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Research Article

PREPARATION DIFFERENTIAL CULTURE MEDIUM FOR *CRYPTOCOCCUS NEOFORMANS* FROM AQUEOUS EXTRACT OF LEAVES AND FLOWERS OF *CHRYSANTHEMUM CINERARIAEFOLIUM*

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ABSTRACT

Objective: A new medium was prepared to isolate and diagnose the yeast *Cryptococcus neoformans* from flower and leaves aqueous extract of chrysanthemum.

Methods: Prepared differential culture medium for *C. neoformans* from aqueous extract of leaves and flowers of *Chrysanthemum cinerariaefolium* and chemical, spectral tests of the extracts were tested, in addition of gas chromatography (GC)–mass was used to diagnose phenolic compounds in both leaves and flowers.

Results: Showed that the yeast was grow with typical colonies on the new medium compared with other media which using in diagnosed of this yeast such as Staib agar and Sabourauds dextrose agar and unlike the yeast *Candida albicans* (as a negative control), which appeared in cream to white on this medium. Furthermore, the colonies are dark brown in color on flower chrysanthemum medium and light brown color on leaves chrysanthemum medium. In addition, the results of the chemical and spectral tests of the extracts confirmed that the plant contains many active compounds such as alkaloids, turbines, tannins, and phenols. The analysis of the extracts of phenolic compounds using GC–mass led to the diagnosis of five compounds in the leaf extract and nine compounds in the flower extract of this plant.

Conclusions: The media was prepared is differential medium that use to diagnosis of *Cryptococcus* such as Staib agar. Moreover, low economic cost, which consists of leaves and flowers of a plant available, abundance and the method of preparation is very simple.

Keywords: *Cryptococcus neoformans*, Fungi, Aqueous Extract, *Chrysanthemum cinerariaefolium,* Differential medium.

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INTRODUCTION

Cryptococcus neoformans are the most widespread opportunistic fungi in the world affecting humans and most animals [1]. The genus *Cryptococcus* belongs to yeast-like, an opportunistic yeast that affects immunocompromised people, which causes high morbidity and mortality, and also affects people who are immunocompetent [2]. Genus *Cryptococcus* contains more than 100 species classified according to the modern classification [3], but the pathogens of humans and animals are very few. The most important are *C. neoformans* and *Cryptococcus gattii*. But *Cryptococcus albidus* and *Cryptococcus laurentii*, are rarely cause disease [4]. *C. neoformans* have four serotypes based on the antigenic properties of it (A, B, C, and D) [5]. Serotype A is the most common type and responsible for the greater proportion of cases of cryptococcosis [6]. The yeast is characterized by spherical to elliptic diameter (6–4) mm, surrounded by a thick capsule of polysaccharides (1–30 μ). Reproduction by budding (a single bud of the base, not true hyphae) and no germ tube [7]. *C. neoformans* cause cryptococcosis, a globally dangerous disease [8]. This disease is more severe in people with immunosuppressive weakness, especially those with T-cell (CD4) cell-mediated immunity and people with human immunodeficiency virus [9]. As well as in healthy people, but in small numbers, almost 95% of cases occur in middle- and low-income countries [10]. Yeast grows on the Sabouraud dextrose agar (SDA) in colonies of creamy, smooth, and mucous form. When the yeast grows on Staib agar, it is formed in spherical, brown, and mucous shaped colonies. The brown color is due to the yeast of the phenoloxidase enzyme, which oxidizes the phenolic compounds found in the plant medium; precipitation of the melanin pigments on the yeast cell gives it brown colonies. Therefore, we used in this study plant (at this 1st time) which contains phenolic compounds in prepared differential medium for this yeast.

METHODS

SDA: Use this medium for comparison and, prepared according to the method of Odds, 1991, melted 65 g of SDA in 1000 mL of distilled water.

Staib agar medium: Used for comparison and prepared according to the method Staib *et al*. [11] by dissolving the following materials:
Black seed niger seed 50 g (obtained from herbal medicine stores in local markets)

- Glucose: 1 g
- Creatinine: 1 g
- KH_2PO_4: 1 g
- Agar: 15 g
- D.W.: 1000 ml.

Chrysanthemum flower agar: Prepared at the 1st time from available and simple materials:

- Chrysanthemum (*Chrysanthemum cinerariaefolium*) flower: 50 g
- Glucose: 1 g
- Agar: 15 g
- D.W.: 1000 ml.

Chrysanthemum leaves agar: Prepared at the 1st time from available and simple materials:

- Chrysanthemum (*C. cinerariaefolium*) leaves: 50 g
- Glucose: 1 g
- Agar: 15 g
- D.W.: 1000 ml.

