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Invitro analysis of antifungal effects of botanicals on *sclerotinia sclerotiorum* causing white mold disease

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Abstract

White mold, *Sclerotinia sclerotiorum*, is a devastating fungal plant pathogen that has affected many crop species worldwide. Using chemicals to control the disease has been practiced over the years, whose prolonged application has negatively impacted the environment, thus finding an organic solution is crucial. The analysis quantifies the effect of 5 different local plants that have been proven to have fungicidal properties; *Artemisia vulgaris* L., *Azadirachta indica* L., *Zingiber officinale* Roscoe, *Allium sativum* L., and *Lantana camara* L. Poisoned food technique was used to study the inhibition effect, carried out by inoculating and growing the fungus on PDA media infused with botanical extracts. The data of mycelial mat diameter was recorded till the control plates were fully occupied. The growth-inhibiting capacity was found as 100% by *Allium sativum*, 28% & 43% by *Azadirachta indica*, 22.44% & 44% by *Zingiber officinale*, and 16.33% & 8.55% by *Lantana camara* at 10% and 20% concentrations respectively. Only a slight difference between the overall inhibition effect of the two concentrations was found with 37.22% inhibition by 20% concentration and 33.38% inhibition by 10% concentration. No inhibition effect was observed from *Artemisia vulgaris* which could be due to heat neutralization of the active constituent during sterilization. Further research needs to be conducted using the botanical with different sterilization techniques. This in-vitro study identified garlic as a critical antifungal alternative to conventional fungicides. Field experiments need to be done to prove its effectiveness.

Keywords: Fungal-growth inhibition, Invitro analysis, Phytochemicals, Phytopathology, Plant extracts, Plant protection, White mold.

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Contents

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Contribution of this paper to the literature
The research provides information that differs in botanicals used and their preparation from other similar research and is a unique study against white mold. The paper adds the knowledge of how different botanicals fare against white mold and guides further research regarding its organic control.

1. Introduction

Sclerotinia sclerotiorum (Lib.) de Bary (Helotiales: Sclerotiniaceae) is known by multiple names but its most common name recognized worldwide is 'White Mold' as it is characterized by cotton-like fluffy white mycelial growth seen on infected plants' stems. The pathogen exhibits its symptoms by creating water-soaked lesions on leaves that move downwards to the stem where it develops into necrotic tissues before developing its characteristic cottony mycelium [1]. *Sclerotinia* is a polyphagous, necrotrophic, homothallic pathogen that is rampant worldwide, especially in temperate regions [2]. It is a devastating plant pathogen that has infected over 75 families, 278 genera, 408 species, and 42 subspecies of plants. A more pronounced effect of *Sclerotinia* is seen in dicot plants like peas, soybean, sunflower, rapeseed, and occasionally in monocot crops and grasses [3]. *S. sclerotiorum* can cause varying degrees of yield losses to different crops, ranging from 20 to 50% loss in canola production [4] and 10 to 50% loss in lettuce production [5]. Plant pests have been interfering with and preventing crops from reaching their full potential yield since the beginning of agriculture. Their damage to plant parts, systems, physiological processes, and ultimately yield has been one of the contributing factors to food scarcity in the world [6, 7]. Almost 40% loss of crop production and 20% loss in post-harvest is incurred every year due to insect pests, weeds, and diseases in the world [8]. To combat the losses, many chemical pesticides have been developed over the years which on one hand have helped reduce the pests' effect while on the other hand have developed a host of problems like resistant pests, persistent pollutants, biological life hazards, and loss of biodiversity due to death of non-target organisms [9, 10]. So, there have been many attempts at developing alternatives to chemical pesticides like biopesticides, botanical extracts, and microbial pesticides. These alternatives have the benefit of being biodegradable, extremely efficient and accurate, and less harmful to the health of humans and the environment [11]. Many studies have been done on controlling *Sclerotinia* using fungicides [12, 13] from which numerous negative effects arise due to their prolonged use [14]. Therefore many studies are being conducted to measure the efficacy of different biopesticides in the control of fungi [15]. So, farmers are in dire need of appropriate and sustainable approaches to the management of such pests and pathogens [16]. The purpose of this research is to evaluate the efficacy of some locally available plants of Nepal with fungicidal effects against white mold in-vitro, which could be later incorporated into the farmers' fields with further processing and development.

