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Efficacies of botanicals in the management of stem end rot disease of mango fruits

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ABSTRACT

The phytopathogenic fungus *Botryodiplodia theobromae* Pat., causes stem end rot disease of mango fruits. Extracts from twenty angiospermic taxa were evaluated for their antifungal activity. The extracts were prepared in water, acetone, ethyl alcohol, petroleum ether, ethyl acetate and methanol. Among the selected plants, essential oil (EO) of *Adenocalymma alliaceum* was found to be effective in controlling the growth of *Botryodiplodia theobromae.* The minimum inhibitory concentration (MIC) of the EO was 100ppm. The oil was found to withstand a high inoculum density. Plant extracts of *Allamanda cathartica*, *Lawsonia inermis*, *Prunus persica* and *Adenocalymma alliaceum* in different solvents showed inhibitory effect on *B. theobromae.* However, Leaf extract of *Adenocalymma alliaceum* in all the solvents namely, water, acetone, ethyl alcohol, petroleum ether, ethyl acetate and methanol was found to have antifungal activity. An enhancement in the shelf life of mango fruits was recorded under *in vivo* trial after treatment with aqueous extract and essential oil of *A. alliaceum.* It is concluded that the aqueous extract and EO of *A. alliaceum* has a great potential in the management of *B. theobromae* damage to mango fruits.

Key words: *Botryodiplodia theobromae* Postharvest diseases, essential oil, stem end rot disease, plant extract, fungicides

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INTRODUCTION

Mango fruits are damaged mechanically and physically during transportation and other post-harvest management methods (Patel *et al.*, 2019). Organic degradation during storage caused by microbial activity is equally responsible for fruit loss. Fungal attack has been identified as a major cause of fruit loss after harvest. About 17.7% mango fruits get rotten due to fungal diseases during transport, storage and sale (Saeed *et al.*, 2017). An important infection that causes mango stem end rot is due to *Botryodiplodia theobromae* Pat. (Maria *et al.*, 2019) and it has been reported as a problem in India (Patel *et al.*, 2019; Le *et al.*, 2022).

Biologically active natural products have been shown to possess the potential to replace synthetic fungicides. Exploitation of these natural products, such as flavor compounds (Wilson, *et al,*, 1987; Utama *et al.*, 2002; Stadelbecher and Prasad, 1974), acetic acid (Molys *et al.*, 1996; Chu *et al.*, 2001), jasmonates (Droby *et al.*, 1999), glucosinolates (Mari *et al.*, 2003), essential oils (Bishop and Reagon, 1998; Bellerbeck *et al.*, 2001; Hidalgo *et al.*, 2002) and plant extracts (Rana *et al.*, 1999; Tripathi and Dubey, 2004) are well documented. Although chemical fungicides provide easy ways of controlling fruit rot after harvest, the continued use of these fungicides has been criticized due to public concerns about their contamination of fruits and vegetables. Chemical fungicides can also cause increased resistance of the pathogen populations. Recently, the exploitation of natural products to control decay and prolong fruit storage life is gaining more attention (Tripathi and Dubey 2004). Research activities to evaluate the efficacy of *A. alliaceum* EO and plant extract in the management of fungal diseases of fruits are

scanty in the literature. This study was carried out therefore, to investigate the antifungal activity of *A. alliaceum* against *Botryodiplodia theobromae* Pat., the causative agent of stem end rot disease of mango fruits.

MATERIALS AND METHODS

Aqueous extracts preparation

Leaves of 20 angiospermic taxa viz. *Adenocalymma alliaceum, Adhatoda vasica, Allamanda cathartica, Annona squamosa, Callistemon lanceolatus, Catharanthus roseus, Celosia cristata, Clerodendrum indicum, Jatropha gossipifolia, Justicia betonica, Lawsonia inermis, Leucas aspera, Ocimum canum, Plumeria rubra, Polygonum glabrum, Prunus persica, Rauvolfia serpentina, Solanum nigrum, Tecoma stans* and *Vitex negundo* were collected from the Banaras Hindu University Campus, Varanasi and were screened against *B. theobromae* as well as other fruit rotting fungi. The Varanasi city is geographically located at $25^{0}00'$ to $25^{0}16'$ N Latitude and $82^{0}50'$ to $83^{0}10'$ E Longitude and altitude 81m (266 feet) above sea level. For the preparation of aqueous extract, leaves were crushed with equal amounts (1:1 W/V) of distilled sterile water in grinder. The filtered extract was then used for the experiments. The extracts were then assayed for the activity against test fungus by modified paper disc technique (Conner and Beachat, 1984). Ten gram leaves of each plant species were extracted separately in five different organic solvents viz. acetone, ethyl alcohol (absolute alcohol), ethyl acetate, methanol, and petroleum ether by macerating them to pulp in a pestle and mortar. The filtrates were assayed separately against the test pathogen by the modified paper disc technique (Tripathi and Shukla, 2018).

