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ANTIFUNGAL EFFICACY OF *PIPER BETLE* L. AND *AZADIRACTHA INDICA* A. Juss LEAVES EXTRACT IN CONTROLLING DAMPING-OFF DISEASES IN GROUNDNUT

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ABSTRACT

The study aimed to investigate the inhibitory potential of betel and neem leaf extracts against the growth of the *Sclerotium rolfsii*, which causes damping-off disease in groundnut plants. The research employed both *in vitro* and *in vivo* methods to determine the antifungal activity of the extracts. The isolation of the pathogen inoculum was done from the base stem of the infected groundnut plants by *S. rolfsii* in the field. The betel and neem leaves were dried at a controlled temperature of 40°C for 7 days, ground, and then immersed in 96% ethanol for 48 hours. The resulting extracts were tested for their inhibitory potential against *S. rolfsii* in vitro by admixing them with PDA media, inoculating with *S. rolfsii*, and measuring colony diameters. The study found that the colony diameter of *S. rolfsii* was significantly influenced by the use of betel leaf extract, neem leaf extract, and a mixture of both extracts at a concentration of 1.5% (v/v). The widest colony diameter was observed in the control treatment on day 5, measuring 7.47 cm. In contrast, the colony diameter of *S. rolfsii* in the treatment with betel leaf extract, neem leaf extract, and a mixture of both extracts on day 5 was 5.58 cm, 4.35 cm, and 1.41 cm, respectively. The addition of each extract to the parameters of colony diameter and inhibition percentage was capable of inhibiting the growth of *S. rolfsii*, with the betel-neem extract treatment exhibiting the highest inhibition percentage of 81.09%. In the in vivo suppression test, the study found that treating groundnut seeds with a 1.5% concentration of betel leaf and neem leaf extracts, as well as a combination of both extracts, significantly reduced the incidence of damping-off disease. The betel-neem extract treatment produced the lowest disease incidence and exhibited the highest percentage of pre-emergence in groundnut seeds. Overall, the study provides evidence that betel and neem leaf extracts have antifungal activity against *S. rolfsii* and could be used as a potential alternative to synthetic fungicides for controlling damping-off disease in groundnut.

Keywords: Antifungal, Fungicide, *In vitro, In vivo*, *Sclerotium*.

INTRODUCTION

As one of the most widely cultivated subsistence crops, groundnuts (*Arachis hypogea* L.), are frequently grown in conjunction with other staple crops such as rice, corn, and soybeans and can serve as an intercrop or companion crop (Hussainy and Vaidyanathan, 2019). In Indonesia, groundnut is ranked as the fourth largest food crop, following rice, corn, and soybeans and serves as a rich source of essential nutrients, including a high concentration of vegetable oil, protein, calcium, phosphorus, iron, vitamin E, and the B-complex vitamins (Altine *et al.*, 2016). The reliance on groundnut imports in Indonesia has been increasing because of the low production of groundnuts domestically. This scenario is primarily attributed to a number of difficulties faced in the cultivation of groundnuts, such as the limited utilization of high-quality planting materials. The absence of adequate planting materials can result in a higher incidence of pest and disease outbreaks, further reducing groundnut production and exacerbating the

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gap between domestic demand and supply (Panth *et al.*, 2020; Ristaino *et al.*, 2021). This situation underscores the necessity for the improvement of groundnut cultivation practices in Indonesia, which should encompass the adoption of good planting materials and the implementation of effective pest and disease management strategies. With a concerted effort in these areas, it is feasible to enhance groundnut production, minimize reliance on imports, and establish a more sustainable and self-sufficient groundnut industry in Indonesia.

