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In Vitro Antifungal Activities of *C. sativum* and *C. aurantifolia* against *Aspergillus sp.* of Onion Bulbs

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ABSTRACT

Phytopathogenic fungi have been a mass problem in agriculture, including black mould disease in Allium cepa. The study objective is to find the antifungal potential of Coriandrum aurantifolia and Citrus sativum against fungal species isolated from onion bulbs. Thus, the antifungal activity of both leaf extracts of C. aurantifolia and C. sativum alone or in combination at different ratios were tested in this study using the food poisoned technique. Phytochemical screening showed that the C. aurantifolia leaf extract has more active metabolites than C. sativum. C. aurantifolia contains all active constituents tested except terpenoids. Infected A. cepa with black mould was used, and the infection was isolated. According to morphological characteristics, fungal was identified as Aspergillus sp. Antifungal activity discovered a synergistic effect between 100 mg/mL of leaf extract (T8), resulting in the highest reduction of mycelial growth and inhibition percentage. The results concluded that C. aurantifolia and C. sativum leaf have potential antifungal applications and are synergistically effective in preventing the growth of Aspergillus sp. They provide a better reduction in mycelial growth than the positive control. The phytochemical compounds in both extracts might contribute to fungal inhibition activities. To conclude, these two extracts were beneficial as a natural antifungal for Allium cepa.

Keywords: *C. aurantifolia; C. sativum; phytochemical screening; Aspergillus sp.; antifungal activity.*

1. INTRODUCTION

Fungi infestation during storage causes significant economic losses crops such as vegetables, fruits, and grains. Upon fungal infections, the nutritional value of the plants reduced, unfit for human consumption [1]. *Aspergillus niger* causes black mould disease in various plants such as onions, tomatoes, and papayas [2]. The fungus is cosmopolitan; it can be found in the soil, organic debris, and crop products [3]. Out of many fungi, *Aspergillus* species has been reported as a mycotoxin producer in which its exposure to humans and animals would cause hazardous effects [4].

Common crop diseases can be controlled effectively by using chemical fungicides. However, the non-eco-friendly characteristic of chemical fungicides and their toxicity have limited their usage widely. Furthermore, current chemical fungicides are associated with the factors causing the emergence of the fungicide resistance population [5]. As a solution, a natural antifungal compound synthesized from plants and herbs could be an alternative to chemical fungicides. Natural products such as plant extracts, essential oils, herbs, and spices highly contribute to

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pharmaceuticals [6]. The natural elements helped to preserve the ecosystem and prevent environmental pollution.

Lime (*Citrus aurantifolia*) and coriander (*Coriandrum sativum*) were examples of plants and herbs with antimicrobial compounds. *Citrus aurantifolia* (*C. aurantifolia*) belongs to Rutaceae, commonly called vital lime or orange bitter [6]. This plant is widely grown in tropical and subtropical regions [7]. Lime is naturally known to possess antibacterial, antiviral, and fungicidal characteristics, and the essential oil of *C. aurantifolia* can control *A. flavus* at 3000 mm/kg [2].

Coriandrum sativum is considered a herb from the family Apiaceae [8]. It is originatedin Mediterranean countries and is widely grown in Italy, India, Morocco, and many other places [9]. Coriander leaves contain volatile oil, protecting against foodborne infections [10]. Synergistic effects are nonlinear accumulative effects of two active ingredients with similar or related outcomes of their different activities or active ingredients with successive or additional events, producing a more significant and outstanding effect [11].

The identification of active compounds found in methanol leaf extract for both *C. aurantifolia* and *C. sativum was* assessed through phytochemical screening. Then, this study focuses on the isolation and identification of *Aspergillus sp.* from onion based on its morphology, and finally, the individual antifungal activities of both extracts and the synergistic effect between extracts were further examined using poison food techniques.

2. MATERIALS AND METHODS

2.1 Sample

Onion bulbs, fresh leaves of lime and coriander were purchased from a local supermarket and stored in room condition.

2.2 Extraction of Lime and Coriander

Fresh leaves of lime and coriander were cleaned, air-dried overnight, and grounded using an electrical blender. The samples were agitated in a shaker for 48 hours at 28°C. The extracts were then filtered and subjected to a rotary evaporator until crude extracts were obtained. Different extracts were prepared (50 and 100 mg/mL) using the dilution method with 90% methanol as solvent. 50 g of powdered sample added in 500 mL of 90% methanol.

