Research Article

OPEN ACCESS

Available on http://www.pjbt.org
Pakistan Journal of Biotechnology
(PJBT)
(P-ISSN: 1812-1837 and E-ISSN: 2312-7791)

ANTIFUNGAL EXPLOITATION OF ESSENTIAL OILS AGINST *Aspergillus niger* CAUSING CHERRY FRUIT ROT

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Article Received 14-10-2023, Article Revised 02-01-2024, Article Accepted 03-01-2024.

ABSTRACT

Cherry is an important fruit being grown throughout world and in Pakistan. This research focused on investigating the occurrence of Cherry fruit rot in various locations within the Quetta district at Balochistan. Infected Cherry fruit samples were collected from various location Viz., Joint Road, Double Road New Adda, Azar Ghangi, and Kansi Road. Isolation and morphological identification procedures were carried out at the mycological laboratory of the Department of Plant Pathology, Balochistan Agriculture College, to determine the causal agent of cherry fruit rot from infected samples. *In vitro* study explored the impact of diverse essential oils on the linear colony growth of *Aspergillus niger* under controlled conditions. Essential oils, such as Rose oil, Neem oil, Coconut oil, and Cinnamon oil, displayed varying inhibitory effects on linear growth of *Aspergillus niger*. These findings enhance our understanding of interactions between agents and the fungus, paving the way for further fungal growth control research. The study contributes practical insights for managing *Aspergillus niger* growth, potentially advancing strategies in the field of fungal growth control and management.

Keywords: *Aspergillus niger*, Essential oils, Antifungal exploitation.

INTRODUCTION

The sweet Cherry, specifically the Ferrovia variety (*Prunus avium L.*), originates from southeastern Europe and western Asia, particularly the region between the Caspian and Black Seas. Some Cherry cultivars require cross-pollination due to self-incompatibility. Botanically, Cherry (*Prunus avium*) belongs to the *Rosaceae* family (ISMEA, 2012). Sweet Cherries are a highly valuable crop, with global production exceeding 2.56 million tonnes across 441,953 hectares, and demand is increasing. Turkey is the top producer with 639,564 tonnes in 2018, followed by the USA. Uzbekistan, Chile, and Iran have also seen significant production growth in the past two decades, becoming key players in sweet Cherry cultivation (FAO, 2020). Cherry (*Prunus avium L.*) is a significant fruit in Pakistan's temperate zone, belonging to the *Rosaceae* family. Pakistan ranks 48th globally in Cherry production, with 6.0 thousand tonnes grown on 2.5 thousand hectares, averaging 2.4 tonnes per hectare. The global trade in fresh Cherries is valued at US$2.3 billion, with Turkey leading in production (0.48 million tonnes), and the United States and Chile leading in exports. China is the top consumer, followed by Russia and the European Union. In Pakistan, Cherry production has grown faster than the global average due to increased cultivation area. Globally, increased production is mainly driven by improved yield per hectare. Pakistan's yield per hectare is below the global average, trailing behind by about 27%. To boost international Cherry exports, Pakistan must address and reduce this yield gap compared to the global norm (Samad, 2018). Cherry cultivation in Pakistan, mainly in temperate zones like Quetta, Kalat, Ziarat, and others, lacks comprehensive information. Pakistan produces over 2,000 tons of Cherries annually but doesn't export those (El-Shazly *et al*., 2013). Cherry cultivation covers about 897 hectares in Balochistan, producing approximately 1,507 tons annually. The prime areas for Cherry cultivation in Pakistan include Quetta, Pishin, Kalat, Zhob, Mastung, and Loralai, but in Balochistan, the focus is mainly on Quetta, Ziarat, and Kalat, with Ziarat cherries being particularly renowned (Ali *et al*., 2003-04). Cherries face postharvest challenges, including water loss, softening, peduncle discoloration, dehydration, and fungal diseases like *Aspergillus niger* (Fruit Rot), *Monilinia spp*. (brown rot), *Botrytis cinerea* (grey mold), *Rhizopus stolonifer* (Rhizopus rot), *Alternaria alternata* (Alternaria rot), and *Penicillium expansum* (blue mold). *Aspergillus niger* is the most damaging, causing up to 30-50% fruit loss during cold storage, especially at 30°C, and is more severe at 100% relative humidity (Romanazzi *et al*., 2008).