2. Materials and Methodology

2.1. Plant Extract Preparation

The plant extracts were produced through a simple crude aqueous extraction similar to the process used by Timila and Manandhar [17]. First, the fresh botanicals were washed to clean any dirt or external chemicals present in them. Then after shade drying, 100 g each of fresh plant parts were taken and ground into paste separately using a mixer. Each resultant paste was mixed with 100 ml of distilled water. Extra care was taken to create a constant weight-to-volume ratio of botanicals to water so that their concentration would be the same throughout each extract. The mixture was then filtered through a muslin cloth and we obtained the botanical extracts in the form of filtrate as shown in Figure 1. The aqueous extracts kept in separate flasks were autoclaved at 121 °C @ 15 psi for 20 minutes to eliminate any microorganisms present within.

Figure 1. Aqueous botanical extracts of (from left) *A. vulgaris*, *A. sativum*, *A. indica*, *Z. officinale* and *L. camara*.

2.2. Media Preparation

The media of choice for the analysis was PDA (Potato Dextrose Agar) prepared by mixing ready-made PDA powder with distilled water. The analysis was carried out using the "Poisoned Food Technique" as described by Balouiri, et al. [18] where two concentrations (10% and 20%) of each plant extract were infused with PDA, creating a total of 10 unique growth media. To create the two concentrations of 10% and 20%, 10 ml and 20 ml botanical extracts were mixed with 90 ml and 80 ml molten PDA respectively. Unaltered PDA media was kept separately as control and four replications for each unique media and control were made by pouring molten PDA 20 ml each in Petri dishes.

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2.3. Fungal Culture Preparation

Sclerotinia for the analysis was isolated from the Garden pea (*Pisum sativum*) pod shown in Figure 2. The PDA media was inoculated with threads of mycelia initially and it was allowed to grow at 24 °C for a week in an incubator. The inoculating processes were repeated two more times from the obtained growth to ensure the exclusion of any other contaminants and obtain a pure culture.

The final pure culture of 7 days was then used to inoculate all the different media and control with the help of a cork borer sterilized in an open flame. Mycelium discs of 5 mm diameter were punched out from the pure culture and placed on the media at the center of each petri dish. The discs were placed with the mycelial side facing down to assist the pathogen in establishing on the new media. The whole inoculation process was done under aseptic conditions inside the laminar air-flow cabinet, and the inoculated dishes were incubated at 24 °C in dark.

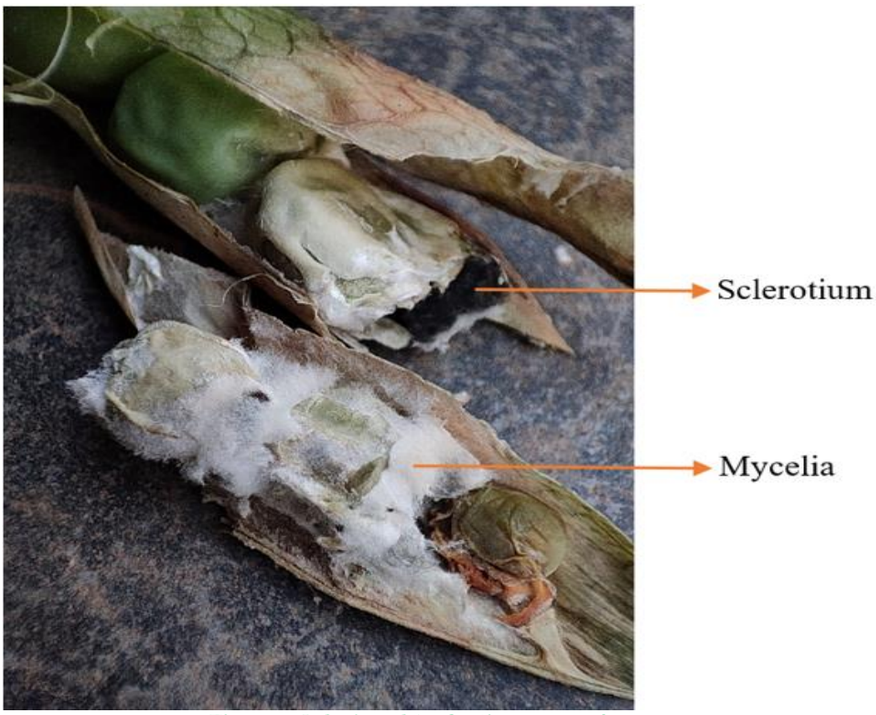

Figure 2. Infection of *S. sclerotiorum* on garden pea.

