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MORINGA OLEIFERA LAM LEAF EXTRACT BASED ANTIFUNGAL HERBAL FORMULATION: *IN VITRO* AND *IN VIVO* STUDY

[1]Ruchi Vyas, [2]Kanika Sharma and [3]Tripta Jain

[1]Research scholar, [2]Rtd. Professor and [3]Assistant Professor

Department of Botany, College of Science, Mohanlal Sukhadia University, Udaipur 313001, Rajasthan, India

Abstract

Fungal disease in plants is still challenging to control and require proper management practices. Fungal diseases cause severe economic losses due to their high speed of infection ability through spores followed by the destruction of plants and crops. However, a wide range of synthetic fungicides are available for the control of pathogens but their improper use can create hazardous effects for animals, humans and the environment. Findings of this study revealed that hydroalcoholic crude extract and partially purified benzene fraction exhibit effective inhibition ability against the test pathogen when the data compared with the values of standard fungicides used in the study as the positive control. Further this partially purified preparation (Benzene fraction) was used to formulte herbal formulation and checked for in vivo disease mangemnet of the host pathogen i.e. Alterneria *alternate*, a causal organism of early blight of tomato. It was done by foliar spray, seed dipping and soil amendment with bioformulation prepared from *Moringa oleifera* leaf extract in combination with neem oilcake and cow dung. . The preventive action was studied as a function of a decrease in disease severity, change in growth characteristics of host plant such as the number of leaves/plants, branches/plant, flower/plant, of fruit/plant, the weight of fruit of a healthy,infected and untreated plant. A significant control of Early blight disease was recorded with bio formulations treatment T3 [seeds were treated with partially purified benzene extract (4 ml): 100% castor oilcake (4 ml): 100% cow dung (2 ml)], T4 [seeds were treated with partially purified benzene extract (2 ml): 100% castor oil cake (4 ml): 100% cow dung (4 ml)], T2 [seeds were treated with 50% alcoholic crude extract (3 ml): 100% castor oil cake (4 ml): 100% cow dung(3 ml)] and T1 [seeds were treated with 50% alcoholic crude extract (2 ml): 100% castor oil cake (2 ml): 100% cow dung (6 ml)] as compared to other bioformulation treatments and its PDI and PEDC were recorded 15.4%, 16.08%, 17.6%, 22.20% and 78.61%, 76.66%, 75.50%,69.16%, respectively. Results showed that treatment with bioformulation treatment T3, notably increased plant height, number of leaves perplant, number of fruits per plant, the weight of fruits per plant, number of branches per plant and number of flowers per plant content followed by formulations number T4,T2,and T3. 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.

Keywords: Fungal disease, bioformulation, extract, target specific, growth parameter, *in vivo*

I INTRODUCTION

Synthetic fungicides, insecticides and herbicides are frequently used in agriculture fields to improve the yield but on the contrary, their negative effects affect the entire ecosystem and their inappropriate use can further possess possible carcinogenic risk. Synthetic fungicides are threatening for human health and also responsible for various environmental pollutions (Gangawane, 1990). Considering their harmful effects on humans

and on the environment as well, there is an emerging need to identify and explore environmentally safe and economically viable tactics for the control of plant diseases which can reduce the use of synthetic agrochemicals (Ghalem and Mohamed, 2008; Mehta and Sharma, 2016). Worldwide researchers are trying to investigate new natural products and their analogs comprising antifungal activities to reduce the economic losses caused by

pathogenic fungal strains. They can be effectively and safely utilized as alternatives to synthetic fungicides. In plants, their secondary metabolites such as phenols, flavonoids, quinones, essential oils, alkaloids, sterols, thymol, coumarins and triterpenoids are untapped reservoirs of various valuable chemicals, can be used for various purposes (Sales et al., 2016;Mehta and Sharma, 2016)

Early blight, of tomato plants commonly known as target spot disease, is commonly caused by fungal pathogen *Alternaria solani*. According to the report of Bessadat et al. (2014), 46-90 % of blight severity in tomato crops is caused by *Alternaria*

alternata in Algeria. The blight disease is considered one of the world's most prevalent and devastating diseases of tomato plants, causes massive damage in plants and significantly reduces the plant yield.

