



**ANTI-PHYTOPATHOGENIC AND ANTIOXIDANT POTENTIAL OF ALCOHOLIC
AND PETROLEUM ETHER EXTRACT OF FLORAL WASTE**

MULYE S¹, MAURYA A¹, KAMBLE S¹, PATIL N² AND JAIN A^{2*}

1: Research Student, Department of Microbiology, Smt. CHM College, Ulhasnagar-3,
University of Mumbai, Maharashtra, India

2: Assistant Professor, Department of Microbiology, Smt. CHM College, Ulhasnagar-3,
University of Mumbai, Maharashtra, India

***Corresponding Author: Ashish Jain: E Mail: ajedri@gmail.com**

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ABSTRACT

Tons of floral waste is generated every year from different religious places. Floral waste has tremendous and largely unexploited potential of being a potent antimicrobial source against pathogenic organisms. In the present study floral waste collected from religious places and utilized for preparation of hot and cold; alcoholic and petroleum ether extracts. These extracts were evaluated for their potential to inhibit growth of fungal phytopathogens isolated from various infected fruits, vegetables and seeds. Hot ethanolic extracts were found most potent among all and inhibited growth of *Penicillium chrysogenum* at 10mg/ml whereas *Aspergillus niger*, *Aspergillus flavus*, *Curvularia sp.*, *Fusarium sp.* and *Alternaria solani* MTCC 2101 were inhibited at 20mg/ml concentration. Safety study with *Lactobacillus curieae* CCTCC M2011381 revealed that extracts do not have any adverse effects on gut beneficial bacteria at minimum inhibitory concentration against fungal pathogens. All extracts exhibited significant antioxidant activity at 0.1mg/ml concentration thus can be used against disorders resulting due to reactive oxygen species and oxidative stress. These extracts could be a promising and safe alternative of chemical fungicides to develop antifungal formulations and to prepare edible coating on fruits and vegetables to extend their shelf life by protecting them from phytopathogens.

Keywords: Floral waste, Floral extract, Phytopathogen, Antifungal, Antioxidant

INTRODUCTION

In traditional and alternative system of medicines, flowers are known for their medicinal properties as they are the source of active antimicrobial, antioxidant and anti-inflammatory agents [1]. India is the land of spiritualism, culture and traditions. One of the most integral parts of Indian system of worship is offering flowers to god [2]. Flowers being offered to god varies from religion to religion [3, 4]. Pilgrim centers share a significant amount of floral waste on daily basis [5]. From past few decades the aquatic ecosystem has been deteriorated due to dumping of floral waste into water bodies [6]. Degradation of floral waste takes more time as compared to kitchen waste degradation [7]. Therefore, treating floral waste generated at various religious places is vital to create an eco-friendly environment. Flowers like marigold, aster, roses gerbera etc., possess antimicrobial potential. It has been estimated that about 80-85% of population in both developing as well as developed countries rely on traditional medicine for primary health care needs as traditional therapy involves the use of plant extracts [8]. Globally about 20% of crop productivity is reduced due to diseases caused by plant pathogens [9]. *Alternaria* spp., *Botrytis cinerea*, *Penicillium* spp., *Collectrotrichum* spp., *Aspergillus* spp., *Fusarium oxysporum* causes diseases in

various crops [10, 11]. Phytopathogens colonize and feed on exudates secreted by plant which ultimately results in decay of growing crop and in the post-harvest as well. Microbiocides available in the market provide primary means of controlling plant pathogens but continuous use of such chemicals has resulted in two major obstacles: resistance in microorganisms towards these microbiocides and soil pollution due to chemical residues accumulated in soil which affects soil fertility [12]. The present study deals with extraction of floral waste generated from religious places to control phytopathogens to prevent loss of crops.

MATERIALS AND METHODS

Chemicals, Reagents Glasswares and Standard Cultures

ABTS (2, 2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)) was purchased from Sigma Aldrich. All the solvents used were of analytical grade and purchased from Thomas Baker. All the culture media used in study were procured from Hi-media (India). All glasswares were of borosilicate while plastic wares and micropipettes were purchased from Tarsons. *Alternaria solani* MTCC 2101 procured from IMTECH, Chandigarh, India and *Lactobacillus curieae* CCTCC M2011381 was procured from culture repository of Microbiology laboratory of

Smt. C.H.M College, Ulhasnagar.

