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Evaluation of antifungal effect of medicinal plants against Panama wilt of Banana caused by *Fusarium oxysporum* f. sp. *cubense*

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ABSTRACT

Panama wilt stands first among all the major fungal diseases affecting banana, by which farmers are facing huge economic losses globally, which is caused by one of the deadly fungus, *Fusarium oxysporum* formal species *cubense* (*Foc*). *Nanjungud rasabale*, which has been given indication tag, is devastated by this fusarium wilt. In the present study, we investigated the *in vitro* biological control of *Foc* using some locally available medicinal plants such as *Prosopis juliflora*, *Piper betle*, *Garcinia indica*, *Callistemon lanceolates*, *Azadirachta indica*, *Decalepis hamiltonii,* and *Combretum indicum*. Soxhlet extraction of selected plants was done using methanol and antifungal activity was determined by poisoned food technique, agar well diffusion, and disk diffusion method. All the botanicals employed in the study reduced the mycelial growth of fungus in different concentrations at various levels. Among them, *G. indica* exhibited highest rank of antifungal activity against the tested plant pathogen *Foc,* thenceforth by *P. betle*. Results revealed that *G. indica* is a potential source of antifungal botanicals; therefore, substantial research is required to take out their active phytochemicals, thus providing a replacement to chemical fungicides and a possible alternative approach to contemporary management practices for Panama wilt of banana.

1. INTRODUCTION

Among the different production risks affecting Banana farming, *Fusarium* wilt is considered as the most important disease globally, particularly in the tropical and subtropical regions [1,2]. This disease is caused by *Fusarium oxysporum* f. sp. *cubense* (Foc). It was first noticed in Australia in 1874. The disease, now, has been reported from the most Banana growing regions [3]. In India, except Nendran and Red Banana, the disease is causing extensive damage in entire Banana cultivation areas particularly affecting nearly all commercial varieties [2,4].

Karnataka is well known for the production of prime quality Bananas specially "Nanjangud rasabale" (NRB) which has its origin from a place called "Nanjangud" in the Mysuru district. Due to its high nutritional content, good fibrous texture, excellent taste, color, and aroma, NRB is most popular and pricey fruit in Karnataka and also in other states. However, the Banana wilt is a big limitation for the profitable production of this elite variety [5].

Foc is a soil-residing filamentous fungus that mainly produces asexual reproductive structures called chlamydospores, micro and macroconidia which are produced on branched or unbranched monophialides [Figure 1]. This enables the fungus to survive for additional 30 years in the soil, even without related host [6].

Through the roots, fungus infects Banana plants and enters xylem (vascular tissue). Then, it blocks the transportation of water and minerals causing certain visible external symptoms such as progressive wilting, gradual yellowing of leaves which spread from outer leaf margins and extend to the middle and from older leaves to younger parts and finally collapsing at the petiole region and splitting of outer leaf tissues takes place longitudinally in the pseudostem of Banana plant [7,8]. The disease shows internal symptoms such as quintessential discoloration ranging from light yellow to dark brown color. It affects vascular tissue particularly in roots, rhizome, and pseudostem and also in the stalk of the fruit [9,10]. In the due course, the disease causes death of Banana plants [8] [Figure 2].

Till date, no control methodology has been introduced to effectively manage the disease. Due to the soil borne nature of *Fusarium* and

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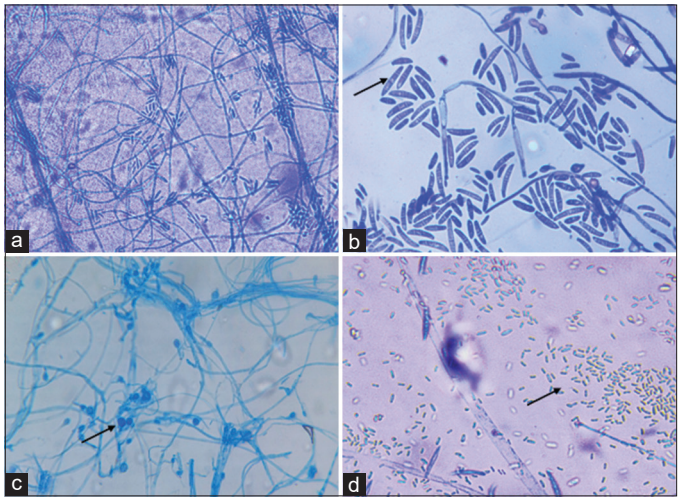

Figure 1: Reproductive structures of *Fusarium oxysporum* f. sp. *cubense*. (a) Mycelium, (b) Macroconidia, (c) Chlamydospores, and (d) Microconidia.

