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In vitro Antifungal Efficacy of Some Plant Extracts against Fungal Pathogens Causing Diseases in Solanaceous Crops

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ABSTRACT

Background: This research aimed to assess the antifungal potential of oil and organic leaf extracts obtained from *Ocimum gratissimum, Kopsia fruticosa* and *Hibiscus rosa-sinensis* against fungal pathogens affecting solanaceous crops.

Methods: Oil of *O. gratissimum* was obtained by hydro-distilled method using Clevenger apparatus. Leaf extracts of *K. fruticosa* and *H. rosa-sinensis* were extracted using three organic solvents namely hexane, ethanol and methanol. The antifungal activity of the oil and organic leaf extracts against the test fungal pathogens was determined by the agar well diffusion method. The efficacy of the oil and organic leaf extracts against the pathogens was evaluated by their minimum inhibitory concentration (MIC), ability to inhibit spore germination and growth kinetic assay.

Result: The oil of *O. gratissimum* (1000 ppm) significantly inhibited the growth of *Cercospora capsici* (81.4%), *Alternaria alternata* (73.7%) and *Fusarium oxysporum* (68.7%). Hexane extract of *K. fruticosa* and methanol extract of *H. rosa-sinensis* (1500 µg/well) displayed strong antifungal activity against *Colletotrichum acutatum* with inhibition percentages of 75.9% and 82.4% respectively. Similarly, ethanol extract of *K. fruticosa* and methanol extract of *H. rosa-sinensis* showed effective inhibition against *C. capsici* and *A. alternata*. The oil of *O. gratissimum* showed MIC values of 50, 100 and 200 µg/mL against *C. capsici, A. alternata* and *F. oxysporum* respectively. Ethanol extract of *K. fruticosa* showed MIC values ranging from 100 to 350 µg/mL, while *H. rosa-sinensis* methanol extract displayed MIC values ranging from 50 to 200 µg/mL against the fungal pathogens. The oil extract also demonstrated strong inhibition of spore germination of the test fungi. Our study suggests the potential of plant-derived essential oils and extracts as effective antifungal agents for crop protection.

Key words: Antifungal activity, Phytopathogenic fungi, Plant diseases, Plant extracts.

INTRODUCTION

Vegetable crops of the Solanaceae family are nutritionally important and contribute substantially to the economy due to their extensive economic significance. However, these crops are often infected by pathogenic fungi causing foliar disease thereby decreasing the yield considerably. In developing nations, losses in global crop yield resulting from fungal infections before and after harvesting may surpass 12% (Negash, 2018). Numerous pathogens such as *Colletotrichum acutatum*, *Cercospora capsici*, *Alternaria alternata* and *Fusarium oxysporum* cause severe diseases in vegetable crops. These diseases reduced the shelf life and market value of the food thereby posing a threat to human well-being. Furthermore, these fungi produce mycotoxins that pose serious health risks to about 4.5 billion people worldwide (Neme and Mohammed, 2017). In order to control and manage plant pathogenic fungi, a wide variety of synthetic compounds, such as benzimidazoles, aromatic hydrocarbons and inhibitors of sterol biosynthesis, have been used as antifungal treatments. The indiscriminate use of chemicals to eradicate plant pathogens has severe negative impacts on both humans and environment (Grewal, 2017). Furthermore, phytopathogens have become resistant to these compounds due to their widespread application. The understanding of these issues by the community has therefore increased the drive to look towards better, more natural alternatives to synthetic chemical pesticides.

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Consequently, there is a greater need to develop fresh, secure and biodegradable substitutes that serve as natural fungicides. Thus, there is a growing interest in the research of the possible utilization of natural products, such as plant-derived essential oils and extracts, which may be less damaging and ecofriendly in disease control (Subramanyam and Hagstrum, 2012).

