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In vitro antimicrobial activity in certain plant products / seed extracts against selected phytopathogens

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Extracts of five different plant seeds- *Syzygium cumini*, *Pheonix sylvestris*, *Manilkara zapota*, *Tamarindus indica*, and *Annona squamosa*- prepared by Microwave Assisted Extraction (MAE) method were screened for their antimicrobial activity against certain phytopathogenic microorganisms. Ethanolic extract of *S. cumini* was found to possess highest average total activity against susceptible microbes. Total activity was found to have a positive correlation with the extraction efficiency. Methanolic extract of *T. indica* exerted bactericidal action against *Agrobacterium tumefaciens* and *Pseudomonas syringae*. Ethanolic extract of *P. sylvestris* was able to protect cabbage leaf against *Xanthomonas campestris*. Acetone extract of *M. zapota* was able to reduce aflatoxin production in *Aspergillus parasiticus* by >50 %. Curcumin proved bactericidal against *X. campestris*.

Keywords: Seeds, Antibacterial, Post Extract Effect (PEE), Phytopathogens, Aflatoxin

Introduction:

A considerable part of the yearly production of agricultural commodities gets destroyed by various pests including bacteria, fungi, viruses, insects, rodents and nematodes. Mycotoxin production of by fungi has added new facets to the gravity of the problem. At times, losses are severe enough to lead to famine in large areas of the densely populated and agriculture-dependent world. Considerable pressure on farmers and consumers to reduce the deployment of synthetic pesticides in agriculture, has triggered renewed interest in botanical pesticides as the alternative and eco-chemical option in pest management. Initially, medically useful antibiotics (e.g streptomycin) were also used for control of plant pathogens (Crueger and Crueger, 1989), but their use is now discouraged due to the problem of drug resistance. The use of plants (e.g *Derris*, *Nicotiana*, and *Ryania*) in control of the pests is an age-old technology (Dubey, 2011).

An increasing number of researchers have demonstrated a large number of plants/plant products to possess antimicrobial property (Dubey et al., 2011). Plant seed extracts have been reported by several researchers for their antimicrobial activity (Nascimento et al., 2000; Kothari

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et al., 2011). Present study was directed at investigation on antiphytopathogenic activity of *Syzygium cumini* L (Myrtaceae), *Pheonix sylvestris* Roxb (Palmae), *Manilkara zapota* L (Sapotaceae), *Tamarindus indica* L (Leguminosae) and *Annona squamosa* L (Annonaceae) seed extracts. The plant pathogens were also challenged with few pure phytocompounds viz. curcumin, quercetin, lycopene, and gallic acid.

Materials and Methods:

Seeds of all the five plants were procured from local market of Ahmedabad city. They were authenticated for their unambiguous identity by Prof. Y.T. Jasrai, Head of Botany Dept., Gujarat University, Ahmedabad.

Extraction: Seeds were extracted in three different solvents (Merck, Mumbai, India)- acetone, methanol, and ethanol (50%), with minor modification in microwave assisted extraction (MAE) method (Kothari et al., 2009) reported earlier by us. One gram of dry seed powder was soaked into 50 mL of solvent, and subjected to microwave heating (Electrolux EM30EC90SS) at 720 W. Total heating time was kept 90, 120 and 70 second for methanol, acetone, and ethanol respectively, with intermittent cooling (reheating duration with methanol was kept 5 sec instead of 10 sec described in our earlier publication). This was followed by centrifugation (10,000 rpm for 15 min), and filtration with Whatman paper # 1 (Whatman International Ltd., Maidstone, England). Solvent was evaporated from the filtered extract and then the dried extracts were reconstituted in dimethyl sulfoxide (DMSO) for broth dilution assay. Reconstituted extracts were stored under refrigeration for further use. Extraction efficiency was calculated as percentage weight of the starting dried plant material.

Microbial strains: Microbial cultures obtained from Microbial Type Culture Collection (MTCC), Chandigarh, used as test organisms are listed in table 1.