2.3 Preliminary Phytochemical Screening

The methanol extracts of lime and coriander were subjected to preliminary phytochemical screening to identify various active metabolites presence, including alkaloids, tannins, saponin, phenols, terpenoids, and flavonoids. The phytochemical screening methods were adopted from [6; 12].

2.3.1 Test for Alkaloid

Extracts are treated with Mayer's reagent (mercuric potassium iodide). The formation of a yellow-coloured precipitate indicates the presence of alkaloids.

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2.3.2 Test for Tannin and Phenol

Three or four drops of ferric chloride test reagent were added to identify tannin or phenol. The intense green colour that appeared was taken as evidence the presence of tannins, and the formation of a yellow colour indicated the presence of phenol.

2.3.3 Test for Saponin

1 mL of extract was diluted with distilled water to 20 mL and shaken in a graduated cylinder for 45 minutes. The formation of a 1 cm layer of foam indicated the presence of saponin.

2.3.4 Test for Flavonoid

Extracts were treated with 5 mL of dilute ammonia solution followed by the addition of concentrated sulphuric acid. The formation of yellow precipitate indicated the presence of flavonoids.

2.3.5 Test for Terpenoid

Salkowski test: A portion of extracts mixed with 2 mL of chloroform and 3 mL of concentrated sulfuric acid was added carefully to form a layer. A reddish-brown colour formed at the interface indicated the presence of terpenoids.

2.4 Isolation of Aspergillus sp. from Infected Onion Bulb

The outer dry scales of the affected bulb were stripped off and sterilized with 1% sodium hypochlorite solution for one minute. The surface was rinsed with distilled water and blotted dry with filter paper. 3 mm^2 of onion tissue from the margins of the infected area was cut and put onto Potato Dextrose Agar (PDA). The plates were incubated at room temperature for seven days (26 ± 2°C). The developing fungal colonies were then sub-cultured continuously on fresh PDA plates to obtain pure fungal cultures. Sterile distilled water was added to the surface of the plate, and spores were scraped. Then, the new PDA plate spread 100 µL of standardized fungal spore suspension. Fungal identification was later identified based on morphological characteristics [3].

2.5 Preparation of Antifungal Assays

The antifungal efficiency of lime and coriander leaf extracts was determined using a poisoned food technique [1, 5]. In this technique, 100 µL of different extract concentrations were added in autoclaved PDA media in the petri dish before solidified. PDA medium with 90% methanol extract was prepared as a negative control, while a medium inserted with synthetic fungicide (Blitox) served as the positive control.

2.6 In vitro Evaluation

Five-millimetre disc of seven old day culture fungi was removed using a cork borer and placed onto a new antifungal medium. Samples were incubated at room temperature, and after 15 days, colony diameter was measured in remillimetres and recorded. Antifungal activities of the extracts were represented by percentage inhibition of mycelia growth and were calculated using the following formula.

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$$\text{Percentage inhibition (\%)} = [(dc-dt)/dc] \times 100 \quad (1)$$

dc = Average increase in mycelial growth in control,
dt = Average increase in mycelial growth in treatment

2.7 Statistical Analysis

The statistical analysis was performed using SPSS software version 20.0 to determine the inhibitory efficacy of different leaf extracts and concentration of *C. aurantifolia* and *C. sativum* on mycelial growth of *Aspergillus sp*.

3. RESULTS AND DISCUSSION

3.1 Preliminary Phytochemical Screening

The phytochemical screening on both extracts was carried out to identify the presence of active compounds. Overall, *C. aurantifolia* was found to have more active compounds than *C. sativum*. The phytochemical screening of *C. aurantifolia* and *C. sativum* leaf extracts is summarized in Table 1.