Many environmental and toxicological problems have been developed by the extensive use of synthetic fungicides. Mancozeb and Bavistin are commonly used fungicides, majorly used for fungal disease control in plants. They are enabled to control fungal diseases and they repress pathogen by various mechanisms like the destruction of the cell membrane, alteration in cell membrane permeability, inhibition of pathogen's metabolic processes and prevention of transcription and translation process in pathogen (Osman and Al-Rehiayam, 2003). Plant-based antifungal formulations are presently gaining attention for disease control. They are effective against disease pathogens and have no antagonistic effects on humans and the environment. These plant-originated antifungal formulations include effective bioactive compounds and inert carrier material (Omer, 2010).

The present study is aimed to evaluate the antifungal potential of *M. oleifera* leaf extracts derived bioformulations, intended to be used as effective biofungicides to control the leaf blight disease in tomato plants. For this alcoholic and aqueous extracts of leaves of *M. oleifera* were prepared and partially purified by soxhlet and successive extractions. Further, these partially purified fractions were assayed for their antifungal activity against A. alternate, the causative agent of Leaf blight disease in tomato plants. The bioformulations tested for their preventive effects against the plant pathogen in the presence of suitable control groups. *In vivo* pot experiments were designed for the assessment of their disease prevention ability. The data obtained were statistically evaluated. These bioformulations have immense potential to be used as effective biofungicides and their commercial production can provide a cost-effective alternative to synthetic fungicides.

II MATERIALS AND METHODS

Collection 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 the crude and partially purified extracts. Cold and hot extraction methods were used to obtain the crude and partially purified fractions of leave powder, respectively.

Cold and Hot Extraction

Crude extracts of *M. oleifera* leaves powder were prepared according to the modified

cold extraction method suggested by Shadomy (1974). For successive separation of different and partially purified organic constituents which are presents in dried plant material, the reflux method of solvent extraction was used (Harborne, 1984; Kokate et al., 1990).

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). All three solvent extracts of *Moringa oleifera* leaves powder were subjected to qualitative phytochemical assay using color reactions (Kokate et al., 1990).

Assessment of antifungal activity of crude and partially purified extracts For the assessment of the antifungal activity of crude extracts of *M. oleifera* leaf extracts viz. 100% alcohol, 50% hydro-alcohol, 100% aqueous extract and partially purified fractions of leaves extract of *Moringa oliefera* were used against *Alternaria alternata*.

Antifungal activity was performed by poison food technique. 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.

Evaluation of MIC and MFC

To evaluate the minimum effective concentration of plant extracts used in the study their MIC value was calculated. Minimum inhibitory concentration (MIC) was determined by the broth dilution method (Collee et al., 1996). To identify the effective minimum fungicidal concentration of the plant extracts a loop full of fungal biomass obtained from all of the tubes used for MIC estimation was streaked separately onto the extract-free PDA slants. The appearance of growth indicates that the extract concentration was just fungistatic (growth inhibitory) and the absence of any fungal growth indicates that the extract concentration used was fungicidal (killed the fungi).

Preparation of pots and soil for plant growth

For the development of tomato seedlings autoclaved and cooled soil was added into the pots having 33 cm height, 30 cm top diameter, 23 cm bottom diameter. Before soil addition pots were sterilized with a 20 % $CuSO_4$ solution. The pre-sterilized pots were filled with sterile soil (10 kg/pot) and further used for tomato seeds germination and seedlings development.

a. Preparation of herbal formulations and solutions of standard drug

For conduction of in vivo study against *A. alternata* various herbal formulations were prepared using the leaf extracts (crude and partially purified benzene 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*

1. 50% alcoholic crude extract + elicitor + binder;
2. Partially purified benzene extract + elicitor + binder;
3. Leaf powder of *M. oleifera*+ elicitor + binder

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.

b. 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 (Jan et al., 2003; Ganie et al., 2013).

