



## Fungistatic Activity of Floral Volatile Compounds of *Alstonia scholaris*

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### Authors' contributions

This work was carried out in collaboration between both authors. Author AM designed the study, performed the fungal growth assays and statistical analysis, wrote the protocol, and wrote the first draft of the manuscript. Author DK conducted all distillation procedures and data recording. Both authors read and approved the final manuscript.

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Short Communication

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### ABSTRACT

**Aims:** The fragrant flowers of *Alstonia scholaris* are rich in volatile compounds or essential oils, which comprise terpenes such as 1,8-cineole and linalool. The effects of the oils on the growth of *Aspergillus niger* were assessed for the first time in the present study.

**Study Design:** Fungal growth assay.

**Place and Duration of Study:** Sample: Department of Botany, St. Xavier's College, Mumbai, between September 2006 and December 2006.

**Methodology:** The volatile compounds of *A. scholaris* were extracted through steam distillation and hydrodistillation by using a Clevenger apparatus. The effect of the steam distillate (10% v/v) on the number of spores of *A. niger* was observed over 5 days. Furthermore, three fractions with distinct fragrances and boiling points were collected through hydrodistillation. The effects of each fraction on mycelial growth and sporulation in *A. niger* were examined. Accordingly, each hydrodistilled fraction was incorporated in growth medium (10% v/v) inoculated with fungal spores. The growth of the fungus was observed over 5 days. Distilled water was used as the control. All experiments were conducted in triplicate.

**Results:** The fungal samples treated with steam distillates showed a significantly lower number of spores than the control after 5 days ( $165 \pm 36$  vs.  $35 \pm 7$  spores/mL) at  $P \leq .05$ . Furthermore, the

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fungal samples treated with the hydrodistilled fractions exhibited a delayed mycelial growth and sporulation compared with the control over 5 days. Fraction 1 was most effective in delaying mycelial growth (Day 4 vs. Day 1). Notably, on Day 4 none of the treated samples but the control sample exhibited sporulation.

**Conclusion:** The volatile compounds of *A. scholaris* flowers clearly suppressed and delayed both mycelial growth and sporulation in *A. niger* but did not inhibit growth entirely. Hence, the floral volatile compounds exhibit fungistatic activity against *A. niger*.

**Keywords:** Floral volatile compounds; terpenes; distillation; *Alstonia scholaris*; *aspergillus niger*; sporulation.

## 1. INTRODUCTION

Floral volatile compounds (FVCs) or essential oils (EOs) are responsible for the strong fragrance of the flowers of *Alstonia scholaris*. The flowers are white, numerous, and arranged in clusters in the inflorescence. The strong fragrance and absence of any bright colors in the flowers indicate that *A. scholaris* is pollinated by nocturnal insects [1]. Thus far, few studies have explored the composition and biological properties of the FVCs of *A. scholaris*. In our previous study [2], the FVCs were extracted through steam distillation of *A. scholaris* flowers, and the components were identified through gas chromatography–mass spectrometry. The identified components included terpenes such as 1,8-cineole, linalool, linalool oxide, and sabinene hydrate. In the present study, the biological effect of the FVCs were examined.

Deterioration of material such as food, fabric, and leather due to fungi is a serious problem worldwide, particularly in tropical countries [3]. Large losses of food and fabric are incurred each year because of fungal spoilage [4]. Conventional methods of controlling or preventing fungal growth involve spraying synthetic fungicides on crops and using preservatives, such as sulfites or salts of organic acids, in packaged foods. However, fungi develop resistance to synthetic compounds and preservatives. Moreover, synthetic compounds can breakdown in food and become potentially toxic. Recently, the use of EOs in food preservation is rapidly gaining scientific attention. EOs are being considered alternatives to conventional fungicides in food. The hydrophobic nature of EOs and their components, particularly terpenes, is responsible for their effects on fungal cell walls, cell membranes, and membrane proteins. The outcomes of the interaction between EOs and fungal cells include cell wall degradation, increased permeability, lesions on the cell

membrane, loss of ergosterol, and loss of chemiosmotic control [5].