2.4. Experimental Design and Antifungal Activity Assay

The experiments were conducted in Completely Randomized Design (CRD) with 5 treatments of 2 concentrations viz: 10% and 20%. Four replications each of treatments and control were kept resulting in a total of 44 Petri plates. The fungicidal effects of botanicals were recorded by daily (24 hours) observation and measurement of the radial growth of mycelium, till the control plates were completely covered by the mycelial mat. For the antifungal activity assay, the percentage of mycelium growth inhibition was calculated by using the formula mentioned by Al-Samarrai, et al. [19]; Bekker, et al. [20] and Javed, et al. [21].

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

Where, C = colony diameter (cm) of the control.

T = colony diameter (cm) of the test plate.

The measurement of mycelial diameter was taken in cm and an average of 4 replications was taken for each treatment. The inhibition percentage was calculated for both concentrations of all the botanical extracts for 3 days until the control plate was fully occupied by the mycelial mat and the results are presented in Table 1.

2.5. Statistical Analysis

The data were recorded in MS Excel 2016, along with calculation of mean and standard error of mean. The Analysis of Variation (ANOVA) was done through R-stat version 4.2.2. Fisher-LSD test was used to compare means at 0.05 significance level.

Table 1. Growth inhibition of *S. sclerotiorum* by the use of botanicals.

Treatments	10% concentration		20% concentration		Mean growth inhibition %
	Av. diameter (cm)*	Inhibition %*	Av. diameter(cm)*	Inhibition %*	
A. indica	6.48	28	5.13	43	35.5
A. vulgaris	9	0	8.88	1.33	0.67
A. sativum	0	100	0	100	100
Z. officinale	6.98	22.44	6.03	33	27.72
L. camara	7.53	16.33	8.23	8.55	12.44
Control	9	---------	9	---------	--------
LSD (0.05)		9.80		3.34	
SE_m		1.45		0.494975	
CV %		19.47		5.95	
Grand mean		33.38		37.22	

Note: * Significant difference at 0.05 probability level; LSD: Least significant difference; SE_m: Standard error of mean; CV: Coefficient of variation.

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3. Result

After 72 hours of inoculation, the control Petri plate was fully covered by the mycelia while both concentrations of *A. sativum* did not have any growth, keeping their inhibition at 100%. *A. indica* and *Z. officinale* inhibited the growth by 28% & 22.44% in 10% concentration and 43% & 33% in 20% concentration respectively. *L. camara* also showed its antifungal property by inhibiting the growth by 16.33% & 8.55% in 10% & 20 % concentrations respectively. But *A. vulgaris* only suppressed the growth by 1.33% in the 20% concentration while no effect was seen in the 10% concentration.

There was little difference between the mean inhibition effect of the two concentrations. 20% concentration was only slightly better with 37.22% mean inhibition compared to 33.38% of 10% concentration. Overall, *Allium sativum* performed the best among all the botanicals, completely inhibiting the growth of *Sclerotinia sclerotiorum*, with *A. indica*, *Z. officinale*, and *L. camara* showing mild to low levels of inhibition against the pathogen with 35.5%, 27.72%, and 12.44% mean inhibition across the two concentrations respectively. The inhibition levels are graphically shown in Figure 3 and the petri plates after 72 hours of inoculation can be seen in Figure 4.

