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FORESTALLING *CLADOSPORIUM SPHAEROSPERMUM* AND *PHOMOPSIS AZADIRACHTAE* INFECTIONS USING CREAM PREPARED FROM ESSENTIAL OIL EXTRACTED FROM *ALLIUM SATIVUM*

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ABSTRACT

The report of pathogenic potentials of *Cladosporium sphaerospermum* and *Phomopsis azadirachtae* in humans, majorly among the farmers is now posing threat to the populace, and the need to search for a preventive measure from readily available, cost effective, ecofriendly, and natural sources will be an ultimate success to be attained. This study was carried out to evaluate the tendency of forestalling *Cladosporium sphaerospermum* and *Phomopsis azadirachtae* infections using cream prepared from essential oil extracted from *Allium sativum*. Soil samples were randomly collected from different farm lands and screened for the presence of *Cladosporium sphaerospermum* and *Phomopsis azadirachtae* using appropriate microbiological techniques. The pathological profile of organism and prophylactic potential of cream were assessed by topically exposing them to albino wistar rats for period of 15 days. The study revealed significant ($P < 0.05$) reduction in the pathological profiles (alopecia, scaly, erythema, macules and ulceration) associated with the dematiaceous fungus, and this was effectively increased in every three days intervals, and more pronounced against *Cladosporium sphaerospermum*. This study has shown that the studied cream protected the rats from *Cladosporium sphaerospermum* and *Phomopsis azadirachtae* infection and could be used as an alternative prophylactic measure to dermatological infection caused by the studied organism.

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Keywords
Pathogenic, *Cladosporium sphaerospermum, Phomopsis azadirachtae, Allium sativum,* Prophylactic, Dematiaceous fungus

INTRODUCTION

Essential oils are complex mixtures which typically consist of a variety of low molecular weight compounds which can range in number up to 100 or more with a select few being the most abundant (Lopes *et al*., 2016; Gupta *et al*., 2017; Lopes *et al*., 2017). The essential oils extracted from medicinal plants had been shown to exhibit antibacterial and antifungal activities both *in vitro* and *in vivo* (Gupta *et al*., 2016). While most subcutaneous infections are not life-threatening and respond well to currently available topical treatment with over-the-counter (OTC) fungal agents, some subcutaneous infections can, however, can be difficult to treat, require prolonged therapeutic regimens, and are increasingly resistant to conventional antifungal therapies. Previous estimates put the total cost for treatment of subcutaneous infections in the US at $1.67 billion (Flores *et al*., 2015). In addition to costs associated with treatment, many currently used antifungal agents have significant side effects thus underscoring the need for identification of therapeutic alternatives including those from natural products such as plant-based bioactive compounds, essential oils, and/or their components (Elaissi *et al*., 2012).

The composition of essential oils can vary due to a number of factors including the extraction method used, the type and species of plant from which they are derived, the composition of the soil, and the exact stage of growth at the time of harvest. For this reason, it is important that careful chemical analyses be performed using methods such as gas chromatography-mass spectrometry (GC-MS) to verify and standardize the composition of essential oils to ensure batch to batch consistency over time (Dias de Castro *et al.,* 2015).

Previous investigators have evaluated the use of essentials against dermatophytes focusing largely on melaleuca, thyme, eucalyptus, oregano, and lavender. Additional studies have been conducted which investigated the antifungal effects of specific components of these and other essentials including mono-, di-, and sesquiterpenes, phenolic terpenes, phenylpropanoids, hydrocarbons, and other cyclic compounds. Some investigations have led to the conclusion that the anti-dermatophytic activity resulted from synergy between major and minor components rather than the result of the presence of a single compound (Dai *et al*., 2016).

Garlic changes its characteristics because of the complexity of its intrinsic chemistry, and processing procedures. Thus, standardization marker compounds are important for ensuring consistent effects. Garlic oil and recently available stabilized allicin, revealed a considerable activity against various phytopathogenic fungi. In a field study with peanut, garlic oil was found to be protective against *Gibberella zeae*, (a fungal parasite of maize).