Table 1. Test organisms

Organism	MTCC no.	[1]Remarks (with inputs from MTCC catalogue)
Pseudomonas syringae	673	Resistant to Cefaclor and Cefadroxil
Agrobacterium tumefaciens	431	Extremely good crown gall producer
Xanthomonas campestris	2286	Was originally collected from cabbage leaves bearing blackrot, Resistant to Cefaclor
Pectobacterium caratovorum	1428	Collected from potato, Resistant to streptomycin, ampicillin, penicillin, cefuroxine, co-trimoxazole, cefaclor, cefadroxil, and amikacin
Pseudomonas marginalis	2758	-
Aspergillus parasiticus	411	Aflatoxin producing strain, test organism for investigating inhibition of mycotoxin production, Resistant to flucanazole and amphotericin B

[1]Antibiotic susceptibility profile was generated in our lab by disc diffusion assay using antibiotic discs from HiMedia.

***MIC determination*:** MIC (minimum inhibitory concentration) determination was carried out using microbroth dilution method as per NCCLS guidelines (Jorgensen and Turnidge, 2003). Assay was performed in a 96-well microtitre plate. Total volume of the assay system in each well was kept 200 µL. Muller-Hinton broth (HiMedia, Mumbai) was used as growth medium for bacteria, and RPMI-1640 for *A. parasiticus*. Methanolic extract of *T. indica* and ethanolic extract of *P. sylvestris* were tested in minimal media to avoid precipitation. Composition of minimal media (per liter) for bacteria (Atlas, 2010): sucrose 15 g, K_2HPO_4 5 g, NH_4Cl 2 g, NaCl 1 g, $MgSO_4$ 0.1 g, Yeast extract 0.1 g. Composition of minimal media for fungi (per liter; http://www.fgsc.net/methods/anidmed.html): $NaNo_3$ 6 g, KCl 0.52 g, $MgSO_4.7\ H_2O$ 0.52 g, KH_2PO_4 1.52 g, glucose 10 g, 2 ml Hutner's reagent, pH-6.5.

Inoculum density of the bacterial suspension was adjusted to that of 0.5 McFarland standard. Turbidity of the fungal inoculum was adjusted between 0.09-0.11 at 530 nm (Espinel-Ingroff and Pfaller, 2003). Broth was dispensed into wells of microtitre plate followed by addition of test extract and inoculum. Extracts (reconstituted in DMSO) were serially diluted into each of the wells. A DMSO control was included in all assays (Wadhwani et al., 2009). Streptomycin (HiMedia) served as positive control for bacteria, and amphotericin B for *A. parasiticus*. Plates with bacteria were incubated at 30°C for 16-20 h, before being read at 655 nm in a plate reader (BIORAD 680). *A. parasiticus* plates were incubated at 30°C for 46-50 h. MIC was recorded as the lowest concentration at which no growth was observed. All MICs were determined on three independent occasions. Concentration at which growth was inhibited by 50% was recorded as IC_{50} value.

After reading the plates for MIC, subculturing was made on nutrient agar plate (incubation was continued for 72 h) from the wells showing no growth, so as to determine whether the extract is bactericidal or bacteriostatic. Growth on the plate indicated bacteriostatic action; absence of growth was interpreted as bactericidal action. Delayed growth as compared to control was interpreted as Post Extract Effect (PEE).

***Total activity*:** Total activity (mL/g) was calculated as (Eloff, 2004): Amount extracted from 1 g (mg) / MIC (mg/mL).
Activity index: Activity index was calculated as MIC of the extract / MIC of positive control.

Determination of time required to kill: In order to determine the time required for a bactericidal extract to kill the susceptible test organism, the test organism was challenged with the extract at its MBC (minimum bactericidal concentration) in a test tube, from which an aliquote of 20 µL was transferred to a nutrient agar plate (devoid of extract) at intervals of 2, 4, 6, 8, 10, and 24 h and was spread evenly. The plates were kept for incubation at 30°C for 72 h. Time corresponding to the plate with no growth was taken as the time required by the extract to kill the bacteria (Pfaller et al., 2004).