The persistent threat posed by the fungal pathogen *Sclerotium rolfsii* continues to hinder groundnut production in Indonesia. This pathogen has been shown to cause significant losses, with estimates suggesting a decline in production of up to 50% (Guclu *et al.*, 2020). The severity of *S. rolfsii* infection has been documented through field surveys, with incidence rates in groundnut crops in Mataram, Saying-saying, and Pamenang in West Lombok Regency ranging from 80-90% (Semangun, 1991). The symptoms of *S. rolfsii* infection include necrotic patches on the base of the plant stem covered in white, cotton-like mycelium, as well as the presence of small, white to brown sclerotia (Bera *et al.*, 2014). These sclerotia have been identified as a key factor in the spread of the disease, due to their ability to persist in soil for long periods. *S. rolfsii* has a wide host range, encompassing over 200 plant species, and is capable of colonizing both wild plants and crop residues (Billah *et al.*, 2017; Dwivedi and Prasad, 2016).

The conventional method of controlling the groundnut damping-off disease caused by *S. rolfsii* has been through the use of synthetic pesticides (He *et al.*, 2022). Synthetic pesticides have been shown to be effective in saving agricultural crops that have been affected by plant diseases, however, they also have negative impacts on the environment and human health (Rani *et al.*, 2021). Botanical pesticides, on the other hand, are pesticides whose active ingredients are derived from plants and other organic materials that can be used to control pests and diseases in crops. The use of botanical pesticides, in particular, is crucial for promoting environmentally sustainable agriculture (Ngegba *et al.*, 2022). Therefore, the utilization of botanical pesticides could be an alternative option for controlling groundnut damping-off disease (Nugroho *et al.*, 2019). Over 2,400 plant species, belonging to 235 families, contain pesticidal substances. Recently, there has been an increasing interest in the use of botanical pesticides, and various medicinal plants can be utilized as botanical pesticides. Plants that contain antifungal compounds are betel and neem leaves (Dalavayi *et al.*, 2021).

The extract from betel leaves has been shown to function as an antifungal agent, capable of affecting the growth and formation of fungal conidia. Betel leaves contain chemical components such as essential oils, alkaloids, and eugenol. Furthermore, the essential oil of betel leaves contains volatile oils (batlephenol), starch, diastase, and substances capable of killing pathogens, functioning as an antioxidant, and serving as a fungicide and antifungal agent (Maimunah *et al.*, 2019). The neem plant, particularly its leaves and seeds, contains several chemicals that are highly beneficial in agriculture. The active ingredients contained in neem leaves include azadirachtin, salanin, meliantriol, nimbin, and nimbidin, which exhibit antibacterial, antiviral, and antifungal properties (Nawaz *et al.*, 2016; Xu *et al.*, 2017). Considering the potential of betel leaves and neem leaves as antifungal agents, as evidenced by the presence of active constituents within them, there exists the prospect that extracts derived from betel and neem leaves could effectively inhibit the growth of *S. rolfsii* fungi, thereby serving as promising botanical pesticides with notable efficacy. Consequently, it is imperative to undertake comprehensive research to assess the effectiveness of betel leaves and neem leaves in managing groundnut damping-off disease caused by *S. rolfsii*.

MATERIALS AND METHOD

Isolation of *S. rolfsii:* Isolation of pathogen inoculum was done from the base stem of the infected groundnut plants by *S. rolfsii* in the field. The process involved cutting 1 cm × 1 cm pieces of stem from the healthy and infected portions of the plant, then soaking in 1% bleach solution for 1 minute and rinsing twice with distilled water for 1 minute each time. The tissue was then air dried and the plant tissue was planted on PDA media (HiMedia, India). The PDA media is formulated by dissolving 39 grams of PDA powder in 1000 mL of distilled water. This mixture is subsequently subjected to sterilization using an autoclave at a temperature of 121°C, under a pressure of 1 atm, for a duration of 20 minutes. After the plant tissue is aseptically plated onto the medium

and then incubated for a period ranging from 4 to 7 days. The pathogen that grew was then purified on new media and characterized microscopically (Fariña *et al.*, 2001).

Pathogenicity Test: A 20 mL suspension of *S. rolfsii* was added to the planting medium in a polybag. One week after the addition of the suspension, groundnut seeds were planted in the inoculated medium. The application of the pathogen to the soil medium facilitated its colonization of the soil and the subsequent infection of the roots and stem of the plant. The development of wilting symptoms in the seedlings was closely monitored and when observed, re-isolation of *S. rolfsii* was performed by cutting the base of the wilted stem and inoculating it onto a new PDA medium. The purified isolate of *S. rolfsii* obtained from this process was used for further testing (Hidayati, 2018).