Numerous reports have demonstrated that phytochemicals derived from plants exhibit antifungal properties (Omojate Godstime *et al*., 2014). Plant extracts contain bioactive compounds belonging to alkaloids,

anthocyanins, anthraquinones, terpenes, steroids, phenols, *etc.* which have been established in a variety of biological activities, including antimicrobial and antifungal effects. The application of various plant extracts to control diseases of fruits and vegetables has been extensively reviewed (Deresa and Diriba, 2023). Therefore, there are various instances to prove that plant-derived essential oils and extracts can be used as alternatives to synthetic fungicides in the management and control of plant pathogens. Considering the myriad of plant species and assuming that each plant has specific chemical substances that have inhibitory effects, the present study aimed to evaluate extracts and essential oils derived from three plant species against fungal pathogens infecting solanaceous crops.

MATERIALS AND METHODS

Plant materials

The leaves of three plant species namely *Ocimum gratissimum*, *Kopsia fruticosa and Hibiscus rosa-sinensis* were collected from Gauhati University Campus, Guwahati, Assam. The plants were identified based on morphological characteristics, *viz*. characteristics of leaves, stems and flowers. The identification of the plant species was authenticated by consulting herbaria and a voucher specimen of each plant species was deposited in GUBH of the Department of Botany, Gauhati University, Assam and accession numbers were obtained.

Extraction of essential oil and plant extracts

For essential oil extract, the air-dried leaves (200 g) of *O. gratissimum* were hydrodistilled for 3 h using a Clevenger-type apparatus. The extracted oil was kept at 4°C in a sealed vial after being dried over anhydrous Na_2SO_4 for further analysis. For plant extracts, air-dried powdered material (50 g) each of leaves from *K. fruticosa* and *H. rosa-sinensis* was extracted with three organic solvents namely hexane, ethanol and methanol separately at room temperature and the solvents were evaporated by vacuum rotary evaporator. The extraction process yielded crude extracts in different proportions, hexane (7.5 g), ethanol (6.6 g) and methanol (6.3 g) extracts. Solvents (analytical grade) for extraction were provided from the Mycology and Plant Pathology Laboratory, Department of Botany, Gauhati University.

Maintenance and culture of fungal pathogens

Pre-isolated fungal pathogens (*Colletotrichum acutatum* KMPS 13423, *Cercospora capsici* KMPS 12423, *Alternaria alternata* KMPS 12623 and *Fusarium oxysporum* KMPS 12023) were obtained from Mycology and Plant Pathology Laboratory of Department of Botany, Gauhati University, Assam India. These pathogens were isolated from diseased samples of solanaceous crops (chilli, tomato and brinjal) causing leaf spot, blight and anthracnose diseases. The pathogens were cultured and maintained on freshly prepared Potato Dextrose Agar (PDA) medium and stored in a refrigerator (4°C) until needed.

Preparation of spore suspension and test samples

The spore suspensions of *C. acutatum* KMPS 13423, *C. capsici* KMPS 12423, *A. alternata* KMPS 12623 and *F. oxysporum* KMPS 12023 were obtained in sterile distilled water from 10-day-old cultures. The spore suspension was collected and subsequently subjected to centrifugation. Using a haemocytometer, a uniform spore suspension of 10^8 spores/mL was obtained. To prepare the stock solutions for the oil and leaf extracts, the oil extract was independently dissolved in dichloromethane, whereas the leaf extracts were dissolved in their respective solvents, namely hexane, ethanol and methanol. Samples of known weights were then further diluted with 5% of their corresponding solvents and a final solvent concentration of 0.5% (v/v) was obtained in the test samples.

Mycelial growth inhibition test

In vitro antifungal activities of the oil extract of *O. gratissimum* and hexane (H), ethanol (E) and methanol (M) leaf extracts of *K. fruticosa* and *H. rosa-sinensis* were assessed based on mycelial growth inhibition. The prepared extracts were determined for their antifungal activity using the Agar well diffusion method (Balouiri *et al*., 2016). The potato dextrose agar (PDA) medium was used as the basal medium for all test fungi. The observation was conducted in both with and without the tested compound. Subsequently, an aseptic perforation of 6 mm in diameter was created with a sterile cork borer. Following this, a specified volume of the essential oil (1000 ppm) and the extracts of hexane, ethanol and methanol (1500 µg/well) were added carefully into the created well. Fungal disks of 5 mm in diameter from an 8-day-old pure culture were placed in the center of the petri dish containing medium under aseptic conditions. The petri dishes were then sealed with parafilm and incubated at 26°C ±2°C for 7 days. The experiments were conducted in triplicates per treatment. The growth of each fungal species was monitored and recorded after one week. Evaluation of antifungal activities was executed by measuring the diameter of growth inhibition zones after one week of incubation. The inhibition percentage of mycelial growth was calculated by the following formula (Bekker *et al*., 2006):

$$\text{Inhibition (\%)} = \frac{C - T}{C} \times 100$$

Where,

C = Diameter (mm) of the fungal colony of the negative control (PDA only).

