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Original Research Article

Comparative Studies of the Antifungal Properties of Mango and Orange Leaves in the Prevention of Gummy Stem Blight in both Water Melon and Cucumber

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ABSTRACT
Gummy stem blight, caused by the fungal pathogen *Didymella bryoniae*, is a significant disease of watermelon (*Citrullus lanatus*) and cucumber (*Cucumis sativus*), leading to reduced yield and economic losses. Fungicides are commonly used to manage this disease; however, they pose risks such as environmental pollution, fungal-resistance and food safety concerns. There is need to explor plant-based alternatives due to their biodegradability, safety and eco-friendliness. This study evaluated phytochemical composition and in vitro antimicrobial potential of ethanolic and methanolic leaf extracts of orange (*Citrus sinensis*) and mango (*Mangifera indica*) against *Didymella bryoniae*, the causal agent of gummy stem blight in watermelon and cucumber. Leaf extracts were prepared using Soxhlet extraction with methanol and water as solvents, and the agar well diffusion method was employed for the susceptibility assay. Preliminary fungal identification was based on conidial and colony morphology, Pathogenicity was conducted on fresh test plants to ensure the organism caused the disease. Phytochemical analysis revealed the presence of tannins, flavonoids, carbohydrates, steroids, and alkaloids in both extracts, with orange leaf extract exhibiting a high concentration of alkaloids and cardiac glycosides (+++). Saponins and anthraquinones were absent in both extracts. Methanolic orange leaf extract demonstrated the highest antifungal activity, with zones of inhibition measuring 88.00 mm and 80.00 mm at concentrations of 200 mg/mL and 150 mg/mL, respectively. In contrast, methanolic mango leaf extract exhibited inhibition zones of 62.23 mm and 57.89 mm at the same concentrations. The lowest concentration (12.50 mg/mL) showed minimal activity (20.21 mm). Fluconazole, used as a control, exhibited inhibition zones of 100 mm at similar concentrations. Statistical analysis indicated significant differences ($P < 0.05$) in the inhibition zones among the various concentrations and between the extracts and the control. The results suggest that orange leaf extract is more effective than mango leaf extract in inhibiting *Didymella bryoniae*.

CITATION
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INTRODUCTION

Tropical fruits are products with excellent global market prospects (Evans and Ballen, 2019). Fruits are rich sources of minerals and vitamins. Hence the need to have it form part of our daily consumption (Bogers *et al*., 2007; Paliyath *et al*., 2008). Cucumber and water melon are rich in antioxidants. They have a lot of commercial benefits. The physiological properties of most fruits makes them highly perishable commodities (Martínez and López, 2021). Postharvest losses can occur at any point in the production and marketing chain, and may range anywhere from >10 % in advanced countries to >30 % in tropical areas and where storage facilities are limited and not readily available (Parfitt *et al*., 2010). Developing countries experiences much more loss (35-50%) due to poor postharvest handling (Paliyath *et al*., 2008).

Gummy stem blight (GSB) is a major disease of many cucurbits, including watermelon, cantaloupe, cucumber, pumpkin, squash, muskmelon, and other melons (Keinath, 2011). Plant diseases on watermelon and cucumber are commonly seen all over the world, and the disease can cause significant production losses when conditions are ideal for the spread of the causal pathogen (Kothera *et al*., 2003). On fruit, this disease is caused by D. bryoniae (anamorph P. cucurbitacearum), is known as black rot whereas the foliage disease is known as Gummy Stem Blight (Keinath, 1995; Keinath *et al*., 1995; Sitterly and Keinath, 1996). Small, water-soaked spots develop on watermelon fruit, enlarge, and exude gummy material. As the symptoms develop, fruiting bodies of the fungus may appear as black specks on the lesions. In the tropics, fruit infection can occur through blossom scars and begin to decay inner fruit tissue, with no visible symptoms on the surface of the host. The decay eventually will progress to the stem end and reach the surface of the fruit (Sitterly and Keinath, 1996).

Biological control of plant pathogens has emerged as a viable disease control strategy (Pal and Gardener, 2006). there are so many factors that are responsible for increasing interest in biological control including the negative effects of fungicides on human health, increased regulatory restrictions, traceability protocols for crop protection practices, nil residue tolerance in some export markets, continued interest in organics, pathogen resistance to commonly used fungicides, and a lack of replacement products (Dawson and Sarris, 2007).