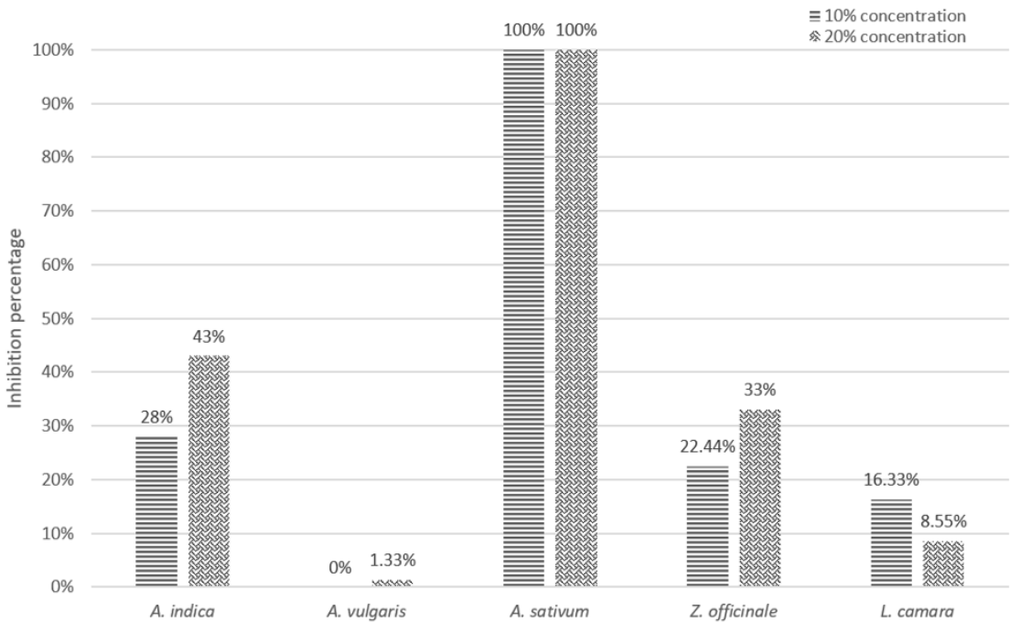

Figure 3. Percentage of inhibition of *S. sclerotiorum* by the use of botanicals (72 hours).

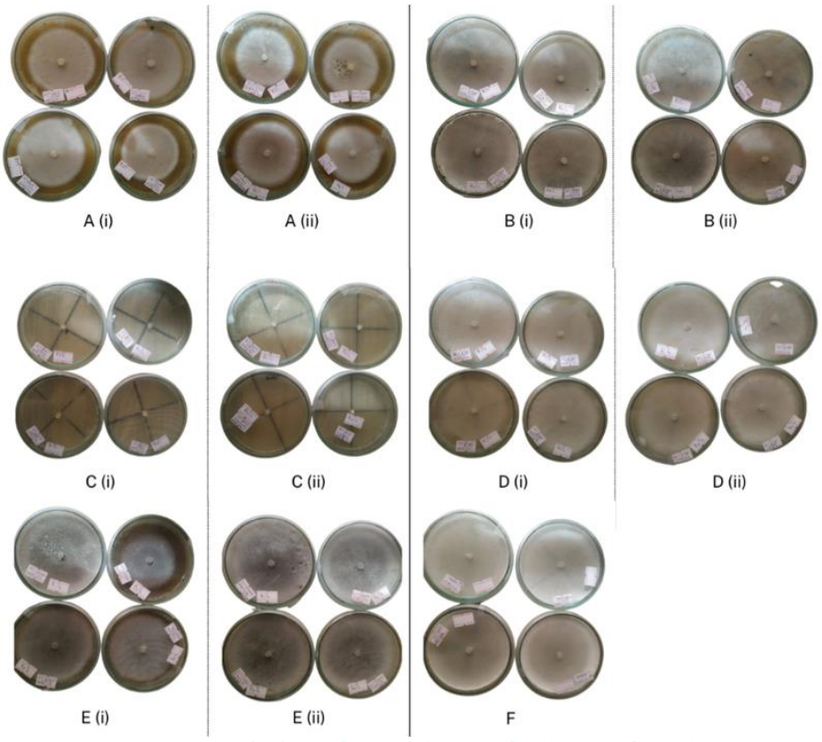

Figure 4. Mycelial growth after 72 hours in **(A)**: *A. indica*, **(B)**: *A. vulgaris*, **(C)**: *A. sativum*, **(D)**: *Z. officinale*, **I**: *L. camara*, **(F)**: Control, where **(i)**: 10% concentration and **(ii)**: 20% concentration.