T = Diameter (mm) of the fungal colony of the test plate (plant extracts).

Minimum inhibitory concentration (MIC)

Determination of minimum inhibitory concentration (MIC) of both essential oil and plant extracts against the test pathogens was carried out by agar dilution method as described by Balouiri *et al.* (2016). To achieve the suitable concentration, the oil and the extracts were meticulously

diluted in DMSO ranging from 50 to 1000 µg/mL (*i.e.*, 50, 100, 200, 350, 500 and 1000 µg/mL). The assay's final DMSO concentrations did not exceed 2%. An aliquot of 10 µL spore suspension (10^8 spores/mL) was introduced in each test tube containing PDB (Potato Dextrose Broth) medium along with different concentrations of the samples prepared. After inoculation, the test tubes were incubated for 3-7 days at 26°C±2°C. The tubes containing only PDB medium with the fungal spore suspension were considered as controls. The MIC expressed in µg/mL was determined as the lowest concentration at which any noticeable growth was not observed.

Spore germination assay

For spore germination assay of *C. acutatum* KMPS 13423, *C. capsici* KMPS 12423, *A. alternata* KMPS 12623 and *F. oxysporum* KMPS 12023, essential oil samples (2 µL) dissolved in 5% dichloromethane were further diluted with water to achieve final concentrations of 50, 100, 200, 350, 500 and 1000 µg/mL of the oil extract, resulting in a final dichloromethane concentration of 0.5%. The samples were inoculated with spore suspension (10^8 spores/mL) of each fungal pathogen. From this, aliquots of 10 µL of the spore suspension were placed onto separate glass slides in triplicate. Subsequently, the slides carrying the spores were placed inside a moisture chamber and incubated at a temperature of 26°C±2°C for 24 h. Following incubation, each slide was stained with lactophenol-cotton blue and then observed under a microscope to determine the spore germination. The percentage of conidial germination was estimated by counting germinated and non-germinated conidia within microscope fields. Conidia were considered germinated when the length of the germ tube equalled or exceeded the diameter of the conidium. The control (0.5% dichloromethane) was tested separately for spore germination of different fungi.

Growth kinetics assay

C. acutatum KMPS 13423 exhibited the highest resistance than other tested fungi when exposed to oil extract in both the mycelial growth inhibition test and spore germination assay and was considered as the test fungus for kinetic study and evaluation of antifungal activity of essential oil. An aliquot of 10 µL spore suspension (10^8 spores/mL) of the fungus was added to the test tubes containing different concentrations of the oil extract (*i.e.*, 50, 100 and 200 µg/mL) and homogeneous suspensions were achieved by gently inverting the test tubes repeatedly for 3-4 times. At specific time intervals of 30, 60, 90, 120 and 150 min, the reaction mixtures were passed through Whatman No. 1 filter paper and the retained spores were subsequently rinsed three times with sterile distilled water. Subsequently, the spores were added in 10 mL of sterile distilled water from which 100 µL of spore suspension was taken on the glass slides and incubated at 26°C±2°C for 24 h. The spores that generated germ tubes were recorded and the percentage of spore germination was calculated. All experiments were conducted in triplicate.

