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A Novel Preparation of Culture Medium from Caper (*Capparis spinosa*) Leaves for the Identification of *Cryptococcus neoformans*, Known as Caper Leaves Agar (CLA)

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Abstract

Cryptococcus neoformans is a yeast pathogen attributed as the causative agent of cryptococcosis, a significant infection disease in humans. Recently, microbiologists have tried to make new media to detect *C. neoformans* from simple natural materials. In this study, a novel medium was prepared from caper leaves as an identification and differential medium for *C. neoformans*. Fifty isolates of *C. neoformans* and *Candida albicans* (as negative control) were cultured on caper leaves agar and incubated at 37ºC for 48 hours. The results of the study revealed that after 48 hours of incubation, the fifty isolates of *C. neoformans* were grown on the caper leaves agar (CLA). The colonies were pigmented by dark brown, while *C. albicans* grow without pigment production. The results of analysis for caper leaves by high-performance liquid chromatography (HPLC) revealed that the caper leaves contained: Myrecelin, Ferrulic acid, Caffeic acid, Quinic acid, Ascorbic acid, Apigenin, and Kaempferol, all those phenol compound considered as substrate to produce melanin as differential pigment. The caper leaves agar (CLA) tested in the current study is a simple, inexpensive, high concentration of phenolic compounds and represents a viable alternative tool for the presumptive identification of *C. neoformans* in environmental and clinical samples.

Keywords: *Cryptococcus neoformans*; Melanin production; Differential medium; caper leaves

وسط زراعي جديد محضر من أوراق القبار لتحديد خمائر المُستخفَّاتُ المُورَمَةُ ، المعروف بوسط اكار أوراق القبار

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الخلاصة

المُستخفَّاتُ المُورَمَةُ ، وهو فطر خميري، يُعزى إليه كونه العامل المسبب لداء المُستخفَّاتُ ، وهو مرض معدٍ مهم في البشر. مؤخرًا، حاول علماء الاحياء المجهرية تطوير أوساط جديدة للكشف عن المُستخفَّاتُ المُورَمَةُ باستخدام مواد طبيعية بسيطة. في هذه الدراسة، تم إعداد وسط جديد من أوراق القبار كوسط انتقائي وتمييزي للمُستخفَّاتُ المُورَمَةُ .تم زراعة خمسين عزلة من المُستخفَّاتُ المُورَمَةُ و المُبيَضّة البيضاء (كعينة تحكم سلبية) على وسط أجار أوراق القبار وتم حضنها عند 37 درجة مئوية لمدة 48 ساعة. كشفت نتائج الدراسة أنه بعد 48 ساعة من الحضانة، كانت العزلات من المُستخفَّاتُ المُورَمَةُ على وسط أجار أوراق القبار قد اكتسبت لونًا بنيًا داكنًا، بينما نمت المُبيَضّة البيضاء دون إنتاج صبغة. أظهرت نتائج التحليل لأوراق القبار باستخدام تقنية الكروماتوغرافيا السائلة عالية الأداء أن محتوى أوراق القبار يتضمن: الميريسلين، وحمض الفيروليك، وحمض الكافيين، وحمض الكينك، وحمض الأسكوربيك، والأبيجنين، والكايمبفيرول. تُعتبر جميع هذه المركبات الفينولية ركائز لإنتاج الميلانين كصبغة تفاضلية. تمثل أكار أوراق القبار التي تم اختبارها في الدراسة الحالية وسيلة بسيطة وغير مكلفة وتحتوي على تركيز عالٍ من المركبات الفينولية، وتعد أداة بديلة فعّالة للتحديد المبدئي لخمائر المُستخفَّاتُ المُورَمَةُ في العينات البيئية والسريرية.

1.Introduction

Cryptococcosis is caused by the fungus *Cryptococcus neoformans,* and it is fatal unless treated with antifungal drugs. *C. neoformans* is a fungus with a protective capsule, known as an opportunistic pathogen that is prevalent worldwide [1]. The immunosuppressor and deficiency individuals were more likely to be at risk of being infected with pathogenic fungi such as *C. neoformans* and other yeast, and those with compromised immune systems were at risk of nerve infection that led to meningitis [2].

Cryptococcus neoformans had various virulence factors, including hydrolysis enzymes such as urease and phospholipase, polysaccharide capsules, and melanin pigments [3,4]. Furthermore, the predominant fungal known to produce the pigment was *C. neoformans*. This pigment was considered a superantigen with more complex structures, such as polymers of indole and phenol units. Melanin has redox properties and can eliminate free radicals [5-7]. The melanin secreted by *C. neoformans* utilized by the laccase encoded by the virulence genes [8].

Melanin pigment is produced from *C. neoformans* when cultured on a medium with phenolic or polyphenolic substrates [9]. This phenolic material is utilized by *C. neoformans* to produce the lactase, which then generates quinones and suffers auto-polymerization, followed by melanin synthesis [10]. The dark pigment showed on the cell wall of yeast due to it retained in the cell wall [11]. *Cryptococcus spp* had a wide range of colors for melanin pigment, from black to light brown, that were deepened in phenolic concentration in the medium [12,13]. Recently, investigations have revealed the presence of pigments in mustard seed agar [14], henna agar [15], apple leaves agar [16], and eggplant leaves agar [17,18].