Figure 2: Symptoms of *Fusarium* wilt of Banana. (a) Plant showing Pseudostem splitting, (b) transversal cut of pseudostem showing an advanced necrosis of vascular tissues, (c) leaf falling by petiole collapse, (d) transversal section of rhizome showing tissue necrosis, and (e) general yellowing and necrosis of leaves ("yellow leaf syndrome") in advance stage of disease.

chlamydospores, it is hard to manage the disease [11]. Quarantine is the only best approach [12-14]. However, these practices could be applied largely in big plantations, not in small holder settings [15].

Keeping in picture of downsides of chemical fungicides using for the control of disease, the use of botanicals for developing management approaches is gaining more importance nowadays. Hence, in the current investigation, seven medicinal plant extracts were tested *in vitro* against the pathogen [16,17].

2. MATERIALS AND METHODS

2.1. Survey on Panama Disease

Period 2017–19, the survey was conducted at various places of Mysuru and Chamarajanagara districts. Type of soil, disease incidence, type of the planting material and cultivar used, and vascular infection of pseudo stem were recorded. Samples from each site were collected for the isolation of pathogen. Random samples were taken in each plot, total number of plants and number of plants affected were calculated as percent disease incidence [18].

$$Percent\ disease\ incidence = \frac{No.\ of\ plants\ showing\ wilting\ symptom}{Total\ number\ of\ plants} \times 100$$

2.2. Pathogen Isolation from Plant Material

The wilt affected parts of Banana plant (rhizome, pseudostem) and soil were collected separately in a polythene bags and labeled. Infected material was sectioned into small pieces and were washed properly under tap water and surface sterilized with 0.1% sodium hypochlorite. The prepared plant pieces were plated on PDA (Hi Media) medium, incubated at 22±1°C for 7–15 days under 12/12 h under U V light and darkness.

The pathogen was identified based on the morphological characteristics. Pure culture was obtained and maintained for further studies [19] [Figure 3].

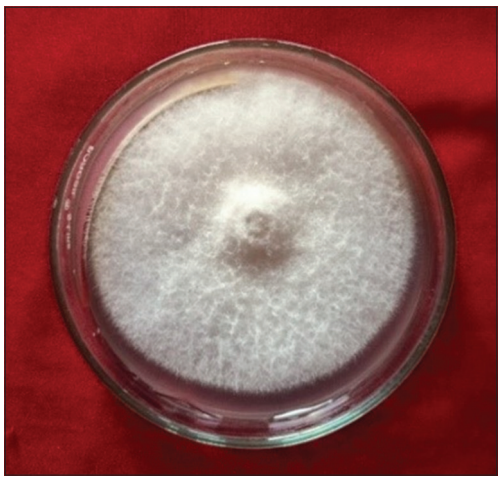

Figure 3: *Fusarium oxysporum* f. sp. *cubense* on PDA media.

2.3. Preparation of Plant Extracts

Seven medicinal plants [Table 1] were selected based on the earlier work conducted in the Center for Innovative Studies in Herbal Drug Technology, Department of studies in Botany and literature review. The plant materials were collected and washed under running tap water, dried, and then homogenized in to a coarse powder and preserved in

Table 1: List of plants used in the study.