Aspergillus niger, a fungus, is known for causing 'black mold' in fruits and vegetables like Grapes, Cherries, Apricots, Onions, and Peanuts. It's a common contaminant in food and can be found in soil and indoor settings. Its colonies appear black, sometimes leading to confusion with the Stachybotrys genus, also called "black mold" (Samson *et al*., 2001). Several essential oils have been studied for their ability to inhibit postharvest fungi under laboratory conditions (Hidalgo *et al*., 2002; Tzortzakis & Economakis, 2007). Existing literature has emphasized the potential of essential oils in prolonging the storage life of fruits and vegetables by mitigating fungal rot (Guynot *et al*., 2003; Jantan *et al*., 2003; Kalemba & Kunicka, 2003). To underscore the significance of essential oils, a comprehensive study assessed the effectiveness of 75 different essential oils against *A. niger*, with some oils having undergone prior evaluation (Kim & Park, 2012). In their research, Kim and Park (2012) explored the impact of 11 essential oils from the *Myrtaceae* family on *A. niger*, noting varying degrees of fungal inhibition, including weak or negligible effects. Among these oils, clove oil stands out as a natural preservative and flavoring agent renowned for its antimicrobial activity, which hinders the growth of molds, yeasts, and bacteria (Matan *et al*., 2006).

MATERIAL AND METHODS

Collection of Infected Cherry Fruit samples: Samples of infected Cherry fruit showing fruit rot symptoms were gathered to document the occurrence of Cherry fruit rot. Sampling was done from Four distinct different locations at quetta Viz., Joint Road, Double Road New Adda, Azar Ghangi, and Kansi Road in the Quetta district of Balochistan. The collected specimens were then transported to the mycological laboratory at the Department of Plant Pathology, Balochistan Agriculture College, where they were subjected to isolation and morphological identification procedures to determine the causal agent. The affected sections, including the fruits, were cut into small pieces measuring 3 to 4 mm in length. These fragments underwent surface sterilization using a 1% solution of commercial bleach (sodium hypochlorite) for 2 minutes. After sterilization, the pieces were rinsed twice with sterilized water and then placed on sterilized blotter paper for drying. Subsequently, the sterilized segments were placed on petri plates containing fresh Potato Dextrose Agar (PDA) medium. Typically, each plate held five sections of infected samples. All petri plates were incubated at a temperature of 25°C±2 for 7 days to facilitate the observation of fungal sporulation and these were further purified using the single spore isolation technique and hyphal tip method. The identification of the fungal colony growth was based on their morphological characteristics, as documented in previous studies (Pitt & Hocking, 1997).

Pathogenicity test: The isolated fungal strains were maintained as pure cultures on Potato Dextrose Agar (PDA) in culture tubes and stored in a refrigerator at 26°C for regular use. These cultures underwent further propagation on PDA medium for a period of two to three weeks. To prepare the inoculum, each isolate was taken and combined with 20 ml of distilled water. Pathogenicity tests were conducted by inoculating healthy Cherry fruits to assess the severity of the disease.

Essential oils: *In vitro* experiments were conducted to assess the effect of essential oils, including Cinnamon, Coconut, Neem, and Rose as prescribed by (Munhuweyi *et al*., 2016). For this study. Four specific Essential oils (EOs) were employed: Rose (*Anibarosa eodora*), Neem (*Azadirachta indica*), Cinnamon (*Cinnamom umverum*) and Coconut (*Cocos nucifera*). These essential oils were emulsified using 5% Tween 20 (v/v) to prepare different concentration of 5%, 10% and 15% which were subsequently used for the experiments (Munhuweyi *et al*., 2016). For this technique, the appropriate quantity was added to individual Petri plates, each containing 20 ml of PDA at a temperature of 25 °C. A 0.5 mm disc of *A. niger* mycelium was positioned on the treated PDA medium, and the plate was subsequently incubated at 24 °C. The extent of radial mycelial growth was assessed at two-day intervals over the course of eight days. The inhibitory percentage (IP) was calculated using the following formula:

$$IP = [(dc - dt) / dc] \times 100$$

Here, '**dc**' represented the mycelium diameter in a control petri dish, and **'dt'** represented the mycelium diameter in the petri dish treated with the essential oil, as measured after each two-days interval (Aminifard &Mohammadi, 2013).