Collection of floral-waste

Floral wastes were collected from religious places located in Mumbai suburbs. Once collected, each batch of samples was first characterized for different types of floral varieties present in waste followed by its cleaning thoroughly with clean water. All debris, rotten leaves and rotten flowers if present in the batches were removed; further the waste was sundried for 18-24 hrs to reduce the moisture content and ground to fine powder using mechanical grinder and stored in air tight containers at room temperature till further use.

Isolation and identification of fungal phytopathogens

For isolation of fungal phytopathogens samples of infected plant parts were collected randomly from forest areas, farms, vegetable-markets and gardens. Infected plant parts were thoroughly washed with distilled water, surface sterilized with 2% Mercuric chloride, were cut into small pieces and inoculated into enrichment tube having 20ml of potato dextrose broth medium for 72-96 hours at room temperature in static condition [13]. Loopful of enriched broth was spot inoculated on potato dextrose agar plates and incubated at room temperature for 72-96 hours. Fungal isolates thus obtained were purified and maintained on potato dextrose agar slants at 4°C. Isolates were

identified on the basis of microscopic and macroscopic characteristics [14].

Preparation of Extracts

For preparing **cold extracts**, 30g of dried floral waste powder of each batch mixed with 300ml of respective solvents (ethanol, methanol and petroleum ether) in 1:10 ratio (W/V). The mixture was subjected to shaking conditions for 50 hours at room temperature in an orbital shaker at 150 rpm. Further the mixture was filtered through Whatman filter paper No-1 and supernatant was separated from the residue. The extracts were concentrated and evaporated to almost dryness under reduced pressure at 50°C. The concentrated extracts were weighed to calculate the yield and were refrigerated at 4°C till further use (Soham et al. 2020). For **hot extraction**, dried floral powder and respective solvents were taken in a ratio of 1:10 (W/V) and were heated at boiling temperatures according to boiling temperatures of solvents under reflux using Soxhlet apparatus till the solvent in siphon tube appeared colorless. After completion of extraction process maximum solvent was recovered and the residual extracts were concentrated by evaporation at 50°C. The concentrates were weighed to calculate the yield and were refrigerated at 4°C till further use [15]. Yield after extraction was calculated using following formula:

$$\text{Percentage yield} = \frac{m \text{ extract}}{m \text{ dried sample}} \times 100$$

Where, $m_{extract}$ = Mass of extract, $m_{dried\ sample}$ = Mass of the dried sample

Preliminary antifungal screening of extracts by modified slide culture method

Preliminary screening of antifungal potential of extracts was determined by modified slide culture method 16. Briefly, small pieces of filter papers (5cm × 5cm in size) were placed into the petri dishes a bent glass rods (9cm) was placed over filter paper and clean grease free slides were placed over glass rod. The whole set up was sterilized. 5ml of sterile water was added on filter paper which helped in upholding humidity. The extracts in 50mg/ml concentrations were added to the 5ml of potato dextrose agar butts and mixed well. Using sterile pipettes, 1ml of potato dextrose agar butts seeded with required concentration of extracts were added onto the sterile slides placed in the petri dishes and was allowed to solidify. A loopful of inoculum was inoculated onto the agar seeded with extracts placed on sterile slides in the petri dishes. Further, the slide culture apparatus was incubated at room temperature for 48-72 hours. The antifungal activity of extracts was determined by observing inhibition of fungal growth on the slide.

Determination of MIC of extracts against fungal phytopathogens

Microorganisms that were sensitive to the extracts at preliminary stage of screening were taken forward for determination of MIC. The concentration range selected for determining MIC was 10mg/ml to 50mg/ml at the interval of 10mg/ml. Further, the extracts those were active at 10mg/ml were then screened at lower concentrations at 0.5mg/ml, 2.5mg/ml, 5mg/ml and 7.5mg/ml by disk diffusion method using potato dextrose agar medium 17. The lowest concentration of the extract that formed clear visible zone of inhibition after incubation was considered as MIC for the respective fungal phytopathogens.

Effect of extracts on beneficial gut flora by agar well diffusion method

Effect of extracts on beneficial gut flora was examined by agar well diffusion method on MRS HiVeg agar medium using *Lactobacillus curieae* CCTCC M2011381 as standard gut flora 18. The lowest concentration of the extract that produced clear visible zone of inhibition after incubation was considered as minimum inhibitory concentration (MIC) for the test organism.