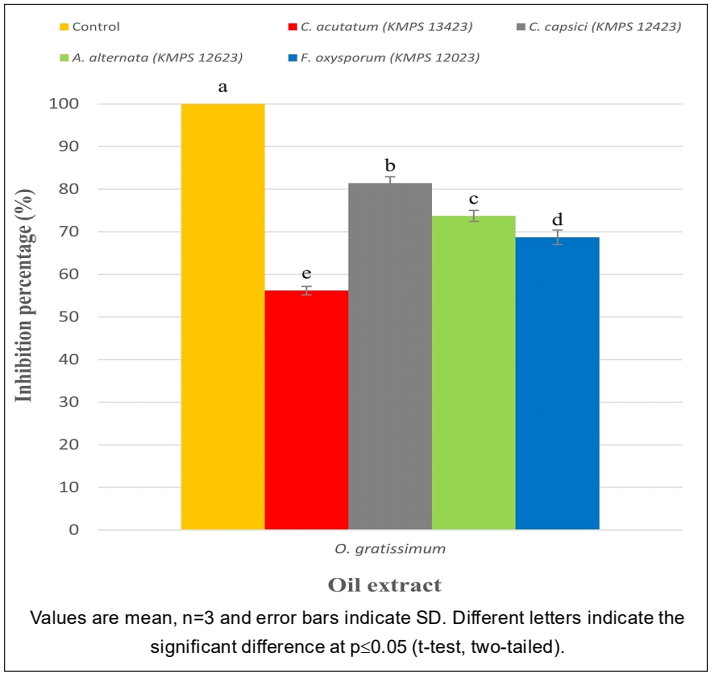
Statistical analysis

The antifungal activity of both essential oil and plant extracts was evaluated and recorded. Each experiment was performed in triplicate and the resulting mean values were computed. A Student's t-test was computed for the statistical significance of the results using SPSS (16.0 Version).

RESULTS AND DISCUSSION

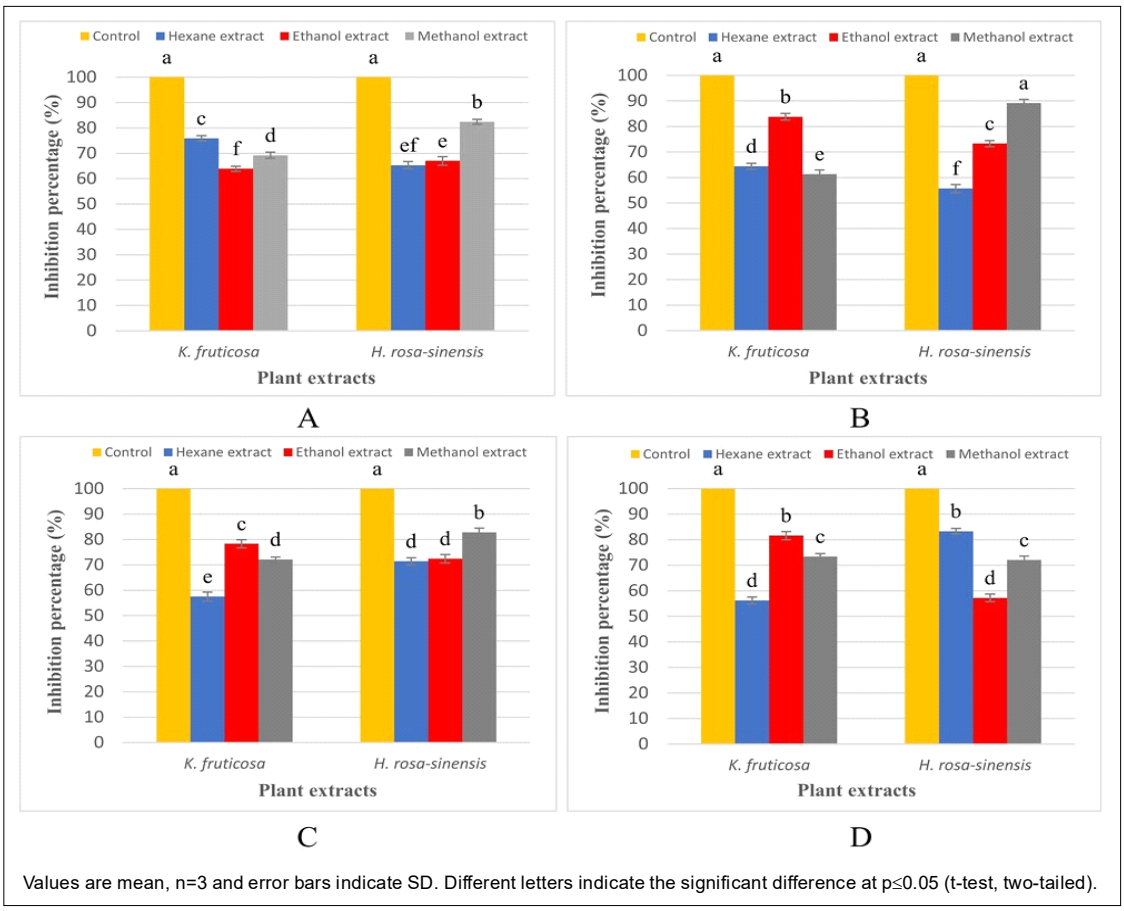
In the present investigation, three plant species identified as *Ocimum gratissimum*, *Kopsia fruticosa* and *Hibiscus rosa-sinensis* were selected to determine the antifungal potential of their extracts against plant pathogens. The results of the antifungal assay revealed that oil extract obtained from *O. gratissimum* exhibited antifungal activity against the test fungal pathogens in varying degrees. The extract showed an inhibitory effect on the radial growth of all phytopathogens and the highest inhibitory effect was observed in *C. capsici* KMPS 12423 (81.4±1.5%) followed by *A. alternata* KMPS 12623 (73.7±1.3%) and *F. oxysporum* KMPS 12023 (68.7±1.7%). The extract showed the lowest inhibitory effect against *C. acutatum* KMPS 13423 (Fig 1). Similarly, organic leaf extracts of *K. fruticosa* and *H. rosa-sinensis* also exhibited notable inhibition of radial growth against all the phytopathogens. It was observed that the hexane extract of *K. fruticosa* and the methanol extract of *H. rosa-sinensis* displayed remarkable antifungal activities against *C. acutatum* KMPS 13423, with an inhibition percentage of 75.9±1.1% and 82.4±1.0%, respectively, while moderate antifungal activity was observed in the ethanol extracts (Fig 2A). Effective inhibition of *C. capsici* KMPS 12423 was observed in the ethanol leaf extract of *K. fruticosa* and methanol extract of *H. rosa-sinensis* while less activity was observed in hexane extracts (Fig 2B). A similar result was observed against *A. alternata* KMPS 12623 in which ethanol leaf extract of *K. fruticosa* and methanol extract of *H. rosa-sinensis* effectively inhibited the pathogen significantly (Fig 2C). It was again observed that ethanol leaf extract of *K. Fruticosa* and hexane extract of *H. rosa-sinensis* effectively inhibited the growth of *F. oxysporum* KMPS 12023 (Fig 2D). From the above results, it was noted that ethanol extract of *K. fruticosa* and methanol extract of *H. rosa-sinensis* effectively inhibited the growth of all the test fungal pathogens.