Assessment of Different Essential Oils for Controlling Linear Colony Growth of Cherry Fruit Rot Fungi *In-Vitro*: In this study, *Aspergillus niger*, was excised from an 8-10-days-old culture plate using a sterile cork borer (5mm) and subsequently placed at the central position of a Potato Dextrose Agar (PDA) plate. These plates were then subjected to incubation at a temperature of 28°C. For comparative purposes, control petri dishes without essential oils were included in the experimental design. Radial colony growth of *Aspergillus niger* was meticulously documented by tracing two perpendicular lines on the underside of the petri plates, intersecting precisely at the center of each plate. Colony growth progression was meticulously recorded in millimeters along these marked lines at 24-hour intervals, until the plates achieved full coverage, across all treatment conditions. Furthermore, the efficacy of the essential oils was evaluated employing the food poisoning method.

RESULT AND DISCUSSION

Isolation And Morphological Identification: Specimens Exhibiting Disease Symptoms Underwent Laboratory Procedures Designed to Isolate and Identify the Etiological Agents. To Accomplish This Goal, The Specimens Were Cultured on Artificial Nutrient Media, Specifically Potato Dextrose Agar (PDA), and Observed for A Period of Seven Days. The Isolated Fungal Species Were Subsequently Sub-Cultured to Ensure the Purification of the Definitive Pathogenic Agent Responsible for the Disease Manifestation.

Colony morphology: The morphology of *A. niger* colony was assessed during a 4-day incubation period at 28°C. The colony exhibited initial white growth, which gradually transformed into a black hue due to the development of deeply pigmented conidia. The reverse side of the colony displayed a pale yellow coloration. Examination under a microscope revealed large, dark brown conidial heads of *A. niger*.

Microscopy: The conidiophores exhibited a dark coloration toward the globule. Conidial heads typically appeared as biseriate brown structures, occasionally with distinct metulae. The vesicles were observed to be globose, dark brown, and characterized by a rough surface.