Based on results obtained from *in vitro* studies six treatments were planned which are mentioned as following

T1: Seeds that have been treated with bioformulation No. 2. (50 % alcoholic crude extract (2mL): 100% castor oil cake (2mL): 100% cow dung (6mL)).

T2: Seeds that have been treated with bioformulation No. 13. (50% alcohol crude extract (3mL): 100% castor oil cake (4mL): 100% cow dung (3mL)).

T3: Seeds that have been treated with bioformulation No. 19 (partially purified Benzene extract (4mL): 100% castor oil cake (4mL): 100% cow dung (2mL)).

T4: Seeds that have been treated with bioformulation No. 24. (Partially purified Benzene extract (2mL): 100% castor oil cake (4mL): 100% cow dung (4mL)).

T5: Seeds that have been treated with *Moringa* leaf powder (50 g): castor oil cake (25g): cow dung (25g).

T6*:* Seeds that have been treated with *Moringa* leaf powder (30 g: castor oil cake (35g) treated seeds: cow dung (35g).

The selection of biomaterials for seed treatment was based upon their antifungal potential which was previously evaluated by in vitro method. Biomaterials (T1- T6) showed effective antifungal activity against *A. alternata* thus selected for in vivo studies. 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.

c. Evaluation of the preventive effect of bioformulations

To evaluate the disease preventive effect of used bioformulations percent disease index (PDI) and percent efficacy of disease control (PEDC) were calculated after the methods of Hussein and Hamideldin (2014). After the successful germination of tomato seeds, 21-day-old healthy seedlings were shifted in plastic plots (3 seedlings per pot) within 30 days. Spore suspension of *A. alternata* containing 1.0 x106 conidia per mL was sprayed on plants (51 days old). After fungal infection (48 h) plants were treated with bioformulations (T1-T6, 25 mL/plant). (For standard control, T5 and T6 bioformulations were combined in the soil and the same approach was used. Not clear please mention according to experimental design). After 15 days of the initial treatment, tomato plants were given a second spray of bioformulation and left for 40 days to measure disease severity. After completion of the treatment period plant height, number of leaves per plant, number of branches per plant, number of flowers per plant, number of fruits per plant, and weight of fruits per plant were recorded for each pot.

d. 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 (Vakalounakis, 1983; Sultana et al., 2009; Zarafi and Moumoudou, 2010; Kumar,and Srivastava, 2013). 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×100 **Equation 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 (Mirkarimi et al., 2013).

e. Statistical analyses

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.

III. RESULT AND DISCUSSION

Chemical fungicides impose severe adverse effects not only on humans but also on animals and the entire ecosystem. Considering the facts of the adverse effects of the chemical fungicides, there is an emerging demand for novel antifungal agents which possess no harmful effects on animals, humans and the environment as well. Biodegradability, target selectivity, a wide range of structural classes, fewer or no side effects are some desirable characteristics of these fungicides (Parajuli et al., 2005). Tomato plants infected with *Alternaria* species show characteristic symptoms of the disease including chlorosis, tissue necrosis, and deterioration in quality and quantity of food which ultimately result in significant economic losses in tomato plants due to infection with the fungi (Gilchrist and Grogan, 1976). *Alternaria alternata*, is responsible for brown patch disease on the leaves and also creates a black pit infection in the tuber (Stevenson et al., 2003). The leaf spot disease was first discovered in tomatoes in China during 2014–15. The disease affected 65 to 85 % of the crops in the inspected region (Ren et al., 2017). The disease

caused necrosis in the leaves and petioles resulted in significant defoliation in plants and reduced yield (Akhtar et al., 2004). For tomato crops, *Alternaria alternata* is considered an extremely destructive fungus. However, sophisticated and effective technologies are available for fungal-based disease control but the majority of them are based on the use of synthetic fungicides.