Determination of antioxidant activity by ABTS radical scavenging assay

The antioxidant potential of extracts was examined using 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) ABTS radical scavenging method 19. The

percentage of ABTS radical scavenging activity was calculated using formula:

$$\text{Percentage inhibition of ABTS radicals} = \frac{A_0 - A}{A_0} \times 100$$

Where, A_0 = absorbance of the control solution (containing all reagents except extracts);

A = is the absorbance of the ABTS solution containing extract.

RESULTS AND DISCUSSION

Collection of floral waste and preparation of extracts

Floral waste collected from religious places (**Table-1**). Total twelve extracts were prepared from collected floral waste. The yield of all extracts is presented in **Table II**. Polar solvents ethanol and methanol showed higher yields than non-polar solvent petroleum ether. The difference in the yield of polar and non-polar solvents is in the accordance with previous published paper 20.

Table I: Sampling details of floral waste collected from different religious places on special occasions

S. No.	Religious place	Date and time of sampling	Geographical location	Type of floral offerings in the waste	Percentage (%) of each type of floral waste
1	St. Bartholomew church	05-11-2017, 11 am	19.2236759° N, 73.138443° E	<i>Rosa chinensis</i> <i>Orchis mascula</i> <i>Gerbera jamesonii</i> <i>Callistephus chinensis</i> <i>Calendula officinalis</i> <i>Tagetes erecta</i>	65.06 % 1.89 % 10.77 % 17.89 % 3.83 % 0.47 %
2	Shiv Temple	14-02-2018, 12 pm	19.2953414° N, 73.2041377° E	<i>Callistephus chinensis</i> <i>Aegle marmelos</i> <i>Tagetes erecta</i> <i>Nelumbo nucifera</i> <i>Rosa chinensis</i> <i>Calotropis gigantea</i> <i>Jasminum sambac</i> <i>Datura stramonium</i>	28.62 % 49.88 % 9.73 % 3.19 % 2.97 % 1.12 % 0.5 % 4.40 %

Table II: Percentage yield of extracts. Key: HEE: Hot Ethanolic Extract, CEE: Cold Ethanolic Extract, HME: Hot Methanolic Extract, CME: Cold Methanolic Extract, HPEE: Hot Petroleum Ether Extract, CPEE: Cold Petroleum Ether Extract, I: Batch, II: Batch

Extracts	Yield of extracts in (g) from 30g of powder	Yield of extracts in (%) from 30g of powder	Extracts	Yield of extracts in (g) from 30g of powder	Yield of extracts in (%) from 30g of powder
HEE I	6.281	20.93	HEE II	5.239	17.46
CEE I	2.352	7.84	CEE II	3.256	10.85
HME I	4.186	13.95	HME II	7.633	25.44
CME I	10.556	35.18	CME II	6.326	21.08
HPEE I	0.6	2	HPEE II	0.521	1.73
CPEE I	0.612	2.04	CPEE II	0.498	1.66

Isolation and identification of fungal phytopathogens

A total of 57 samples comprising of infected leaves, fruits and vegetables were collected from various places like markets, gardens, forests and farms of Thane district. After processing of samples, total 29 fungal isolates were obtained. **Table III** represents sources and diseases caused by isolated fungal phytopathogens under study. **Figure 1** represents percentage wise distribution of identified fungal phytopathogens. Fungal isolates selected for the study are potent phytopathogens causing diseases like black knot, blight, panama disease, wilt, leaf blister etc. *Fusarium* spp. causes diseases like panama disease in banana, crown rot and stem browning in wheat and barley, whereas anthracose, fruit rot, leaf spot, steam-end rot in papaya [21-23].

Aspergillus flavus causes fruit rot, powdery mildew in tomato and mango [24]. *Curvularia* spp. causes spongy rot disease in sweet potato and fruit rot, dry rot and watery soft rot in tomato [25]. *Penicillium* spp. causes diseases in apple, orange, banana, mango grape etc., the most common disease caused by penicillium is brown rot in nectrines and in plums [26, 27]. *Aspergillus niger* is one of the most common pathogen found infecting onion causing bulb rot and black molds on the surface of onion [28, 29]. It also affects oranges, pawpaw watermelon, pineapple, tomatoes etc., [30]. *Alternaria solani* infects potatoes, tomatoes, cereals, cabbage, broccoli etc., causing early blight, leaf blight, steam blight, apical fruit rot and forming necrotic lesions on leaves [31].