Essential oil isolation

Since the leaves of *A. alliaceum* were having strong aromatic odor, the volatile fungicidal fraction of the leaves was isolated by hydro distillation through Clevenger's apparatus (Tripathi and Shukla, 2018). The isolated fraction showed two distinct layers-an upper oily layer and a lower aqueous layer. Both layers were separated and the moisture from the oily layer was removed by adding anhydrous sodium sulphate.

Culture of fungi

Cultures of the fungi *Botryodiplodia theobromae* Pat, *Botrytis cinerea* Pers *ex* Fr., *Ceratocystis paradoxa* (Dade) C. Moreau, *Colletotrichum gloeosporioids* Penz., *Monilinia fructicola* (Wint.) Honey, *Penicillium digitatum* (Pers) Sacc., *P. expansum* Link *ex* S.F Gray, *P. italicum* Wehmer and *Phomopsis citri* Fawe., were obtained from the Indian Agricultural Research Institute, New Delhi. The Cultures of *Aspergillus niger* Van Tiegh and *Rhizopus stolonifer* (Ehren. *ex* FR.) Lind was isolated from the infected mango fruits in the laboratory. All the fungal cultures were maintained on PDA medium. The Czepeks agar medium was used throughout the study period.

Antifungal and Minimum inhibitory concentration (MIC)

Fungitoxicity of the extracted oil was tested by the poisoned food technique (Pandey *et. al.*, 1982; Perrucci *et al.,* 1994). The concentration of the essential oil was prepared by dissolving the requisite amounts in 0. 5mL of 0.1% Tween-80 and then mixing with 9.5 mL of Czepeks agar medium to produce 500ppm, 400ppm, 300ppm, 200ppm, 100ppm concentrations. The control sets were prepared similarly using equal amounts of sterilized distilled water in place of the oil.

Nature of toxicity of the extracts and essential oil

The nature of toxicity (fungitoxic/fungicidal) of the extracts (leaves extracted in water, acetone, ethyl acetate, ethyl alcohol, methanol and petroleum ether) and essential oil was tested against the fungus following the method of Thompson (1989).The inhibited fungal discs of the extracts and oil treated sets were re-inoculated into fresh medium and revival of their growth was observed.

Fungitoxic properties of the oil

The effect of increased inoculum densicity of the test fungus on fungitoxicity of the oil was studied following the method of Moleyar and Pattisapu (1987). The effect of storage on the fungitoxicity of leaves of *A. alliaceum* was studied by keeping 2kg leaves at room temperature ($28\pm1^{o}C$) in a sterilized paper bag

for 30 days. The fungitoxicity of the leaves was tested after regular interval of 2 days.

Physicochemical properties of the oil

The oil was standardized through GLC and physicochemical properties viz. specific gravity, specific rotation, refractive index, solubility in different organic solvents, acid number, saponification value, ester value, phenolic content and carbonyl content following the method of Plaza *et al.* (2004).

Comparison of *A. alliaceum* with some prevalent synthetic fungicides

The efficacy of the oils was compared with some fungicides *viz.,* benzimidazole (benomyl), diphenylamine, phenylmercuric acetate (ceresan) and zinc dimethyl dithiocarbamete (zirum) by the usual poisoned food technique.

***In vivo* testing**

Mature healthy fruits of medium size (Dashehari) obtained from local market were used for the experiment. The fresh mango fruits surface was disinfested. To prepare spore suspension, spores were harvested from 7 day old culture and suspended in sterile distilled water and a wetting agent 0.01 %Tween 80. Fruits were wounded and inoculated with 40 µL of spore suspension (10^5 spores/mL) of *B. theobromae*. The inoculated fruits were kept in desiccators. The fruits of treatment sets were given dip treatment in aqueous extract of *A. alliaceum* (1:1w/v) for 5 min, 15 min and 30 min. The fruits of control were dipped in water. Six fruits were taken in each control and treatment sets and the experiment was repeated thrice. The observation was based on the mean values.