Mango leaves have been traditionally used in different cultures for their medicinal properties. Studies have demonstrated the antimicrobial activity of mango leaf extracts against fungi and both Gram-positive and Gram-negative bacteria. For instance, a study by Mahesh and Satish (2008) found that mango leaf extract exhibited significant antibacterial activity against Escherichia coli, Staphylococcus aureus, and Pseudomonas aeruginosa. The antimicrobial activity was attributed to the presence of bioactive compounds such as flavonoids, tannins, and phenolic acids in the leaves.

Orange leaves, similarly, have been studied for their antimicrobial potential. Research has shown that extracts from orange leaves possess inhibitory activity against various pathogenic bacteria and fungi. In a study by Yang et al. (2010), orange leaf extract displayed antibacterial activity against Salmonella enterica and Listeria monocytogenes. The antimicrobial properties were attributed to the presence of compounds like limonoids, flavonoids, and terpenoids in the leaves. This study therefore compares the antimicrobial activity of mango and orange leaves on gummy stem blight of cucumber and watermelon.

MATERIALS AND METHODS

The study was carried out in the Department of Science Laboratory Technology University of Jos. The sample of water melon and cucumber infected with gummy stem blight (black rot) as seen in Plate 1 and Plate 2 were obtained separately in sterile polythene bag form Farin Gada and Terminus market and were transported to the laboratory for analysis. 100g of orange and mango leaves where collected separately and were also transported to the laboratory for analysis.

Plate 1: Diseased Cucumber sample obtained from Terminus market

Plate 2: Diseased Watermelon sample obtained from Terminus market.

Media preparation

All the media used in the experiment were prepared according to the manufacturer's instruction.

plant preparation and extraction

The leaves were air dried at 30^{0}C and grinded into powdery form. 150g of the powdered leaves was subjected to extraction with 600 ml of methanol. The extract was separately filtered using Whitman's no1 filter paper. Then the extract was concentrated in vacuum using a rotary evaporator at 40^{0}C. The methanol remaining in the extract was removed by placing it at room temperature overnight to give a residue weighing 8g.

Phytochemical Screening of Successive Extracts

The presence of glycosides, tannins, flavonoids, alkaloids, saponins, carbohydrates, proteins and water soluble vitamins in the reconstituted leaf extract was examined by standard phytochemical methods (Harbone, 1998).

Pathogen Isolation, Identification and Pathogenicity

Sections (0.5 cm in diameter, six pieces per friuts) were cut from the margins of necrotic or symptomatic tissue with a sterile scalpel, surface disinfested in 0.6% sodium hypochlorite for 10s, it was rinsed twice in sterile distilled water, and dried on sterile filter paper. The tissue sections were then plated on half-strength potato dextrose agar (PDA; Difco Laboratories, Detroit, MI) amended with 0.5 g/L of streptomycin sulfate to prevent bacteria growth. Petri dishes were incubated at 23°C in the dark for 5 to 7days. The isolates were transferred onto water agar (WA), and hyphal tips from the margin of actively growing cultures were removed with a sterile scalpel and plated on full strength PDA to generate pure cultures. The fungal strains were subjected to lactophenol cotton blue staining for morphological studying. Preliminary identification of the isolates was based on conidial and colony morphology and pigmentation PDA, respectively (Leslie and Summerell 2006).

Pathogenicity test was carried out on healthly cucumber and water melon fruits to confirm that the organism was pathogenic and caused the same symptoms as was seen on the disesead plant.

Determination of Antifungal Activity of the Extracts

The crude extracts were screen for its antifungal activity i.e. determination of zone of inhibition against tested organism by agar well diffusion method as described by (Perez, *et al*., 1990). Sterile PDA gar plates were inoculated with prepared inoculum with sterile cotton swab. Then with the help of sterile cork borer no. 6, wells were made in the inoculated media plate. 50 µl of the working solution/ suspension of different concentration were transferred into the well with the help of micropipette. The control was also placed in the separate well at the same time. After proper incubation, the plates were viewed for the zone of inhibition, which is suggested by clear areas without growth around the well.

Determination of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

The crude extract which showed antifungal activity, were subjected to two-fold serial dilution method to determine minimum bactericidal concentration (MBC). A set of 12 screw capped test tube containing 1ml nutrient broth was required. The test tubes were labeled as positive growth control, negative growth control and numbers 1 to 10. Different dilution of crude extract as 75%, 50%, 25% and 5% was made. Then two fold serial dilution of the extract was prepared each containing equal volume but decreasing concentration. 50µl of culture inoculum of test fungi was added to each tube with the help of micropipette except negative control.

All the tubes were incubated at 15^{0}C for 3days and observed for turbidity by comparing with +ve and –ve control.