In the present study, the effects of the floral distillates on the growth of *Aspergillus niger* were examined for the first time. The effects of the FVCs on fungal growth were assessed by determining the effect of the FVCs on spore count and that on the time of onset of sporulation. Both spore count [6] and sporulation onset [7] were considered indicators of fungal growth. The results of the study showed that the FVCs significantly reduced the number of spores and delayed sporulation in *A. niger*. Reducing spore number and delaying sporulation is likely to considerably reduce the production of mycotoxins and eventually reduce losses and wastage of food because of spoilage and contamination with mycotoxins.

## 2. MATERIALS AND METHODS

### 2.1 Collection of Plant Materials

Fresh inflorescences of *Alstonia scholaris* in full bloom were collected from trees in the Rang Bhavan locality of South Mumbai between October and November 2006. The flowers were stored airtight containers at 4°C until further use.

### 2.2 Hydrodistillation

Approximately 80 mL of distilled water was taken in a 250-mL round-bottom flask. Glass beads were used to prevent bumping of water during distillation. Approximately 50 g (fresh weight) of corolla tubes were added to the flask. A Clevenger apparatus (Borosil, Mumbai) was used for hydrodistillation at atmospheric pressure. All joints were coated with silicone grease (Merck, Germany) for sealing. A calibrated heating mantel (Labline, Mumbai) was used for heating to ensure gradual and controlled heating for approximately 45 min to reach 70°C.

The build-up of steam was prevented by means of a vent. The heating was then stopped when the vapors reached the bent tube connecting the still head to the condenser. The entire apparatus was cooled until vapors stopped rising from the water in the round-bottom flask. Then, the flask with distilled water and corolla tubes was replaced by a flask containing only distilled water. The temperature was then increased to 100°C. The steam rising from the flask flushed the fragrant vapors toward the condenser for approximately 10 min. Subsequently, three fractions with different odors were collected at intervals of approximately 5 min.

Fraction 1: Distinctly floral, slightly green, and spicy odor

Fraction 2: Mildly floral, mildly spicy, and slightly woody odor

Fraction 3: Distinctly herbal and mildly woody odor

The collection of fractions was stopped as soon as a vegetable-like odor was perceived (after approximately 75 min).

### 2.3 Effect of Steam Distillates on Number of Spores in *A. niger*

In a previous and closely related study, the composition of the steam distillate was analyzed [2]. The GC–MS analysis revealed the presence of components such as 1,8-cineole and linalool, which have been reported to show antifungal activity [8]. The effect of the steam distillate on spore production in *Aspergillus niger* ATCC 16404 was observed. A suspension of the spores ( $10^6$  spores/mL) [9] was inoculated in Sabaroud's dextrose broth (HiMedia, Mumbai) containing either distilled water (control) or 10% (v/v) steam distillate (test).

The cultures were incubated at 25°C under dark and static conditions for 5 days until sporulation was observed. The fungal mats were dabbed dry and transferred to sterilized petri dishes (Borosil, Mumbai) under sterile conditions and flooded with 10 mL of a sterile spore sampling solution (0.1% saline + 1% Teepol). The fungal mats, which were formed by fusion of colonies, were scraped uniformly by using sterilized spatulas to ensure that the spores were suspended in the sampling solution. The hemocytometer method [10] was used to count the number of spores in a unit volume of the sampled solution. The experiment was run in triplicate ( $n = 3$ ).

### 2.4 Effect of Hydrodistillates on Mycelial Growth and Sporulation in *A. niger*

Based on the reduction in spore number caused by the steam distillates, the effects of the hydrodistillates (fractions) on sporulation in *A. niger* were tested.

A fixed volume (0.2 mL) of distilled fractions 1, 2, or 3 (10% v/v) was mixed with culture medium to obtain a final volume of 2.0 mL (consisting of culture medium, distillate, and spore suspension). The experiment was run in triplicate ( $n = 3$ ).

### 2.5 Statistical Analysis

Data are reported and means and standard deviations. All data were analyzed statistically using SPSS (version 19.0). The number of spores in samples obtained from cultures treated with the steam distillate was compared with that in the control samples by using the unpaired t-test. Values of  $P < .05$  were considered significant. The time in days to the first appearance of mycelia and spores (even beyond the 5-day observation period) in the fungal cultures treated with hydrodistillation fractions and control were compared using one-way analysis of variance followed by post hoc Duncan's multiple range test. In all graphical representations, means assigned different alphabets in a given data series are significantly different from each other.