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

Type of leaves extract	Alkaloids			Carbohydrate		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	Liberman's test
PE	+ve	+ve	+ve	+ve	+ve	-ve	-ve	-ve	-ve
Benzene	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	-ve
Chloroform	+ve	+ve	+ve	-ve	-ve	+ve	+ve	+ve	-ve
Acetone	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	-ve
Ethanol	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	-ve
Methanol	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	-ve
Aqueous	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	-ve

Phytochemical analysis of *M. oleifera* leaves extracts Phytochemical screening of different leaf extracts of *M. oleifera* was performed qualitatively. Phytochemical screening provides information about the existence of various bioactive components in the extracts used for the study. These metabolites exhibit different bioactivities and can play a significant role in disease prevention. Table 1 shows the presence of different phytochemicals in the leaves extracts of *M. oleifera*. 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. Similarly, Ojikao (2019) reported antimicrobial activity in ethanol, ethyl acetate and n-hexane leaves extract of *M. oleifera* plant. Phytochemical analysis of the extracts showed the presence of tannins and alkaloids in all three extracts. Phlobatannins were present only in n-hexane extract, while they were not reported in both ethanol and ethyl acetate extracts. On the contrary saponins and phenols were present in ethanol and ethyl acetate extracts but they were absent in nhexane extract. The quantitative analysis of phytochemicals showed tannins were present in maximum amount (8.22%) followed by saponins (1.75%), alkaloids (0.42%) and phenols (0.19%). The presence of secondary metabolites presumably affects the inhibitory potential of the plant extracts (Goel and Sharma, 2013; Amenu, 2014).

Assessment of antifungal activity of M. oleifera leaves extracts

Crude, as well as partially purified leaf extracts of the *M. oleifera* plant, were evaluated for their antifungal potential against *Alternaria alternata*. Part of leaves extract (10mg/mL) was added into the PDA media and 7 days old culture of pathogenic fungi was allowed to grow upon it. After 7 days of incubation average diameter of the fungal colonies was measured against control and percent mycelial growth inhibition was calculated. Table 2 and 3 depicted the antifungal potential of leaves extracts of *M. oleifera* plant. The inhibitory activity of the extracts was compared with standard fungicides like bavistin, mancozeb and water, which were used as positive and negative controls, respectively (Table 4). As results showed, maximum antifungal activity was observed with hydroalcoholic crude extract (29.1 mm colony diameter and 68.31% growth inhibition) followed by alcohol extract (61.97% inhibition). Whereas, partial purification of the leaves extracts further 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. Inhibition activity of partially purified acetone, alcohol and methanol extract was measured as 61.96%, 59.86% and 57.75% respectively. Aqueous, petroleum ether and chloroform fractions showed less inhibition against A. alternata. The experimental results were compared with water used
as negative control and standard fungicides like mancozeb, bovistin which served as the positive control. The hydroalcoholic extract is less effective than the used standard drugs whereas the affectivity of benzene fraction is more than the standard fungicide Bovistin. Negative control water, showed the maximum diameter of fungal colonies (78.9 mm) assuming no inhibitory effect projected by the negative control against the pathogenic fungi.
In the present study crude and partially purified leaves extracts of *M. oleifera* Lam. have been assessed for their antifungal potential against A. alternate, responsible for disease in tomato plants (early blight disease). The poison food technique was used for the assessment of antifungal activity of different fractions. Cold and hot extraction methods were used for the preparation of extracts in different organic solvents, which were recycled
subsequently with rotary vacuum evaporator. As the results showed, maximum percent extractive value in terms of yield was obtained with the alcoholic extract whereas maximum fungal inhibitory activity was observed with hydroalcoholic crude extract

and partially purified benzene extract against *A. alternata*. Mancozeb and bovistin were used as standards and the inhibitory effects of hydroalcoholic and benzene extracts were comparable with
them. Results of antifungal activity suggest that *M. oliefera* leaf extracts possess antifungal activity against A. alternata and can be used to develop a biocontrol agent. As their effectivity is comparable with standard fungicides used in the study thus they can potentially replace the synthetic fungicides used in agricultural fields for disease control. Zaffer et al. (2015) assessed antifungal activity methanolic and ethyl acetate leaves extracts of *M. oleifera* plant. Their phytochemical analysis revealed the presence of flavonoids, alkaloids, tannins, triterpenoids, glycosides, and steroids in the extracts. Aqueous and ethanolic leaf extracts of *M.oleifera* plant were evaluated for their antifungal properties by Patel et al.,(2014). *Saccharomyces cerevisiae*, *Candida albicans* and *Candida tropicalis* strains were used for the assessment of antimicrobial activity of plant extracts.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. 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.