The results were interpreted on the basis of the fact that growth occurs in the positive control and any other tube in which the concentration of the extract is not sufficient to inhibit growth and lowest concentration of the agent that inhibits growth of the organism as detected by lack of visible turbidity as designated the minimum inhibitory concentration (MIC). However, in some cases it was difficult to identify whether the turbidity was due to the growth of bacteria or due to the turbidity of extract itself, the tubes were subcultured on nutrient agar plate with proper label followed by incubation at 15^{0}C for 2-3days. Then they were examined for the growth of fungi. The antifungal activity of both plant extract were observed and compared to determine the best plant extract which can be used to inhibit the fungi Causing gummy stem blight of cucumber and water melon.

Statistical analysis of data

The statistical analysis of the data and comparison of the means was done using the SAS software and the Duncan's multiple range tests, respectively. Transformation of the data was done as needed.

RESULTS AND DISCUSSION

Results

Result of photochemical analysis of orange and mango leaf extract (Table 1), reveals the presence of tannins, flavonoids, Carbohydrates, Steroids and Tarpones and absence of Saponin and Anthraquinones. There was high concentration of Alkaloid and Cardiac glycoside of (+++) in the orange extract. The percentage yield of the methanolic extract of orange and mango peels was 37.60% and 21.10% respectively. Subsequently, the methanol extracts of each plant material was selected for the antimicrobial activity based on the percentage yield produced by the solvent.

The pathogen was isolated on PDA medium and morphological characters of the isolated fungal pathogen were studied on culture plates as well as under microscope. Table 2-5 indicates the morphological characteristics of the fungi associated with gummy blight of both cucumber and water melon. Colony morphology on culture plates was compared with the characteristics of the causal agent of GSB disease explained by Keinath *et al.,* (1995). *Didymella bryoniae* was identified as the fungi associated with the disease after Preliminary identification of the isolates based on conidial and colony morphology and pigmentation in all the culture plate.

The result shows the susceptibility patterns of the test isolate to the varying concentrationsas seen in figure 1. At concentration higher antibacterial activity was exerted on

the test organisms by all the extracts. The methanolic extract of orange leaf at a highest concentration of 200mg/mL and 150mg/mL was 88.00mm and 80.00 mm respectively on the test isolate with 50.00mm as the least at 12.50mg/mL while the methanolic extract of mango leaf extract at a highest concentration of 200mg/mL and 150mg/mL has a zone of inhibition of 62.23mm and 57.89 respectively, the least concentration 12.50mg/ml has a concentration of 20.21mm. The control fluconazole at a highest concentration of 200mg/mL and 150mg/mL has a zone of inhibition of 100mm as shown in table 6.

Table 7 represents the Minimum Inhibitory Concentration (MIC) of the extracts against the organisms. A constant MIC value of 12.50mg/ml was obtained for the methanolic extracts against test isolate; the methanolic extracts had MIC values varying between 10.25-20mg/ml on the isolate. The methanolic extracts of both lemon and lime showed fungicidal effects on the test organisms with value as low as 11.00mg/ml for all the isolates.

Table 1: Phytochemical analysis of orange and mango leave

Constituents	Mango leaf	Orange leaf
Alkaloid	_	+++
Saponin	_	_
Tannin	+++	++
Flavonoids	+++	+++
Carbohydrates	++	++
Steroids	++	++
Tarpones	++	++
Anthraquinones	_	_
Cardiac glycoside	+	+++
% yields	37.60%	21.10%

Key:
(+) = Present in trace amount
(++) = Present in moderate amount/ concentration
(+++) = Present in high amount/ concentration
(-) = Absent

Table 2: Cultural and Morphological characteristics of *Didymella bryoniae*

Cultural characteristics	Morphology characteristics	Isolate
White to olive to dark green with flat Elevation	White substract mycelium and white hairy aerial mycelium. Pycnidia and psuedothecia present. Conidia and septa are present. Mean dimension of conidia ranging from 6.2 μm to 9.3 μm in length and 0.05 to 3.63 μm in the width.	*Didymella bryoniae*

Table 3: Cultural and Morphological characteristics of watermelon samples isolate collected from faringada market

Cultural characteristics	Morphology characteristics	Isolate
White to olive to dark green with flat Elevation	White substract mycelium and white hairy aerial mycelium. Pycnidia and psuedothecia present. Conidia and septa are present. Mean dimension of conidia ranging from 7.2 μm to 10.5 μm in length and 0.15 to 3.63 μm in the width.	*Didymella bryoniae*

Table 4: Cultural and Morphological characteristics of cucumber samples isolate collected from terminus market

Cultural characteristics	Morphology characteristics	Isolate
White to olive to dark green with flat Elevation	White substract mycelium and white hairy aerial mycelium. Pycnidia and psuedothecia present. Conidia and septa are present. Mean diamension of conidia ranging from 5.6 μm to 9.4 μm in length and 0.11 to 1.63 μm in the width.	*Didymella bryoniae*

Table 5: Cultural and Morphological characteristics of watermelon samples isolate collected from terminus market.