Considering the inhibitory effects shown by the oil and plant extracts, Minimum Inhibitory Concentration (MIC) was determined for all the test fungal pathogens. The result indicated that oil extract of *O. gratissimum* could able to inhibit *C. capsici* KMPS 12423, *A. alternata* KMPS 12623 and *F. oxysporum* KMPS 12023 at MIC values of 50, 100 and 200 µg/mL, respectively. The oil was less effective against *C. acutatum* KMPS 1342 and showed inhibition at MIC value of 500 µg/mL (Table 1). Furthermore, the susceptibility of various organic leaf extracts of *K. fruticosa* and *H. rosa-sinensis* was also assessed. Notably, the ethanol extract of *K. fruticosa* and the methanol extract of *H. rosa-sinensis* displayed higher susceptibility compared to the other extracts against the tested fungi, as presented



Values are mean, n=3 and error bars indicate SD. Different letters indicate the significant difference at p≤0.05 (t-test, two-tailed).

Fig 1: Growth inhibition (%) of test pathogens by oil extract of *O. gratissimum*.



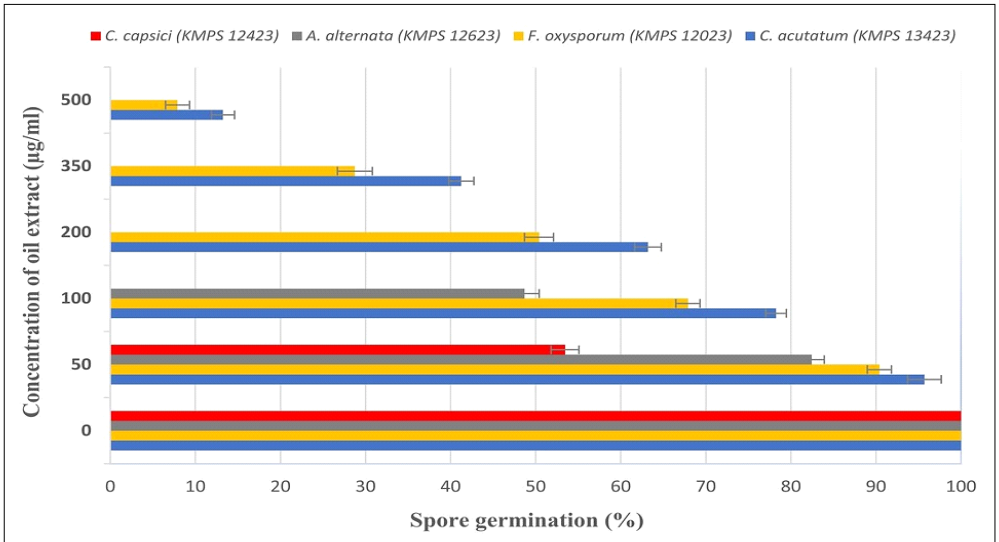
Values are mean, n=3 and error bars indicate SD. Different letters indicate the significant difference at p≤0.05 (t-test, two-tailed).

Fig 2: Growth inhibition (%) of pathogenic fungi by various organic leaf extracts of *K. fruticosa* and *H. rosa-sinensis* against (A) *C. acutatum* (KMPS 13423); (B) *C. capsici* (KMPS 12423); (C) *A. alternata* (KMPS 12623) and (D) *F. oxysporum* (KMPS 12023).

in Table 1. The ethanol extract of *K. fruticosa*, showed MIC values ranging between 100–350 µg/mL against the phytopathogens, *C. acutatum* KMPS 13423, *C. capsici* KMPS 12423, *A. alternata* KMPS 12623 and *F. oxysporum* KMPS 12023. Further, the hexane and methanol extracts demonstrated antifungal activity against the same fungi with MIC values ranging from 200-500 µg/mL each. In the case of *H. rosa-sinensis*, the methanol extract exhibited MIC values ranging from 50-200 µg/mL against *C. acutatum* KMPS 13423, *C. capsici* KMPS 12423, *A. alternata* KMPS 12623 and *F. oxysporum* KMPS 12023. Conversely, the hexane and ethanol extracts displayed antifungal potential against the aforementioned fungi, each displaying MIC values of 100-350 µg/mL.

In the present investigation, it was found that oil extract obtained from *O. gratissimum* was effective in inhibiting the fungal pathogens. Therefore, in order to observe the further efficacy of the oil extract in growth reduction potential spore germination and growth kinetics assay was undertaken for all the test fungal pathogens. Results obtained for the oil extract on the spore germination assay of each of the test fungi are shown in Fig 3. DMSO (0.5%, v/v) was used as a negative control and it did not inhibit the spore germination of any of the plant pathogens tested. There was a significant inhibition of fungal spore germination shown by the oil extract at different concentrations. Complete inhibition (100%) of fungal spore germination was observed in *C. capsici* KMPS 12423 at 100 µg/mL concentrations while in *A. alternata* KMPS 12623 inhibition was shown at 200 µg/mL concentrations. The oil also exhibited a potent inhibitory effect on the spore germination of *F. oxysporum* KMPS 12023 and *C. acutatum* KMPS 13423 in the range of 50-80% at concentrations ranging from 200 to 500 µg/mL.

The antifungal kinetics assay was determined only against *C. acutatum* KMPS 13423 and the result of the oil extract against the pathogen is shown in Fig 4. Exposure of *C. acutatum* KMPS 13423 spores to different concentrations of the essential oil for 30-150 min caused varying degrees of inhibition of spore germination. As the exposure time and concentration increased, there was an observed increase in fungicidal activity. The oil extract at 50 µg/mL showed antifungal activity but did not inhibit completely and about 50% inhibition was observed at an exposure time of 120 min. However, there was a marked increase in the killing rate at 100 and 200 µg/mL after 30 min of exposure and 95 to 100% inhibition of spore germination was observed on 150 min exposure, respectively. At low concentrations, the significant rate of inhibition was the characteristic feature of the oil extract.