Botanical name	Common name	Family	Part used
Piper betle	Betel	Piperaceae	Leaves
Prosopis juliflora	Algaroba	Mimosaceae	Leaves
Garcinia indica	Kokum	Clusiaceae	Dried Fruit rind
Callistemon lanceolatus	Bottle brush	Myrtaceae	Leaves
Azadirachta indica	Neem	Meliaceae	Leaves
Decalepis hamiltonii	Makali beru	Apocynaceae	Roots
Combretum indicum	Rangoon creeper	Combrataceae	Leaves

sealed bottles. About 50 g of coarsely powdered plant material was subjected to Soxhlet extraction using methanol as a solvent. Extract was collected and dried using rotary evaporator and preserved for future studies [20].

2.4. Extract Preparation for Antifungal Assay

The samples were solubilized at different concentration (50 mg/ml, 100 mg/ml, and 200 mg/ml) in 20 % methanol and sonicated for 30 min. Dissolved samples were centrifuged [21].

2.5 Poisoned Food Technique

For poisoning food technique, PDA media was prepared, autoclaved, and poured into glass petri plates (10 ml/plate). Plant extract was added to PDA at concentration of 50 mg/ml, 100 mg/ml, and 200 mg/ml and allowed to solidify. Round disk of 6 mm from 7-day-old culture with grown mycelium was taken and was inoculated in center of media plate upside down. Chlorhexidine was used as a commercial standard and methanol is used as blank. After inoculation, plates were incubated at 30°C for 3–7 days [22,23]. Zone of inhibition (ZOI) was measured (in mm) and percentage of growth inhibition was determined using the formula:

$$\% \text{ of Growth inhibition} = \frac{dc - dt}{dc} \times 100$$

where, dc = Average increase in mycelial growth in control.

dt = Average increase in mycelial growth in treated media.

2.6. Agar-Well Diffusion Method

Test plates were prepared with 20 ml of PDA for well diffusion method. After media get solidified, 100 µL of *Foc* suspension of 0.5–2.5×10^3 concentration was added and uniformly spread over plates using L shaped rod. For well diffusion assay, wells (about 6mm diameter) were prepared and 40 µL with different concentration of the plant extract were added. 30 µL Chlorhexidine (200 µg/ml) was used as a commercial standard. 20% methanol was loaded as blank. A loading was completed, the plates were kept in aseptic conditions to allow the full absorption of plant extracts employed in the study. After that, plates were incubated at appropriate growth conditions for 24–48 h. After the period of incubation, plates were examined for the inhibition zone of fungal growth and measured in mm [24].

2.7. Disk Diffusion Method

Test plates were prepared with 20 ml of PDA for disk diffusion method. After media get solidified, 100 µL of *Foc* suspension of 0.5–2.5 × 10^3 was added and uniformly spread over plates using L shaped rod. Sterile disks were placed and loaded with 40 µL different concentration of the plant extract step by step with a time gap of 20 min for sample diffusion. 30 µL Chlorhexidine (200 µg/ml) was used as a commercial standard. 20% methanol was loaded as blank. once loading was complete, plates were kept in aseptic conditions to allow the complete absorption of plant extracts employed in the study. After that, the plates were incubated at appropriate growth conditions for 24–48 h. After the period of incubation, plates were examined for the inhibition zone of fungal growth encircling the well were measured in mm and recorded [24].

2.8. Minimum Inhibitory Concentration (MIC) Assay

The MIC was determined based on the broth dilution method using 96 well plates [25]. For the determination of MIC, inoculum suspension was prepared from broth cultures. *Foc* culture was adjusted to 0.5 McFarland turbidity standards (1.5 × 108 CFU/ml) and 10 µL of diluted suspensions of fungal culture was added to 50 µL of various concentrations of methanolic extract of *Garcinia indica* in to the well. The 40 µL of extract from 50 mg/ml stock was added in the three columns of first row. The extract was serially diluted from first row to last row. The sample concentration ranged from 20 mg/ml, 10 mg/ml, 5 mg/ml, 2.5 mg/ml, 1.25 mg/ml, 0.625 mg/ml, 0.3125 mg/ml, and 0.15625 mg/ml for samples from first to last row. 30 µL chlorhexidine from 200 µg/ml stock was added in the first row, 3 column of standard. The standard was serially diluted from the first to last row. The standard concentration ranged from 60 µg/ml, 30 µg/ml, 15 µg/ml, 7.5 µg/ml, 3.75 µg/ml, 1.875µg/ml, 0.9375 µg/ml, and 0.469 µg/ml. Sterile broth with 20% methanol served as a media blank and only *Foc* culture without treatment was considered as control. Plates were incubated at 25±2°C for 48 h. After incubation, optical density was taken at 600 nm to analyze the inhibition of *Foc*.