## 3. RESULTS AND DISCUSSION

### 3.1 Effect of Steam Distillates on Number of Spores in *A. niger*

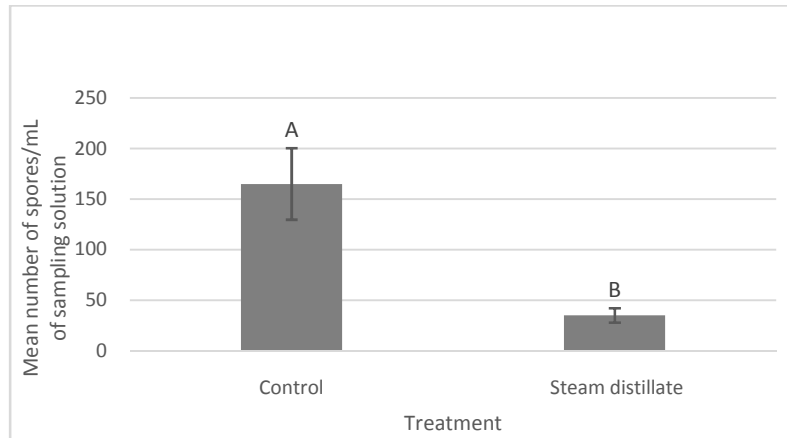
The *A. niger* culture exhibited small colonies spread out across the petri dish. Sporulation began after approximately 96 hours, by which time, the fungal colonies had fused to form mats. The mean number of spores in the sample derived from fungal cultures grown in the presence of steam distillates was significantly lower than that in the control sample ( $35 \pm 7$  vs.  $165 \pm 35$  spores/mL of sampled solution) as shown in Fig. 1.

### 3.2 Effect of Hydrodistillates on Mycelial Growth and Sporulation

Fungal growth appeared to be adversely affected in the presence of the hydrodistillates over the 5-day observation period (Table 1). The initial inoculum (spore suspension) was visible in all

samples. The control sample exhibited the first signs the mycelial growth on Day 1. The samples containing Fractions 2 and 3 exhibited first signs the mycelial growth on Day 2. However, in the sample containing Fraction 1, the first appearance of mycelial growth was observed on Day 4. On Day 4, the control sample showed the first signs of sporulation. However, none of the

samples treated with the hydrodistillates exhibited signs of sporulation until the end of the observation period (5 days). All the samples were observed for an additional period of 3 days. As shown in Fig. 2, the samples treated with Fractions 3, 2, and 1 respectively, exhibited the first signs of sporulation on Days 6, 7, and 8 (mean duration).

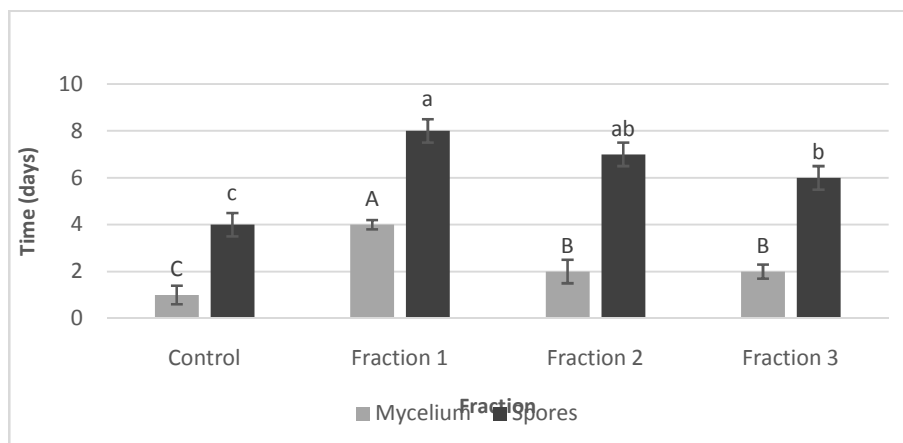


**Fig. 1. Effect of steam distillate on number of spores produced in *A. niger***  
 Mean ± standard deviation of triplicates are reported (n = 3). The difference in mean number of spores is significant at  $P \leq .05$