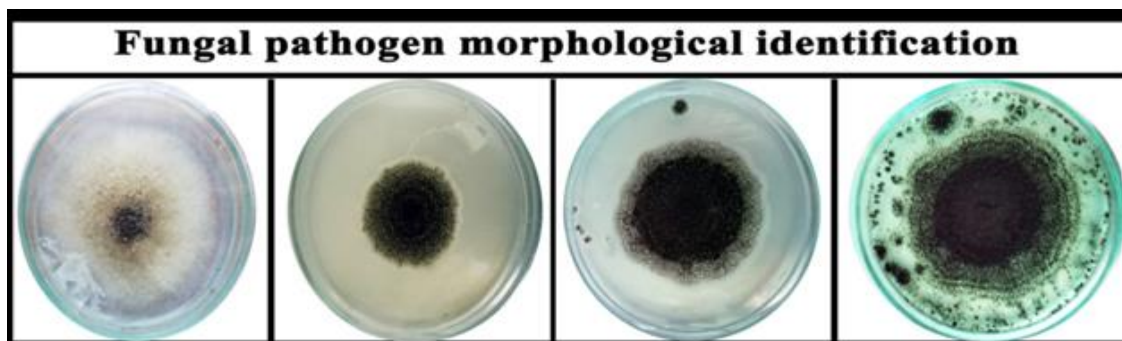

Figure 1 *Aspergillus niger* colony morphology

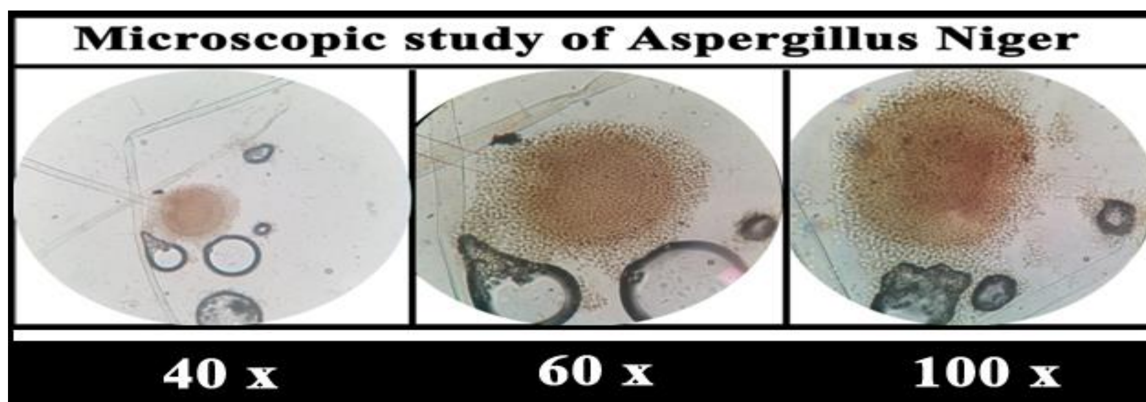

Figure 2 Microscopic observation of *Aspergillus niger* spores

Pathogenicity assays: Pathogenicity tests were performed on Cherry fruits using three distinct methods: cut, injection, and dip techniques. The observed symptoms resembled those initially identified on the affected fruits. These symptoms included the emergence of water-soaked lesions that gradually enlarged into soft, brown. Additionally, a notable characteristic was the presence of white mycelia covering the affected areas. After 7 days of inoculation, the disease incidence varied based on the method employed, while control fruits showed no symptomatic manifestations.

Among the three methods employed, the injection method exhibited the highest level of infection, followed by the cut method with a moderate level of infection, and the dip method with the lowest level of infection. Notably, no infection was observed in the control group.

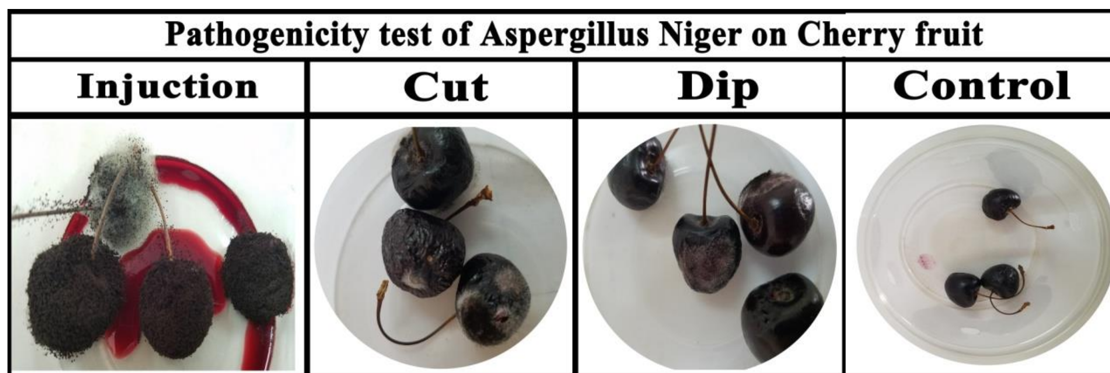

Figure 3 Pathogenicity test was conducted over Cherry fruit by Injection, Cut and Dip method.

Essential oils

In Vitro Antifungal Effects of Essential Oils on Mycelial Radial Growth: The evaluation of antifungal effectiveness involved conducting an in vitro contact assay that quantified the inhibition of hyphal growth. This specific assay method, referred to as the "solution method" (SM), was adapted for the essential oil treatment on a Potato Dextrose Agar (PDA) medium, following the methodology established by Özden and Bayindirli in 2002.

The experimental design encompassed a range of treatments, each conducted in vitro. Four distinct essential oils Rose, Cinnimum, Lemon and Neem were employed, and each oil was subjected into three distinct concentrations (5%, 10%, and 15%).

Assessment of Antifungal Activity of Essential Oils: In vitro experiments were conducted to evaluate the antifungal properties of the mentioned essential oils (EOs) at different concentrations. The assessment was carried out using the poisoned food technique.

Comparison of mean effects of Rose essential oil types on the radial growth of *A. niger* fungi under in vitro conditions.: The results of the mean comparison analysis regarding the influence of different types of essential oils on fungal growth unveiled noteworthy trends. Notably, Rose essential oil exhibited also the lowest fungal growth rate at a 15% concentration on the 2nd, 4th, 6th, and 8th days, demonstrating average measurements of 6.33 mm, 9.66mm, 13 mm and 14.66 mm, respectively. This pattern persisted, though to a slightly lesser extent, at a 10% concentration, where the fungal growth rates on the 2nd, 4th, 6th, and 8th days averaged at 7.33 mm, 15 mm, 19.66 mm and 26.33 mm. The 5% concentration of Rose essential oil also resulted in continuous growth, with measurements of 9.33 mm,16 mm, 26.33 mm and 37.33 mm on the same respective days. These findings stood in contrast to the outcomes of other essential oil treatments. In contrast, the control group demonstrated consistent growth over time, contributing to fungal growth measurements of 13 mm, 27 mm, 34 mm, and 45 mm on the 2nd, 4th, 6th, and 8th days, respectively. Notably, treatments involving Rose essential oil exhibited lowest fungal growth rates, surpassing those of other essential oil treatments, up to the eighth day.