4. Discussion

The world's increasing population demands greater food production every year. This arises a challenge to protect the crops and reduce food loss, especially from pests and pathogens. Globally 30% of crop products are lost due to fungal plant pathogens, and the toxins produced by the fungal growth and changes in color, odor, and taste, degrades food on a large scale [22]. Fungicide use can decrease such losses of food, but in the wake of organic food

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in recent years, their usage has been limited [23]. Therefore, many attempts have been made in finding organic solutions to the fungal problem, one of which is the use of plant botanicals.

The botanicals selected for the analysis have potential antifungal properties which have been shown by various studies throughout the years; *Zingiber officinale* [24] *Azadirachta indica* [25, 26] *Artemisia vulgaris* [27] *Allium sativum* [28] *Lantana camara* [29]. These plants have been used for our plant pathological needs and purposes through multiple generations spanning thousands of years [30]. In that course of time, their foundation, and the key constituents responsible for their beneficial uses have been documented.

The compounds responsible for the antifungal nature of the botanicals are presented in Table 2. The concentration, amount, and quality of these phytochemicals depend vastly on the genotype, environmental condition, soil condition, plant parts harvested, harvesting season, and the stage at which they are harvested [31]. The plant parts which have been used for this analysis have all been collected in the locality of Rupandehi, Nepal, and so these parameters are likely to change along with the efficacy against *Sclerotinia* in comparison to native plants of any other region.

Table 2. Chemical constituents of the botanicals used for the analysis.

Plant	Family	Key Constituent	References
Artemisia vulgaris (Mugwort leaf)	Asteraceae	α-thujone, Ascaridole β-thujone 1,8-cineole	Satyal, et al. [32]; Blagojević, et al. [33] and Cetin, et al. [34]
Azadirachta indica (Neem leaves)	Meliaceae	Quercetin Azadirachtin	Sithisarn and Gritsanapan [35] and Mahmoud, et al. [36]
Zingiber officinale (Ginger rhizome)	Zingiberaceae	Gingerols Terpenes	Park, et al. [37] and Yeh, et al. [38]
Allium sativum (Garlic cloves)	Amaryllidaceae	Allicin Ajoenes	Leontiev, et al. [39] and El-Saber, et al. [40]
Lantana camara (Leaves)	Verbenaceae	Germacrene-D, E-caryophyllene Bicyclogermacrene, isocaryophyllene	Passos, et al. [41] and Sousa, et al. [42]

The most effective among all the botanicals used was found to be *Allium sativum*, followed by *Azadirachta indica*, *Zingiber officinale*, and *Lantana camara*. Similar results have been obtained in many experiments done worldwide [15, 17, 43]. *A. vulgaris* did not show any significant effect and failed to inhibit fungal growth. The antifungal property of *Artemisia* species, just like the other botanicals, is governed by its secondary metabolites. One of the secondary metabolites present in *Artemisia* is a compound Ascaridole [32, 44]. It is a thermolabile compound [45] so it decomposes when subject to extreme heat. This could be the reason for the negative inhibition shown by *A. vulgaris* as the compound could have been destroyed when it was being autoclaved for sterilization.

5. Conclusion

The world is battling with food insecurity and the use of chemicals. While the short-term immediate benefits are favorable, continued chemical usage can be devastating for our food supply in the future and the use of bio-rational compounds is imperative for sustainable agriculture. So, it is important in identifying the best organic alternative to some of the major diseases of crops. These identified botanicals can be then used as a source to isolate the specific chemical constituent and develop a much more effective remedy for agricultural diseases. Such analysis can help identify hidden control measures of our locality and further aid in developing new medicines for diseases of plants. The obtained result of the antifungal nature of the botanicals used here, needs to be further confirmed in the field condition. Many other local botanicals can be examined for their efficacy against *Sclerotinia sclerotiorum* and efforts can be made to isolate the chemical compounds. Further experimentations with different extraction and application methods need to be conducted before ruling out any potential plant.

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