Table 2: Antifungal activity of crude extract of *M.oliefera* leaves extractagainst *A. alternata*

S. No.	Type of Extract	Growth diameter of fungal colonies after 7 days (mm) ± SD	% Mycelial growth inhibition
1	Alcohol	30 ± 1.52	61.97
2	Aqueous	34.5 ± 1.15	56.27
3	Hydroalcoholic (1:1)	29.1 ± 1.73	68.31

Table 3: Antifungal activity of various 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	Petroleum ether	59.33± 1.15	24.87
2	Benzene	21.37 ± 1.00	72.53
3	Chloroform	48.27 ± 0.57	38.82
4	Acetone	30.01 ± 1.15	61.96
5	Alcohol	31.67 ± 1.15	59.86
6	Methanol	33.33 ± 2.08	57.75
7	Water	35.07 ± 1.15	55.55

Table 4: 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

Evaluation of MIC and MFC values of benzene fraction

Preliminary studies showed the highest affectivity of benzene fraction against *A. alternata*. Benzene fraction inhibited the mycelial growth of test fungi by 72.53% thus this fraction was further used for assessment of minimum inhibitory concentration i.e. MIC and minimum antifungal concentration i.e. MFC. These values are important to identify the minimum dose of the studied extract which can effectively control the growth of test fungi. There are various techniques used for antimicrobial susceptibility tests. In the present study the poison food technique was used after the method of Collee et al., (1996) for the assessment of antifungal activity of various leaf extracts of M. oleifera plant. The antifungal activity assessment is based on the inhibition of microorganism growth, which indicates the sensitivity of microorganisms towards the substrate used for assessment. The activity is measured as a function of the diameter of the growth zone, the lesser the zone more is the activity (Dhingra and Sinclair, 1985).
The MIC and MFC values of the benzene fractions are presented in Table 5. To assess the MIC value of benzene extract 0.019 mg/mL to 10 mg/mL of benzene extract was used and 2.5 mg/mL was found as the minimum concentration of the extract where inhibition was observed. Thus MIC of the benzene extract was 2.5 mg/mL whereas, MFC for the benzene extract was 5 mg/mL against *A. alternata*. Thus hydroalcoholic crude extract and the partially purified extract (benzene extract) of *M. Oleifera* plant leaves possess effective inhibitory potential against *A. alternata* and thus can be further explored as bio fungicides to control the leaf blight disease in tomato plants.

Table 5: MIC and MFC values of benzene Fraction of *M. oleifera* leaves extract.

S.No	Test pathogen	MIC (mg/ml)	MFC (mg/ml)
1	*Alternaria alternata*	2.5	5

Determination of percent disease index (PDI) and percent efficacy of disease control (PEDC)

To calculate the disease severity in the plant after treatment with bioformulations (T1 –T6) and control treatments (C1-C4) 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 bioformulation T3 (78.61%) with PDI 15.40% the values are comparable and slightly higher than standard fungicide mancozeb C1 (76.11%) with PDI 17.20%. After T4 the second highest activity was observed for T4 (76.66%) with PDI 16.8%, then for T2 (75.50%) with PDI 17.6%, T5 (73.88%) with PDI 18.8%, T6 (72.77) with PDI 19.6% and least activity was observed for bioformulation T1(69.16%) with PDI 22.2%. For control treatments maximum PEDC was recorded for C4 (88.88%) with PDI 9.4% followed by C1 (76.11%) with PDI of 17.2%. The PEDC and PDI values of T3 i.e. bioformulation no. 19, prepared from Partially purified benzene extract (4mL): 100% castor oil cake (4mL): 100% cow dung (2mL) were comparable with control C1 (standard fungicide mancozeb). 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. Treatment with Mancozeb was found more effective than treatment with T4, T2, T1,T5 and T6. Maximum disease control efficiency was observed in T3 (Fig.1). 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.