Extraction of Betel and Neem Leaves: The betel and neem leaves were subjected to a drying process at a controlled temperature of 40°C for a period of 7 days. Once the leaves were dried, they were meticulously ground and sifted through a 100 mesh sieve. A quantity of 500 g of each leaf's powder was carefully immersed in 1 L of 96% ethanol for a duration of 48 hours. The solvent was then efficiently evaporated through the use of a rotary evaporator at a constant temperature of 40°C. This meticulous extraction process was repeated several times until the desired sample quantity was achieved for the subsequent tests (Hoesain *et al.*, 2021).

In Vitro Inhibition Test of Betel Leaf and Neem Leaf Extracts against *Sclerotium rolfsii:* The test involved the admixture of PDA media with extracts obtained from each leaf, until a concentration of 1.5% (v/v) was attained. The resulting media was subsequently dispensed into Petri dishes of 9 cm diameter. Upon solidification of the media, a 6 mm *S. rolfsii* inoculum was placed at the center of each dish and incubated for a period of 5 days. The inhibitory potential of betel leaf and neem leaf extracts against the growth of *S. rolfsii* colonies was determined based on measurements of colony diameters. The growth of both mycelial and sclerotial colonies was assessed by daily observations from the day of inoculation until the fourth day thereafter. The colony diameter was calculated by creating a vertical and horizontal line, intersecting at the midpoint of the fungal colony located at the outer edge of the Petri dish base. The inhibitory value was determined by comparing the colony diameter of *S. rolfsii* in the treated group with that of the control group (Bhuiyan *et al.*, 2012).

In Vivo Suppression Test of Betel Leaf and Neem Leaf Extracts against *S. rolfsii:* The sterilized planting media was placed in 20 germination trays measuring 34 cm × 27 cm × 14 cm. Each germination tray was filled with approximately 5 kg of planting media, and then the pathogen was inoculated by adding 20 ml of *S. rolfsii* suspension to each germination tray. The In Vivo method employed to reduce the incidence of damping-off disease involved treating seeds with a 1.5% concentration of betel leaf and neem leaf extracts, as well as a combination of betel and neem leaf extracts. The control treatment consisted of seed immersion in aquades. Each treatment was immersed for a period of one hour. The treated groundnut seeds were planted at a depth of 3-5 cm with one seed per planting hole in each of the germination trays. A total of 30 groundnut seeds were included in each germination tray, and observations were conducted daily to monitor disease symptoms during the pre-emergency and post-emergency stages (Akgul *et al.*, 2011; Bhatia *et al.*, 2005).

DATA ANALYSIS

The data obtained were subjected to analysis of variance (ANOVA) and significant differences among treatments were further tested using the Duncan Multiple Range Test (DMRT) at a significance level of 5% (Hoesain *et al.*, 2021).

RESULTS

Pathogen Isolation: The present study successfully isolated fungi associated with damping-off disease in groundnut in the field. These fungi exhibited similar characteristics to *S. rolfsii*, as evidenced by symptoms such as stem base rot, gradual wilting, and eventual death (Figure 1a). Further identification of the fungus was carried out by culturing it on PDA media macroscopically, which revealed white hyphae that did not form spores, with a colony shape resembling fur and solid clumps resembling cotton (Figure 1b). Microscopic identification at 100× magnification (Figure 1c) confirmed the presence of *S. rolfsii*, as indicated by the presence of hyphae and clam connections on colonies aged 6-8 days. The clam connections were observed in old hyphae with a width of 8.75-11.25 µm and a height of 6.25-12.50 µm.