Values are mean, n=3 and error bars indicate SD.

Fig 3: Effect of different concentrations (µg/mL) of the oil extract of *O. gratissimum* on spore germination of all the tested fungi.

Table 1: Minimum inhibitory concentrations (MIC) of oil and leaf extracts of selected plants against phytopathogenic fungi.

Fungal pathogens	*O. gratissimum*	*K. fruticosa* (µg/mL)			*H. rosa-sinensis* (µg/mL)		
	(µg/mL)	H	E	M	H	E	M
C. acutatum (KMPS 13423)	500	200	350	350	350	350	100
C. capsici (KMPS 12423)	50	350	100	500	100	200	50
A. alternata (KMPS 12623)	100	500	200	200	200	200	100
F. oxysporum (KMPS 12023)	200	500	100	200	100	100	200

H: Hexane extract; E: Ethanol extract and M: Methanol extract.

As the global population continues to grow, ensuring food security becomes a prominent issue, especially in developing countries. Nonetheless, these food supplies entail risks from a variety of pathogens that not only lessen the usefulness and economic value of food products but also make them unfit for consumption, thus negatively impacting human health and well-being. Over time, a wide range of synthetic chemicals have been employed as antifungal agents to curb the proliferation of plant pathogenic fungi. However, these chemicals are well-known for causing adverse effects on living organisms and the environment. Thus, at present attention is shifting towards other alternatives that consumers perceive as natural and eco-friendly. This has resulted in research activities dedicated to formulating safer antifungal solutions, including those utilizing plant derived oils and their extracts to counteract plant pathogens in agriculture. Studies have demonstrated that the antifungal activity of plant extracts and compounds against phytopathogenic fungi could be good alternatives for controlling plant diseases (Deresa and Diriba, 2023).

In our present study, oil and organic solvent extracts obtained from three plant species namely *O. gratissimum*, *K. fruticosa* and *H. rosa-sinensis* have shown antifungal activities against plant pathogens causing diseases in solanaceous crops. The oil of *O. gratissimum* successfully inhibited mycelia growth and spore germination of *C. capsici*, *A. alternata* and *F. oxysporum*. Similar studies made by Faria *et al*. (2006) reported that essential oil obtained from aerial parts of *O. gratissimum* inhibited the growth of several fungi including *Botryosphaeria rhodina*, *Rhizoctonia* and *Alternaria* sp. Previous investigations have noted that essential oils obtained from *O. gratissimum* exhibit antimicrobial characteristics. It is documented that the volatile oil extracted from this plant is primarily composed of phenolic compounds, with special emphasis on thymol (Oliver, 1960; Sainsbury and Sofowora, 1971). These phenolic constituents have been attributed for the documented antimicrobial properties to the oil. The organic solvent extracts obtained from *K. fruticosa* and *H. rosa-sinensis* also showed inhibitory effects on the mycelia growth of *C. capsici*, *A. alternata* and *F. oxysporum* in different concentrations. Similarly, Debjani *et al*. (2017) observed that plant extracts obtained from Ginger, *Polyalthia* and *Clerodendrum* showed a good inhibitory effect on *Rhizoctonia solani* and also inhibited the growth of *Colletotrichum capsici* at different concentrations**.** Ethanol extract obtained from *K. fruticosa* has been reported to exhibit antifungal, antimicrobial and cytotoxic activities (Long *et al*., 2018). However, there is no report on leaf extract obtained from this plant against phytopathogenic fungi to date. The extract obtained from this plant revealed novel indole alkaloids, specifically kopsifolines G-K, alongside a recognized alkaloid, kopsifoline A. Therefore, the antifungal efficacy can be ascribed to the presence of these alkaloids. *Hibiscus rosa-sinensis*, commonly referred to as the Chinese hibiscus, is a widely recognized medicinal plant employed in addressing diverse health conditions (Magdalita and San Pascual, 2022). There are reports of antimicrobial activity of this plant extract against pathogenic bacteria (Ruban and Gajalakshmi, 2012; Patel *et al*., 2012). However, information on this plant extract against pathogenic fungi is still lacking. Our study showed that organic solvent extract obtained from *H. rosa-sinensis* leaf showed effective inhibition against plant pathogens namely *F. oxysporum, C. acutatum*, *C. Capsici* and *A. alternata*. The organic leaf extract of this plant has been reported to contain various phytochemicals like flavonoids, tannins and phenols (Farasayu *et al*., 2021). In many instances, these phytochemicals demonstrated antibacterial effects by inhibiting the synthesis of nucleic acid, biofilm formation, energy for bacterial metabolism and the function of the cytoplasmic membrane. Therefore, the