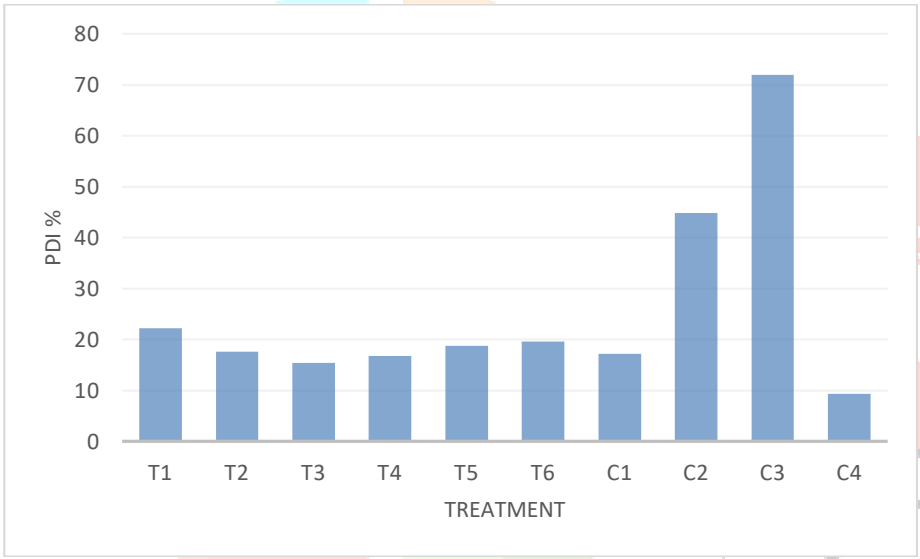

Figure 10.1. Percent Disease Index for different bioformulations (T1-T6) and controls (C1-C4) used in the study.

Growth Parameters

To evaluate the overall efficacy of prepared bioformulations and control groups against the early blight disease in tomato plants different growth parameters of tomato plants were evaluated (tables 10.2-10.7).

1. Effect of treatments on plant height

Table 6 show the effect of different treatments and synthetic fungicides on the height of tomato plants. Treatment with bioformulations and mancozeb reduced the detrimental effects of fungi and the height of the plant was increased in comparison to control groups. Maximum plant height observed with C1 (42.66cm) followed by T3 (40.66 cm), T5 (38.33cm), T4(38 cm), T2(35.33 cm), T6(34.33 cm), and T1(32 cm).

Table6: Effect of bioformulations and control treatments on height of tomato plants.

S.No.	Treatments	Height of tomato plant(cm)			Average ± SD
		R1	R2	R3	
1.	T1	34	31	31	32±1.73
2.	T2	32	38	36	35.33± 3.05
3.	T3	43	43	36	40.66 ±4.04
4.	T4	39	39	36	38±1.73
5.	T5	39	38	38	38.33±.57
6.	T6	32	36	35	34.33±2.08
7.	C1	42	42	44	42.66±1.75
8.	C2	33	36	35	34.66±1.52
9.	C3	27	29	30	28.66±1.57
10.	C4	32	32	31	31.66±.57
11.	t				1.54
12.	df				28
13.	Significance*				Non significant

p>0.05(non significance)

2. Effect of treatment and control groups on numbers of leaves/plants

The effect of different treatments and synthetic fungicides along with other control treatments on the number of leaves per plant was evaluated and data is shown in Table 7. The data indicate a slight reduction in the number of leaves per plant due to foliar infection by pathogenic fungi. However, a significant increase in the number of leaves per plant was observed due to treatment with bioformulations and mancozeb treatment. The maximum number of leaves were observed with T3 (45.33) followed by T4 (42.66), T2 (41.66), T1 (40.66), T6 (39.33) and T5 (36.33).