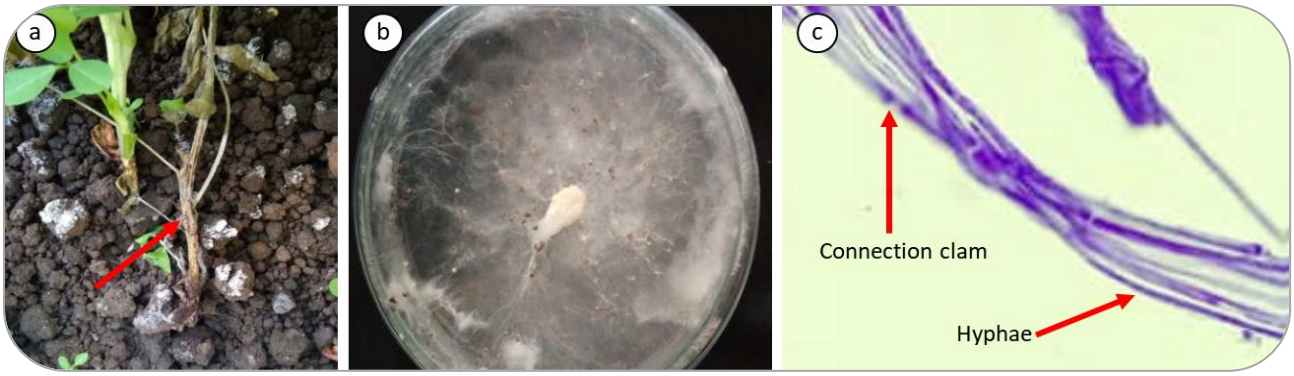

Figure 1. (a) Symptomatic groundnut plant stems; (b) pure isolate of *S. rolfsii* on PDA media; (c) microscopic view of *S. rolfsii.*

The pathogenicity test results of *S. rolfsii* on groundnut seedlings revealed that the fungus was indeed pathogenic to groundnut seeds. This was evident from the occurrence of damping-off disease symptoms in groundnuts, characterized by the appearance of white mycelium that resembled fine cotton on the stem base of the plant, where it meets the soil. These symptoms were observed on the stem portion of the groundnut seedlings, as illustrated in Figure 2.

Figure 2. Damping-off disease symptoms that indicate the pathogenicity of the *S. rolfsii* isolate in this study.

The Growth Rate of *S. rolfsii* Fungi In Vitro: The use of betel leaf extract, neem leaf extract, and a combination of both extracts at the same concentration had a significant impact on the colony diameter of *S. rolfsii*. As depicted in Figure 3, the colony diameter of *S. rolfsii* increased substantially from day 1 to day 5 of observation. The growth rate of the colony diameter varied for each extract treatment, with the widest colony diameter of 7.47 cm observed in the control treatment on day 5. Conversely, on day 5, the colony diameter of *S. rolfsii* in the betel leaf extract treatment, neem leaf extract treatment, and a combination of both extracts treatment measured 5.58 cm, 4.35 cm, and 1.41 cm, respectively.

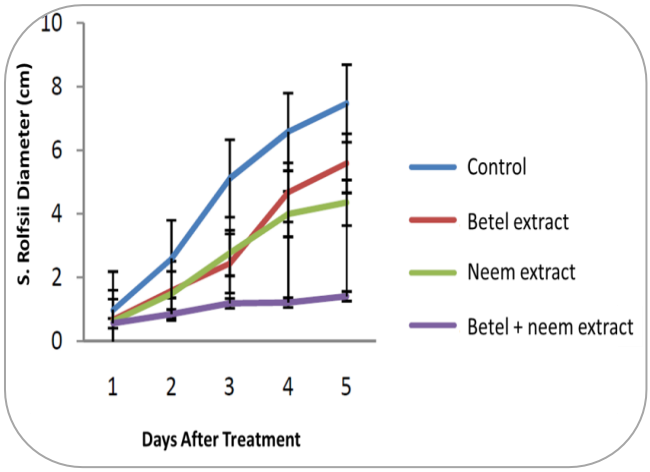

Figure 3. The growth of *S. rolfsii* colony diameter

The experimental results also revealed that the use of the combination of extracts effectively prevented the fungal growth on PDA media from fully covering the surface of the Petri dish. This observation indicates that the combination of extracts has the potential to inhibit the growth of *S. rolfsii* colonies, as opposed to the control treatment, which showed complete coverage of the entire Petri dish by the fungal colony, indicating unhindered growth (Figure 4). These findings provide compelling evidence for the inhibitory effect of the combination of betel leaf and neem leaf extracts on the growth of *S. rolfsii*.