Caper (*Capparis spinosa*) is a shrub that grows on walls and in quarry areas. It belongs to the Capris family and includes approximately 40-50 genera and 700-900 species [19]. This perennial thorny plant features numerous evergreen branches adorned with thick, soft leaves resembling thorns. In the morning, it boasts beautiful white flowers that undergo a captivating transformation to a dark red hue in the afternoon. Additionally, the plant yields pear-shaped fruits, as indicated by references [20,21]. Caper contains glucosides such as rutin, myrosinase enzyme (capric and lactic acids, quaternary alkaloids such as stcadherin, and sugars and volatile oils with a smell similar to the smell of garlic due to the sulfur, soapy, and gelatinous substances they contain, sterols, organic and fatty acids, and coumarins. Using the

caper leaves immediately after picking is recommended, as it loses effectiveness when left for a period longer than one day after picking [22,32].

Flavonoids are one of the most versatile natural polyphenol products due to their complex pharmacological effects ranging from anti-diabetic and anti-inflammatory to anti-cancer effects [24]. Anti-cancer clioquinols consist of a group of natural compounds containing glucose derivatives and amino acids [25]. They are structurally made of compounds with special functional groups of sulfur and nitrogen, which give plants their characteristic aromas and biological activity [26].

The aim of this current study is to prepare a new medium from caper leaves (*Capparis spinosa*) for the identification of *C. neoformans*.

2. Material and Method.

2.1. Yeast isolates

Twenty-five isolates of *C. neoformans* and an equal number of *Candida albicans* yeast isolates were obtained directly from the mycology laboratory in the Department of Biology at the College of Science, University of Al- Mustansiriyah. These isolates were cultured on slants of Sabourauds dextrose agar (SDA) medium until they were needed for study. They had previously been isolated from the oral cavities of patients with diabetes mellitus and identified using the VITEK 2 System.

2.2. Preparation of caper leaves agar (CLA) medium.

The samples were collected in the summer from orchites of Salman Al-Muhammadi in Baghdad province. The Capparis *spinosa* that had the best characterization were collected, including a height of 40 cm and a total spread, including all branches of 70 cm, and growth in high temperatures with dry conditions. Next, the samples were dried at room temperature for 3 to 5 days then grinding using an electric grinder. Fifty grams of dry powder were dissolved in 1 liter of distilled water (DW) and subsequently boiled for 30 minutes. Afterward, the mixture was allowed to cool and then filtered through gauze. Five hundred ml of final volume was adjusted to 1 liter with distilled water. The pH was set to 6.0, and 20 grams of agar-agar were incorporated into the mixture, which was then autoclaved at 121°C for 15 minutes. After cooling the medium to 45 ºC, 250 mg/1000ml of chloramphenicol was added to prevent bacterial contamination. The prepared medium was poured directly into sterile dishes [15,16].

2.3. Test of melanin production on caper leaves agar (CLA) medium by C. neoformans.

The caper medium was inoculated with *C. neoformans* isolates and *Candida albicans* isolates (as negative control), and the sunflower seed agar (SSA) medium was inoculated with *C. neoformans* as a positive medium. Plates were incubated at 37°C for 2-7 days to observe any change in the color of the colonies [17]. The cultivation of yeasts was done in the laboratory with a minimum biological safety level 2 (BSL-2).

2.4. Measuring the concentration of phenolic compounds in caper leaves.

2.4.1. Extracting of phenolic compounds from caper leaves:

Fifteen microliters of 80% ethanol were added to 0.2 gm of dried caper leaves, mixed well using an ultrasound device for 30 sec. at 500 revolutions/sec., and centrifuged at 7500 rpm for 15 min. after that, the sediment was discarded, and the eluent was concentrated using a stream of nitrogen. The samples were again suspended in 1 ml of HPLC grade methanol and mixed with 0.5 ml of potassium metabisulfite (0.5%), filtered using 0.22µl filter paper. Twenty µl were injected into the HPLC device under the separation conditions set by the manufacturer [27].

2.5.1. Quantitative and qualitative calculation of phenolic compounds

A high-performance liquid chromatography (HPLC) device was used to estimate the quantity and quality of phenolic compounds in the caper extract and compare it with standard samples. 10 microliters of samples were injected into a separation column (ODSc18) with dimensions (250 x 4.6) mm I.D., a particle size of 5µm, and a temperature of 30 ºC. [28].

3. Results and Discussion.

3.1. Detection of C. neoformans on the caper leaves agar (CLA) medium

Figure 1 showed significant production of melanin pigments after 48 hours at 37 ºC, and also, the colony was pigmented by brown color. Additionally, the yeast produces the pigments in large amounts in the medium.