3. RESULTS

The survey data collected during 2017–2019 in the main Banana growing regions of Mysuru and Chamarajanagar are presented in Table 2. In Chamarajanagar district, the incidence of wilt disease was highest (95.4%) in Singanalluru, Kollegala (T) Chamarajanagar (D) followed by Duggahalli (90%) Nanjanagudu (T) Mysuru (D).

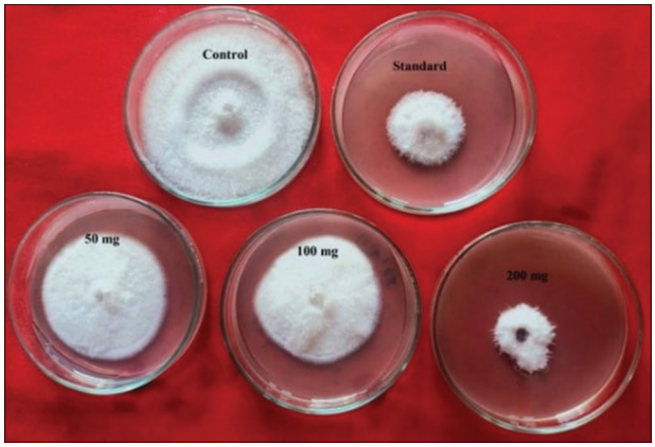

Figure 4: Poison food technique showing antifungal activity of *Garcinia indica* against *Foc.*

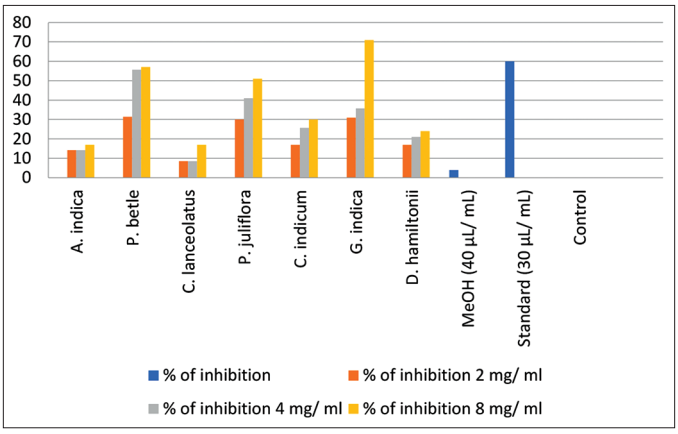

Figure 5: Percentage of inhibition in poison food technique.

Results of poisoned food technique for determining antifungal efficacy of seven methanolic plant extracts against Panama wilt pathogen are shown in Table 3. All the seven plant extracts showed inhibition of *Foc in vitro* with different percentage.

Among all the different treatments used, results showed that highest inhibition, that is, 71% was recorded in 200 mg/ml methanolic plant extract of *G. indica* (20 mm) [Figure 4]. About 57% were recorded in 200 mg/ml methanolic extract of *Piper betle* which showed 30 mm ZOI. The least inhibition was observed in *Callistemon lanceolatus* (17%). Moderate inhibition was observed in methanolic extracts of *Prosopis juliflora* (51%), *Combretum indicum* (30%), and *Decalepis hamiltonii* (24%) [Figure 5].

Results of disk diffusion method showing antifungal efficacy of seven methanolic plant extracts against Banana wilt pathogen are presented

Table 2: Survey for incidence of Panama wilt of Banana.