The results of the mean comparison regarding essential oil concentration demonstrated that an escalation in essential oil concentration corresponded to diminished fungal growth rates in comparison to the baseline. Specifically, from the second to the eighth day, the most substantial fungal growth rate was recorded in the treatment without essential oil (control). In contrast, the lowest fungal growth rates were associated with Rose essential oil concentrations of 15%, 10%, and 5% from the second to the eighth day, respectively

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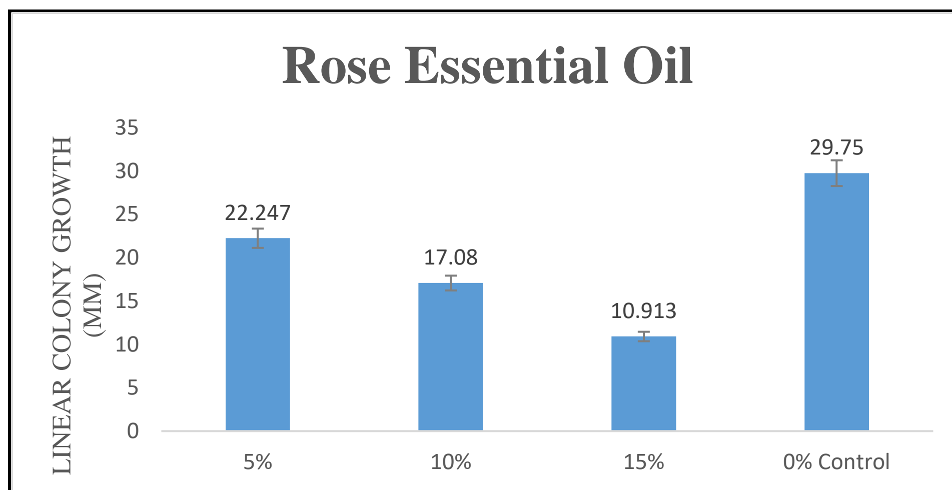

Figure 4 Comparing Linear Colony Growth (%) of *Aspergillus niger* at Various Rose Essential Oil Concentrations with a Control Group

Comparison of mean effects of Coconut essential oil types on the radial growth of *A. niger* fungi under in vitro conditions.: The results of the mean comparison analysis regarding the influence of different types of essential oils on fungal growth unveiled noteworthy trends. Notably, Coconut essential oil exhibited the moderate fungal growth rate at a 15% concentration on the 2nd, 4th, 6th, and 8th days, demonstrating average measurements of 7 mm, 10.33 mm, 14 mm and 19.66 mm, respectively. This pattern persisted, though to a slightly lesser extent, at a 10% concentration, where the fungal growth rates on the 2nd, 4th, 6th, and 8th days averaged at 9.33 mm, 16.33 mm, 22.66 mm and 29.66 mm. The 5% concentration of Coconut essential oil also resulted in continuous growth, with measurements of 9.66 mm, 19.33 mm, 31.66 mm and 39.66 mm on the same respective days. These findings stood in contrast to the outcomes of other essential oil treatments.

In contrast, the control group demonstrated consistent growth over time, contributing to fungal growth measurements of 13 mm, 27 mm, 34 mm, and 45 mm on the 2nd, 4th, 6th, and 8th days, respectively. Notably, treatments involving Coconut essential oil exhibited sustained fungal growth rates, surpassing those of other essential oil treatments, up to the eighth day.