The *in vivo* efficacy of the oil of *A. alliaceum* was tested by fumigating the inoculated fruits. The inoculation was done by the same method as used with the extract. In the treatment a set, the requisite amount of oil was introduced in desiccators by soaking in a piece of cotton so as to give concentration of 100 ppm (V/V).The initiation of rotting of the fruits was observed. Six replicates were kept for treatment and control sets and the experiment was repeated thrice.

RESULTS

Out of the 20 plant species tested against *B. theobromae*, extracts from the leaves of *A. alliaceum* (water, acetone, ethyl alcohol, ethyl acetate, methanol and petroleum ether) showed potent activity that inhibited the mycelial growth of the mold completely (100%) (Table 1). With the exception of *A. alliaceum*, five other plants namely, *Adhatoda vasica* (ethyl acetate), *A. cathartica* (ethyl acetate, methanol), *L. inermis* (ethyl acetate), *Plumeria rubra* (ethyl acetate) and *Prunus persica* (ethyl acetate) were also found to show antifungal activity against the pathogen but, their activity was found to be confined to only one or two organic solvents. None of them was found to be effective as an aqueous extract. Ethyl acetate was found to be a highly desirable solvent in the extraction of EOs from these 6 plants which showned 100% efficacy. The antifungal activity of the other 5 plants was suspended (Table 1).

Leaf extract poisoning persisted for 8 days in the aqueous extract while in the organic solvents were usually up to 25 days for ethyl acetate, 10 days for methanol and alcohol and 5 days for acetone and 2 days for petroleum ether. The sweet smell of the leaves disappeared after 5 days. The fat content of the plant was 0.5%. The oil completely prevented the growth of fungal mycelial at 100ppm showing its MIC at 100 ppm. At 50ppm it inhibited mold growth by 85% (Table 2).

Toxic substances found static in water, acetone, petroleum ether and whole alcohol, showed a weak growth of hyaline mycelial in the medium. However, it was cidal in methanol and ethyl acetate (Table 1). The fungitoxicity of essential oils found to be naturally cidal in the toxic environment (100ppm) and hypertoxic concentrations (200ppm) (Table 2). It was noted that the oil that inhibited fungal growth of treatment sets containing even 64 discs of the test fungus showing the ability of the oil to withstand a very high level of the inoculum (Table 2).

The oil was found to have a long shelf life of up to two years. The oil was naturally warm as it remained toxic to mold at different temperatures between 10 and 80 ºC (Table 2). GLC analyses of the essential oil showed that it was a mixture of 7 major components and 15 small components. Various physichochemical features

namely; specific gravity, specific rotation, refractive index, melting of various organic solvents, saponification value, ester value, phenolic content and carbonyl percentage of the oil are listed in Table 3.

Table 1. Antifungal activity test for different angiospermic taxa against *B. theobromae*

Angiospermic Plants	**Solvents used**					
	Water	**Acetone**	**Ethyl acetate**	**Ethyl alcohol**	**Methanol**	**Petrolium ether**
Adenocalymma alliaceum	100	100	100*	100	100*	100
Adhatoda vasica Nees	0	5.00	100	12	25	5
Allamanda cathartica Linn	10	90	100	95	100	15
Annona squamosa	0	0	0	0	0	0
Callistemon lanceolatus	0	10	60	50	10	5
Catharanthus roseus Linn G.Don	0	0	0	0	0	0
Celosia cristata Linn	0	0	0	10	0	0
Clerodendrum indicum (Linn.)	0	0	0	0	0	0
Jatropha gossipifolia Linn	20	25	50	45	55	15
Justicia betonica Linn	0	0	0	0	0	0
Lawsonia inermis	50	70	100	95	80	40
Leucas aspera	0	0	0	0	0	0
Ocimum canum Sims	5	24	50	35	30	10
Plumeria rubra	20	50	100	40	50	0
Polygonum glabrum	0	0	0	0	0	0
Prunus persica (L.) Stockes	60	30	100	80	90	50
Rauvolfia serpentina	0	0	0	0	0	0
Solanum nigrum	0	0	0	0	0	0
Tecoma stans (Linn) H.B.ε K	0	0	0	0	0	0
Vitex negundo Linn.	*5*	*0*	*25*	*0*	*0*	0

