# Bean Seed Cover Agar, New Culture Medium For Cultivation and Identification of Cryptococcus Neoformans

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**Abstract**
Melanin production by *Cryptococcus neoformans* is widely used for isolation and identification of *C. neoformans*. In this study, we used the new medium prepared from fava bean seed cover extract. The isolates of *C.neoformans* and *Candida albicans* (as negative control ) were cultured on fava bean seed cover agar, Also Sunflower Seeds agar (*Helianthus Annuus*) inoculated with *C. noformans* as positive control. results showed that at 48 hours the isolates of *C. Neoformans* was pigmented on fava bean agar and produce dark brown colonies, while *C. Albicans* grow, but don't produce pigment. The analysis of seed cover by high performance liquid chromatography (HPLC) showed that the fava bean seed cover content: Myrecelin, Ferrulic acid, Caffeic acid, Ascorbic acid, Apigenin and Kaempferol.The present phenolic compound confirms fava bean seed cover agar useful as a media for the rapid identification of *C. neoformans*.
Keyword: Cryptococcus neoformans , melanin pigment , Vicia faba, bean seed cover

## 1. Introduction

Cryptococcosis is caused by two main species of the genus Cryptococcus, C. neoformans, comprising serotypes A, D, AD, and C. gattii comprising serotypes B and C. The genus Cryptococcus belongs to class Basidiomycetes, C. neoformans is one of the main opportunistic fungi that infects humans, causing many clinical infections, the most dangerous of which is meningoencephalitis in people with low immunity, including people infected with AIDS. Different Studies indicate that cryptococcal meningitis are responsible for hundreds of thousands of deaths each year (1,2,3,4).
C. neoformans has three main virulence factors: the ability of yeast to grow at 37°C, the presence of a polysaccharide capsule and the production of melanin pigment. The presence of the capsule and the production of melanin pigment have a great effect on the virulence of yeast because both have protective effects and lead to damage to the host as virulence factors (5).
The ability of C. neoformans to produce melanin was discovered at the beginning of the sixties of the last century (6,7), when this yeast was grown on Niger seed agar medium containing Guizotia abyssinica seed extract. The enzyme responsible for building the melanin pigment was identified as Phenoloxidase ( Laccase), which is attached to the yeast cell wall (8).
C. neoformans cannot produce melanin from internal sources because it lacks the enzyme Tyrosinase responsible for the production of Dihydroxyphenols compounds from internal sources, so this C. neoformans needs to acquire phenol compounds from its growth environment (9). C. neoformans pigment production requires the presence of exogenous substrates such as ortho- and para-diphenols and catecholamines including dopamine, epinephrine, norepinephrine and 3,4 dihydroxyphenyl alanine (DOPA) (10–12).
The bean plant (Vicia faba L.) belongs to the Fabaceae family. It is cultivated in different regions of the world and has many benefits. Fresh and dry bean seeds are used for human consumption as well as feed, because of the high nutritional value of its seeds and contains a high percentage of protein (up to 35% in dry seeds), and it is a good source of many nutrients, such as K, Ca, Mg, Fe and Zn (13,14). Moreover, consists of Several compounds, such as polyphenols (15), carotenoids (16), and carbohydrates (17).
Based on the above, the objective of this study was to prepare a new medium from the seed cover of fava bean and used to isolate and characterize C.neoformans .

## 2. Materials and methods

### 2.1Isolates

*Cryptococcus neoformans* and *Candida albicans* isolates were obtained from mycology laboratory, Department of biology of the sciences college ,Mustansiriyah university. This isolates was maintained on sabourauds dextrose agar until used.

### 2.2 Preparation of fava bean seed cover agar

Seeds were collected from the bean plant, the bean seed cover was removed, cover was collected and dried in the shade for 3-5 days, then it was ground with an electric grinder. 50 g of dry powder was weighed and a liter of distilled water was added to it, then boiled for 30 minutes, cooled and filtered through medical gauze and the volume was

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completed to a liter using distilled water, pH was adjusted to 6 and 20 g of agar-agar was added to the medium, medium was sterilized with autoclave at 121 °C for 15 minutes, cool the medium to 45-55°C, then add the chloramphenicol as antibacterial agent prevent bacterial contamination and pour it directly into sterile dishes (18) , (19). The medium of sunflower seeds was also prepared in the same way above, except for replacing the bean seed cover with sunflower seeds for this medium used as a positive control to compare the characteristic growth of *C. neoformans* on it compared with the new medium.

### 2-3Testing the ability of *C.neoformans* to produce melanin on bean seed cover agar

The bean seed cover agar was inoculated with *C. neoformans* and *Candida albicans*, then incubated at 37°C for 48 hours, the results were read, plate were left for 7 days in the incubator to notice any change in the color of the colonies. The sunflower seed agar was also inoculated with *C.neoformans* and incubated at 37°C for 48 hours(20) .