Several researchers have studied the effects essential oil extracted from different plant materials against different fungi isolated different ecological sites Studies but little or no published study has been conducted on the effect of organic cream or any form of cream prepared from essential extracted from *Allium sativum* (garlic) on filamentous fungi, mostly the dematiaceos fungi. This shows that there is paucity of information on this aspect of mycological study. This study was carried out to evaluate the tendency of forestalling *Cladosporium sphaerospermum* and *Phomopsis azadirachtae* infections using cream prepared from essential oil extracted from *Allium sativum*

MATERIALS AND METHODS

Isolation and Characterization of Test Isolate from Soil Sample

Sample Collection: This was carried out using the method described in the study published by Iheukwumere *et al.* (2021). The litter from the soil surfaces was carefully scrapped out using sterile stainless spoon. The soil auger was derived to a plough depth of 15 cm in the farm land, and soil sample was drawn up to 10 samples from each sampling unit into a sterile tray. The samples were thorough mixed and foreign materials such as roots, stones, pebbles and gravels were carefully removed. The soil sample was then reduced to half by quartering the sample. Quartering was carried out by dividing the soil sample into four equal parts and the two opposite quarters were discarded and the remaining two quarters were mixed. The process was repeated for the rest of soil samples used for this study. The samples were carefully labeled and then kept in a disinfected cooler, to maintain its temperature and stability of the number of the isolates. The samples were transported to the laboratory for analysis.

Isolation of the Fungal Isolate: This was carried out using the method described in the study published by Iheukwumere *et al.* (2021). One gram of the soil sample was weighed into a 50 mL beaker (Pyrex) using analytical weighing balance (JJJ430BC), little normal saline (0.85% NaCl) was added; this was shake thoroughly and made up to 10 mL using the normal saline. The sample was aseptically plated on Sabouraud Dextrose Agar (SDA) prepared according to the manufacturer's direction, supplemented with chloramphenicol antibiotics (0.05%). This was incubated at room temperature (30±2ºC) for 7-10 days. The fungal isolates that were darkened on the reverse side were aseptically sub cultured on SDA containing chloramphenicol antibiotics (0.05%) and incubated at room temperature (30±2°C) for 7-10 days.

Identification of Fungal Isolate: The fungal isolate was identified to the genus/species level based on macroscopic and microscopic characteristics of the isolate obtained from pure cultures (Iheukwumere *et al*., 2020).

Macroscopy: The colony was carefully examined for fungal characteristics. The rate of growth, color, shape, texture, consistency of the growth and other peculiar features of the colony were observed according to the method described in the work published by Iheukwumere *et al.* (2020).

Slide culture technique: This was carried out using the method described in the study published by Iheukwumere *et al.* (2020). A filter paper was cut and placed on the bottom of Petri-dish. Two slides were crossed over each other on top of the filter paper and the filter paper was moistened. The set-up was sterilized by autoclaving at 121^0C for 15 minutes. Approximately one centimeter square agar block was cut from already prepared Potato Dextrose Agar (PDA) and placed on the intersection of the two slides. The four edges of the agar block were inoculated with the test organisms. It was then covered with sterile cover slip and incubated at room temperature for 5 days. After 5 days of growth, the cover slip was removed and inverted over a slide containing a drop of lactophenol cotton blue (LCB). The agar block was removed and discarded. A drop of LCB was also placed on top of the adherent colony on the slide and covered with sterile cover slip. The edges of the cover slip were sealed with nail polish to prevent evaporation of the stain. The slides were examined under the microscope using x10 and x40 objective lenses.

Prophylactic Potential of the Prepared Cream against Superficial Infection caused by Test Isolate

Preparations of Plant Materials: The fresh fruits of *Monodora myristica* (African Nutmeg) were collected from Onitsha, Anambra State, Nigeria. The plant material was authenticated appropriately Dr B. Garuba, in Botany Department, Michael Okpara Federal University of Agriculture, Umudike. The plant material was washed and dried under shade at room temperature for 14 days. The dried plant material was ground to powder form using sterile electric grinder. (Iheukwumere *et al.,* 2020).