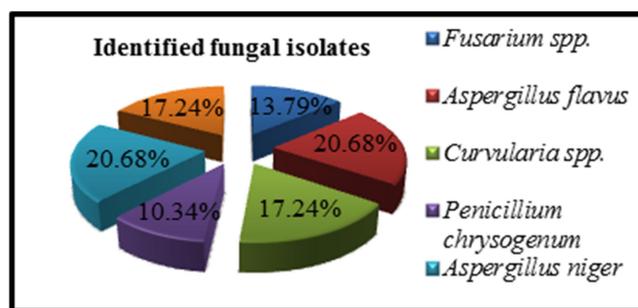


Figure 1: Percentage wise distribution of identified fungal pathogens

Table III: Sources and diseases caused by isolated fungal phytopathogens

S. No.	Host plant	Plant part	Disease	Sampling site	Isolated organism
1	<i>Carica papaya</i>	Leaf	Fusarium fruit rot	Market, Ulhasnagar	<i>Fusarium</i> sp.
2	<i>Mangifera indica</i>	Leaf	Leaf spot	Forest area, Titwala	<i>Aspergillus flavus</i>
3	<i>Solanum lycopersicum</i>	Whole fruit	Watery soft rot	Market, Ulhasnagar	<i>Curvularia</i> sp.
4	<i>Citrus X sinensis</i>	Leaf	Fruit rot	Forest area, Titwala	<i>Penicillium chrysogenum</i>
5	<i>Allium sativum</i>	Bulb	Aspergillois	Market, Ulhasnagar	<i>Aspergillus niger</i>

Preliminary screening of antifungal potential of extracts

Among all the screened extracts HEE I, CEE I, HME I, CME I, HEE II, CEE II and CME II displayed antifungal activity at 50mg/ml against all the fungal phytopathogens under study whereas, HPE I, CPE I, CME II, HPE II and CPE II failed to display antifungal potential against any of the fungal phytopathogens under study at 50mg/ml concentration. The extracts were concluded to be positive if the growth was not observed on the agar seeded with extracts (Table IV and Table V).

Minimum inhibitory concentration (MIC) against fungal phytopathogens

Extracts that possessed antifungal potential in preliminary stage of screening were taken forward for determining minimum inhibitory concentration against selected fungal phytopathogens. The results revealed that all the selected extracts were potentially effective in suppressing the growth of phytopatogens under study with variable potency. All the extracts from Batch I arrested the growth of test organism between the concentration range of

10mg/ml-40mg/ml whereas, all the extracts from Batch II retarded the growth of test organisms between 20mg/ml-50mg/ml concentration (Table VI).

Effect of extracts on beneficial gut flora

The results of safety study revealed that all the extracts show MIC against representative gut beneficial flora *Lactobacillus curieae* CCTCC M2011381 at 30mg/ml or higher concentration. At lower concentration both batch I and II extracts do not exert any significant adverse effects on beneficial gut flora (Table VII).

Safety of extracts toward normal intestinal flora further ensure their suitability to use in spray and edible coating to protect from phytopathogens and increasing shelf life of fruit and vegetables. **Antioxidant activity of extracts**

All the extracts displayed potential antioxidant activity at concentration of 0.1mg/ml (Table VIII). In the present study the order of scavenging activity is as follows: HEE I > CME I > CME II > CEE I > HME I > HME II > HEE II > CEE II > CPEE I > CPEE II > HPEE II > HPEEI

Table IV: Preliminary screening of extracts at concentration of 50mg/ml by ditch plate method. Key : HEE: Hot Ethanolic Extract, CEE: Cold Ethanolic Extract, HME: Hot Methanolic Extract, CME: Cold Methanolic Extract, HPEE: Hot Petroleum Ether Extract, CPEE: Cold Petroleum Ether Extract, I: Batch, II: Batch. Key: (+) = Inhibition of Growth, (-) = Growth observed

S. No.	Organisms	Extracts					
		HEE I	CEE I	HME I	CME I	HPE I	CPE I
1	<i>Aspergillus niger</i>	+	+	+	+	-	-
2	<i>Aspergillus flavus</i>	+	+	+	+	-	-
3	<i>Penicillium chrysogenum</i>	+	+	+	+	-	-
4	<i>Fusarium sp.</i>	+	+	+	+	-	-
5	<i>Curvularia sp.</i>	+	+	+	+	-	-
6	<i>Alternaria solani MTCC 2101</i>	+	+	+	+	-	-

Table V: Preliminary screening of extracts at concentration of 50mg/ml by ditch plate method. Key : HEE: Hot Ethanolic Extract, CEE: Cold Ethanolic Extract, HME: Hot Methanolic Extract, CME: Cold Methanolic Extract, HPEE: Hot Petroleum Ether Extract, CPEE: Cold Petroleum Ether Extract, I: Batch, II: Batch. Key: (+) = Inhibition of Growth, (-) = Growth observed