Table 1: Phytochemical composition of lime and coriander leaf methanol extracts

Active Constituent	*C. aurantifolia*	*C. sativum*
Alkaloid	+	-
Tannin	+	+
Saponin	+	+
Flavonoid	+	-
Phenol	+	+
Terpenoid	-	-

+ indicates the presence of the active constituents
- indicates the absence of the active constituents

Similar work has also been pursued by Namani *et al.* [13] on *C. aurantifolia.* The leaf extracts showed the presence of major classes of phytochemicals such as alkaloids, carbohydrates, tannins, flavonoids, and steroids. The biological activity of medicinal plants is attributed to secondary plant metabolites. Polyphenolic compounds exhibit a wide array of biological actions, including antioxidant activity. Phenolic compounds primarily neutralize or scavenge free radicals by donating electrons [14].

A recent study conducted by Al-Aamri *et al.* [15] has reported that the essential oil of *C. aurantifolia* leaves showed excellent antibacterial activity against *Staphylococcus aureus*. Limonene was found as the primary compound in this study. Alkaloids are also known to have good antimicrobial and antifungal activities, consistent with phytochemical screening. The presence of hydroxyl moiety in the alkaloids could enhance the cytotoxic, antimicrobial, and antifungal activities [16].

Total phenolics and flavonoid levels and their antioxidant effects have recently been discovered in *C. sativum* [17]. Besides, this finding was paralleled with the results by Farah *et al.* [18], which stated that the *C. sativum* leaves had higher amounts of phenolic compounds than the seeds.

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3.2 Identification of Aspergillus sp.

To identify microorganisms responsible for spoilage of onion bulbs during storage, samples were collected from onion bulbs' surfaces infected with black mould (Figure 1a). The samples collected were inoculated on PDA media and were allowed to proliferate, followed by subculturing to obtain a pure culture. The purified fungal isolates were then identified using microscopic evaluation. After seven days of culture, the pure colony was identified as *Aspergillus sp.* (Figure 1b). The fungal identification refers to the salt and pepper colony's physical appearance. The microscopic view of conidia shows globose and subglobose shapes with 3.5-5.0 um in diameter (Figure 1c). The isolated fungal also has a dense layer of dark brown to black conidial heads. Aspergillus sp. conidial heads are large, globose, and radiate [19]. Conidia are microscopically shown as dark brown to blacku, with a rough-walled structure [20].

According to Figure 1d, the isolated sample of *Aspergillus sp.* hyphae shows that the structure is septate, and the conidiophore is long and brown. Its vesicle consists of a round and radiate head. *Aspergillus sp.* conidial heads initially radiate, splitting into columns in age [19]. Meanwhile, Figure 1e shows a biseriate structure of phialides. This finding is in excellent agreement with Sharma [20], which stated that the biseriate phialides are borne on a brown and septate metula.