Place	Variety/cultivar	Soil type	Planting material used	Incidence of disease (%)	Pseudostem vascular infection	Stage of crop (Months)
Singanalluru Kollegala Chamarajanagar	Ney Poovan (AB) (Yalakki bale)	Red loamy soil	Sucker	95.4	5	6
Near temple T Narasipura	Ney Poovan (AB) (Yalakki bale)	Red soil	Tissue cultured	81.8	4	7
Thumbala, T. Narasipura Mysuru	Ney Poovan (AB) (Yalakki bale)	Red soil	Tissue cultured	16.6	4	7
Mallupura, Nanjangud Mysuru	Ney Poovan (AB) (Yalakki bale)	Red loamy soil	Sucker	25	4	6
Vatalpura T. Narasipura Mysuru	Ney Poovan (AB) (Yalakki bale)	Red loamy soil	Sucker	22.2	5	10
Alathur Nanjangud Mysuru	Ney Poovan (AB) (Yalakki bale)	Red loamy soil	Sucker	10	3	8
Pillahalli, Mysuru	Rasthali (AAB) (Rasabale)	Red loamy soil	Tissue cultured	28	3	6
Basavanapura Chamarajanagar	Ney Poovan (AB) (Yalakki bale)	Black soil	Sucker	10	2	5
Duggahalli (Plot 1) Nanjanagudu Mysuru	Ney Poovan (AB) (Yalakki bale)	Red loamy soil	Sucker	36.6	4	7
Duggahalli (Plot 2) Nanjanagudu Mysuru	Ney Poovan (AB) (Yalakki bale)	Red loamy soil	Sucker	90	6	10

Pseudostem Vascular Infection Scaled range from 1 to 6, 0: No disease, 1: Corm completely clean, no vascular discoloration, 2: Isolated points of discoloration in vascular tissue, 3: Discoloration up to 1/3rd of vascular tissue, 4: Discoloration of between 1/3rd and 2/3rd of vascular tissue, 5: Discoloration greater than 2/3rd of vascular tissue, 6: Total Discoloration of vascular tissue

Table 3: Poison food technique showing antifungal activity.

Samples	ZOI (cm)	% of inhibition	ZOI (mm) of sample 2 mg/ml	% of inhibition 2 mg/m	ZOI (mm) of sample 4 mg/ml	% of inhibition 4 mg/ml	ZOI (mm) of sample 8 mg/ml	% of inhibition 8 mg/ml
Azadirachta indica	-	-	60	14.2	60	14.2	58	17
Piper betle	-	-	48	31.4	31	55.7	30	57
Callistemon lanceolatus	-	-	64	8.5	64	8.5	58	17
Prosopis juliflora	-	-	49	30	41	41	34	51
Combretum indicum	-	-	58	17	52	25.7	49	30
Garcinia indica	-	-	**48**	**31**	**45**	**35.7**	**20**	**71**
Decalepis hamiltonii	-	-	58	17	55	21	53	24
Control	7	-	-	-	-	-	-	-
MeoH (40 µL/mL)	6.7	4	-	-	-	-	-	-
Standard (30 µL/mL)	2.8	60	-	-	-	-	-	-

ZOI: Zone of inhibition; Bold value: Plant extract showing significant activity

Table 4: ZOI of samples against *Fusarium oxysporum* f. sp. *cubense* using Disk diffusion.

Samples	*ZOI (mm) of Standard (6 µg/well)	ZOI (mm) of sample 50 mg/ml	ZOI (mm) of sample 100 mg/ml	ZOI (mm) of sample 200 mg/ml
Azadirachta indica	15	0	0	10
Piper betle	15	11	15	20
Callistemon lanceolatus	16	11	17	21
Prosopis juliflora	17	19	20	21
Combretum indicum	17	15	17	20
Garcinia indica	17	18	25	29
Decalepis hamiltonii	16	0	0	0

*ZOI: Zone of Inhibition

Table 5: ZOI of samples against *Fusarium oxysporum* f. sp. *cubens* using well diffusion.