Extraction of the essential oil: A 2000 mL Soxhlet extractor that has three main sections: a percolator (boiler and reflux) which circulates the solvent, a thimble (usually made of thick filter paper) which retains the solid to be extracted, and a siphon mechanism, which periodically empties the thimble was used for process. Twenty grams (100 g) of the plant material to be extracted was placed inside the thimble. The thimble was then loaded into the main chamber of the Soxhlet extractor. Then 1000 mL of n-hexane was placed in a 1000 mL distillation flask. The flask was placed on the heating mantle (2000 mL, 220 V, 500 W). The Soxhlet extractor was placed at the top of the flask. A reflux condenser was placed at the top of the extractor. When the n-haxane was heated to reflux, the solvent vapour travelled up a distillation arm, and flooded into the chamber housing the thimble of solid. The condenser ensured that any solvent vapour cooled, and dripped back down into the chamber housing the solid material. The chamber containing the solid material slowly filled with warm solvent. When the Soxhlet chamber was almost full, the chamber was emptied by the siphon. The solvent then returned to the distillation flask. The thimble ensured that the rapid motion of the solvent did not transport any solid material to the still pot. This cycle was allowed to repeat many times for 12 h. After extraction, the solvent is removed, typically by means of a rotary evaporator to collect the essential oil.

Preparation of organic cream: The shea butter (one full 500 mL beaker) and Almond oil (50 mL) were mixed in a small stainless pot (2 litres capacity). They were stirred thoroughly and heated using cooking gas until all the ingredients were completely melted. This was followed by the addition of essential oil (100 mL) into the mixture and these were stirred and mixed properly. Then the mixture was enriched with vitamins (A, D and E), colourant and fragrance. The cream was then dispensed (20 mL) into a container and allowed to solidify (Iheukwumere *et al*., 2021).

Test Isolate: The test isolate was prepared using 0.5 MacFarland matching standard prepared by mixing 0.05 mL of 1 % $BaCl_2.2H_2O$ and 9.95 mL of 1 % conc. H_2SO_4. The test isolate was prepared using normal saline (0.85 % NaCl). The culture plates were flooded with the normal saline and these were thoroughly macerated and filtered using watman no 1 flter paper. The filtrate was further diluted to match with turbidity of the prepared 0.5 MacFarland standard (1.5×10^8 cells/mL).

Experiment Animal: The laboratory animal use in this study was albino wistar rats purchased from animal house at university of Nigeria, Nsukka (UNN). The rats were transported to the animal house at Department of Biochemistry, Faculty of bioscience, Nnamdi Azikiwe University (NAU) Awka. The rats were randomly examined for their suitability for the study .Those that was not suitable for the test was excluded in the study.

Animal study: This was carried using the method described in the study published by Iheukwumere *et al.* (2021). A total of 12 albino wistar rats were used for this study. The body of each rat was disinfected with 70% ethanol. Three locations were selected and the hairs in each location were thoroughly removed, and these were labeled A, B and C. Location A was topically administered 0.5 mL test isolate, B was applied cream and later applied 0.5 mL test isolate whereas C was left without applying anything as control. The procedure was repeated for the remaining rats. The pathological parameters of the test isolates were monitored and healing potential of the cream was also monitored in every three days for 15 days.

Statistical Analysis

The data obtained in this study were presented in tables. Chi square(x^2) was used to determine the significance of the sample sources 95% confidence level. Pairwise comparison was carried out using student "t" test (Iheukwumere *et al*. 2021).

RESULTS

The characteristic features of the fungal isolate are shown in Table 1 and Plates 1 and 2. The study revealed the macroscopic characteristics of the isolate such as the texture of the colony, shape of the colony, colour of the colony, reverse side of the colony and growth rate of the colony. The microscopic characteristics such as the nature of the hyphe, nature of conidiophore, nature of conidia, their shapes, texture, and colours were also revealed in Table 1 and Plates 1 and 2.

The sample of organic cream prepared using essential oil extracted from *Allium sativum* (garlic) was shown in Plate 3. The prepared cream had good consistency, soft, dark colour, satisfactory fragrance and moderate oily. One of the major advantages of this cream was that the cream exhibited similar quality of emulgel by possessing the gel and penetrating qualities.

The clinical features exhibited by *Cladosporium sphaerospermum* and *Phomopsis azadirachtae* such as alopecia, discoloration, scaly, swelling, erythema, patches, macule, erosion, formation of vesicle and ulceration are shown in plates 4 and 5. The study revealed that alopecia, discoloration, patches, swelling and erythema were mostly seen as the clinical manifestation of the organism. The study also revealed that the essential cream that was used as prophylaxis significantly ($P < 0.05$) reduced the clinical manifestation of the isolate to minimal or 0% level as shown in plates 4 and 5.

