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ANTIFUNGAL EFFICACY OF BENZENE FRACTION OF *MORINGA OLEIFERA* LAM LEAF EXTRACT IN NATURAL CONDITION

Botany

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ABSTRACT

Now a days, in pandemic it is very important to search alternatives of organic control of pathogens in crops. These control methods protect environment as well as give perfect healthy nutrients to wellbeings that is very important for immunity. In this context present research also rely on same to protect tomato crops from pathogen *A. alternata* which known to causes severe loss in quality and quantity of tomato. Partially purified benzene extract that is vaccum evaporated and neutralized using cow dung and castor oil cake. This extract showed best inhibition of test pathogen both in lab condition (in vitro) as well as in natural condition (in vivo). Furthermore this extract known to possess good stability under various physical conditions applied. Natural means of crop pathogen control is very healthy and ecofriendly mean to protect environment as well as living beings.

KEYWORDS

Extarct, crop, pathogen, Alternaria, tomato, environment, living being

INTRODUCTION:

Fungicides mainly used in field have several detrimental effects on living beings. These chemicals enter the food chain and affect each and every body consume it. In order to protect environment as well as living beings, it is very important to focus research on searching alternatives that safely protect the crops from fungal infection without causing health effects on dependent living beings (Paranagama et al., 2003).

In this way, several microbial based as well as plant based microbial growth inhibitors have been searched. Literatures suggested that these antifungal not only provide protection but also provide some important nutrients employed in healthy growth. Several workers have worked on development of ecofriendly antifungal agent (Numpaque et al.,

2011; Shin et al., 2014; Villanueva Bermejo et al., 2015; Gavaric et al., 2015). Mostly plant based herbal formulation had successfully been incorporated in field trial. These formulations did not have residual effect and microbes could also not developed resistance against same. These benefits lead the increase in demand of herbal formulations and to fulfil needs it is important to screen plants for these properties.

The fungal pathogen *Alternaria* spp. is known for the early blight disease in tomato and other solanaceous crops which cause serious damage to the crop mainly during the fruiting period (Neeraj and Verma, 2010). The infection with pathogen generates leaf spots, tuber blight on potato, fruits rot and stem lesions on tomato fruits, results in a massive reduction in the quantity and quality of fruits produced (Cerkauskas, 2005; Momel and Pemezny, 2006).

Moringa oleifera is a medicinal plant that is rising as a promising and additional attuned substitute to existing chemical antifungals. Plant parts in addition to roots, flowers, bark, stem, leaves, seeds and essential oils have antimicrobial effect hence being employed for medicinal and other purposes (Dwivedi and Enespa, 2012). Analysis of these plant parts revealed that they possess noteworthy minerals, vitamins, protein and phytochemical substances that antimicrobial efficacy and can be employed to inhibit the growth of microorganisms (Arora and Onsare, 2014; Satish et al., 2013; Gurjar et al., 2012).

Present research is also based on in vitro and in vivo antifungal efficacy of partially purified benzene faction of *Moringa* leaf extract against *Alternaria alternata* causing significant yield in tomato plant. Study also involved formulation of herbal mixture with natural elicitor and binders and efficacy of same in natural environmental condition.

MATERIALAND METHODS

Collection and processing of plant material

- Leaves of Moringa oleifera plant used for the study were collected from Botanical
- Garden, College of Science, M.L.S. University, Udaipur, roadsides, local gardens and other adjoining areas of Udaipur. Dried leaves powder was used for further experimental work to prepare partially purified extracts.

Phytochemical Screening of *Moringa oleifera*

- Standard preliminary phytochemical analysis tests were performed for freshly
- prepared different leaf extracts of Moringa oleifera plant. Phytochemical analysis of leaf
- extracts helped in the identification of the presence of various known secondary plant
- metabolites in the extracts (Khandelwal, 2008).

