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Original paper

Isolation and identification of yeasts from UTI patient's exhibit susceptibility to *Agaricus gennadii* extract

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ABSTRACT. Antifungal resistance represents a major clinical challenge to clinicians responsible for treating invasive fungal infections due to the limited arsenal of systemically available antifungal agents. Currently, fungal invasive infections are related to antifungal resistance by pathogenic fungi. The current study assumed to use of natural ingredients such as the fruiting bodies of large fungi, specifically *Agaricus gennadii*. This study is also a new initial step in the field of producing an antifungal that has a promising and safe future. However, most of the used antifungals such as polyenes, azoles, echinocandins, and flucytosine have side effects and may lead to damage to the human body. To achieve the goal of this study, 120 mid-stream urine samples were collected from urinary tract infection (UTI) patients, who attended Samarra General Hospital, the primary care sector and some medical clinics in the city of Samarra/Salah al-Din during the period from 11-1-2020 to 1-2-2021. Many laboratory examinations were performed including: microbiological, biochemical, and molecular tests of 70 samples of UTI patients, which develop yeast colonies on culture plates and were considered as positive results**.** Our results showed that the extract of the *Agaricus gennadii* fruit bodies contained a number of organic compounds including phenolics, flavonoids, saponins, terpenoids and alkaloids. Regarding the susceptibility of the isolated yeast species, many concentrations (100%, 75%, 50% and 25%) of the fungal extract were investigated. Data analysis of the obtained results showed that among all tested yeasts, *Trichosporon mucoides* and *Candida parapsilosis* were susceptible to the fungal extract at all concentrations, however, no effect of the fungal extract on the rest of the studied yeasts. Also, our results demonstrated that the susceptibility was increased with the increase of the fungal extract concentration. More studies are needed to separate and test the exact role of these compounds in the inhibition of fungal growth.

Keywords: yeast, fungi, *Candida* spp., *Agaricus gennadii*, antimycotics

Introduction

Recently, fungal infections have become a life-threatening source for many individuals, causing a large proportion of mortality and morbidity throughout the world [1]. Urinary tract infections (UTIs) have been a widespread disease for a long time and can be considered a public health problem, as in the world about half of the world's population suffers from urinary tract infections in all age groups [2]. Yeasts are the most common cause of fungal infections in the urinary tract of humans compared to filamentous fungi [3]. The most common yeasts causing disease are *Candida* and *Cryptococcus*. [4]. In an attempt to avoid the failures of antibiotics and to overcome the emergence of resistant strains, most recent studies have turned to nature as an alternative to chemicals. Among the alternatives that exist in nature are large fungi as well as plants, and large fungi are meant to be fungi that produce fruit bodies that are visible to the naked eye and are belonging to two divisions. (Basidiomycota and Ascomycota) [5,6].

Globally, *Agaricus* spp. are among the most important commercial mushrooms. Thus far, the antibacterial activities of many *Agaricus* spp. have

been reported [7]. Most of the studies available in the literature focused on the screening of antifungal activity of mushroom extracts. Additionally, no studies that investigate antifungal activities of *Agaricus* spp. in particular *Agaricus gennadii* were reported in Iraq so far. The current study aimed to estimate the susceptibility of several yeast spp. included in this study to the *A. gennadii* fruit bodies extract.

Materials and Methods

Sample collection

One hundred and twenty mid-stream urine samples were collected from patients who were clinically diagnosed as UTI cases and attending Samarra General Hospital, the primary care sector and some medical clinics in Samarra/Salah al-Din, during the period from 1 November 2020 to 1 February 2021. Many investigations were used including microbiological, biochemical, and molecular examinations showed that 58.34% (70 samples) of UTI patients revealed fungal growth on culture plates.