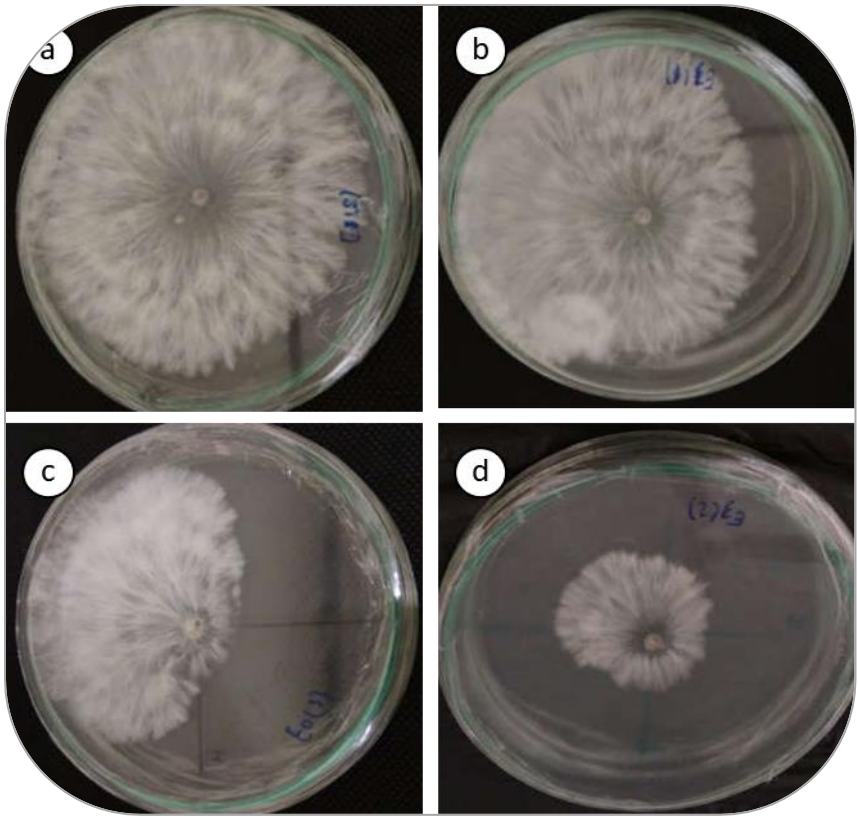

Figure 4. Resistance test of the extract against S. rolfsii on PDA media: (a) Control treatment, (b) Betel leaf extract treatment, (c) Neem leaf extract treatment, (d) Betel and neem leaf extract treatment.

The test results showed that adding each extract had a significant effect on the colony diameter and growth inhibition percentage of *S. rolfsii* (Table 1). The betel and neem leaf extract treatments were significantly different from the control, with colony diameters of 7.47 cm and suppression diameters of 5.58 cm and 4.35 cm, respectively. Nonetheless, the betel, neem, and betel-neem extract treatments demonstrated their potential to hinder pathogen development. The betel-neem extract treatment was the most effective, with the smallest colony diameter of 1.41 cm. Moreover, the inhibition percentage parameter revealed that the betel-neem extract treatment had the highest percentage of 81.09%, which was significantly different from the neem leaf extract treatment at 42.10% and the lowest percentage observed in the betel leaf extract treatment at 24.85%.

Table 1. Percentage of Inhibition of Betel Leaf and Neem Leaf Extracts on the In-vitro Growth of *S. rolfsii*

Treatments	Diameter (cm)	Percentage of Inhibition (%)
Control	7.47 ± 0.72 a	0.00 ± 0.00 a
Betel	5.58 ± 0.38 b	24.85 ± 1.29 b
Neem	4.35 ± 0.61 c	42.10 ± 2.16 c
Betel + neem	1.41 ± 0.13 d	81.09 ± 2.86 d

Note: Numbers in the column followed by the same letter were insignificantly different at p-value of 0.05 (Duncan Multiple Range Test).