Samples	ZOI (mm) of Standard (6 µg/well)	ZOI (mm) of sample 50 mg/ml	ZOI (mm) of sample 100 mg/ml	ZOI (mm) of sample 200 mg/ml
Azadirachta indica	15	0	09	1
Piper betle	15	16	21	25
Callistemon lanceolatus	17	14	18	23
Prosopis juliflora	16	18	19	22
Combretum indicum	14	13	14	16
Garcinia indica	**15**	**21**	**26**	**29**
Decalepis hamiltonii	16	0	0	0

ZOI: Zone of inhibition; Bold value: Plant extract showing significant activity

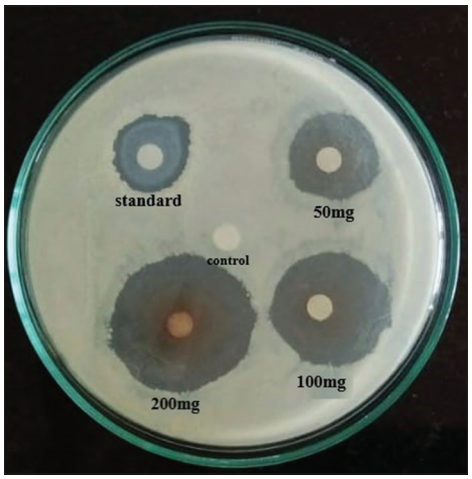

Figure 6: Zone of inhibition of *Garcinia indica using* disk diffusion method.

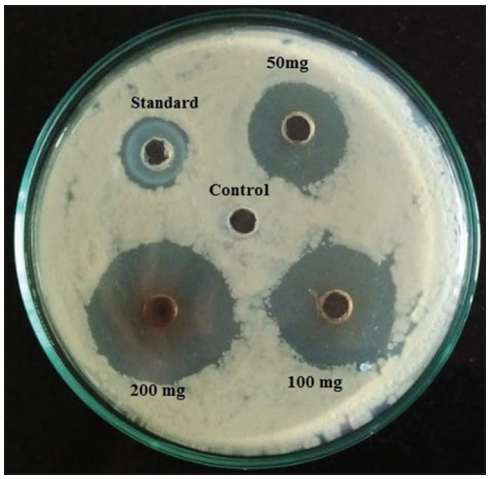

Figure 8: Zone of inhibition of *Garcinia indica using* well diffusion method.

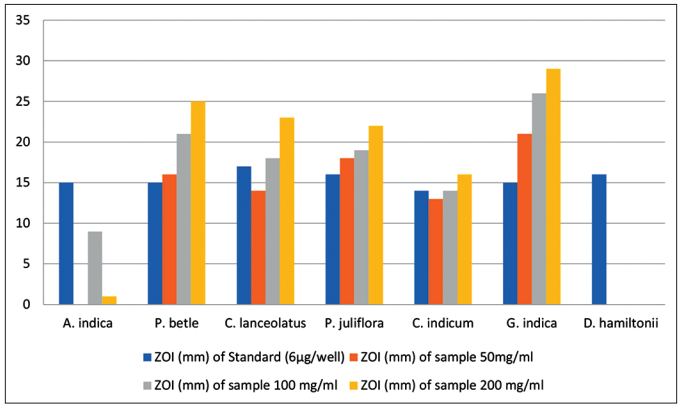

Figure 7: Zone of inhibition in disk diffusion method.

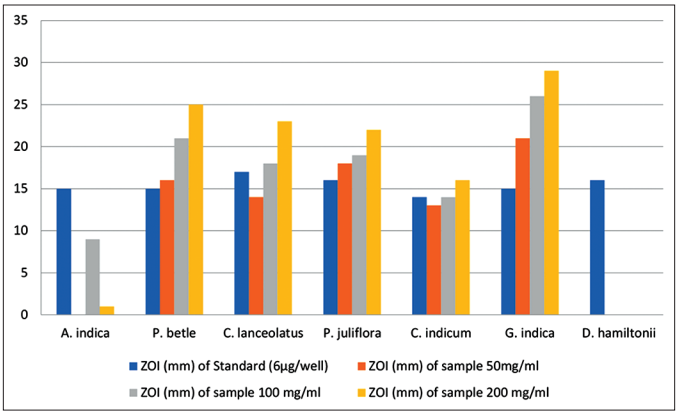

Figure 9: Zone of inhibition in well diffusion method.

in Table 4. Among seven extracts employed for the study, *G. indica* exhibited significant antifungal activity against *Foc* with the maximum ZOI of 29 mm at the concentration of 200 mg/ml [Figure 6]. Other two concentrations of *G. indica* also showed good efficacy against *Foc* with the ZOI 25 mm and 18 mm at the concentration of 100 mg/ml and 50 mg/ml, respectively. *P. betle* methanolic extract also demonstrated good results against Banana wilt pathogen with the ZOI of 21 mm at the concentration of 200 mg/ml [Figure 7].