Cultural characteristics	Morphology characteristics	Isolate
White to olive to dark green with flat Elevation	White substract mycelium and white hairy aerial mycelium. Pycnidia and psuedothecia present. Conidia and septa are present. Mean diamension of conidia ranging from 8.2 μm to 12.5 μm in length and 0.05 to 3.63 μm in the width.	*Didymella bryoniae*

Table 6: Mean zone of inhibition of methanolic extract of orange and mango leaf on *Didymella bryoniae*

Isolate	Extract	Concentration (mg/ml)					
		200	150	100	50	25	12.5
Didymella bryoniae	Orange leaf extract	89.0±1.2	80.0±0.4	76.2±0.4	70.9±0.2	64.1±0.4	50.1±1.5
	Mango leaf extract	62.2±1.1	57.9±1.0	50.3±0.4	43.4±0.3	39.8±1.1	20.2±1.2
	Positive control (fluconazole)	100±0.0	100±0.0	100±0.0	94.1±0.5	94.1±0.2	94.1±0.3

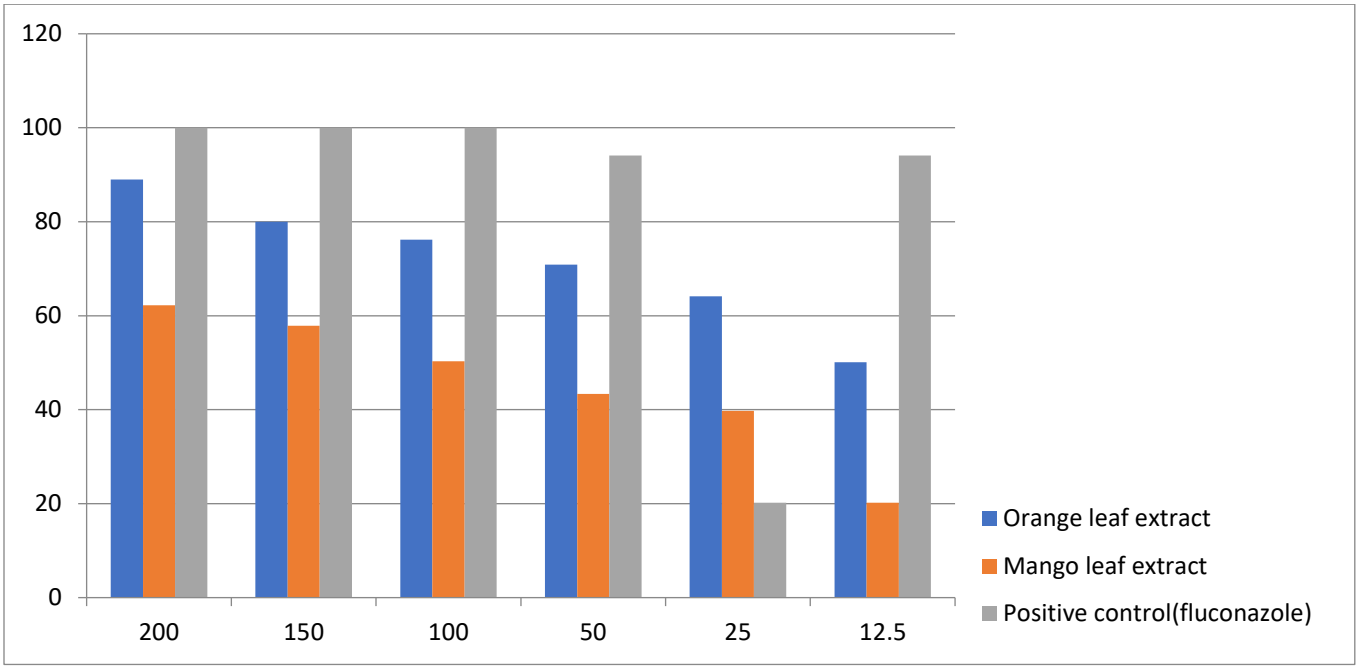

Figure 1: Graphical representation of the Mean zone of inhibition of methanolic extract of orange and mango leaf on *Didymella bryoniae*

Table 7: Results showing the test for the minimum inhibitory concentration as well as the Minimum Fungicidal Concentration of Methanol Extracts against test organisms.