*Cidal nature of extracts

Table 2. Fungitoxic properties of *A. alliaceum* essential oil

Inhibitory action		**Fungi toxicity (cidal/ static**		**Inoculums density**		**Thermo stability at one hr**		**Storage effect**	
MIC (ppm)	MGI (%)	MC (ppm)	MGI (%)	Fungal discs	MGI (%)	Temperature (^{0}C)	MGI (%)	Months	MGI (%)
50	85	100	100*	2	100	10	100	2	100
100	100	100	100*	4	100	20	100	4	100
200	100			8	100	30	100	6	100
300	100			16	100	40	100	8	100
400	100			32	100	50	100	10	100
500	100			64	100	60	100	12	100
								14	100
								16	100
								20	100
								24	100
								26	100

MIC-Minimum Inhibitory Concentrations; MC-Minimum Concentration; MGI-Mycelial Growth Inhibition; *- Cidal nature

Table 3. Physicochemical properties of the oil extracted from the leaves of *A. alliaceum*

Parameters	Observations
Yield of oil	0.5 %
Colour	Pale yellow
Odour	Pungent
Specific gravity at 28 ºC	0.6832
Specific rotation	+23.0º
Refractive index	1.621
Solubility	
Acetone (1:1Conc)	Soluble
Absolute alcohol (1:1Conc)	Soluble
90 % alcohol (1:1Conc)	Soluble
Ethyl acetate (1:1Conc)	Soluble
Benzene (1:1Conc)	Soluble
Chloroform (1:1Conc)	Soluble
Hexane 1:1Conc)	Soluble
Methanol (1:1Conc)	Soluble
Acid number	2.83mg
Saponification value	58.21mg
Ester value	45.35mg
Phenolic content	nil
Carbonyl percentage	2.00

The MIC of synthetic fungicides benzimidazole, diphenylamine, phenylmercuric acetate and zincdimethyl dithiocarbamate were found to be 200, 800, 500 and 400ppm respectively which was higher compared to the *A. alliaceum* oil (100ppm). Hence essential oil was found to be more potent than the synthetic fungicides. In vivo efficacy of the aqueous extract and oil of *A. alliaceum*

The fruits of control sets were completely covered with water soaked brown appearances with ash colored blackish mycelial growth after 6 days of storage showing complete rotting of the fruits. The fruits given 5 min dip treatment did not show any enhancement of shelf life and showed similar symptoms of rotting after 6 days. The initiation of rotting in fruits which were given 15 min and 30 min dip treatments in aqueous extract of *A. alliaceum* leaves was after 8 days and 11 days respectively. Therefore the respective enhancement of shelf life was up to 2 days and 5 days in each type of the dip treatments (Figure 1).

The oil treated fruits were found to enhance the shelf life as there was no fungal growth on the treated fruits compared to the control fruits. Though, the initiation of rotting was started after 14-days of storage, showing 8-days of enhancement of shelf life, the treated fruits peel showed browning coloration (Figure 1).

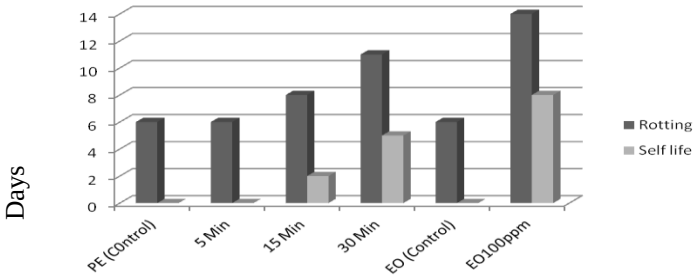

Figure 1. Initiation of rotting after days and self-life enhancement of fruits under *in vivo* treatment of plant extract and essential oil

DISCUSSION

The conservative nature of some extinct plants has been known for centuries and there has been a renewed interest in antimicrobial properties extracted from fragrant plants. Some plants extracted with different solvents have shown anti-fungal activity for different storage periods (Nigam *et al.,* 2021; Tripathi *et al.,* 2021). In the present study extracts of *A. alliaceum* in water, acetone, ethyl acetate, ethyl alcohol and methanol were found to exhibit strong toxicity against *B. theobromae*. Activity was static in water, acetone, whole alcohol and petroleum ether and regenerated mycelium showed weakness with hyaline mycelium. The extracts in ethyl acetate and methanol were found to be cidal as the reconstituted fungal discs were not regenerated when transferred to a new location. The extracts in water and all organic solvents has shown strong potency of *A. alleaceum* leaf extract which can be used as a potent botanical fungicide in controlling fruit fungal pathogen after harvest, during storage and transportation.