Results of agar-well diffusion method for seven methanolic plant extracts against Banana wilt pathogen are tabulated in Table 5. Figure 8 shows the inhibition zone diameters for *G. indica* which showed the maximum efficacy against *Foc* with the ZOI of 29 mm at the concentration of 200 mg/ml, this is followed by *P. betle* which showed the ZOI 25 mm at 200 mg/ml concentration [Figure 9].

In the present study, it was found that the methanolic extract of fruit rind of *G. indica* at all the concentrations in all the three methods showed significant antifungal activity against Banana wilt pathogen – *Foc*. Hence, *G. indica* was used for MIC assay.

The MIC is defined as the lowest concentration that inhibited the fungal growth to an absorbance lower than 0.05 at 600 nm. The MIC of the methanol extract of fruit rind of *G. indica* was found to be at 0.625 mg/ml.

4. DISCUSSION AND CONCLUSION

Banana is one of the main food and fruit worldwide. Fusarium wilt is increasingly threatening the Banana production. Traditional methods like chemical control of *Foc* are employed, but they are not adequate to control the disease. Besides, the methods are hazardous to environment [26]. Therefore, the environment friendly approaches are required to control the wilt pathogen [27].

The hunt for antifungals from natural sources like plant has gained much importance nowadays and attempts have been put into point out compound in plants that can act as convenient antifungal agent to replace the chemical pesticides. Phytochemicals procured from plant products set out as an example to evolve less harmful or nontoxic and more efficient medicine for managing the growth of microorganism [28,29].

In the present work, antifungal efficacy of seven plant extracts was tested against the wilt fungus. It is noticeable that, all the seven methanolic extracts of plants are efficient in inhibiting the growth of mycelia of *Foc.*

Results of the present study depict that *G. indica* fruit rind extract showed very good antifungal effect in minimum concentration in comparison with the commercial standard used in all the three techniques employed in the study. *P. betle* also showed good efficacy against the pathogen by showing inhibition of *Foc in vitro*. These results are in accordance with the study of Gnanashekaran *et al*., [29] who tested five plants against *Foc*, among them, *P. betle* plant extract exhibited maximum antifungal activity.

In the study of Mengane and Kamble [16], *Azadirachta indica* showed maximum inhibition of the wilt pathogen followed by *Eucalyptus* and *Artessemia*. In our work, *Azadirachta* also showed inhibition of *Foc*. Ul Qurat and Shahzad [30], work using some Gymnospermic cone extracts against Banana wilt fungus, showed up to 70% inhibition of the test fungus. Kubara *et al*. [31], found that *Acacia nilotica* leaves extracts are good in inhibiting the pathogen. Among the three plant extracts examined at the concentration of 75%, 50%, and 25% plant extracts suppressed the disease in seedlings with the survival rate of 50%, 49%, and 48%.

From the current work and review of literature, we can find that plants can be excellent source of phytochemicals and would definitely yield better antifungal products that could be more friendly with environment and can be biodegradable.

From the results of present study, it is concluded that the fruit rind extract of *G. indica* is effective against panama wilt pathogen *Foc*. Further, isolation and characterization of bio active compounds in the extract and field experiments are to be carried out to recommend the herbal remedy against the Banana wilt.

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6. AUTHORS' CONTRIBUTIONS

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work. All the authors are eligible to be an author as per the international committee of medical journal editors (ICMJE) requirements/guidelines.

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8. CONFLICTS OF INTEREST

The authors report no financial or any other conflicts of interest in this work.

9. ETHICAL APPROVALS

This study does not involve experiments on animals or human subjects.

10. DATA AVAILABILITY

All data generated and analyzed are included within this research article.

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