Table 1: Macroscopic and microscopic characteristic of the fungal isolate

Macroscopic Characteristic	Microscopic Characteristic	Possible Isolate
The colony was woolly, olivaceous brown in front and dark on the reverse side. The growh rate was moderate	The hyphae were septate. It produced unicellular, long chains of conidia that had lemon shaped. The conidia were brown without	*Cladophialophora bantiana*

to rapid	attachment scar.	
The colony was whitish brown to brown with pale to pink margin, and dark on the reverse side .The growh rate was slow to moderate	The hyphae were profusely branched, septate and pigmented. The conidiophore was short and simple, bearing alpha and beta conidia. The conidia were subglobose with thick wall	*Phomopsis azadirachtae*

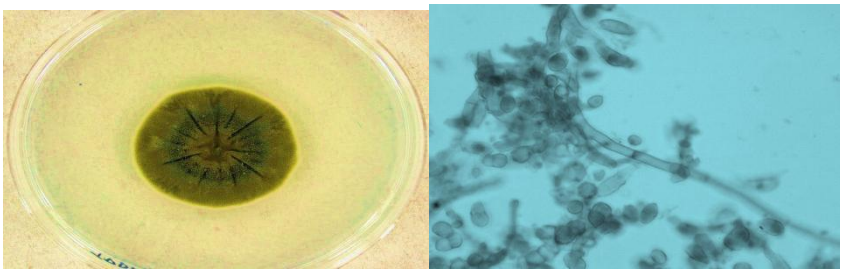
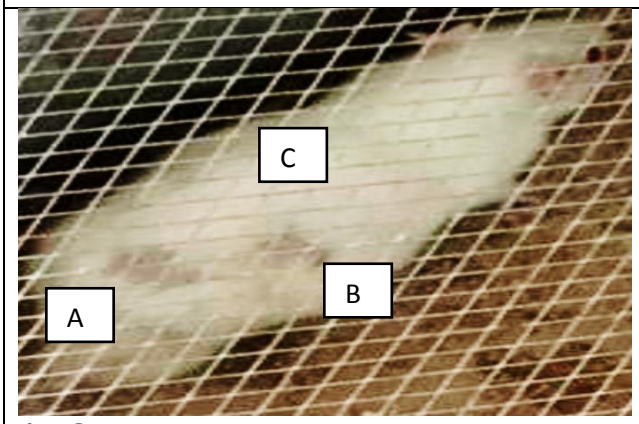


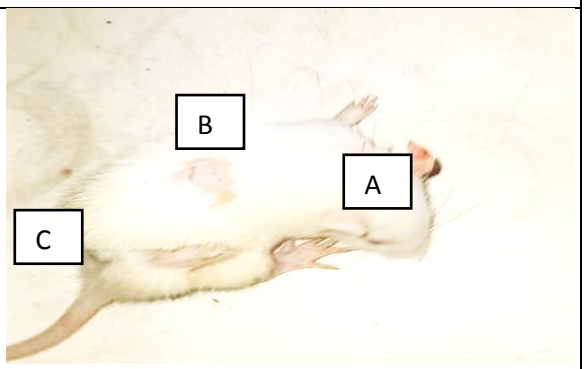
Plate1: Macroscopic and microscopic feature of *Cladosporium sphaerospermum*

Plate 2: Macroscopic and microscopic feature of *Phomopsis azadirachtae*

Plate 3: Organic cream prepared with essential oil extracted from *Allium sativum*