S. No.	Organisms	Extracts					
		HEE II	CEE II	HME II	CME II	HPE II	CPE II
1	<i>Aspergillus niger</i>	+	+	-	+	-	-
2	<i>Aspergillus flavus</i>	+	+	-	+	-	-
3	<i>Penicillium chrysogenum</i>	+	+	-	+	-	-
4	<i>Fusarium sp.</i>	+	+	-	+	-	-
5	<i>Curvularia sp.</i>	+	+	-	+	-	-
6	<i>Alternaria solani MTCC 2101</i>	+	+	-	+	-	-

Table VI: Minimum Inhibitory concentration of extracts (in mg/ml) against fungal phytoapthogens by agar well diffusion method. Key: HEE: Hot Ethanolic Extract, CEE: Cold Ethanolic Extract, HME: Hot Methanolic Extract, CME: Cold Methanolic Extract, HPEE: Hot Petroleum Ether Extract, CPEE: Cold Petroleum Ether Extract, I: Batch

S. No.	Organisms	Extracts						
		HEE I	CEE I	HME I	CME I	HEE II	CEE II	CME II
1	<i>Aspergillus niger</i>	20	40	30	20	40	30	30
2	<i>Aspergillus flavus</i>	20	30	40	20	20	30	40
3	<i>Penicillium chrysogenum</i>	10	30	20	20	40	30	30
4	<i>Fusarium sp.</i>	20	30	40	40	40	30	40
5	<i>Curvularia sp.</i>	20	20	30	30	30	20	40
6	<i>Alternaria solani MTCC 2101</i>	20	20	30	20	30	20	30

Table VII: Minimum Inhibitory concentration of extracts against *Lactobacillus curieae* CCTCC M2011381 in (mg/ml) by Agar well diffusion method. Key: HEE: Hot Ethanolic Extract, CEE: Cold Ethanolic Extract, HME: Hot Methanolic Extract, CME: Cold Methanolic Extract, HPEE: Hot Petroleum Ether Extract, CPEE: Cold Petroleum Ether Extract, I & II: Batch

S. No.	Extracts	Organism	Extracts	Organisms
		<i>Lactobacillus curieae</i> CCTCC M2011381		<i>Lactobacillus curieae</i> CCTCC M2011381
1	HEE I	30	HEE II	30
2	CEE I	30	CEE II	30
3	HME I	30	HME II	30
4	CME I	30	CME II	30
5	HPEE I	40	HPEE II	40
6	CPEE I	40	CPEE II	40

Table VIII: Percentage antioxidant activity of extracts by ABTS scavenging assay Key: HEE: Hot Ethanolic Extract, CEE: Cold Ethanolic Extract, HME: Hot Methanolic Extract, CME: Cold Methanolic Extract, HPEE: Hot Petroleum Ether Extract, CPEE: Cold Petroleum Ether Extract, I: Batch, II: Batch

Extracts	Antioxidant activity in (%)	Extracts	Antioxidant activity in (%)
HEE I	99.88 ± 0.04	HEE II	98.77 ± 0.15
CEE I	99.65 ± 0.07	CEE II	98.49 ± 0.02
HME I	99.11 ± 0.019	HME II	99.10 ± 0.02
CME I	99.77 ± 0.05	CME II	99.69 ± 0.02
HPEE I	30.34 ± 0.03	HPEE II	31.54 ± 0.06
CPEE I	74.14 ± 2.52	CPEE II	42.24 ± 0.09

CONCLUSION

Present study was aimed to assess the effectiveness of the extracts prepared from collected floral waste from various religious places against potent fungal phytopathogens and to evaluate its antioxidant potential. Alcoholic extracts prepared exhibited antifungal potential against all the fungal phytopathogens under study. All the extracts displayed excellent antioxidant potential indicating it can prevent damage to cells caused by free radicals and help in boosting up overall health. Safety study performed revealed the extracts won't have any adverse effects on gut beneficial bacteria since inhibiting at higher concentrations. These results explore the possibilities of finding our active ingredients in the extracts responsible for its inhibitory action. These extracts can be used in preparing microbiocides to prevent the diseases caused by phytopathogens and in increasing the shelf life of crops. This is the first ever study reported on the extracts prepared from floral waste and exploring its potential against phytopathogens.

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