Table7: Effect of different bioformulations and control treatments on leaves of tomato plants

S. No.	Treatments	Number of leaves/plants			Average ± SD
		R1	R2	R3	
1.	T1	42	41	39	40.66±1.52
2.	T2	40	45	40	41.66±2.88
3.	T3	45	46	45	45.33±0.57
4.	T4	41	43	44	42.66±1.52
5.	T5	36	39	36	36.33±2.57
6.	T6	42	40	36	39.33±3.05
7.	C1	37	40	38	38.33±1.52
8.	C2	41	40	40	40.33±0.57
9.	C3	20	28	25	24.33±4.04
10.	C4	33	35	32	33.33±1.52
1.	T				2.98
2.	Df				28
3.	Significance*				significant

p<0.01 and p<0.05 significant at both level (significant)

Table7 (b): Statistical analysis of results of number of leaves on tomato plants for control groups

Control Group	N	Mean	SD	F	df	Result
C1	3	38.33	1.52	102.57	3,8	SIGNIFICANT
C2	3	40.33	0.57			
C3	3	24.33	4.04			
C4	3	33.33	1.52			

3. Effect of treatment and control groups on numbers of branches per plant

The effect of different bioformulations and control treatments on the numbers of branches per plant has been illustrated in table no8 . The data indicate a slight reduction in the number of branches per plant due to foliar infection. The numbers of branches in treatment groups are comparable with the control group i.e. C1 (mancozeb treated plants). The maximum average number of branches were obtained with T6 (13.33) followed by C1 (11.66) T3 (12), T2 (11), T5 (11), T4 (9.33), T1 (9.33), C2 (8), C3 (7.33) and C4 (6.66).

Table 8: Effect of different bioformulations and control treatments on branches of tomato plants

S. No.	Treatments	Number of branches/plants			Average ± SD
		R1	R2	R3	
1.	T1	10	8	10	9.33±1.15
2.	T2	12	10	11	11±1
3.	T3	10	8	10	12±1
4.	T4	10	9	9	9.33±.57
5.	T5	12	10	11	11±1
6.	T6	15	12	13	13.33±1.52
7.	C1	11	11	13	11.66±1.15
8.	C2	10	7	7	8±1.73
9.	C3	8	8	6	7.33±1.15
10.	C4	6	8	6	6.66±1.15
11.	**t**				4.08
12.	**df**				28
13.	Significance*				SIGNIFICANT

$p<0.01$ and $p<0.05$ significant at both level (significant)

4. Effect of bioformulations and control treatment on numbers of flowers per plant

Table 9 depicted the data showing the effect of different bioformulations and control groups on the numbers of flowers per plant. Due to foliar infection reduction in flower numbers was observed. The flower setting was also affected as the disease progressed and became more severe. Maximum numbers of flowers were observed with T3(11.33) the value was exactly equal to the control group C1(11.33) treated with mancozeb fungicide. The treatment group T3 showed an average flower number 11.33 whereas other groups C2, T4, T1, C4, T6, T2, C3 and T5 had 9.66, 10.33,9, 8.33, 7.33, 7, 6 and 5.33 average numbers of flowers/plant, respectively.

Table 9: Effect of different bioformulations and control treatments on number of flowers of tomato plants

S. No.	Treatments	Number of flowers/plants			Average ± SD
		R1	R2	R3	
1.	T1	9	9	9	9±0
2.	T2	7	7	7	7±0
3.	T3	12	11	11	11.33±0.57
4.	T4	9	11	11	10.33±1.15
5.	T5	5	6	5	5.33±.51
6.	T6	9	6	7	7.33±1.52
7.	C1	13	13	8	11.33±2.88
8.	C2	8	9	12	9.66±2.08
9.	C3	6	6	6	6±0
10.	C4	9	9	7	8.33±1.15
11.	t				1.08
12.	df				28
13.	Significance*				NS

p>0.05(non-significant)

5. Number of fruits per plant affected by treatment with bioformulations and control groups

The number of fruits per plant with respect to the treatment with bioformulations and control groups has been mentioned in Table no. 10.Foliar infection caused a slight reduction in fruit numbers. The maximum number of fruits observed with C1 (9.33) and the results are comparable with the treatment group T3 (8.66).