Fungi	Minimum Inhibitory Concentration (MIC) mg/ml	Minimum fungicidal Concentration (MFC) in mg/ml
Didymella bryoniae	12.50	11.00
Positive control	25.00	25.00

Discussion

The use of plant extracts to control plant diseases is a means of bicontrol that is explored in plant pprotection. This is due to the toxic nature of chemical fungicites and their harmful effects to the comsumers. Secondary metabolites in citrus plants have been identified as therapeutic agents in the management of several diseases. Phytochemical analysis of Citrus sinensis has revealed the presence of carbohydrates, flavonoids, glycosides, coumarin glycosides, volatile oils, organic acids, fats and fixed oils (Raza *et al*., 2019). Tannins, flavonoids, saponins, phenolic compounds and essential oils are believed to be the phytochemicals responsible for the antimicrobial effects of plants. Flavonoids have been linked to several biological activities including antibacterial, antioxidant and inflammatory activities. They are also known to possess the capacity to modulate enzymatic activities and inhibit cell proliferation. In plants, they are known to play a defensive role against invading pathogens. Tannins form complexes with prolinerich proteins that inhibit cell protein synthesis. Synergistic action of tannins, flavonoids, alkaloids and saponins are known to inhibit the growth of pathogens (Rahman *et al*., 2011).

Phytochemical studies of orange and mango leaf methanolic and ethanolic extract which indicates the presence of presence of tannins, flavonoids, Carbohydrates, Steroids and Tarpones with saponin and Cardiac glycoside in high concentration in the orange leaves extract also agreed with the studies by Nayak *et al*. (2012).

The Identified fungi associated with the gummy stem blight disease after Preliminary identification of the isolates based on conidial and colony morphology and pigmentation in all the culture plate collection from both terminus and faringada market showed *Didymella bryoniae* as the causal agent of gummy stem blight disease. Result of the morphological identification of the isolate is similar to work of Babu *et al*., (2015) who characterize *Didymella bryoniae* infecting water melon and other cucurbits in Florida and Georgia.

This study also revealed that the methanolic extracts of orange and mango leaves possesses high antifungal activity as it showed the broadest spectra against *Didymella bryoniae* tested. This could be attributed to the presence of a high concentration of phytochemicals which in turn is facilitated by the solubility capabilities of methanol used as an extraction solvent. Similar reports in concordance with this phenomenon include the works of (Khushwaha *et al*., 2012).

The susceptibility patterns of the test isolate to the varying concentrations. At concentration higher antibacterial activity was exerted on the test organisms by all the extracts. The methanolic extract of orange leaf was 89.00mm followed by 80.00mm at a highest concentration of 200mg/mL and 150mg/mL respectively on *Didymella bryoniae* with 50mm having the least inhibition at 12.50 mg/ml. Meanwhile, susceptibility rates of the methanol extract of the mango leaf showed the highest zones of inhibitions of 62.23mm and 57.89mm all on *Didymella bryoniae* at a concentration of 200mg/ml and 150 mg/mL respectively. The results of this study are consistent with those of Badar *et al*.,(2008) that some plants contain active compounds that have the ability to inhibit certain microorganisms, the researcher explained that these compounds differ in their chemical structures and thus vary in inhibitory activity in the growth and survival of fungi .

Low MIC values were obtained for the extracts, with a constant value of 12.5mg/ml for the methanolic extract. This result also emphasizes the high antimicrobial potency of extracts of orange peel and mango peels because a low MIC indicates a high efficacy of the extract. At a low concentration of 12.5mg/ml, the methanolic extract exerted fungicidal effects on *Didymella bryoniae*. The methanolic extracts also had MFC values of 11.00mg/ml-25mg/ml. Hence, extracts of orange and mango leaves can effectively lyse fungi cells at low concentrations, thus confirming the aforementioned high antimicrobial efficacy of the plants. However, all the test plant extracts at varying concentrations inhibited mycelia growth. The inhibitory effect of the test plants increased with concentration of the extract. The degree of control of the test organism by different plant extracts varied and was significant ($P < 0.05$).

CONCLUSION

The significant growth inhibitions of the test organisms by the two plants extracts suggests their possible use in controlling these organisms in disease-causing situations and opportunistic human infections. Methanol extract of the orange leafs extract was the most effective, revealing its potential efficiency for the treatment of diseases arising from this organism. The antifungal profile of these plant

extracts underlines their recognition and application as medicinal plants. The results obtained from this research proved that the extracts of orange and mango leaves have varying degree of fungal activity against the test organisms. This suggested that extracts of orange and mango peels can be useful in developing a new drug, which can be used in treating fungi causing gummy stem blight of both cucumber and water melon infections caused by the test organisms in this study.

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