Growth and culture

The collected mid-stream urine samples were grown on Sabouraud Dextrose Agar (SDA), HiCrome^{TM} Candida Differential Agar to distinguish *Candida* species, and *Cryptococcus* differential agar containing antibacterial chloramphenicol at a concentration of 10 µg/ml. All culture media were purchased from HiMedia Laboratories Pvt. Ltd, Mumbai, India. The inoculated culture plates were incubated at 37°C for 24–48 h [8]. For positive fungal cultures, biochemical and biological examinations were performed including a ureas test [9] and germ tube formation test [10,11]. The used media (mentioned above) were prepared according to the instructions of the manufacturers. Then, the isolates were inoculated in the Sabouraud Dextrose Agar (SDA), afterwards, the plates were incubated at 30°C for 24–48 h. *Cryptococcus* differential agar was used to distinguish *Cryptococcus* species [12]**.** The features of the growing colonies were macroscopically and microscopically described [13,14].

Urease test

To investigate the ability of the isolated yeast species weather produce urease enzyme, a urease medium was prepared and inoculated with the isolated yeasts. The result will be considered as positive if the medium colour (yellow) is turned to pink, which is mean that the yeast produces the urease enzyme and can digest urea [11].

Germ tube formation test

To distinguish *C. albicans* and *C. dubliniensis*, 0.5 ml of human serum was placed in a sterile test tube, then a small portion of the developing colony on SDA was added and then the tube was incubated at a temperature of 35–37°C for 2 h, then a drop of the suspension was placed on a glass slide and covered with a coverslip, and then examined under the microscope (40×) [10,11].

Stains

To examine the microscopical features, some stains were used including methylene blue for observing the yeast cells, budding cells, and pseudohyphae [15], and India ink to observe the presence of the capsule or not. This stain (India ink) was used to distinguish *C. neoformans* from other yeasts that cannot form a capsule [16].

Collection Agaricus gennadii

Agaricus gennadii fruit bodies were collected from Elam, Salah al-Din region and sent for identification to the Department of Life Sciences/College of Education for Pure Sciences/Tikrit University. Distilled water was used to remove soil and other material to avoid contamination, afterwards, it was dried for 15 days away from sunlight, in a well-ventilated room. Later, the propagules were ground with an electric grinder and stored as a powder in sterilized dark containers until use [17].

Preparation of the alcoholic ethanolic extract of Agaricus gennadii

Following the method used by Hu et al. [18], the fungal powder was extracted then dried using a rotary evaporator device to get rid of the alcohol, then placing the filtrate in glass Petri dishes, and then placing the dishes in the incubator at a temperature of 35°C for 24 h to get rid of the alcohol. The process was repeated several times to obtain a sufficient amount It was extracted and kept in the freezer at –54°C.

Minimum fungicidal concentration MFC

The minimum fungicidal concentration of the fungus *Agaricus gennadii* fruitbody extract was

determined for all isolated yeasts using the extract of the fungus, different concentrations were prepared at dilutions of 25%, 50%, 75%, 100% in water. In order to estimate the minimum fungicidal concentration, the apparent growth diameters are compared to the diameters of the growing colonies on the minimum extract-free media (control), however, the concentration at which the growth was stopped was specified as the minimum fungicidal concentration [19].

Chemical detection of active compounds in the fungus Agaricus gennadii

Estimation of total alkaloid content

To detect the alkaloids in the fungal extract, Dragendroff's method was used [20]. Using the Soxhlet extractor device, in which the appearance of a precipitate is evidence of the presence of alkaloids. Then, the concentration of alkaloids was determined. Several concentrations were prepared and measured at a wavelength of 470 nm.

Estimation of total phenolic content

To estimate of total phenolic content, the fungal extract was dried in the shade at room temperature for 24 h, then grind to a fine powder in a Kirby mixer. Following, using a Soxhlet extractor device the extract was processed to detect by filtration and was concentrated using a rotary evaporator under reduced pressure at 40°C and then stored at 4°C until the analysis was done. Phenols were detected according to the Folin method using gallic acid and Folin-Ciocalteu reagents The mixture was tested using a spectrophotometer at a wavelength of 765 nm. The concentration of total phenols is calculated relative to the titration curve for gallic acid and in units of mg/g dry weight) [21].

