. *C.limon, C. sinensis* and *C.limetta* were found effective as fungal suppressant of *Mucor* and *Rhizopus spp.* The interesting observation was that ethanolic extract of all plants were found more effective then aqueous extract of all the taken *Citrus* species.

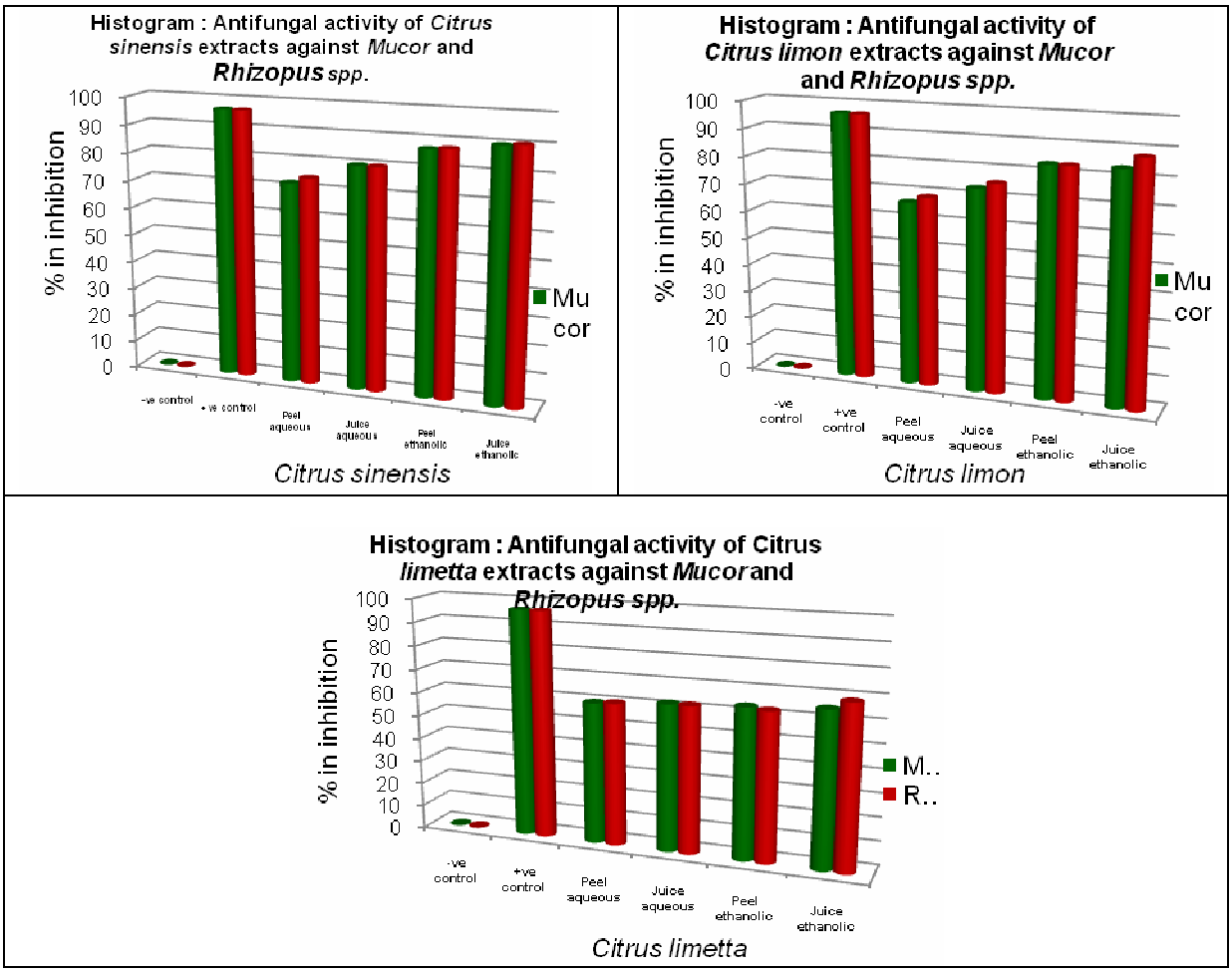
Juice extract was comparatively more effective then peel extract. Among the investigated plants extract *C.sinensis* showed significant mycelial growth inhibition of (90%) followed by *C.limon* (83.56%), *C.limetta* (65.33%) against Mucor species. The mycelia inhibition of *Rhizopus* species followed the same trend with individual variability. Normal growth of spoilage fungi was significantly hindered by plant extract treatment. The mycelia growth was almost completely inhibited by sodium benzoate (positive control) and profuse mycelia growth observed in (negative control).Among the three species *Citrus sinensis* was found most effective. Detailed reading as depicted in Table-1. The antifungal activity of *Citrus sps*. is in agreement with results of effect of ethanolic extract of lime compared with garlic and ginger found that lime is effective against some gram positive bacteria and some fungi.(Khan et al, 2007).