**Table 1. Growth of *A. niger* treated with the hydrodistillates**

Days→	Day 1		Day 2		Day 3		Day 4		Day 5	
Sample ↓	M	S	M	S	M	S	M	S	M	S
Control	+	-	++	-	+++	-	+++	+	+++	++
Fraction 1	-	-	-	-	-	-	+	-	+	-
Fraction 2	-	-	+	-	++	-	++	-	++	-
Fraction 3	-	-	+	-	++	-	++	-	++	-

M: mycelium; S: spores; +: present; -: not detected



**Fig. 2. Time until the first appearance of new mycelia and spores in *A. niger***  
 Mean ± standard deviation of triplicates are reported (n = 3). The difference in mean number of days is significant at  $P \leq .05$ . The bars on the left and right indicate the number of days until appearance of mycelia and spores, respectively. Upper-case and lower-case alphabets have been used to indicate significantly different means in the mycelium and spore series, respectively

In the present study, the distillates of *A. scholaris* significantly reduced the number of spores and delayed the appearance of sporulation. The antifungal activity of several EOs has been documented, particularly in various species of *Aspergillus* [2,3,8]. For example, EOs derived from lemon grass, cassia, clove, and cinnamon reduce spore formation in species of *Aspergillus*. Furthermore, EOs obtained from lemon grass and oregano inhibit spore germination [5]. The antifungal properties of EOs are now being explored for use in preservation of food and fabric. EOs are considered acceptable food additives by consumers because of their natural origin. A majority of EOs are generally considered safe for human use; consequently, the search for safe and effective natural alternatives to synthetic preservatives is gaining momentum [3], and EOs are among the most suitable alternatives. Our previous study on the FVCs of *A. scholaris* revealed that the steam distillate comprises terpenes such as 1,8-cineole, linalool oxide, linalool and sabinene hydrate in addition to others. The boiling points (at 760 mmHg) of the aforementioned terpenes are 176.4°C, 188°C, 198°C and 200°C, respectively [11]. The literature suggests that the extraction of linalool and 1,8-cineole by steam distillation and hydrodistillation are similar [12]. Hence, it is reasonable to assume that the Fraction 1 contained 1,8-cineole. Furthermore, 1,8-cineole exhibits strong antifungal activity against *A. niger* [13]. Hence, in the present study, the fungal samples treated with Fraction 1 exhibited delayed appearance of mycelia (4 days vs. 1 day) and spores (8 vs. 4 days) compared with the control. Similarly, linalool, which may be assumed to be in Fraction 2 on the basis of boiling point, exhibits antifungal activity [14]. As expected, the fungal samples treated with Fraction 2 also exhibited delayed appearance of mycelia (2 days vs. 1 day) and spores (7 vs. 4 days) compared with the control.

Sporulation is a crucial stage in the life cycle of sporogenic fungi. The production of mycotoxins is strongly associated with sporulation [7,15]. *Aspergillus* spp. produce mycotoxins such as aflatoxins and ochratoxin [16]. In general, mycotoxins are highly toxic to humans and animals. Clearly, food material that is contaminated by *Aspergillus* spp. is likely to contain mycotoxins and is unfit for consumption. Consequently, contamination of food by *Aspergillus* spp. causes large losses of food grains and processed foods every year [3].

In the present study, the FVCs of *A. scholaris* were reported to delay sporulation for the first time. It may be reasonable to assume that the FVCs also interfered with mycotoxin production. Because the FVCs comprise monoterpenes such as linalool and 1,8-cineole, the interaction between fungal cells and the FVCs is likely to have caused disturbances in the cell wall and cell membrane as well as the pathways involved in spore formation and those involved in mycotoxin biosynthesis [13]. The actual mechanisms underlying the observed delay in sporulation warrant detailed investigation from a molecular perspective in subsequent studies.

#### 4. CONCLUSION

The findings of the present study indicate that the FVCs of *A. scholaris* can be considered candidates for natural food preservatives owing to their fungistatic activity against *A. niger*. However, their safety for consumption and effects on the aroma and flavor of foods should be assessed thoroughly before use.