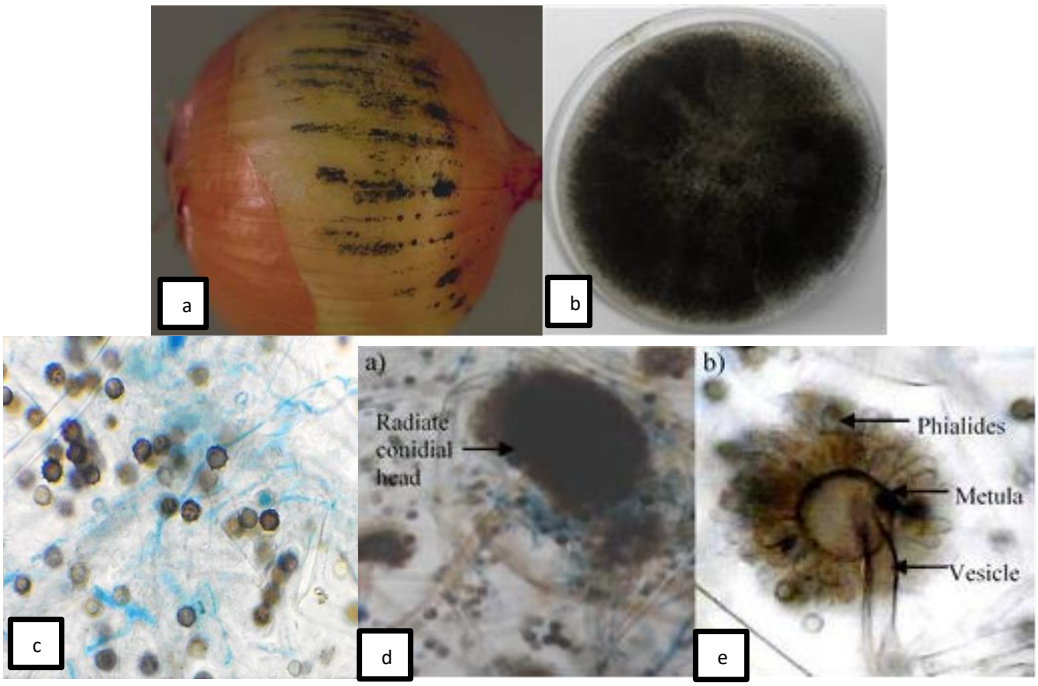

Figure 1 a) Early infection of *Aspergillus sp.* on onion bulb [21] b) Microscopic view of a pure colony of *Aspergillus sp.* cultured for seven days c) Conidia of *A.niger* under 100x magnification d) conidiophore and e) biseriate structure of phialides in *Aspergillus sp.* under 40x magnification

3.3 Evaluation of Antifungal Activities

The poisoned food technique was employed to determine the inhibitory efficacy of different leaf extracts of *C. aurantifolia* and *C. sativum*. The result shows the potential of leaf extracts as natural antifungals the mycelial growth of *Aspergillus sp.* isolated from *A. cepa*. Both extracts in antifungal treatments of *Aspergillus sp.* effectively reduces mycelial growth (Table 2).

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Increased extract concentrations in each leaf sample increase fungal inhibition. For single extract treatment, results found that the lowest average mycelial growth diameter was found at the highest extracts concentrations (100 mg/ml) of *C. aurantifolia and C. sativum* with 15.5 mm (T2) and 18.9 mm (T4), respectively. Single treatment of extracts shows potential in inhibition of the mycelial growth diameter. *C. sativum* contains a broad range of bioactive compounds and an abundance of biological properties, including antifungal, antimicrobial, and act as conventional antibiotics [22]. While for *C. aurantifolia*, the bioactive compound in the plant tissues inhibits mycelial growth and spore germination of *Aspergillus sp.* [23].

C. aurantifolia (T2) presented the lowest mycelial growth in a single treatment. *C. aurantifolia* extract has a higher presence of active metabolites than *C. Sativum*. According to research conducted by Pathan *et al.* [6], *C. aurantifolia* hydro-alcoholic leaf extracts can inhibit the growth of *Aspergillus sp.* up to 66.6 %. The data shows a good agreement with Laribi *et al.*[22] that *C. aurantifolia* has a high potential as an antifungal.

Table 2: Average diameter of mycelial growth (mm) in each treatment

Treatment	*C. sativum* extract (mg/mL)	*C. aurantifolia* extract (mg/mL)	Average Diameter of Mycelial growth (mm)
T1	0	50	31.8 ± 0.40^{g}
T2	0	100	15.5 ± 0.26^{d}
T3	50	0	31.0 ± 0.09^{g}
T4	100	0	18.9 ± 0.12^{f}
T5	50	50	17.5 ± 0.23^{e}
T6	50	100	5.8 ± 0.09^{b}
T7	100	50	5.2 ± 0.18^{b}
T8	100	100	3.6 ± 0.09^{a}
Negative control			54.3 ± 0.17^{h}
Positive control (Blitox 2 mg/L)			12.7 ± 0.09^{c}

*mean with the same letter were not different ($p < 0.05$, Tukey test)

In the combined treatment of both extracts, it was discovered that mycelial growth was highly inhibited compared to the single treatment. The range of mycelial diameter was found from 17.5 to 3.6 mm. Table 2 shows that an increment in extracts concentration had significantly reduced the mycelial growth diameter. This is explained by increasing the concentration of extracts in T6 and T7 reduces mycelial growth to 5.8 mm and 5.2 mm, respectively. T8 is the optimum treatment with the lowest mycelial growth (3.6 mm). *Aspergillus sp.* growth is significantly affected by the extract's concentration. The extract combination at high concentration successfully gave a synergistic effect towards mycelial growth. A mixed combination of phytochemicals may give better antifungal activities than a single phytochemical [23].