Estimation of total flavonoid content

The total content of flavonoids in the crude extract was determined by the aluminium chloride measurement method, and then the absorbance of the model was recorded at a wavelength of 510 nm. The total flavonoid concentration was calculated relative to the titration curve for rutin and in units of mg/g dry weight [22].

Estimation of total saponins content

The saponin content in the sample was determined by the extraction method [23]. The saponins were extracted using butanol and washed with 5% NaCl solution and evaporated to dryness in a pre-weighed evaporating dish dehydrated at 60°C in the oven and re-weighed after cooling in the desiccator. The process was repeated two more times to obtain a defined mean. The saponin content varies according to the percentage of the original sample as the following:

Percentage of saponins =

(W2–W1/Wt. of the sample) × 100

W1= weight of evaporating dish;

W2 = weight of evaporating dish + sample

Estimated percentage of terpenoids

A standard solution of linalool is prepared in several concentrations, which were analysed using the spectrophotometer at a wavelength of 538 nm. The readings were recorded and then the percentage of turbines was calculated [24].

Method of propagation of the pits

Yeast suspension (0.1 ml) at a concentration of 1.5×10^8 was spread on a plate-containing medium. The suspension concentration was prepared by comparing the turbidity of the test suspension with that of the McFarland constant solution [25]. Then left for 30 min at room temperature to dry. Later, drills of 5 mm in diameter were done in the culture medium with a sterile cork borer and 0.2 ml of the prepared gradient concentrations of antifungals were added. The plates were incubated at 37°C for 48 h. Two replicates were performed for each plate. Then, the antifungal inhibitory effect of all concentrations was determined by measuring the diameter of the fungal-growth free (growth inhibition) zone [26].

Results

One hundred and twenty mid-stream urine samples were collected from UTI patients, who attended Samarra General Hospital, the primary care sector and some medical clinics in the city of Samarra/Salah al-Din during the period from 11-1-2020 to 1-2-2021. Laboratory culture, microscopic, biochemical and molecular examination of 70 samples of UTI patients, which develop yeast colonies on culture plates and considered as positive results**.**

Chemical detection of active compounds in mushrooms Agaricus gennadii

To determine the active compounds in the fungal extract, many organic compounds were investigated

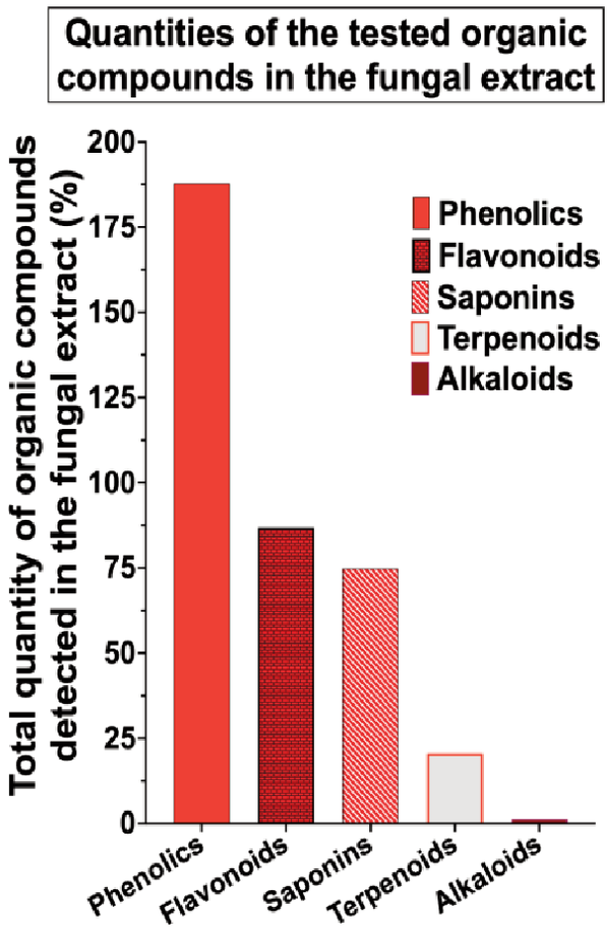

Figure 1. Total quantities of the investigated organic compounds in *Agaricus gennadii* fruit bodies extract