Recent findings on the effectiveness of essential oils such as biodegradable and ecofriendly fungitoxicants have shown the potential for their exploitation as a natural fungicide (Nigam *et al.,* 2021; Sempere *et al.,* 2021; Tripathi *et al.,* 2021). The fungal activity of oils found in *Ocimum, Thymus, Origanum, Anethum, Eucalyptus, Foeniculum* and Citrus against several post-harvest germs reveal the marked fungicidal activity of carvacrol (thyme, origanum oil) and p-anisaldehyde (anethol

oxidation products in anise oil) (Caccioni and Gizzardi 1994). Some volatile aromatic compounds produced by the fruit during ripening also showed antifungal activity. Acetaldehyde has been found to be effective in the post-harvest protection of apples (Stadelbacher and Prasad 1974) and stone fruits (Mari *et al.,* 2004). Hexanal and benzaldehyde, produced by stone fruit metabolism, also have fungistatic/fungicidal activity when used in post-harvest treatment against *Monolinia laxa* and *Rhizopus stolonifer* (Bonaterra *et al.,* 2003).

In the present study volatile fungitoxic principles were discovered in the form of essential oil from *A. alleaceum*. Yield of oil was 0.5%. The oil was yellow in color with a strong odor. Fragrant aroma, although the characteristic of the plant is due to the presence of a combination of alkenyl sulphide and thiosulfonates, coproducts made from akylcysteine sulfoxides, which are precursors to stable plant materials (Zoghbi *et al.,* 1984). Although the leaves of the *A. alliaceum* (garlic tree) quickly produce splendor, garlic as a fragrance when treated, the smell disappears after a few days of drying.

The oil was found to be naturally fungitoxic at 100ppm and hypertoxic at concentrations of 200ppm. The oil was found to be resistant to high inoculum congestion as it showed antifungal activity in the treatment sets containing 64 fungal discs. This is another oil potential that should be used as a botanical fumigant. The oil remained toxic for 2 years, with a long shelf life .The oil was stable to heat naturally as it was able to withstand temperatures up to 80ºC without losing toxins. GLC analysis showed that it was a mixture of seven major and fifteen minor components indicating that these components were responsible for the toxicity of the oil. The oil MIC was more effective than suynthetic fungicides as MIC oil was found to be lower (100ppm) compared to synthetic fungicides.

Several fungitoxicants of plant origin have been found to be harmless in treated products and some have shown improvement in their health. In this study, extracts and essential oils have proven to be efficaceous by improving the shelf life of mango fruits during storage. The treatment for 15 min enhanced the shelf life up to 2 days, while the dipping of fruits for 30 min enhanced the shelf life up to 5 days. The oils were used as fumigants at 100ppm (MIC).The fumigated fruits of treatment sets showed enhanced shelf life up to 8 days. The aqueous extracts did not show any adverse symptoms on the fruit peel. Though the essential oils enhanced the shelf life of fruits, the color of the fruits was turned to brown.

The use of these substances as antimicrobial agents can be an interesting field of investigation as the toxicity to mammals is very low, and their flexibility allows them to be used in cold or active packaging. Extracts of *A. alliacea* with strong fungal toxins, moldastatic (in water, petroleum ether and acetone) and fungal environment (in ethyl acetate and methanol), long shelf life and a broad spectrum of fungus may be recommended as a botanical fungitoxicant. More essential oils with low toxicity MIC compared to synthetic can provide antiseptic environment against the experimental fungi and other decaying fruit fungi. An environmentally friendly condition and effective resistance against stem end rot of mango fruits are provided by the extracts and EOs of *A. alliaceum*. The EOs have the potential to be used as botanical fungicidal fumigant.

The *in vivo* experiments with oils have shown a negative effect in the form of brownish colouration on the peel of the fruit. Similar damage to citrus fruits treated with thyme, cinnamon and essential oils have been reported by Plaza *et al.* (2004). Therefore, the potential use of essential oils to control postharvest diseases require a detailed examination of their biological activity, dispersion in fruit tissues, and the development of a formula which inhibits the growth of pathogens at nonphytotoxic concentrations. Investigations on the mode of action and practical applicability of such plant products is required so as to maximize their use in the control of postharvest diseases.

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