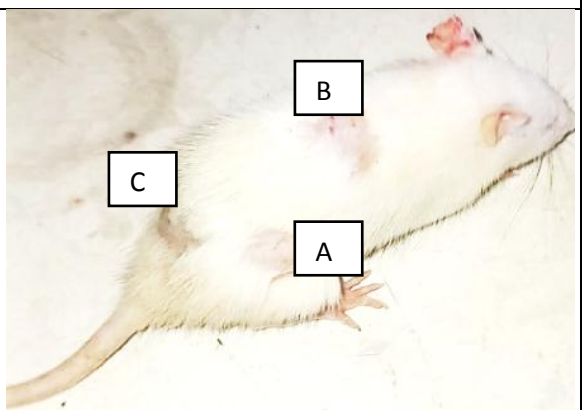


A= Organism
B = Cream
C = Control
DAY 3



A= Organism
B = Cream
C = Control
DAY 6

A= Organism
B = Cream



A= Organism
B = Cream

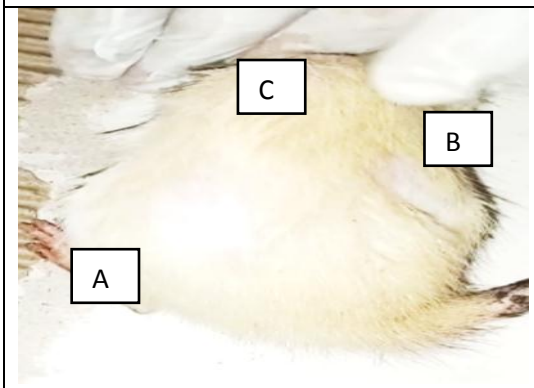
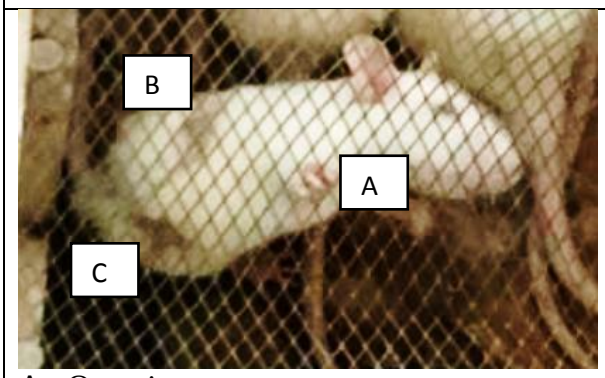
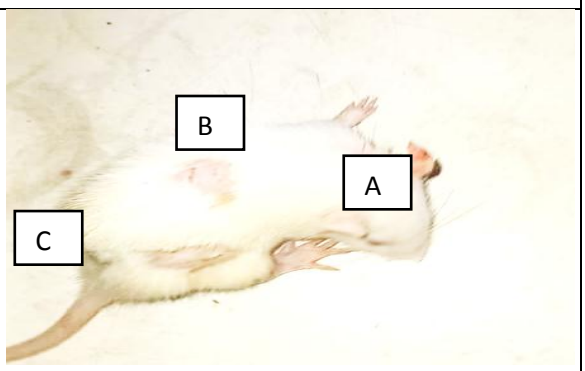
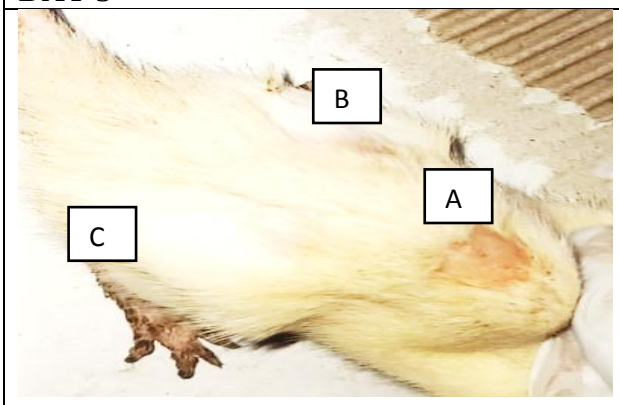
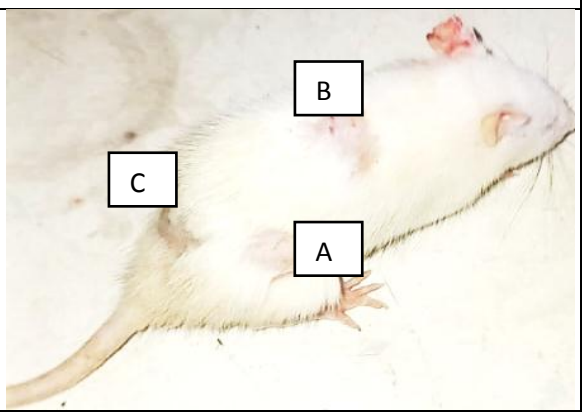
C = Control DAY 9	C = Control DAY 12
 A= Organism B = Cream C = Control DAY 15	

Plate 4: Effect of the cream prepared from *Allium sativum* essential oil on the infection caused by *Cladosporium sphaerospermum*

GARLIC CREAM / ORGANISM C	
 A= Organism B = Cream C = Control DAY 3	 A= Organism B = Cream C = Control DAY 6
	