Assessment of antifungal activity of crude and partially purified extracts

- For the assessment of the antifungal activity of crude extracts of M. oleifera leaf
- partially purified fractions of leaves extract of Moringa oliefera were used against Alternaria alternata. Antifungal activity was performed by poison food technique. After completion of incubation period, the size fungal colonies (average diameter in mm) was measured on and the mycelial growth inhibition (%) was calculated by the following formula

Mycelial growth inhibition (%) = gc-gt / gc× 100

Where, gc = Diameter of fungal colony in control set after subtracting the diameter of inoculums disc; gt = Diameter of fungal colony in treatment set after subtracting the diameter of inoculum disc.

Effect of physical factors on the antifungal activity of bio formulations: effect of different physical factors like heat, sunlight, pH and storage times was observed on benzene fraction and herbal formulation.

Preparation of herbal formulations: Herbal formulations were produced by using partially purified fraction of benzene, elicitor (castor oil cake) and binder (cow dung) in the different ratio (Table 1). A total number of 15 bioformulation were prepared by using partially purified benzene extract in combination with elicitor and binder.

Table1:*Invitro*AntifungalActivityofBioformulationsAgainst*Alternariaalternata*

Ratio No.	FormulationType	Ratio (ml)	GrowthDiameterafter 7 days(mm) ±SD	% Mycelial Growth Inhibition
1	Benzene extract: castor oilcake: cow dung	1:1:8	19±0.23	75.91

2	Benzene extract: castor oilcake: cow dung	2: 2:6	18±0.14	77.18
3	Benzene extract: castor oilcake: cow dung	3:3:4	27±0.24	65.77
4	**Benzene extract: castor oilcake: cow dung**	**4:4:2**	**15.33±0.15**	**80.57**
5	Benzene extract: castor oilcake: cow dung	5: 3:2	24.02±0.23	69.55
6	Benzene extract: castor oilcake: cow dung	8:1:1	23.43±0.12	70.30
7	Benzene extract: castor oilcake: cow dung	6:2:2	26.12±0.57	66.89
8	Benzene extract: castor oilcake: cow dung	4:3:3	22.66±0.54	71.28
9	**Benzene extract: castor oilcake: cow dung**	**2:4:4**	**16±0.14**	**79.72**
10	Benzene extract: castor oilcake: cow dung	2:5:3	25.04±0.17	68.26
11	Benzene extract: castor oilcake: cow dung	1:8:1	24±0.56	69.58
12	Benzene extract: castor oilcake: cow dung	2:6:2	18±0.14	77.18
13	Benzene extract: castor oilcake: cow dung	3:4:3	27±0.57	65.72
14	Benzene extract: castor oilcake: cow dung	4:2:4	26±0.57	67.04
15	Benzene extract: castor oilcake: cow dung	3:2:5	26.03±0.57	67.00
16	Control (water)		78.90±0.56	

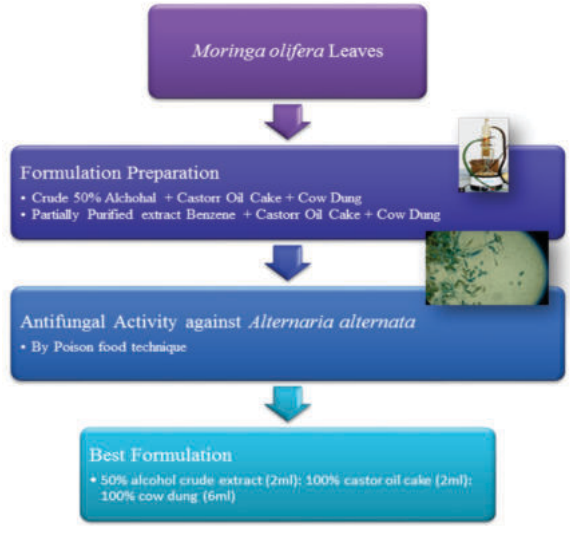

Figure 1: Bioformulation

Preparation of herbal formulations and solutions of standard drug

For conduction of in vivo study against *A. alternata* using herbal formulations were prepared by partially purified benzene leaf extract of *M. oleifera* plant. Leaf extracts were mixed with elicitor and binder (castor oil cake and cow dung, respectively) for the enhancement of the antifungal activity of extracts. Following formulations were prepared for antifungal activity assessment against *A. alternata* As an elicitor, castor oil cake was used to increase the activity of the extract against the pathogen. Cow dung was used as a binder which helps in binding of elicitor and extracts together for the preparation of a cohesive mixture. As standard antifungal drug mancozeb (a dithiocarbamate non-systemic agricultural fungicide with multisite, protective activity on contact) was used in sterile water with a concentration of 10 mg/mL.