The severity of Damping-off Disease: The inoculation experiment revealed the presence of damping-off symptoms in groundnut plants, both before and after emergence (Figure 5). Pre-emergence symptoms were observed in the seeds before germination, which included rotting, turning brownish-black in color, and being covered with white mycelium of *S. rolfsii* on their surface. In contrast, post-emergence symptoms were seen in the seedlings after emergence, wherein the fungus infected the seeds, leading to plant collapse caused by stem base rot. These symptoms indicate the severe impact of *S. rolfsii* on plants, highlighting the importance of implementing effective management strategies to prevent the spread of damping-off disease and minimize its adverse effects on crop yield and quality.

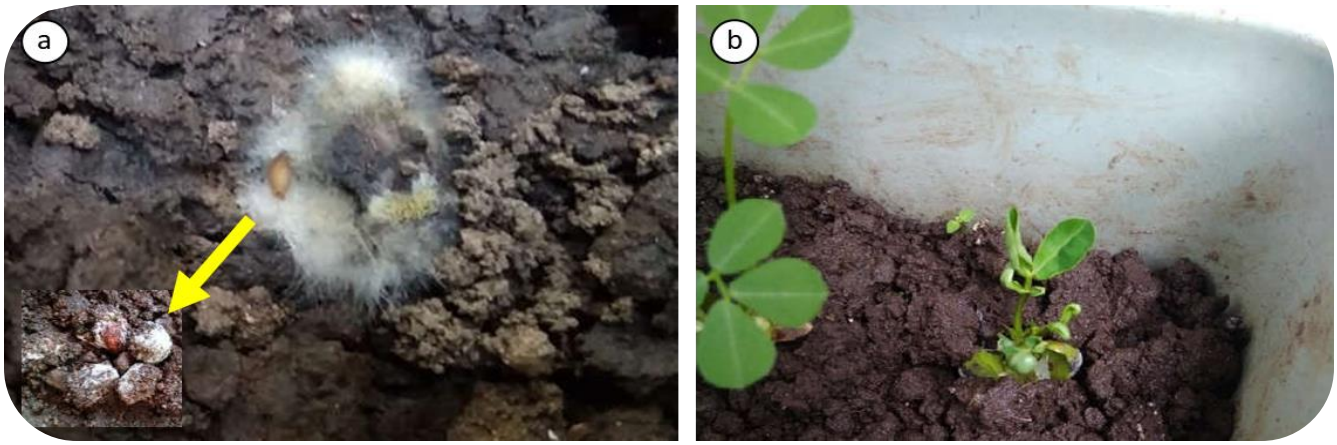

Figure 5. Damping-off Symptoms in Groundnut Plants: (A) Pre-emergence, (B) Post-emergence

The advanced analysis of pre- and post-emergence percentage of damping-off disease in groundnut seeds revealed that the application of betel and neem leaf extracts resulted in the lowest disease severity of 5.33% and 33.1%, respectively (Table 2). The findings indicate a significant difference in disease severity between the betel and neem leaf extract treatments and the control treatment, which had disease severities of 24.66% and 43.5%, respectively. However, there was no significant difference between the betel leaf treatment (24%) and the neem leaf treatment (14.66%) for pre-emergence and between the betel leaf treatment (35.11%) and the neem leaf treatment (34.23%) for post-emergence. These results suggest the potential effectiveness of using betel and neem leaf extracts in managing damping-off disease in groundnut plants, thereby improving crop yield and quality.

Table 2. The severity of damping-off diseases in groundnut seeds treated with extracts of betel leaf and neem leaf using seed treatment

Treatments	Diseases Severity (%)	
	Pre-emergence	Post-emergence
Control	24.66 ± 1.23 a	43.50 ± 2.67 a
Betel	24.00 ± 0.96 ab	35.11 ± 1.87 b
Neem	14.66 ± 2.14 b	34.23 ± 1.24 b
Betel + neem	5.33 ± 0.73 c	33.10 ± 1.73 bc

Note: Numbers in the column followed by the same letter were insignificantly different at p-value of 0.05 (Duncan Multiple Range Test).