Experiment with host plant: Ability of ethanolic extract of *P. sylvestris* to inhibit growth of *X. campestris* on cabbage leaf was also investigated. Pieces of fresh cabbage leaf were treated with 1 % $HgCl_2$ for 60 s, followed by 60 s treatment with 95% ethyl alcohol (Satyanarayana and Vargese, 2007). Then wash with sterile distilled water was given to remove any traces of previously used chemicals. Twenty µl of ethanolic extract of *P. sylvestris* (300 µg/ml) was evenly applied on surface of the disinfected cabbage leaf, followed by application of 20 µl of *X.*

campestris suspension. Extract was replaced with DMSO to prepare negative control. Cabbage leaf inoculated with test organism (with no extract applied) served as growth control. One disinfected leaf piece (with neither extract nor organism applied on it) was also kept for comparison. Incubation was carried out at 30°C for 5 days, with visual observation at regular intervals.

Aflatoxin estimation: *A. parasiticus* was grown in Czepk dox medium (HiMedia), with and without test extract, contained in 25 ml flasks; incubation was continued for 5 days at 30°C under static condition with intermittent shaking. This was followed by separation of mycelium from the broth; mycelium was subjected to dry weight determination after drying at 55°C till constant weight was achieved. Aflatoxin was extracted in a mixture of toluene:acetonitrile (9:1) (Nesheim and Stack, 2001), which was mixed with the broth remaining after removal of mycelia, and kept on shaker for 12 h at room temperature. This was followed by separation of solvent layer and measurement of aflatoxin at 350 nm. Amount of aflatoxin was calculated using following formula:

$$\text{Aflatoxin } (\mu g/mL) = A \times mw \times 1000/\varepsilon.$$

Where, A: absorbance at 350 nm; mw: molecular weight of aflatoxin; ε: molar absorptivity

Thin-layer chromatography (TLC): TLC separation of methanolic extract of *T. indica* was carried out on silica gel 60 F_{254} plates (20X20 cm; 0.25 mm thickness; Merck). Solvent system applied was- chloroform: acetone (90:10). Separated components were identified by observing the plate under UV light (254 and 365 nm; Uvitec-LF-206.LS, UK).

Results and Discussion:

Extraction: Extraction efficiency of all the extracts is recorded in table 2. Highest and lowest extraction efficiency was obtained for ethanolic extract of *S. cumini* and *A. squamosa* respectively.

Table 2. Extraction efficiency of all the extracts

	Solvent	Seed	Extraction efficiency (%)
1	Ethanol (50%)	*A. squamosa*	6.04
2	Acetone		12.11
3	Acetone	*M. zapota*	7.47
4	Methanol		7.02
5	Ethanol (50%)	*P. sylvestris*	6.65
6	Methanol	*T. indica*	14.43
7	Ethanol (50%)	*S. cumini*	24.33
8	Methanol		17.34

Antibacterial assay: Results of broth dilution assay of the all seed extracts against different organisms are shown in table 3. Methanolic extract of *T. indica* was able to inhibit all the five test organisms. It proved bactericidal against *A. tumefaciens* and *P. syringae* with MBC of 625 and 400 µg/ml respectively. It required 8 h of incubation of the organism in the medium containing extract at these concentrations for killing to occur, as determined by plating at regular intervals from the experimental well. MBC/MIC ratio of this extract against *A. tumefaciens* and *P. syringae* was 1 and 1.14 respectively. A small MBC/ MIC ratio (< 4 to 6) is usually expected for

bactericidal agents (Choi et al., 2010). This ratio between 1 to 2 has been suggested as indicator of bactericidal mode of action (Brown et al., 2008). Earlier we have reported the same extract for its cidal action against human pathogenic bacteria (Kothari and Seshadri, 2010). This extract tested positive for alkaloids when mixed with Dragondroff's reagent.