Fungal strain

C. neoformans and *Candida albicans* isolates were obtained from the Micro. Laboratory, Department of Life Sciences, Faculty of Science/

University of Al-Qadisiyah. These isolates were saved on slant of SDA until to use.

Preparation of new media

Prepared by a weight of 50 g of dried flowers and leaves of *C. cinerariaefolium* separately and then added to one liter of distilled water and boiled for half an hour, then filtered through a medical gauze and complete the volume of the solution by distilled water to one liter and then add 1 g of glucose and 15 g of agar sterilized with autoclave. After sterilization, added chloramphenicol (250 mg/mL) and distributed in sterile dishes.

Cultivation of fungal strain

By streaking the isolates (that previously cultured on SDA at 37°C for 48 h) on the each medium which was prepared from leaves and flowers media. *C. albicans* was also developed as a negative control. The isolates were also studied on the Staib agar and SDA as a positive control and incubated at 37°C for 7 days and any chromatic change of colonies was followed.

Analysis of the components of the leaves and flowers of *C. cinerariaefolium*

Preparation of water extract, not flowers and leaves of the C. cinerariaefolium

Prepare the water extract of the leaves and flowers by a weight of 20 g of crushed leaves and flowers separately in 200 ml of distilled water and then put each in a stirrer incubator 120 rpm and 50 m for 16 h. Then, the extracts were filtered 100% (leaves and flowers) and were storage of extracts to be used [12].

Chemical detection of some active components of water extracts

Some chemical components were chemically detected using the following reagents [13] Table 1.

Extraction of phenolic compounds from the leaves and flowers of C. cinerariaefolium

Phenolic compounds were extracted according to the method was mentioned by [13,14]:

- Mix 20 g of crushed leaves and flowers separately with 400 ml of acetic acid solution (1%).
- Extraction using the inverter condenser in water bath at a ËšC of 70 m for 8 h. Then left to cool down.
- Filtered the mixture with a piece of gauze and then with the filter paper Whatman No. 1 and transfer to the funnel and add the same size of n-propanol and then the amount of salt (sodium chloride) until reaching saturation. It is composed of two layers, the upper and the organic layer containing the phenolic compounds, while the lower is eliminated.
- Propanial extract was collected and dried with rotary evaporator at a ËšC of 45 C and then left to dry at room ËšC then collected the resulting material and kept for use.

Spectral tests

The ultraviolet (UV) and infrared spectra of phenolic compounds for both leaves and flowers were measured separately using the UV-visible spectrophotometer and Fourier transform infrared, respectively.

Table 1: Chemical components of water extracts

No.	Compound	Reagents	Result
1.	Alkaloids	Tannic acid	The appearance of turbid white nailed
2.	Phenols	Aqueous ferric chloride (1%)	Appearance of green precipitation
3.	Turbines	Liebermann–Burchard	A brown ring
4.	Foam	Foam test	Foam is dense over the surface of the extract and lasts long
5.	Tannins	1% lead acetate	White deposit gelatinous

Diagnosis phenolic compounds

The gas chromatography (GC)–mass analysis was performed using a GC Clarus 500 PerkinElmer instrument with a mass spectrometer and a silica capillary column with dimensions of 30×0.25×1 µm. The carrier gas used is helium with a drop of 1 ml/min. The injector is operated at 250°C and the oven was programmed at 110°C for two min and then gradually increased to 280°C in 9 min. The components were determined based on the data of the National Institute of Technology, where the results were compared with the range of known components.

RESULTS

Growth on the developed media

The results showed that the yeast of *C. neoformans* was grown on the SDA at 37°C in white circular colonies and mucus smooth, as in Fig. 1, and these results are consistent with [15,16]. When the yeast of *C. neoformans* was grown on the Staib agar at 37°C, the results showed that the yeast had grown in spherical colonies, smooth mucus, and brown structure. The browned *C. neoformans* were colored brown due to phenoloxidase (phenoloxidase), which oxidizes the phenolic compounds found in the medium, depositing the melanin pigment on the wall of the yeast, giving colonies of brown, and distinct colonies from *C. albicans* Fig. 2.

Analysis of the components of the leaves and flowers

The results of the chemical extracts for the leaves and flowers of the plant (Table 2) showed that the extract contains phenolic compounds, tannins, and turbines.