DISCUSSION

S. rolfsii is a fungal plant pathogen that can cause significant damage to groundnut plants. Upon infecting the plant, the hyphae of *S. rolfsii* can secrete cellulolytic enzymes and oxalic acid, both of which can contribute to the softening of stem tissue and eventual plant death (Dwivedi and Prasad, 2016). Cellulolytic enzymes can break down cellulose, which is a major component of plant cell walls, into simpler sugars that the fungus can use as a nutrient source (Kumar *et al.*, 2017). This enzymatic breakdown can weaken the structural integrity of the cell wall, making the plant more susceptible to collapse. The hyphae of *S. rolfsii* secrete these enzymes into the plant tissue upon infection, causing the cellulose in the cell walls to break down. As a result, the structural integrity of the cell wall is compromised, and the stem tissue of the plant becomes softer and more pliable, leading to eventual collapse (Lachke and Deshpande, 1988).

In addition to cellulolytic enzymes, oxalic acid is another compound secreted by the hyphae of *S. rolfsii* that can contribute to the softening of plant tissue. This acid can dissolve calcium, an important component of plant cell walls, which can lead to the weakening of the cell wall structure. As a result, the stem tissue of the groundnut plant can become more pliable and eventually collapse, leading to the symptoms of the disease caused by *S. rolfsii* (Sarma *et al.*, 2002; Schilling *et al.*, 2000). The obstruction of xylem tissue in transporting water and nutrients is another consequence of *S. rolfsii* infection. The collapse of the stem tissue can block the flow of water and nutrients to other parts of the plant, ultimately leading to plant death (Koike, 2004; Mahadevakumar *et al.*, 2018). The combined effect of cellulolytic enzymes and oxalic acid on the structural integrity of the cell wall and the blockage of water and nutrient flow can cause significant damage to groundnut plants and pose a significant threat to crop yields (Kwon *et al.*, 2012).

In this study, it was found that extracts from betel and neem leaves can suppress the growth of *S. rolfsii*, resulting in a reduction in the severity of the disease caused by the infection. The betel leaf is known to possess multiple

antifungal properties owing to the presence of various bioactive compounds like eugenol, chavicol, and terpenes. Scientific studies have indicated that eugenol and chavicol exhibit potent antifungal activity against diverse fungal species, including *Aspergillus*, *Candida*, and *Trichophyton* (Datta *et al.*, 2011; Pawar *et al.*, 2017). The research findings of Didehar *et al*. (2022) demonstrate that eugenol reduces fungal germ tube formation, resulting in decreased nutrient absorption from host tissues. However, it also leads to elevated levels of lipid peroxidation and reactive oxygen species, inducing oxidative stress and increasing permeability in the fungal cell membrane. In a separate study, Costa *et al*. (2015) reported that Chavicol inhibits fungal growth by disrupting the cell membrane and enzyme activity, while also stimulating the production of reactive oxygen species and altering gene expression within fungal cells. Additionally, Zore *et al*. (2011) have stated that terpenes can hinder the growth of *Candida albicans* by affecting membrane integrity and causing cell cycle arrest. Moreover, terpenes present in betel leaf have also been reported to demonstrate antifungal activity by hindering fungal growth and inhibiting spore germination (Zhang *et al.*, 2022). The antifungal characteristics of the betel leaf position it as a natural remedy with potential therapeutic value against fungal infections (Madhumita *et al.*, 2020).

Likewise, neem leaf has been reported to possess strong antifungal properties due to the presence of bioactive compounds such as nimbin, nimbidin, and gedunin. The antifungal properties of neem leaf are believed to arise from its ability to disrupt fungal cell membranes, inhibit fungal enzyme activity, and interfere with fungal metabolism (Raghavendra and Balsaraf, 2014; Suleiman, 2011). Scientific evidence supports the antifungal activity of neem leaf against several fungal species (Ezeonu *et al.*, 2018). The antifungal properties of both betel leaf and neem leaf make them promising candidates as botanical fungicides.