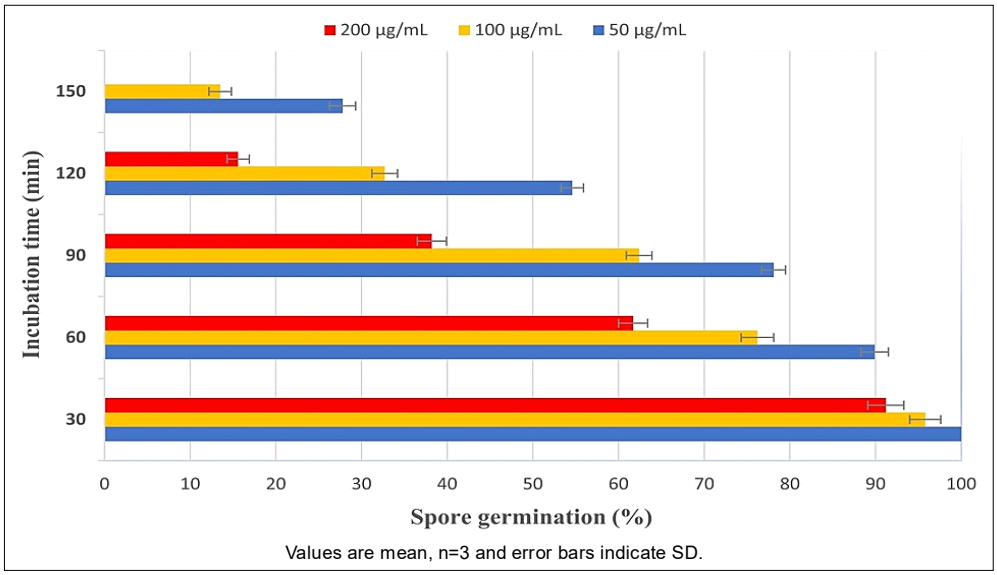


Fig 4: Kinetic growth inhibition (%) of *C. acutatum* (KMPS 13423) spores by the oil extract of *O. gratissimum*.

antifungal effect of this plant may also be due to the presence of these secondary metabolites. Our studies have demonstrated that extracts derived from medicinally important plants could be successfully used for the control and management of plant pathogens in eco-friendly and more economical ways as compared to synthetic fungicides.

CONCLUSION

In the present investigation, the oil extract of *O. gratissimum* and organic leaf extracts obtained from *K. fruticosa* and *H. rosa-sinensis* exhibited varying degrees of antifungal activity against plant pathogenic fungi. Exploring the effects of these essential oils and plant extracts against fungal pathogens could offer valuable insights for the development of innovative antifungal agents. Such agents could prove pivotal in managing critical fungal infections affecting plants, animals and humans alike. Consequently, the findings suggest that harnessing plant derived oils and their extracts might serve as an alternative to synthetic fungicides within agro-industries. Additionally, our results have also highlighted the potential for identifying naturally derived fungicides from plant origin, which could play a pivotal role in mitigating the detrimental impact of various phytopathogens causing diseases on crops, vegetables and ornamental plants.

Statements and declarations

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Authors contributions

S. Saha and T.F. Yesmin: Investigation and analysis of the experiment, S. Saha and A. Sarma: Preparation of original draft of the manuscript; C. Mili and P.K. Mishra: Preparation of figures and table, statistical analysis; K. Tayung: Supervision.

Conflict of interest

The author declares that there is no conflict of interest for this submission.

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