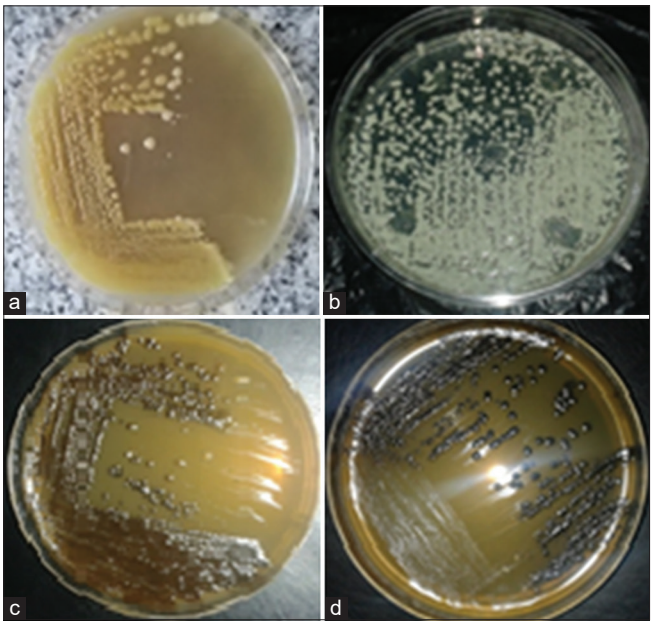

Fig. 1: Growth of *Cryptococcus neoformans* on various media, (a) Staib agar, (b) SDA, (c) leaves medium, (d) flower medium

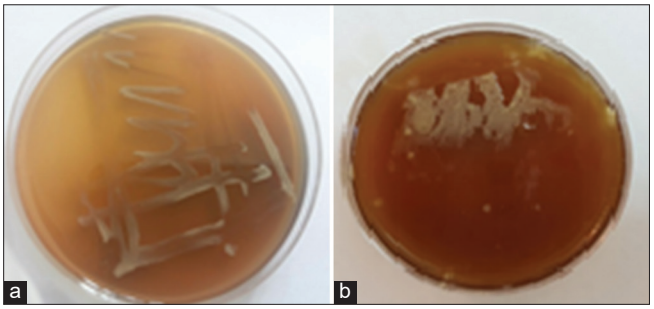

Fig. 2: *Candida albicans* (negative control) on the media (a) leaves, (b) flowers

Spectral tests

Figs. 3 and 4 show the UV spectrum of the phenolic extract of both leaves and flowers, respectively. It is noted that there is a peak at 328 nm. This is due to the hydroxyl group. The second beak was at 260 nm. Advanced analysis in food strategies, which stated that λ max for phenol lies between 211 and 270 nm. While Figs. 5 and 6 represent the red-ray spectrum of the phenolic extract for each of the leaves and flowers of the plant, respectively. The figures show that there is a spectral frequency at 3811 in Fig. 5 and 3865 in Fig. 6 which belongs to the OH group. The frequencies at 2923 in Fig. 5 and 2931 in Fig. 6 refer to aromatic CH and frequencies at 1627 in the two forms; they belong to the double bonds of (C=C).

Table 2: Chemical tests of active compounds in the water extract of leaves and flowers of plant

No.	Compound	Reagent	Treatment	
			Leaves	Flower
1.	Alkaloids	Tannic acid	+	+
2.	Phenols	Aqueous ferric chloride (1%)	+	+
3.	Turbines	Liebermann–Burchard	–	+
4.	Foam	Foam test	+	+
5.	Tannins	1% lead acetate	+	+