Figure 2 shows the percentage of mycelial inhibitions of each treatment. . In this study, there are significant relationships between *C. aurantifolia* and *C. sativum* in inhibiting the growth of *Aspergillus sp.* isolated from the infected onion. According to Figure 2, the reduction of mycelial growth percentage was highly found in combined treatment. With 93.3% inhibition, T8 had the highest percentage of inhibition.T7 and T6 followed this with 90.4% and 89.3%, respectively. The lowest inhibition was found in T1 with 41.4%. The findings revealed interaction of bioactive compounds in both *C. aurantifolia* and *C. sativum* enhances the fungal inhibitory activity. This could be the phenolic ingredient in both extracts contributing to *Aspergillus sp.* inhibition [24]. Similar study done by Karmegam [25] reported that the

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antimicrobial activity of combination extract of *Aegle marmelos* (stone apple), *Coreopsis auriculuta* (lobed tickseed) and *Cissus quadrangularis* (veld grape) revealed more assertive inhibition zones than the individual extracts [24].

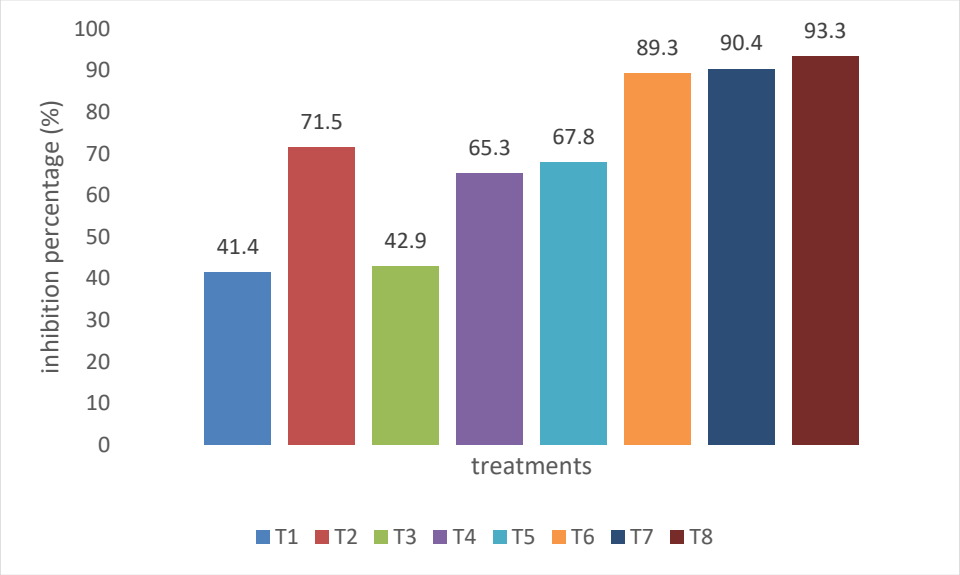

Figure 2: Percent inhibition of mycelial growth

3. CONCLUSION

In conclusion, *C. aurantifolia* showed the presence of major classes of phytochemicals tested than *C. sativum*, namely alkaloids, tannins, saponin, flavonoids, and phenols. This study has successfully isolated and identified *Aspergillus sp.* from infected *A. cepa*. The antifungal activities of *C. aurantifolia* and *C. sativum* were done, and both extracts are effective against the fungus *Aspergillus sp.* either alone or synergistically. In a single treatment, *C. aurantifolia* has a higher antifungal potential than *C. sativum*. However, the combined treatment was more effective in treating *Aspergillus sp*. T8 synergises both extracts in inhibiting *Aspergillus sp.* with 3.6 mm mycelial growth diameter and 93.3% inhibition percentage. The extracts have shown significant potential as a natural fungicide. The present study proves that plants and their products are valuable alternatives to prevent and control the growth of phytopathogenic fungi such as *Aspergillus sp.*, which is responsible for black mould disease of onion during pre-harvest and post-harvest season. It is widely known that fungi can develop resistance to chemical fungicide, which leads to higher dose applications to kill them effectively. The implications of this action towards the environment can be lethal to other organisms. Instead of using chemicals, natural products are more environmentally safe, readily available, have less adverse effects on plant growth, and are less expensive.

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