### 2.4 Determination of the phenolic compounds in the bean seed cover

Weigh 0.2 g of dry bean bean seed cover and add 15 ml of 80% ethanol to it and mix well using an ultrasonic device for 30 seconds at a speed of 500 r/s, then centrifugal at a speed of 7500 rpm for 15 minutes. Remove the Supernatant and concentrated with a stream of nitrogen (N2) .The samples were then suspended in 1 ml of HPLC grade methanol, then filtered with Millipore 0.22 µl . 20 µl was injected into the HPLC device under the separation conditions established by the factory .

HPLC, high-performance liquid chromatography (HPLC) was used to estimate the quantity and quality of phenolic compounds in the extract of bean seed cover and compared to the standard samples. 10 µl of samples were injected into a separating column (ODSc18) with dimensions (250 × 4.6) mm I.D, 5 µm particle size, and a temperature of 30 ° C. And under the following conditions: - Mobile phase: Solvent A (formic acid 1%), Solvent B (acetonitril), flow speed: 0.8 ml/min, wavelength 254 nm, temperature: 30 °C. The readings were recorded on the wavelength and according to the retention time Rt of the standard solutions and the studied sample, and Identification of compounds was a achieved by comparing their spectra and retention times of standards when available, each standard was 5µg/ml. The concentration of phenolic compound calculation as follows: [21.22]

Concentration of compound µg/ml= (area of compound/area of standard) ×concentration of standard ×dilution factor

## 3. Results and discussion

The ability of *C. neoformans* to make dark pigments in certain agar was discovered by Staib in the 1960s and has been used to identify this fungus8. Melanin is an important virulence factor for several microorganisms, including *Cryptococcus neoformans* and *Cryptococcus gattii*, thus, the assessment of melanin production and its quantification may contribute to the understanding of microbial pathogenesis (23).

*Cryptococcus neoformans* and *Candida albicans* were inoculated on bean seed cover agar ,all isolates of *C. neoformans* showed brown colonies on bean seed cover agar at 48 hours, post inoculation, while those the colonies of *C.albicans* remained white up to a maximum period of incubation (7days) figure1, 2 , moreover on sunflower seed agar, as positive control, all isolates of *C.neoformans* produced a brown pigment (figure3).

The results showed that, *C.neoformans* and *C.albicans* grow well on the medium of bean seed cover agar and the characteristics of its typical colonies ,while *C.neoformans* produced brown melanin pigment.

Pigment formation is catalysed by a laccase enzyme which is believed to convert substrate to melanin through a Mason-Raper scheme (24,25).

Growth of *C. neoformans* in substrates such as L-DOPA results in the production of a black pigment that is cell associated. The time of melanization is variable and depends on substrate concentration and factors that can affect laccase activity such as glucose and temperature (26).

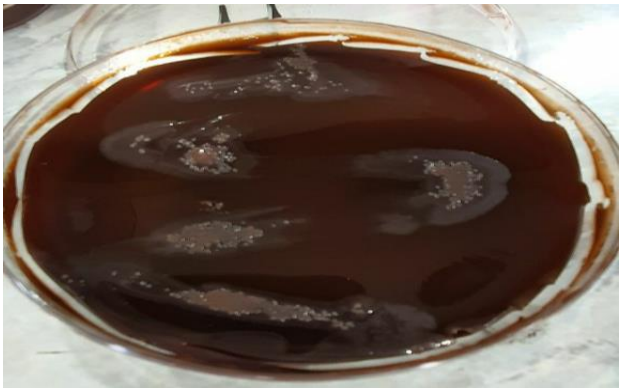

*Fig. 1. Dark Brown pigment of Cryptococcus neoformans , on fava bean seed cover agar after 24 hours at 37°C.*

*Fig.2.White colonies of Candida albicans on fava bean seed cover agar after 24 hours at 37°C.*

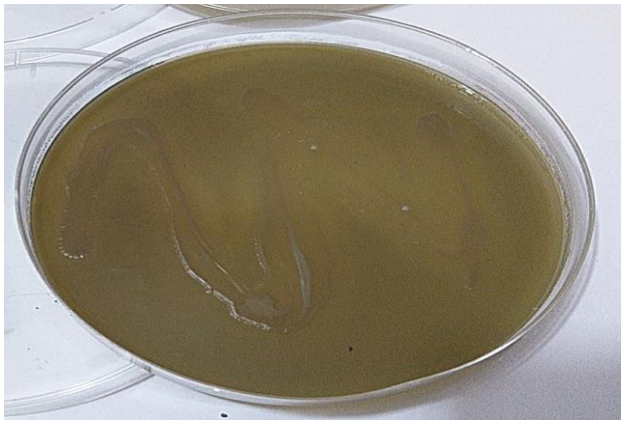

*Fig. 3.Brown pigment of Cryptococcus neoformans on sunflower seed agar after 24 hours at 37ºC.*