(+): Positive, (–): Negative

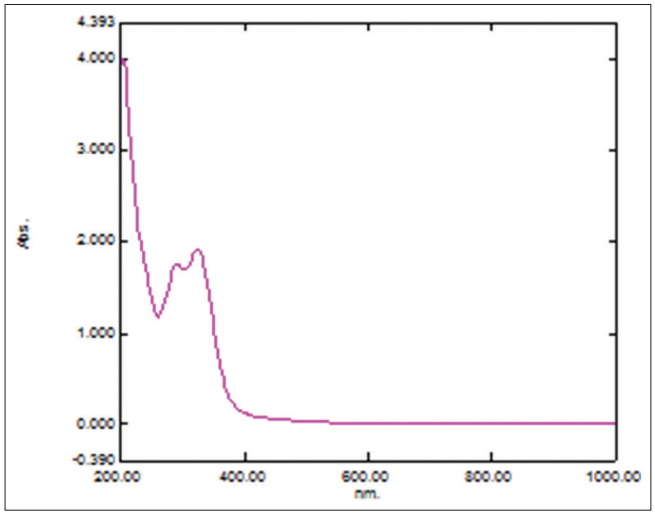

Fig. 3: Spectroscopy of the ultraviolet radiation of phenolic extract of flower

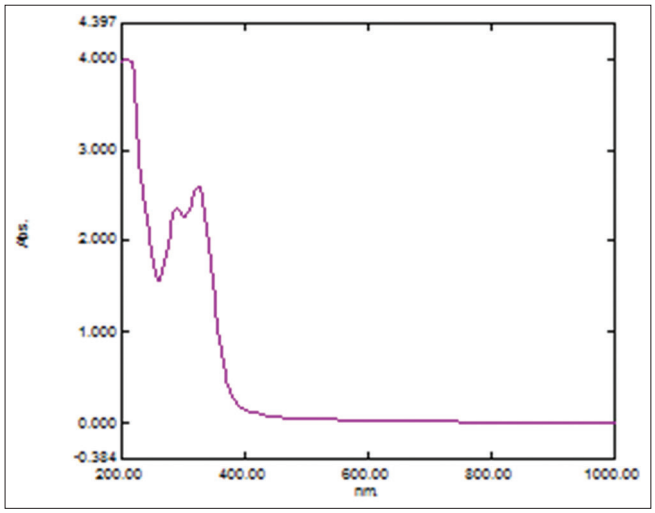

Fig. 4: Spectroscopy of the ultraviolet of the phenolic extract of leaves

Diagnosis of phenolic compounds using GC mass

Tables 3 and 4 and Figs. 7 and 8 show the results of the analysis using GC mass for the phenolic extract of the leaves and flowers of the plant, respectively. Extracts were found to contain a number of chemical compounds. Five compounds appeared in the leaves extract and nine compounds in the flower extract with some compounds present in both extracts but with a slightly different retention time.

DISCUSSION

The literature reported that high centration's from phenolic compounds in the leaves and flowers of *Chrysanthemum morifolium*, therefore colony of yeast appear with brown colour as a results of phenolic oxidation, these results are consistent with Lungran *et al*. [17]. The test of the ability of the yeast *C. neoformans* to grow on the new media of the flowers and leaves; the results showed that the yeast can grow easily and specifications of colonies typical of the above media and can be distinguished colonies easily by brown color, unlike the yeast *C. albicans* (as a negative control), which appeared in cream to white because the plant is a container of high concentrations of phenolic compounds, and the results showed that colonies of yeast growing on the medium of the flowers are dark brown compared with their colonies on the medium of leaves are light brown and can be attributed to the different concentrations of phenolic compounds between flowers and leaves. As shown in Fig. 1, the results are in line with Dulaimi *et al*., [18], Minhas *et al*. [19], Katiyar *et al*. [20], Ajah [21] of their use of media contained a phenolic compound. Plants are a potential source of new paradigms of antibiotics such as alkaloids, flavonoids, glycosides, terpenoids, and tannins [22]; results of active compounds appear the extracts contain many types of these compounds; this result is in line with Kareem and Amran [23]. Spectra test of both extracts shows phenolic compounds; our results are in coincidence