Table 3. Results of broth dilution assay of all the extracts

	Extract	Organism	IC_{50} (µg/ml)	MIC (µg/ml)	Total activity (ml/g)	Average total activity (ml/g)
1.	*S. cumini* (MeOH)	*P. syringae*	300	535	330.28	550.52
		X. campestris	150	225	770.76	
		A. tumefaciens	-	>1000	NA	
		P. marginalis	1000	>1000		
		P. caratovorum	-	>1000		
2	*S. cumini* (EtOH)	*P. syringae*	100	400	608.25	946.16
		X. campestris	100	150	1622	
		A. tumefaciens	100	400	608.25	
		P. marginalis	1000	>1633	NA	
		P. caratovorum	>1000	>1633	194.68	
3	*T. indica* (MeOH)	*P. syringae*	150	350	249.12	289.74
		X. campestris	-	425	366.65	
		A. tumefaciens	-	625	380	
		P. marginalis	200	600	259.58	
		P. caratovorum	700	800	194.68	
4	*P. sylvestris* (EtOH)	*P. syringae*	>500	>922	259.58	210.21
		X. campestris	-	150	466	
		A. tumefaciens	200	850	74.35	
		P. caratovorum	500	700	NA	
		P. marginalis	>600	>858	194.68	
5	*M. zapota* (Acetone)	*P. syringae*	-	>868	NA	NA
		X. campestris	-	>723		
		A. tumefaciens	-	>868		
		P. marginalis	-	>579		
		P. caratovorum	-	>579		
6	*M. zapota* (MeOH)	*P. syringae*	-	>1050		
		X. campestris	>500	>1050		
		A. tumefaciens	>500	>1050		
		P. marginalis	-	>1050		
		P. caratovorum	-	>1050		
7	*A. squamosa* (EtOH)	*P. syringae*	>400	>771		
		X. campestris	-	>771		
		A. tumefaciens	>257	>771		
		P. marginalis	>200	>771		
		P. caratovorum	-	>771		
8	*A. squamosa* (Acetone)	*P. syringae*	-	>475		
		X. campestris	>285	>475		
		A. tumefaciens	>285	>475		
		P. marginalis	-	>475		

Ethanolic extract of *S. cumini* was found to possess highest average total activity (946.16 ml/g). Total activity of this extract against *X. campestris* was 1622 ml/g, which means if one gram of this extract is diluted to 1622 ml, still it would be able to inhibit the test organism. Total activity is a measure of the amount of material extracted from a plant in relation to the MIC of the extract, fraction or isolated compound. It indicates the degree to which the active fractions or compounds present in 1 g can be diluted and still inhibit growth of the test organism (Eloff, 2004).

In this study total activity was found to have a strong positive linear correlation (r = 0.93) with extraction efficiency. In our earlier work with the same extracts against human pathogenic bacteria also, these two quantities were found to have a positive linear correlation (Kothari, 2011). This suggests the importance of applying an efficient extraction method while screening crude extracts for their bioactivity. MAE has been reported to be suitable for extraction of antimicrobial and/or antioxidant compounds from plant materials (Kothari et al., 2009; Kothari and Seshadri, 2010; Upadhyay et al., 2011; Pasquet et al., 2011) while simultaneously maintaining denaturation of heat-labile phytocompounds at a minimum.

Both the extracts of *S. cumini*, and ethanolic extract of *P. sylvestris* were found to be bacteriostatic against susceptible organisms. But interestingly ethanolic extract of both the seeds significantly reduced the ability of *X. campestris* to revive growth on a extract free nutrient medium, which can be described as Post Extract Effect (PEE), whose duration was determined to be 36 h, i.e. visible growth on nutrient agar (after plating from wells containing inhibitory concentration of the test extract) was obtained only after 36 h of incubation (it took 24 h when contents were plated from control well). Similar effect for antibiotics termed as Post Antibacterial Effect (PAE)/ Post Antifungal Effect (PAFE) has been described in literature (Pfaller et al., 2004). Agents exhibiting a PAE/PAFE require extended incubation following subculture in either time kill or MLC (minimum lethal concentration) determinations in order to ensure the detection of slow growing but not dead organisms.