MIC Values

The MIC of the plant extracts is presented in Table – 2 and 3. The results show that MIC of *C.sinensis* ethanolic juice extract against *Mucor* sp and *Rhizopus* sp.were same ie. 6.25 mg/ml showing the strongest value. MIC of aqueous juice extract of *C.sinensis* was 12.5mg/ml for both Mucor and Rhizopus. While MIC for *C. limon* ehanolic juice extract was 6.25 mg/ml for Mucor and 12.5 mg/ml for Rhizopus.

Table.1 Antifungal activity of three species of *Citrus* against spoilage fungi by poisoned food technique

Plant Samples	Plant parts used in different solvents with control		Mycelial growth (mm) Mucor	% inhibition	Mycelial growth (mm) Rhizopus	% inhibition
C..sinensis	Negative Control		90	0	90	0
	Positive Control		3	96.6	3	96.6
	Aqueous	Peel	25	72.22	23	74.44
		Juice	18	80	18	80
	Ethanolic	Peel	11.5	87.22	11	87.77
		Juice	9	90	8.5	90.55
C.limon	Aqueous	Peel	30	66.6	28	68.88
		Juice	24	73.33	22	75.55
	Ethanolic	Peel	15	83.33	15	83.33
		Juice	14	83.56	11	87.77
C.limetta	Aqueous	Peel	36	60.00	35.5	60.55
		Juice	34	62.22	34	62.22
	Ethanolic	Peel	33	63.66	33.5	62.7
		Juice	31	65.33	28	68.88



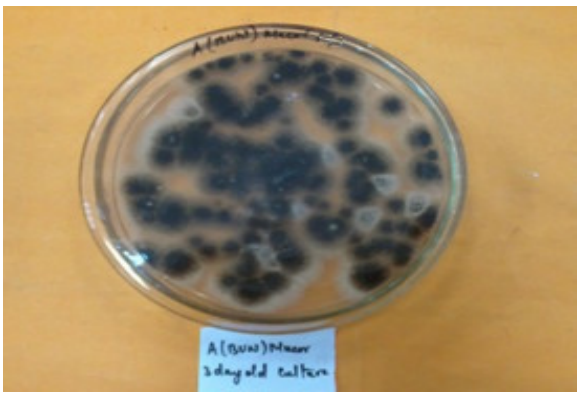


Fig. 1
Mucor sp. on SAB agar plate at 72 hr. at 30 ^{o}C
(Negative Control)

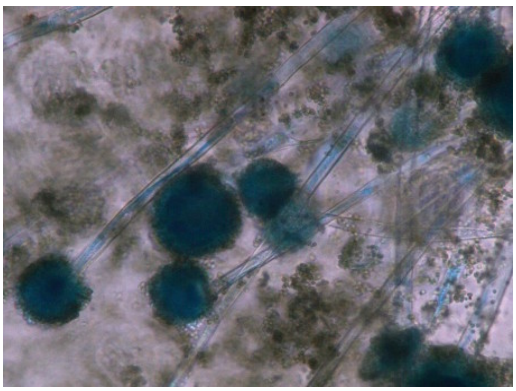


Fig. 2
Microscopic image of *Mucor sp.*
(LPCB 10X x 40X

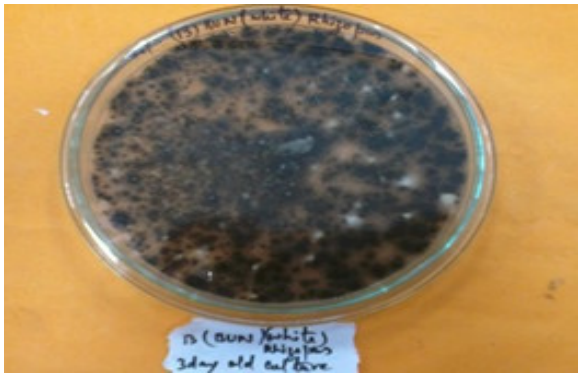


Fig. 3
Rhizopus sp. on SAB agar plate at 72hr. at 30 ^{o}C
(Negative Control)