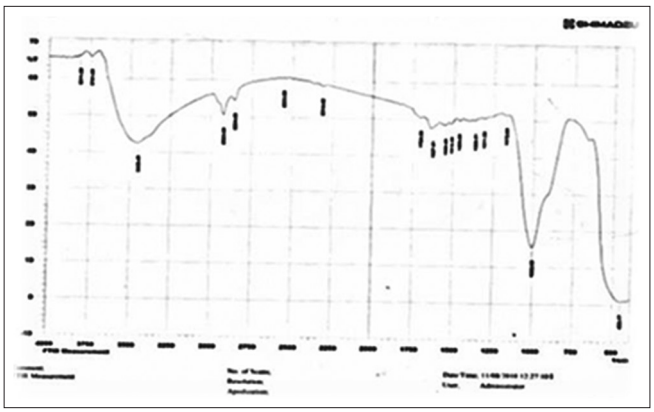

Fig. 5: Fourier-transform infrared spectrometry of phenolic extract of leaves

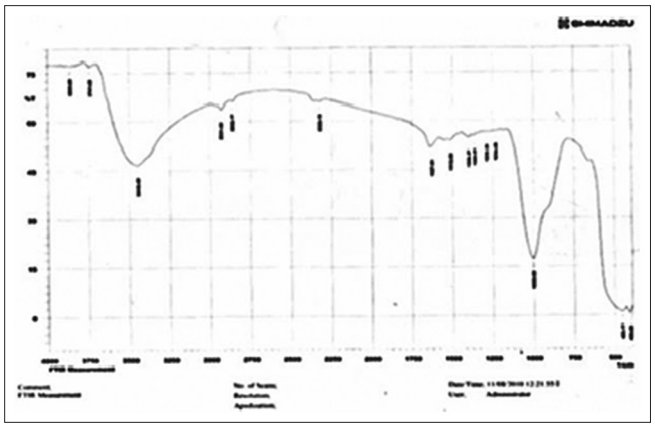

Fig. 6: Fourier-transform infrared spectrometry of phenolic extract of flowers

Table 3: Chemical compounds in phenolic extract of leaves of plant

No.	Compound	Retention time
1.	Butanoic acid	10.11
2.	3,7,11,15-tetramethyl-2-hexadecen -1-01	12.63
3.	2-(2-methylpropyl)-	13.55
4.	3-Buten-2-one, 4-(2-hydroxy-2,6,6-trimethylcyclohexyl)-	18.48
5.	Thujone	23

Table 4: Chemical compounds in phenolic extract of flowers of plant

No.	Compound	Retention time
1.	Acetic acid	6.88
2.	Chlorogenic acid	9.50
3.	2-Butenic acid-	10.50
4.	Hexadecanol	12.11
5.	3,7,11,15-tetramethyl-2-hexadecen -1-01	12.65
6.	3,6-octadien-1-01,3,7-dimethyl	16.82
7.	3-Buten-2-one, 4-(2-hydroxy-2,6,6-trimethylcyclohexyl)-	18.40
8.	Thujone	22.85
9.	Luteolin-7-β-glucoside	24.20

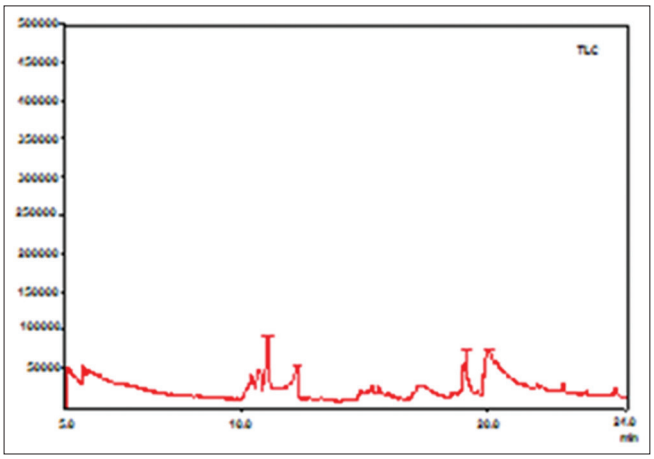

Fig. 7: Chromatogram for leaf extract

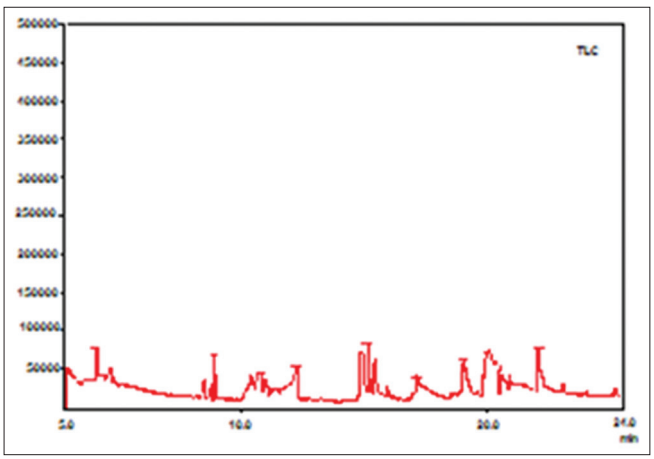

Fig. 8: Chromatogram for flower extract

with Silverstein *et al.* [24]. Gas chromatography–mass spectrometry used to identify different substances within a test sample such as plants sample [25] in this study GC–mass results appear that leaves and flowers of the plant were tested, rich with phenolic compounds this result is consistent with Han *et al.* [26] and Sheikh *et al.* [27].

CONCLUSIONS

This medium is characterized by low economic cost, which consists of leaves and flowers of a plant available, abundance and the method of preparation is very simple. It is similar to the medium of chrome agar of *Candida* in terms of containing the phenolic compounds, which acts as a color detector, which supports the presence of phenoloxidase enzyme and works to deposition of melanin giving dark brown color. Furthermore, the media were prepared which is progress of media that use to diagnosis of *Cryptococcus* such as Staib agar.

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AUTHORS' CONTRIBUTIONS

The author declares that this work was done by the author named in this article.

CONFLICTS OF INTEREST

There are no conflicts of interest we alone responsible for the content and writing of this article.

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ETHICS STATEMENT

No ethical approval was required as the research in this article related to microorganisms.

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