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#### COMPETING INTERESTS

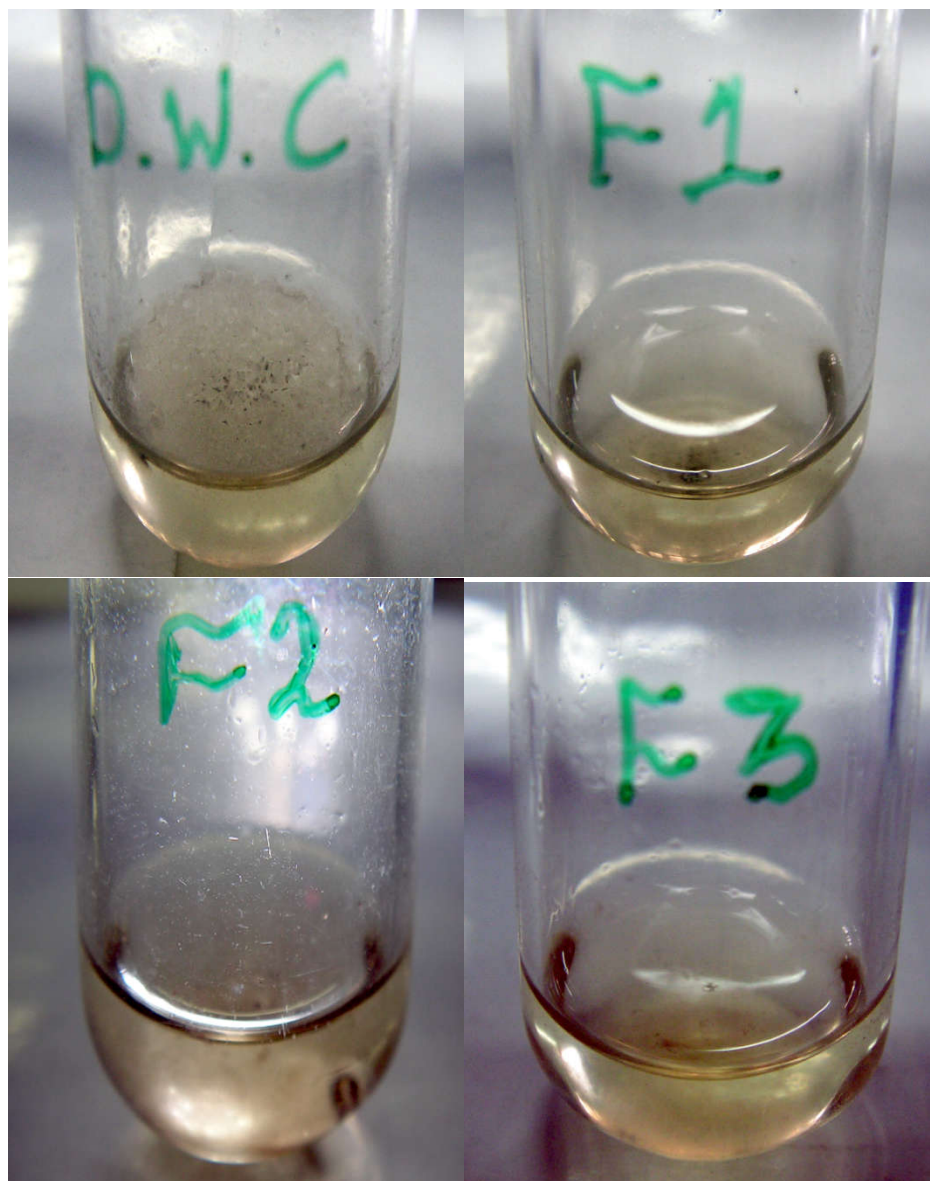
Authors have declared that no competing interests exist.

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## APPENDIX



**Supplementary Fig. 1. Growth (mycelia and spores) of *Aspergillus niger* on Day 5 after inoculation**

A: Control; B: Fraction 1 (10% v/v); C: Fraction 2 (10% v/v); D: Fraction 3 (10% v/v). Initial inoculum is visible in all test tubes. Spores are visible only in the A. Slight mycelial growth in B. Mycelial growth visible in C and D.

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