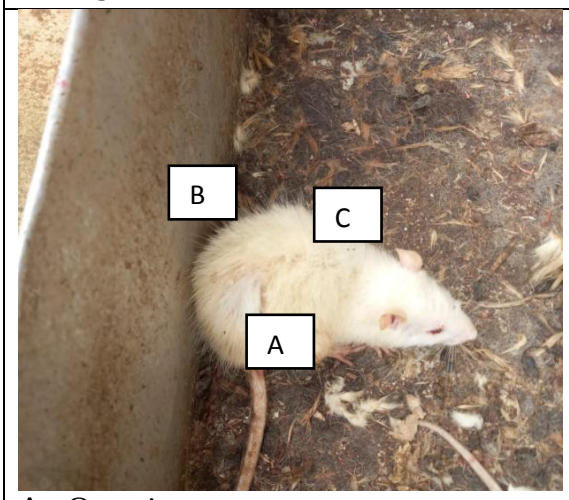
A= Organism B = Cream C = Control DAY 9	A= Organism B = Cream C = Control DAY 12
 A= Organism B = Cream C = Control DAY 15	

Plate 5: Effect of the cream prepared from *Allium sativum* essential oil on the infection caused by the *Phomopsis azadirachtae*

DISCUSSION

The characteristic features of *Cladosporium sphaerospermum* and *Phomopsis azadirachtae* observed in the present study shared similar characteristic features of dematiaceous fungi isolated and characterized by other researchers (Revankar, 2007; Revankar and Sutton, 2010; Yew *et al.,* 2014; Ozgok and IlShan, 2020). The pathological features associated with the implicated fungi agrees with the findings of Revankar and Sutton (2010), Derber *et al.* (2010), Brandt *et al*. (2013) and Yew *et al*. (2014). Different species of dematiaceous fungi were isolated and characterized from different ecological soil samples by many other researchers (Nagano *et al*., 2008; Sudhadham *et al*., 2010; Bensch *et al.*, 2012; Giraldo *et al*., 2014; Asl *et al*., 2017)

The pathologic potential of this fungus is its ability to produce skin lesions as a result of its extracellular enzymes. Nosanchuk *et al*. (2015) reported production of melanin significantly enhanced the virulence of the dematiaceous fungi, and also aid them to survive in diverse hostile environment. The pronounced activity of the essential cream supported the fact that the active; the plant extract contained some bioactive substances that showed both healing and cidal activity on the implicated lesions. The pathological features observed from some dematiaceous fungi isolated and characterized in the present sudy corroborated with the findings of many researchers (Zeng *et al*., 2007; Rossetto *et al.*, 2010; Queiroz-Telles *et al*., 2009; Welsh *et al.,* 2007; Hoffmann *et al*., 2011). Hoffmann *et al.* (2011) recorded the ability of dematiaceous fungi to produce lesions in laboratory animals. Zeng *et al*. (2007) observed that dematiaceous fungus was responsible for subcutaneous infection of the lower limbs. Silva *et al.* (2005) also documented the subcutaneous infection known as phaeohyphomycosis on the scrotum caused by dematiaceous fungus, which was characterized by dark patches on the scrotum Aala *et al*. (2010)

investigated *in vitro* antifungal activity of alicin extracted from *Allium sativum* and recorded optimum inhibition of dermatophytes. Amin *et al*. (2005) observed a significant inhibitory activity of *Allium sativum* against bacterial and fungal pathogen s in India. An *et al*. (2009) documented a synergistic property of *Allium sativum* with amphotericin B in tackling infection caused by *Candida albicans*. Armstrong-Janes *et al.* (2017) documented the efficacy of *Allium sativum* in treating fungal infection using immunotherapeutic approaches. Behbahani *et al.* (2017) recorded optimum antimicrobial potentials of *Allium sativum* essential oils against pathogens. Borlinghaus *et al*. (2014) investigated the chemistry and biological properties of Allicin and recorded a significant antimicrobial activity. Khodavandi *et al*. (2010) recorded a synergistic property of allicin with azole drugs in treating *Candida* infections.

CONCLUSSION

The study has shown that *Cladosporium sphaerospermum* and *Phomopsis azadirachtae* isolated from soil samples showed significant dermatological lesions in albino wistar rats, and these were effectively prevented among the rats topically applied essential cream produced from *Allium sativuum.*

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