Antifungal agents represent a group of medications that are widely used for the treatment and prevention of fungal infections. These agents exhibit different modes of action, that is, they operate in various ways to inhibit the growth and proliferation of fungi (Arif *et al.*, 2009). One common mode of action is the inhibition of fungal cell wall synthesis. Fungal cell walls are vital structures that provide structural support and protection to the cell. Certain antifungal agents, such as caspofungin and echinocandins, function by targeting enzymes involved in cell wall biosynthesis, thus impeding the synthesis of new cell wall material (Carrillo-Munoz *et al.*, 2006). Another mode of action is the disruption of fungal cell membranes. The cell membrane is a critical component of fungal cells that helps to maintain the integrity of the cell. Azoles, polyenes, and allylamines are examples of antifungal agents that work by binding to ergosterol, which is an essential component of the fungal cell membrane. This binding disrupts the membrane, causing it to become more permeable, ultimately leading to cell death (Alcazar-Fuoli and Mellado, 2013; Arockianathan *et al.*, 2019).

Antifungal agents can also inhibit fungal nucleic acid synthesis. Nucleic acids constitute the building blocks of genetic material in fungi. Flucytosine is an example of an antifungal agent that operates by interfering with the synthesis of DNA in fungal cells, ultimately leading to cell death (Odds *et al.*, 2003). Griseofulvin is an example of an antifungal agent that works by interfering with fungal metabolism by inhibiting fungal cell division and protein synthesis. These agents are particularly effective against dermatophyte fungi that cause skin and nail infections (Al-Obaidi *et al.*, 2019).

The extracts of betel and neem leaves are believed to act as antifungal agents by destroying the fungal cell wall, causing lysis, and thus suppressing the severity of damping-off diseases caused by the *S. rolfsii* in groundnut. These extracts contain several essential oil compounds, azadirachtin, and phenols, which are known to possess antifungal properties (Singh *et al.*, 2005). The neem tree is known to produce a diverse range of biologically active compounds, with over 140 compounds identified from different parts of the tree. The tree's fresh leaves were the source of the first purified polyphenolic flavonoids, including quercetin and ß-sitosterol, which have been found to exhibit antibacterial and antifungal properties (Ahmad, 2012).

Quercetin is a flavonoid compound known for its various biological activities, including antifungal properties. It has been found to exhibit inhibitory effects against a wide range of fungal pathogens. The antifungal activity of quercetin is believed to be due to its ability to interfere with fungal cell membrane integrity, cell wall synthesis, and DNA replication, ultimately leading to fungal cell death (Oliveira *et al.*, 2016). Several studies have reported the antifungal activity of quercetin against various fungal pathogens, including *Candida albicans*, *Aspergillus niger*, and *Penicillium expansum* (Sadeghi-Ghadi *et al.*, 2020).

ß-sitosterol is a phytosterol that has been reported to have antifungal activity. It is a natural steroid found in plants, including neem, and is structurally similar to cholesterol. The antifungal activity of ß-sitosterol is believed to be due to its ability to disrupt the fungal cell membrane, leading to membrane damage and ultimately cell death (Kiprono *et al.*, 2000). It has been suggested that ß-sitosterol causes the leakage of intracellular material, resulting in the collapse of the fungal cell wall. Moreover, ß-sitosterol has been shown to have synergistic effects with other antifungal agents, including fluconazole and amphotericin B, making it a promising candidate for the development of new antifungal agents (Mahmoud *et al.*, 2011).

CONCLUSION

In conclusion, the combination of betel leaf extract and neem leaf extract has been found to be the most effective in inhibiting the growth of the *S. rolfsii*. The colony inhibition percentage has been reported to be 81.09%. Moreover, this combination has also demonstrated significant potential in suppressing the development of Pre-emergence disease, with a reduction rate of 5.33%, as well as Post-emergence disease, with a reduction rate of 33.10%. The results suggest that the mixture of betel leaf and neem leaf extracts may have promising applications in the prevention and treatment of *S. rolfsii* infections.

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