Seed treatment plan:

To identify the antifungal activity of biofomulations by in vivo method plants were treated with bioformulations and a study was conducted from October 2020 to March 2021. For evaluation of the preventive effect of biofomulations the seed dipping and foliar spray methods were used [27,28].

Formulation:

Seeds that have been treated with bioformulation No. 19 (partially purified Benzene extract (4mL): 100% castor oil cake (4mL): 100% cow dung (2mL)).

Healthy seed dipped in respective bioformulations were sowed in pre-sterilized soil pots containing 10 kg soil infested with *Alternaria alternata* inoculum (20 mL/pot).

Four different controls were also maintained similarly which are as follows:

C1: Mancozeb-treated seeds with concentration 10mg/mL (served as positive control)
C2: Healthy untreated seeds sown in unsterilized and uninoculated soil
C3: Untreated healthy seeds sown in sterilized soil which was inoculated with *Alternaria alternata*
C4: Untreated healthy seeds in uninoculated sterilized soil.

Assessment of Disease Severity

Disease assessment was performed weekly. For this quantitative assessment was performed where the number of affected plants and leaves were conducted till 120 DAS (days after sowing). The fungicide mancozeb was used as a control. The total number of leaves per plant, the height of the plant, the number of blooms on each plant, the number of fruits on each plant, and the weight of each fruit per plant were also recorded. Twenty leaves of each plant were selected, and the diseased area was assessed on a 0-5 scale and reported as a percent disease index. The degree of early blight disease severity on the leaves of the treated and unsprayed plants was measured on a scale of 0 to 5. Where 0= no Infection; 1= infection up to 10%; 2= 11-25% infection; 3= 26-50% infection; 4= 50-75% infection; and 5= more than 75 % infection (measurement was based upon leaf area affected or leaf abscised [30,31,32,33]. The severity of disease was calculated by Percent Disease Index (PDI) and percent efficiency of disease control (PEDC) by the following equations.

PDI = P (n×v)/5N×100Equation 1

Where P = disease percent, n = number of infected leaves on the plant, v = numerical rate of infected leaves (0-5), N = total number of leaves

PEDC = {(Disease Index in control – Disease index in treatments)/ Disease Index in control} × 100 Equation 2

Data were subjected to analysis of ANOVA (one-way ANOVA), student t-test and Post –Hoc Comparisons. Five replicates were maintained with each experiment [34,35].

STATISTICALANALYSES

The IBM SPSS Statistics Ver. 20 program was used for all statistical analyses and calculations. The statistical data was reported as the mean of three independent replications along with the standard error (SE) for at least three replicates. Further, the coefficient of variation was calculated, followed by a range test at the P 0.05 significance level, to see the consistency of the results. The trials were carried out in triplicates, and each experiment was repeated twice using a completely random design.

RESULT AND DISCUSSION:

Plants have rich contents of antimicrobial secondary metabolites like alkaloids, saponins, volatile oil which confers protection from various microbes causes severe yield deterioration. Most of the research now a day's focus on exploring such natural content from plants that are effectively control plant disease and has no residual effect. A systematic research on same will be very useful to develop formulations with economically inherit property of disease control.

Table 1. Phytochemical screening of different leaves extracts of M. oleifera plant.