Table10: Effect of different bioformulations and control treatments on number of fruits on tomato plants

S. No.	Treatments	Number of fruits/plants			Average ± SD
		R1	R2	R3	
1.	T1	10	6	6	7.33±2.03
2.	T2	5	8	8	7±1.73
3.	T3	9	8	9	8.66±0.57
4.	T4	5	10	9	8±2.64
5.	T5	6	6	5	5.66±.57
6.	T6	8	8	8	8±0
7.	C1	10	9	9	9.33±.57
8.	C2	6	5	3	4.66±1.52
9.	C3	2	3	4	3±1
10.	C4	4	5	3	4±1
11.	t				0.91
12.	df				28
13.	Significance*				NS

p>0.05(non significance)

6. Effect of treatment and control groups on total fruit weight per pot

The data in Table 11 show the effect of treatment and control groups on the fruit weight of tomatoes per pot. In control groups, C2, C3, and C4, a significant reduction in fruit weight were observed due to fungal infection. Whereas significant improvement in fruit weight was observed which were treated with bioformulations. Maximum fruit eight was found with T3 (81.17g) followed by T1 (71.49g), C1 (70.62 g), T2 (65.88g), T6 (62.01g), T4 (60.56 g), T5 (57.93 g), C2 (43.36 g), C4 (34.86 g) and C3 (26.52g).

Table 11:Effect of different bioformulations and control treatments on weight of tomato fruits

S.No.	Treatments	Weight of tomato fruits (g)			Average ± SD
		R1	R2	R3	
1.	T1	69.12	75.20	70.15	71.49±3.25
2.	T2	59.02	58.63	64.05	60.56±3.02
3.	T3	80.85	80.33	82.33	81.17±1.07
4.	T4	65.30	64.15	68.19	65.88±2.08
5.	T5	60.20	55.37	58.23	57.93±2.42
6.	T6	63.51	62.51	60.01	62.01±1.80
7.	C1	69.25	68.61	74.01	70.62±2.95
8.	C2	44.62	43.21	42.27	43.36±1.18
9.	C3	26.20	25.52	27.84	26.52±1.19
10.	C4	33.52	36.02	35.05	34.86±1.26
11.	**t**				5.57
12.	**df**				28
13.	Significance*				SIGNIFICANT

$p<0.01$ and $p<0.05$ significant at both level (significant)

The plant-based bioformulations use various mechanisms to control diseases in plants such as prevention in fungal multiplication, prevent transcription and translation process in fungal pathogen and also can cause membrane damage in pathogen (Senthilraja et al., 2013). On the other hand, they support antioxidant and defense enzyme activity of plants and also boost the plant immune responses thus help plants in disease management [58,59,60,61,62,63]. In a pot experiment, tomato seeds were treated with several bio formulations and later on infected with *A. alternata* spore suspension by foliar spray. Efficacy of bioformulations compared with control groups and fungicide treated group of plants. It was observed that plant extract-based bioformulations significantly improved the growth parameters of tomato plants in the presence of the pathogen and also inhibits the disease progression by preventing the spread of the pathogen. Statistical analyses revealed that bioformulation number T3 had a significant rise in plant height, the number of leaves per plant, the number of branches per plant, the number of flowers per plant, the number of fruits per plant, and the weight of fruits per plant, followed by formulation numbers T4, T2, and T1. In the present study use of elicitor and binder was aimed to enhance the disease-preventing potential of plant extracts. However, in the advanced studies, nanoparticle-based bioformulations are presently being highly explored to improve the disease controlling ability of plant extracts (Meena, et al., 2017; Meena et al., 2019; Khare and Arora,2010; Khare and singh, 2011; Choudhary et al., 2017). Nanoparticle-based approaches are effective but their cost expensiveness and complex reaction chemistry making their use difficult by the low-income groups of the society. In this context, the present approach of using a combination of plant extract with elicitor and binder is a cost-effective and easy-to-use technology that can be used for plant disease management.

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