### Determination of phenolic compounds in the bean seed cover

After separation using HPLC, the following phenolic compounds were found in the bean cover: Myrecelin, Ferrulic acid, Caffeic acid, Ascorbic acid. Apigenin, Kaempferol. Figures 4 and 5 show the sequence of compounds for the Standard and bean seed cover

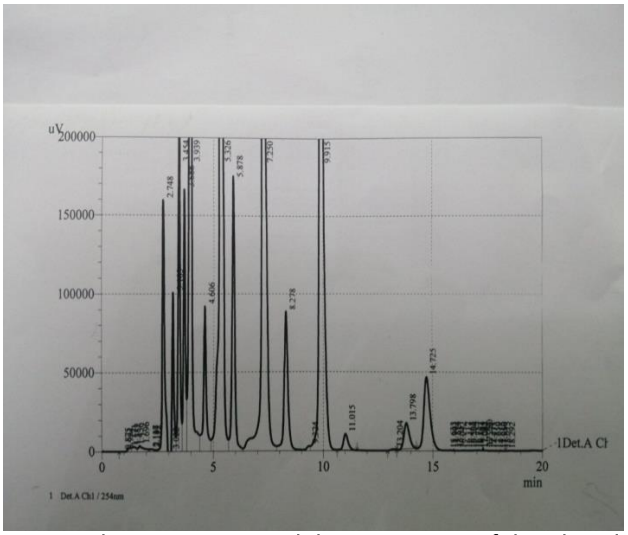


*Fig. 4.Chromatogram and the sequences of the eluted material of the standard, each standard was 5µg/ml, detection at254nm*

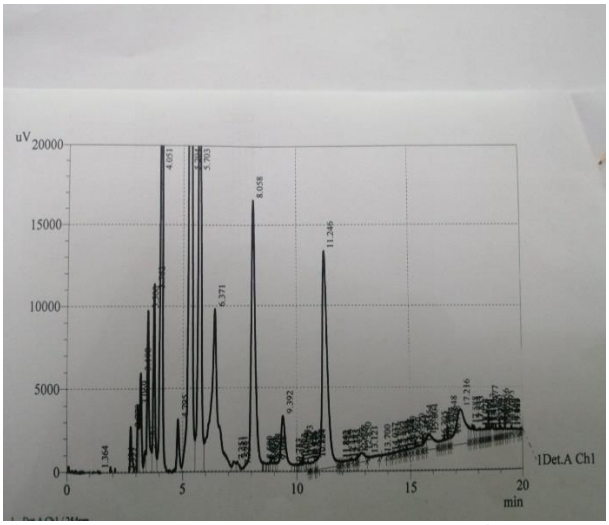


*Fig. 5.Chromatogram and the sequences of the eluted material of seed cover of fava bean extract ,detection at 254nm.*

Melanin is a negatively charged pigment, usually brown to black, polymerised from phenolic and/or indolic compounds. It is a hydrophobic pigment of high molecular weight, usually associated with proteins, and often with carbohydrates(27,28).
other culture media have been described to induce melanin production by C. neoformans, such as cowitch (Mucuna pruriens) seed agar(29). These seeds induce fungal melanisation because they contain L-DOPA, an important precursor for melanin production(30).
The cover of the bean seeds usually contains many carbohydrates and protein such as amino acids, which are a source of nitrogen, as well as phenolic compounds, and from here it was possible to prepare a local medium with the same specifications as the manufactured media, but at a lower cost and with high efficiency in isolation and identification of C. neoformans .
Several selective culture media were used to isolate and characterize C.neoforman from different materials (18, 31,32,33.). But the main problem appears in the unavailability of these materials in the market and their high price as well as the difficulty of preparing them, so the use of the bean seed cover to prepare simple medium containing bean seed cover only ,and does not need other additives except for agar to harden the medium moreover, bean is available and It is grown in all countries.
On the other hand, the bean seed cover can be preserved for long periods of time and used when needed because they are not damaged by storage. Moreover, the bean seed cover of the plant does not affect the country's economy.

## 4. Conclusion

Fava bean (*Vicia faba* ) seed cover agar developed for the rapid identification of cryptococcus *neoformans* based on melanin produced by the phenoloxidase activity .*C. neoformans* produces brown pigmented colonies, when grown on agar media made from a seed cover extract of fava bean ,while *Candida albicans* did not produce the reaction product. The analysis of fava bean seed cover by high performance liquid chromatography ( HPLC) showed that the fava bean seed cover content high concentration of phenolic compound that confirms fava bean seed cover agar as cuture medium for rapid identification of *C. neoformans.*

## 5. Aknowledgements

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