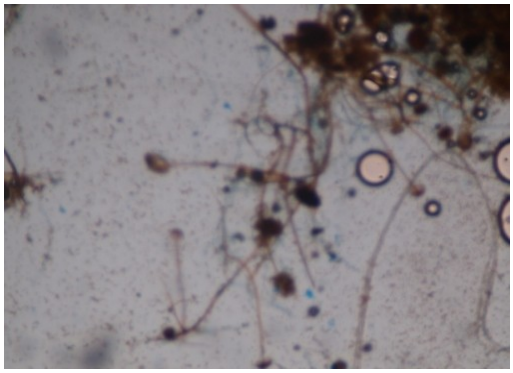


Fig. 4
Microscopic image of *Rhizopus sp.*
(LPCB 10X x 40X

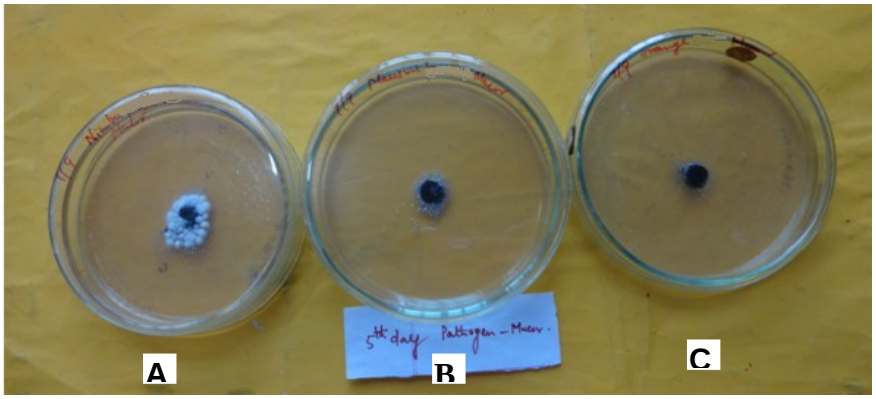


Fig. 5
in vitro antifungal activity of the ethanolic extract of *Citrus spp.* against *Mucor sp.*

A – *C. limetta*
B – *C. limon*
C – *C. sinensis*

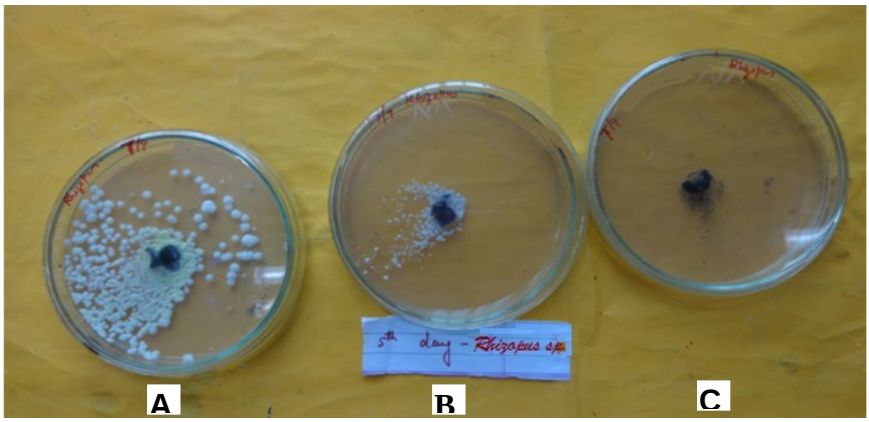


Fig. 6
in vitro antifungal activity of the ethanolic extract of *Citrus spp.* against *Rhizopus sp.*

A – *C. limetta*
B – *C. limon*
C – *C. sinensis*

MIC recorded for aqueous juice extract against Mucor and Rhizopus were 12.5 and 25 mg/ml respectively. MIC of Ethanolic and aqueous juice and peel extract of *C. limetta*, as well as peel extract of *C.sinensis* and *C.limon* were not experimented as it comparatively exhibited less efficacy as an antifungal agent (Table-1). It is worth mentioning that lower MIC is correlated with higher antimicrobial activity. MIC value of 6.25 mg/ml infers the extract having potentiality as potent antifungal agent. It can be easily exploited as preservative in

food to increase its shelf value. The findings are in accordance with the studies by Conte A. et al. 2007.

Increased interest in bio preservation of food systems has recently led to development of new natural anti fungal compounds having different origin. It may be suggested that all the Citrus species used in the experiment (ethanolic extract especially) can be used as a potential source of natural preservative which if incorporated in processed foods. Further research can be done to test the toxicity related to standardization of dose of plant extracts before it can be commercialized in form of nutraceutical foods.

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