The results of the mean comparison regarding essential oil concentration demonstrated that an escalation in essential oil concentration corresponded to diminished fungal growth rates in comparison to the baseline. Specifically, from the second to the eighth day, the most substantial fungal growth rate was recorded in the treatment without essential oil (control). In contrast, the Moderate fungal growth rates were associated with Coconut essential oil concentrations of 15%, 10%, and 5% from the second to the eighth day, respectively

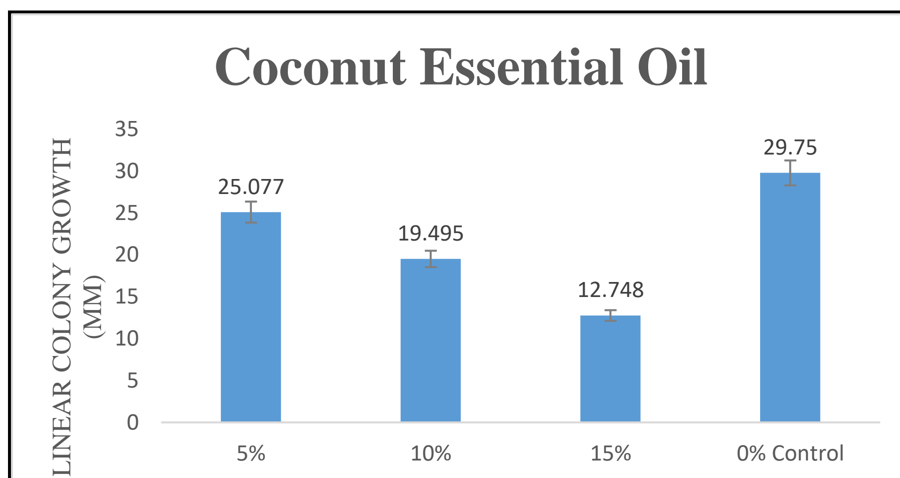

Figure 5 Comparing Linear Colony Growth (%) of *Aspergillus niger* at Various Coconut Essential Oil Concentrations with a Control Group

Comparison of mean effects of Cinnamon essential oil types on the radial growth of *A. niger* fungi under in vitro conditions: The results of the mean comparison analysis regarding the influence of different types of essential oils on fungal growth unveiled noteworthy trends. Notably, Cinnamon essential oil exhibited also the moderate fungal growth rate at a 15% concentration on the 2nd, 4th, 6th, and 8th days, demonstrating average measurements of 8.33 mm, 13mm, 18.33 mm and 28 mm, respectively. This pattern persisted, though to a slightly lesser extent, at a 10% concentration, where the fungal growth rates on the 2nd, 4th, 6th, and 8th days averaged at 9.33 mm, 17.33 mm, 24.66 mm and 32.66 mm. The 5% concentration of Coconut essential oil also resulted in continuous growth, with measurements of 11.33 mm, 18.66 mm, 29.66 mm and 40.33 mm on the same respective days. These findings stood in contrast to the outcomes of other essential oil treatments**.**

In contrast, the control group demonstrated consistent growth over time, contributing to fungal growth measurements of 13 mm, 27 mm, 34 mm and 45 mm on the 2nd, 4th, 6th, and 8th days, respectively. Notably, treatments involving Cinnimum essential oil exhibited sustained fungal growth rates, surpassing those of other essential oil treatments, up to the eighth day.

The results of the mean comparison regarding essential oil concentration demonstrated that an escalation in essential oil concentration corresponded to diminished fungal growth rates in comparison to the baseline. Specifically, from the second to the eighth day, the most substantial fungal growth rate was recorded in the treatment without essential oil (control). In contrast, the moderate fungal growth rates were associated with Cinnimum essential oil concentrations of 15%, 10%, and 5% from the second to the eighth day, respectively

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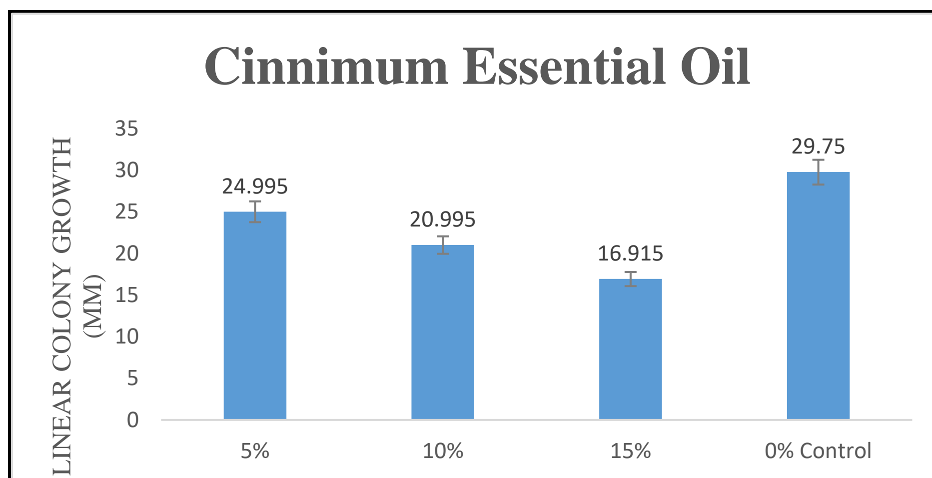