Type of leaves extract	Alkaloids			Carbo hydrate		Tannins	Saponins	Flavonoids	Phytosterols
	Wagner's Test	Mayer's Test	Hager's Test	Molish's Test	Fehling Test	Ferric chloride test	Foam test	Conc. H2so4 test	Liebermann's test
Benzene	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	-ve

The given table depicted that partially purified fractions of benzene leaves extract exhibit all types of phytochemicals/secondary metabolite was present is used for the study whereas Saponins,

Flavonoids and carbohydrate the metabolite was obtained in Acetone, Ethanol, Methanol, Aqueous leaves extract. Elangovan et al., (2014) reported the presence of alkaloids, flavonoids, steroids, tannins, saponins and glycosides in chloroform and petroleum ether extracts of M. oleifera leaves. Antibacterial activity of the studied extracts was reported against Gram-positive and Gram-negative bacteria. Antioxidant activities of the extracts used in the study were also reported by the authors. Similarly, Ojikao (2019) reported antimicrobial activity in ethanol, ethyl acetate and n-hexane leaves extract of M. oleifera plant.

Table 2: Antifungal activity of partially purified fractions of *M.oliefera* leavesextract against *A. alternata*

S. No.	Type of Extract	Growth diameter of fungal colonies after 7 days (mm) ± SD	% Mycelial growth inhibition
1	Benzene	21.37 ± 1.00	72.53

The inhibitory activity of the extracts was compared with standard fungicides like bovistin, mancozeb and water, which were used as positive and negative controls, respectively (Table 3).

Partial purification of the leaves extracts enhanced their antifungal activity as partially purified benzene fraction showed 72.53% growth inhibition against A. alternata with a colony diameter of 21.37 mm.

Table 3: Antifungal activity of standard fungicides and water served as control against *A. alternata*

S. No.	Standard fungicides and water control	Growth diameter of fungal colonies after 7 days (mm) ± SD
1	Mancozeb	19.1 ± 1.52
2	Bavistin	27.5 ± 1.52
3	Water	78.90 ± 1.15

As partially purified benzene fraction is as effective as standard fungicides used in the study thus they can potentially replace the synthetic fungicides used in agricultural fields for disease control. Such biocontrol agents do not possess any hazardous effect on the environment, animals and human beings as well. The presence of various secondary metabolites in the leaves extracts of M. oliefera plant (as screened in the present study) further establishes a correlation between the secondary metabolites and their antifungal potential (Fasogbon et al., 2021). Thus, this plant is a rich source of bioactive natural products which can be used in various activities and also for the development of new pharmaceutically important value-added products.

The effect of different physical factors on the antifungal activity of *M. oleifera* leaf extracts and their screened bio formulations have been assessed by the food poison technique. The exposure to sunlight was given for 15 hrs and 30 hrs. It was observed that there were no significant changes in the activity of bio formulations after the sunlight exposure for 15 hrs and 30 hrs. Antifungal activity remained sustainable for benzene extract as well. When samples were treated with wet heat (treated in a water bath) for 50 °C and 100 °C the activity of benzene extract remain same. The effect of pH on the antifungal efficacy of the selected materials was evaluated. Benzene extract of *M. oleifera* leaves showed a similar effect of pH in their antifungal activity. Minimum loss of activity was found with formulation-19 (Benzene extract, showed a reduction in activity from73.59% to 72.54% . The effect of antifungal activity of leaf extracts of *M. oleifera* and their bio formulations was assessed against the storage time of biomaterials. All the biomaterials were stored at room temperature for 12 months and their antifungal activity was assessed in 6 months intervals. For all the studied biomaterials it was observed that the storage time did not affect their antifungal activity as no significant changes in the growth of fungal colonies and percent mycelial inhibition activity was observed with increased storage time . Hada and Sharma (2017) also suggested that active principles present in Cassia fistula are highly susceptible to change in physical environment. However it is found that extract and herbal formulation of Cassia fistula can be stored for 12 months, remains stable at alkaline pH, can stand with exposure to sunlight and high temperature. Hence a little favourable manipulation of physical conditions could improve its shelf life and can be used as fungicides for controlling microorganisms. Antifungal efficacy in natural condition. To evaluate the disease severity in the plant after treatment with bioformulations and control treatments their PDI and PEDC were calculated. The more effective the treatment is, the less is the disease severity. The effectiveness of bioformulation is inversely proportional to the disease severity. Maximum percent efficiency of disease control (PEDC) was observed with partially purified benzene formulation (80.87%) with PDI 15.40% the values are comparable and slightly higher than standard fungicide mancozeb C1 (76.11%) with PDI 17.20%. A higher PEDC value of T3 (78.61%) than C1 PEDC (76.11%) indicates the effectiveness of T3 formulation against *A. alternata*. Results show that this formulation can be used for effective control of early blight disease in tomato plants. Statistical analysis was performed with Student t-test, one-way ANOVA and posthoc comparisons analysis. Significance was measured at $p<0.001$ and values were found significant.