Ability of ethanolic extract of *P. sylvestris* to inhibit growth of *X. campestris* on cabbage leaf was also investigated. *X. campestris* was not able to cause any damage to the cabbage leaf in presence of this extract. The piece of leaf which was inoculated with *X. campestris* but not treated with extract was damaged due to growth of test organism on it (fig 1). *X. campestris* is known to cause black rot of cabbage and cauliflower (Bhardwaj and Laura, 2009), and is capable of rapid spread. Cabbage cultivation is a multi-billion dollar industry worldwide, reflecting its value as a vegetable crop, source of vegetable oil, component of fodder crop for livestock feed, and ingredient in condiments and spices (http://usda.mannlib.cornell.edu/MannUsda/viewDocumentInfo.do?documentID=1397). Black rot is considered the most important disease of cabbage and other crucifers (http://ipm.illinois.edu/vegetables/diseases/black_rot/index.html).

In addition to above seed extracts, certain pure phytocompouds (gallic acid, quercetin, curcumin, and lycopene) were also evaluated against the same test organisms. Curcumin was able to inhibit three of the test organisms with the MIC falling in the range 30-70 µg/ml (table 4). It proved bactericidal against *X. campestris* at 30 µg/ml (requiring 6 h to exert its killing

effect), whereas bacteriostatic against *P. marginalis* and *P. caratovorum.* Antibacterial ativity of curcumin against *Aeromonas hydrophila* was earlier reported by us (Kothari et al., 2011). Bacterial cytokinesis in *Bacillus subtilis* was indicated to be inhibited by curcumin (Rias et al., 2008). Curcumin was able to reduce growth of *A. parasiticus* by ~50% at 100 µg/ml. It has earlier been reported as a promising antifungal agent (Martins et al., 2009), and is known for its ability to bind a variety of proteins and inhibit the activity of various kinases (Goel et al., 2008). Quercetin was effective against all the five bacteria but not against *A. parasiticus.* Quercetin in lotus leaves (Mingyu and Zhuting, 2008), and *S. cumini* extracts (Kothari et al., 2011) was reported as a potential antibacterial agent. Gallic acid at 90 µg/ml was able to reduce growth of *A. tumefaciens* by 70%. Lycopene failed to notably inhibit any of the test bacteria up to 60 µg/ml.

Table 4. Broth dilution assay of pure compounds

Compound	Organism	MIC (µg/mL)	Activity index[2]	MBC (µg/mL)
Curcumin[1]	*X. campestris*	30	3	30
	P. marginalis	70	7	static effect
	P. caratovorum	50	-	
Quercetin	*X. campestris*	40	4	
	P. marginalis	100	10	
	P. caratovorum	100	-	
	A. tumefaciens	100	10	
	P. syringae	150	15	

[1] An IC_{50} of 200 µg/ml was registered for curcumin against *A. parasiticus*.
[2]Streptomycin was taken as positive control for all organisms except *X. campestris*. For latter ampicillin was used.

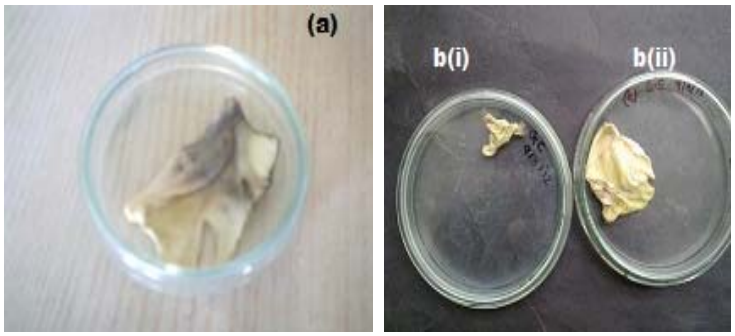

Fig 1. Protection conferred on cabbage leaf against *X. campestris* by application of *P. sylvestris* ethanolic extract
(a) Untreated cabbage leaf inoculated with *X. campestris* after 3 days. **b (i)** Untreated cabbage leaf inoculated with *X. campestris* after 5 days. **b (ii)** Cabbage leaf treated with *P. sylvestris* extract and inoculated with *X. campestris* (after 5 day incubation)

Effect of plant products on aflatoxin production: Among all the extracts tested, only acetone extract of *M. zapota* was able to significantly reduce aflatoxin production/secretion by *A. parasiticus* (table 5). It inhibited total aflatoxin production and aflatoxin B1 in particular to almost identical extent. Interestingly methanolic extract of *T. indica* heavily enhanced aflatoxin production with simultaneous reduction in mycelial growth.