and their quantities were determined. Results of the current study showed that the extract of the *Agaricus gennadii* fruit bodies contained a number of organic compounds including phenolics, flavonoids, saponins, terpenoids and alkaloids. More studies are needed to separate and test the exact role of these compounds in the inhibition of fungal growth (Fig. 1).

To determine the susceptibility of the isolated yeast species, many concentrations of the prepared fungal extract 100%, 75%, 50% and 25%, and applied on culture plates inoculated with the isolated yeast species and the inhibition zone was measured after incubation for 48 h at 37°C (Fig. 2). The results of this test showed that *Trichosporon mucoides* and *Agaricus gennadii* were susceptible to the fungal extract at all concentrations, however no effect of the fungal extract on the rest of the studied yeasts. Also, our results revealed that the inhibition zone was increased with the increase of the fungal extract concentration, which at a concentration of 100% was the highest with *Trichosporon mucoides*

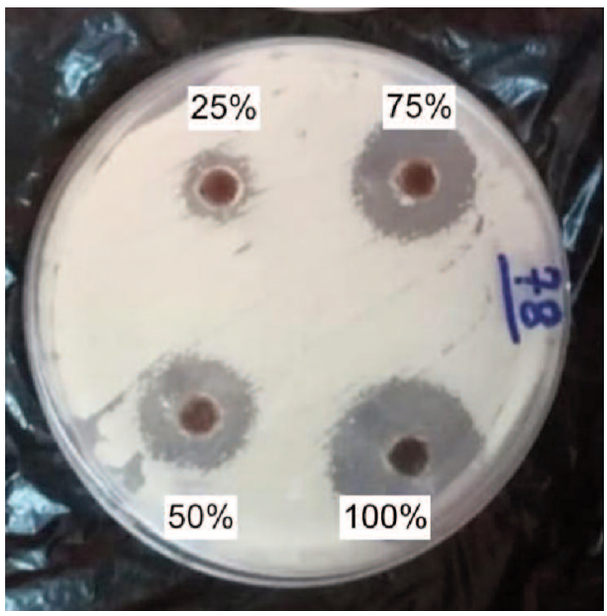

Figure 2. Inhibitory activity of *Agaricus gennadii* extract at different concentrations against yeast species

and *Candida parapsilosis* and scored the highest inhibition (60.33 and 61.13 mm, respectively). However, the effect of lower concentrations (75, 50, and 25%) have an approximately similar effect on

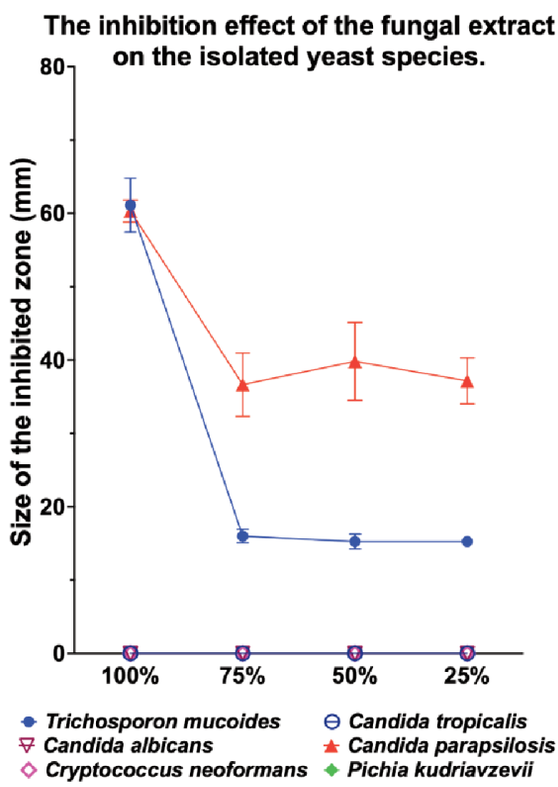

Figure 3. The inhibition effectiveness of *Agaricus gennadii* extract with different concentrations against the isolated yeasts: the size of the inhibition zone (mean in mm)