Figure 6 Comparing Linear Colony Growth (%) of *Aspergillus niger* at Various Cinnimum Essential Oil Concentrations with a Control Group.

Comparative Analysis of Neem Essential Oil on the in Vitro Radial Growth Inhibition of *Aspergillus niger* fungi: The results of the mean comparison analysis regarding the influence of different types of essential oils on fungal growth unveiled noteworthy trends. Notably, Neem essential oil exhibited the highest fungal growth rate at a 15% concentration on the 2nd, 4th, 6th, and 8th days, demonstrating average measurements of 8.33 mm, 13.33 mm, 18 mm and 22 mm, respectively. This pattern persisted, though to a slightly lesser extent, at a 10% concentration, where the fungal growth rates on the 2nd, 4th, 6th, and 8th days averaged at 10.33 mm, 18 mm, 26.33 mm and 34.66 mm. The 5% concentration of Neem essential oil also resulted in continuous growth, with measurements of 13.33 mm, 23.66 mm, 31 mm and 38.66 mm on the same respective days. These findings stood in contrast to the outcomes of other essential oil treatments.

In contrast, the control group demonstrated consistent growth over time, contributing to fungal growth measurements of 13 mm, 27 mm, 34 mm and 45 mm on the 2nd, 4th, 6th, and 8th days, respectively. Notably, treatments involving Neam essential oil exhibited sustained fungal growth rates, surpassing those of other essential oil treatments, up to the eighth days.

The results of the mean comparison regarding essential oil concentration demonstrated that an escalation in essential oil concentration corresponded to diminished fungal growth rates in comparison to the baseline. Specifically, from the second to the eighth day, the most substantial fungal growth rate was recorded in the treatment without essential oil (control). In contrast, the highest fungal growth rates were associated with Neam essential oil concentrations of 15%, 10%, and 5% from the second to the eighth day, respectively.

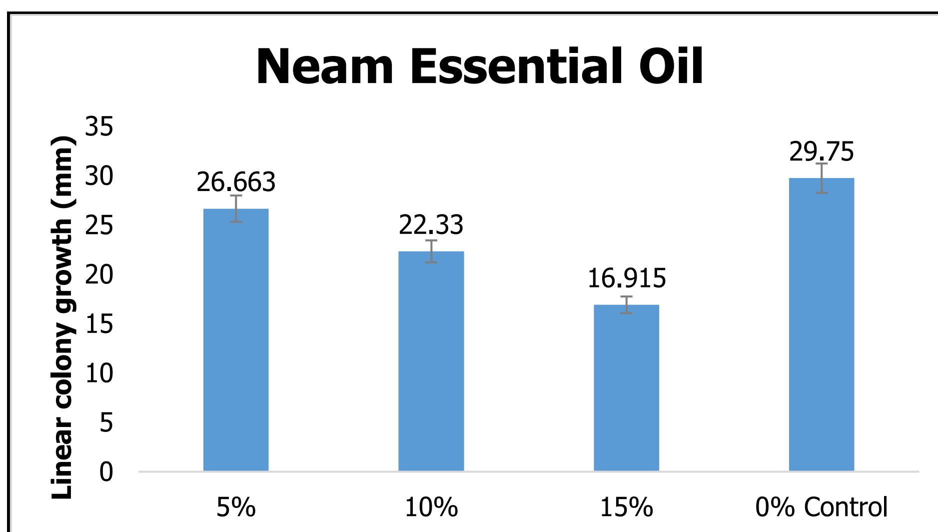

Figure 7. Comparing Linear Colony Growth (%) of *Aspergillus niger* at Various Neem Essential Oil Concentrations with a Control Group

CONCLUSION

Different essential oils exhibited varying impacts on *Aspergillus niger's* colony growth and conclusion of the study is as follow; Rose oil was showed effective results in terms of controlling fungal colony growth.

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