Table 5. Effect of extracts on mycelial growth and aflatoxin production by *A. parasiticus*

Plant extract	Control mycelial weight (g)	Experimental mycelial weight (g)	% Difference	Aflatoxin B1 in control (µg/mL)	Aflatoxin B1 in experimental (µg/mL)	% Difference	Total Aflatoxin in control (µg/mL)	Total Aflatoxin in experimental (µg/mL)	% Diff.
A. squmosa (MeOH)	0.09±0.00	0.10±0.08	+16.48	2.00±0.77	2.89±0.00	+ 44.5	8.55±3.31	12.36±0.00	+44.56
A. squamosa (EtOH)	0.09±0.00	0.09±0.00	0.00	9.39±0.11	12.47±4.06	+32.80	40.15±0.49	46.82±7.81	+16.61
S. cumini (MeOH)	0.09±0.00	0.08±0.00	-14.73	3.04±0.07	2.84±0.02	-6.57*	13.44±0.25	12.14±0.10	-9.67
M. zapota (Acetone)	0.06±0.00	0.05±0.00	-19.11*	7.31±1.01	3.14±1.16	-57.04*	31.29±5.01	13.42±4.94	-57.11*
T. indica (MeOH)	0.09±0.00	0.07±0.00	-13.18*	1.87±0.19	5.75±0.04	+207.48*	7.43±1.63	24.61±0.19	+231.22*
P. sylvestris (EtOH)	0.05±0.00	0.06±000	+15.25	1.13±0.18	4.36±0.08	+285.84*	4.82±0.73	18.64±0.39	+286.72*
M. zapota (MeOH)	0.04±0.02	0.03±.021	-5.00	2.61±0.60	1.46±0.02	+44.06	11.16±2.58	6.41±0.07	+42.56
S. cumini (EtOH)	0.03±0.00	0.03±0.01	+5.88	0.51±0.11	0.80±0.16	+56.86	2.95±0.64	3.41±0.72	16.78

'+ 'or '–' sign indicates increase or decrease over control; * p <0.05; Aflatoxin and mycelial weight are expressed as mean±SD of three independent replicates.

TLC separation: TLC separation of methanolic extract of *T. indica* seeds yielded three fractions with R_f value 0.15, 0.96, and 1 (table 6). TLC profile can be considered an important component in overall characterization of bioactive extrctas.

Table 6. TLC separation of *T. indica* methanolic extract

Fraction no.	Appearance of separated spot		R_f	hR_f
	365 nm	254 nm		
1	Translucent	Not visible	0.15	15
2	Translucent	Not visible	0.96	96
3[a]	Red	Brown	1.0	100

[a] In daylight only fraction 3 of methanol extract was visible as a light green coloured component.

Our study has identified certain plant products possessing promising antibacterial activity against gram-negative bacteria *viz. X. campestris, A. tumefaciens, P. caratovorum, P. marginalis,* and *P. syringae.* Gram-negative bacteria are generally much harder to find "hits" against, presumably as a result of their outer membrane in cell wall which greatly decreases permeability and because they are intrinsically resistant through the expression of membrane bound efflux pumps (Gibbons, 2008). *X. campestris* is believed to be responsible for more than 50 % of crop loss in crucifers in India (Singh et al., 2011). *P. syringae* is believed to be responsible for crop losses of kiwi fruits in Japan, Korea, and Italy amounting to 2 million euros (www.eppo.int/quarantine/ P. syringae pv actnidiae. html). Chemical pesticides alone cannot be relied upon for effective control of plant pathogens. A comprehensive integrated pest management program with natural antimicrobial plant products as one of its integral components is likely to be more fruitful. Natural plant products that are biodegradable and eco-friendly are receiving the attention worldwide as competent alternatives to synthetic pesticides.

Natural anti-phyopathogenic plant products may be of special significance to the developing/underdeveloped world, such as Africa and India, where synthetic pesticides are too expensive for subsistence farming (Pretarius and Watt, 2011).

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