the inhibition of the yeast growth (*Trichosporon mucoides*: 16, 15.25, and 15.25 mm, respectively, and *Candida parapsilosis*: 36.67, 39.83, and 37.27 mm, respectively (Fig. 3).

Discussion

Due to the absence of studies on the effective compounds of *Agaricus gennadii* and the few scientific references regarding its being an antifungal or in medical uses [27]. Also, because of the importance of active compounds in large fungi, the study clarified its biological role, and it could be an effective candidate in biomedical and pharmaceutical applications, and it is included in clinical treatments against pathogenic fungi. It is part of the natural flora in Iraq, so the study aimed to identify its medical importance. Regarding the problems associated with the resistance of fungi to antibiotics, this study focused on using natural ingredients such as the fruiting bodies of large fungi, specifically *Agaricus gennadii*, and a crude alcohol extract was prepared with different concentrations of 100%, 75%, 50% and 25%, and applying it on some of the fungal species that were studied in this study.

Currently, it is possible to produce new antifungals from large fungi because they contain active substances capable of inhibiting an important part of pathogenic fungi and to develop drugs that lead to high efficiency and low toxicity, which gives positive results in the treatment of patients with fungal diseases, and these treatments can serve the principles of dynamics pharmacokinetics/ pharmacokinetics (DK/PD) Antifungals are among the mechanisms that serve the near future to avoid the use of antibiotics that contain high cytotoxicity and side effects.

The results of the current study showed the inhibition of the fungi *Candida parapsilosis* and *Trichosporon mucoides* in all concentrations, where the concentration was 100% the highest among all prepared concentrations. These results agreed with data was reported by other studies [28,29]. About what gave the genus *Agaricus* spp. an inhibitory activity against antimicrobial microbes. It indicates that the extract of *A. gennadii* inhibited the growth of the two types of *T. mucoides* and *C. parapsilosis* may be due to the sensitivity of the two mentioned types according to the active chemical compounds contained in the fungus *A. gennadii* [27]. was exhibited by a clear zone appearing on the culture plate. These results are agreed with the study Al-Zubaidi [30], where the concentration of 100% recorded the highest percentage of inhibition compared to other concentrations. It may indicate the concentration and strength of the active substances contained in the fungus, which is attributed to the inhibitory effect on the growth of the two fungi.

The lack of effect of *A. gennadii* extract on other species *C. tropicalis*, *C. krusei*, *C. albicans*, *Cry. neoformans*, *Cry. gattii* and *P. kudriavzevii* may be due to the virulence of these species and their ability to overcome the toxicity contained in the extract *A. gennadii*. In the study [17], this type was recorded for the first time in Iraq in Salah al-Din Governorate, which was recorded in Iran [29]. In a study conducted by Shakeri [7], it was confirmed that the genus *Agaricus* contains sugars that had immune and anti-cancer activities. As indicated in a study by Janknegt [27], the chemical content and the pharmaceutical and medical importance of this species have not been studied so far. Therefore, through this study, the effective content of mushrooms and their medical role in inhibiting fungal pathogens were identified. It was recommended in this study to conduct further studies. In the effect of extracts of large fungi as alternatives to the currently prevailing antifungals, then conducting clinical studies to reveal the antifungal efficacy of active compounds against pathogenic fungi. As well as activating the aspect of diagnosing fungi within mycology laboratories at hospitals and health care centres, since most of the workers at these institutions have no knowledge of fungal diseases or methods that are required to detect pathogenic fungi.

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