Table 4: Estimation of disease severity in terms of PDI and PDEC

#Treatment/ # Control	Plant	Total Leaves Assessed	Total Leaves infected	0	1	2	3	4	5	PDI	Average PDI	PEDC
T3	1	20	7	13	1	2	1	2	1	21	15.33	80.57
	2	20	7	13	2	1	2	1	1	19		
	3	20	6	14	2	2	1	1	0	13		
	4	20	6	14	2	2	1	1	0	13		
	5	20	5	15	2	1	1	1	0	11		
C1 (Mancozeb)	1	20	13	7	8	4	1	0	0	19	17.2	76.11
	2	20	12	8	6	3	2	1	0	22		
	3	20	11	9	7	4	0	0	0	15		
	4	20	10	10	4	6	0	0	0	16		
	5	20	10	10	3	3	0	0	1	14		
C2 (Uninoculated, unsterilized soil	1	20	15	5	2	1	2	5	4	52	44.8	37.77
	2	20	14	6	4	1	0	5	4	46		
	3	20	13	7	3	1	2	3	4	43		
	4	20	12	8	2	2	2	3	3	39		
	5	20	13	7	2	2	1	5	3	44		
C3 (Inoculated, sterilized soil)	1	20	18	2	3	3	2	3	7	62	72	-
	2	20	20	0	4	0	4	3	9	73		
	3	20	19	1	3	4	0	2	9	68		
	4	20	18	2	0	0	4	8	6	74		
	5	20	20	0	1	1	2	6	10	83		
C4 (Uninoculated, sterilized soil)	1	20	2	18	1	0	0	0	1	6	9.4	88.88
	2	20	1	19	0	0	0	0	1	5		
	3	20	4	16	0	0	2	0	2	16		
	4	20	3	17	0	1	0	0	2	12		
	5	20	2	18	0	0	1	0	1	8		

The PDI and PEDC were measured via a pot experiment on tomato plants to test the antifungal efficiency of bio formulations against the *A. alternata*. Before the application of biofungicides in the agricultural field, it is important to evaluate their disease control ability and toxic effects if so, ever on host plants. The Standard Blotter Method is popularly used for assessment of the toxicity of the prepared formulations and the effect is measured in terms of percent seed germination ability (Masangwa et al., 2017; Meena et al., 2017; Meena et al 2017).

The findings revealed their efficacy in disease management and control in the real agricultural field conditions. Their natural origin, eco-friendly nature and biodegradability are other additional advantages of using these bioformulations as potential fungicides. In the present study leaf extracts of *M. oleifera* plants have been combined with castor oil cake and cow dung for preparation of effective bioformulations against *A. alternata* fungi for leaf blight disease control in tomato plants. In the presence of suitable control groups, prepared bioformulations were evaluated for their antifungal potential by in vivo studies. Pot experiments have been conducted with tomato plants and disease severity in terms of PDI and PEDC has been measured for treated and control groups. The reduction in disease severity after treatment with bioformulations was found comparable\with synthetic fungicides mancozeb, used as control. T3 treatment (Formulation no.19) had maximum antifungal potential and was found effective against *A. alternata* in both in vivo and in vitro experiments. The prepared bioformulations also improved the growth parameters of plants in the presence of the pathogen. These bioformulations can be further explored for their target-specific disease prevention strategy. More and intense field experiments are required for their commercial and viable use in agricultural field conditions. Owing to their efficacy in the control of leaf blight disease of tomato plants, their industrial and commercial production for

widespread application can be initiated in near future.

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