Figure 1: *Cryptococcus neoformans* exhibits brown pigment colonies on caper leaves agar (CLA) after 48 hours of incubation at 37 ºC.

3.2. Comparison between negative control and positive control medium

The positive result of *C. neoformans* was to produce melanin pigments on the seed sun agar, which pigmented the colonies with brown. The protocol depended on the study by Patel [28]. As for negative control, there was no change in the medium and colonies pigments. The results are shown in Figures 2 and 3.

Figure 2: *Candida albicans* with creamy color on caper leaves agar (CLA) after 48 hours of incubation at 37 ºC (negative control)

Figure 3: *Cryptococcus neoformans* with brown pigment colonies on sunflower seed agar (SSA) after 48 hours of incubation at 37 ºC (positive control).

3.3. Detection of the quality and quantitate of the phenol compound.

The result in Table 1 revealed phenolic compounds in the leaves of *caper*. The chromatography analysis showed six types of phenol compounds in the leaves, with a higher concentration of gallic acid (0.371±.0.05 ng/ml) and ascorbic acid (0.144 ±0.01ng/ml) than other compounds.

Table 1: Concentration of phenol compounds from leaves of caper.

Phenol compound	Concentration ± SE (ng/ml)
Gallic acid	0.371 ±0.05
Ascorbic acid	0.144 ±0.01
Ferrulic acid	0.012 ±0.011
Myrecelin	0.002 ±0.001
Apigenin	0.021 ±0.010
Kaempferol	0.006 ±0.001

The results of the culture on CLA medium and SSA control shown in Figures 1 and 2 revealed good growth and a good selection of colonies for *C. neoformans*, which were associated with differential growth by the production of brown melanin pigment in the CLA medium. The present investigation has identified a distinct characteristic related to melanin production through phenoloxidase activity in *C. neoformans* and *Cryptococcus gattii*. Consequently, an agar medium containing a melanin pigment precursor holds the potential for differentiating and identifying cryptococcal colonies from other yeast varieties. Additionally, C. *neoformans* isolates displayed brown colonies on caper agar medium within 48 hours post-inoculation, facilitating easy identification. Conversely, colonies of *C.albicans* remained creamy in caper agar medium throughout the entire 5-day incubation period [29].

Furthermore, all isolates of *C. neoformans* exhibited the production of a brown pigment when cultured on sunflower seed agar (SSA), which served as the positive control medium.

Earlier studies have documented the emergence of brown-pigmented *C. neoformans* colonies using diverse culture media containing L-dopa (L-3,4-dihydroxyphenylalanine), caffeic acid, as well as prepared medium from extracts of various plants [30,31]. Moreover, other studies used the remains of some plants to prepare culture media that were used to isolate and identify

C. neoformans, such as pods of fava beans and seed covers of fava beans [17,18].

According to HPLC analyses presented in Table 1, the current study affirms that media incorporating caper leaf extract, devoid of additional carbon or nitrate sources, support the growth of *C. neoformans* and facilitate the formation of brown colonies. This observation highlights the potential of this novel medium. Conversely, fresh caper leaves contained the highest concentration of gallic acid and ascorbic acid. Our findings align with previous studies emphasizing the crucial role of melanin as a virulence factor in the basidiomycete fungal pathogen *C. neoformans*. In the presence of phenolic compounds like L-DOPA, *C. neoformans* produces brown eumelanin through laccases (Lac1 and Lac2) [32]. These laccases, once expressed, are loaded into secretory vesicles and deposited as spherical particles within the cell wall, using chitin as an anchoring molecule, or they are secreted extracellularly [33-35].

Sundry studies have demonstrated the appearance of colonies with brown pigmented to the *C. neoformans* species complex when using different cultured media [36,37]. Our study confirms that a plant-based medium, without any additional carbons, nitrate, or vitamins, can support the growth of *C. neoformans* yeast. This finding is consistent with previous studies demonstrating that *C. neoformans* and *C. gatti* species can grow on distinct plant material, like *Arabidopsis thaliana*, *Pseudotsuga menzieesii,* and other conifers [38].

Several selective media were used for isolating and identifying the *C.neoformans* provided by various companies and suppliers. However , these media were often costly and not readily available in low-income countries. The medium described in the current study is based on leaves, which are inexpensive, widely accessible, and require no additives, making them an optimal choice for laboratories with limited resources. Additionally, this study broadens the range of plant-based substrates that support the growth of cryptococcal yeasts and highlights these pathogens potential to colonize plants containing phenolic and polyphenolic compounds.

Conclusions

Melanin stands out as a crucial virulence factor in *C. neoformans*. In the presence of external phenolic compounds, *C. neoformans* synthesizes brown melanin through laccases. When cultured in agar media derived from caper leaf extract, melanin-producing *C. neoformans* colonies display a distinctive brown color, setting them apart from *C. albicans*, which does not yield brown-pigmented colonies. Notably, the caper leaves agar (CLA) medium proves effective for isolating *C. neoformans* from clinical and environmental samples.

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Conflicts of interest
There are no competing interests.

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