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Two New Media Apple Leaves Agar and Eggplant Leaves Agar for Identification of *Cryptococcus neoformans*.

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Abstract

The fungus *Cryptococcus neoformans* possess a phenoloxidase enzyme ,an enzyme which catalyses the oxidation of phenolic substrates to melanin pigment .In this study ,we used two new media made from an extract of apple leaves and eggplant leaves and compared with classic medium sunflower seed agar. Results obtained after the culture of five isolates of *C. neoformans* and two isolates of *Candida albicans* seven days at37°C , showed that at48hours all isolates of *C. neoformans* were pigmented on both two media and produce brown colonies, while both isolates of *C. albicans* do not produce brown pigmented colonies on two media. Since *C.albicans* isolates do not produce the pigment ,apple leaves agar and eggplant leaves agar are useful as differential isolation media for the rapid identification of *C. neoformans.*

Key words: *Cryptococcus neoformans* ,Melanin production,Apple leaves agar, Eggplant agar.

1. Introduction

Cryptococcus neoformans is an encapsulated opportunistic yeast like fungus that is a relatively frequent cause of meningoencephalitis in immunocompromised patients, and also occasionally causes disease in apparently healthy individuals (Mitchell and Perfect, 1995). The laboratory identification of medically important *Cryptococcus* species takes into account the particular characteristics of this genus. The majority are yeasts that produce capsules, able to grow at 37 ° C, and produce enzymes urease and laccase (Lacaz *et al.*,1991).

C. neoformans when cultured in media containing phenolic or polyphenolic substrates ,they form a pigment called melanin(Lacaz *et al*.,1991) . Electron micrographs of cells incubated in L-DOPA showed deposition of the pigment in the cell wall (Shaw and Kapica ,1972). Enzyme phenoloxidase (laccase) present in yeast act on these phenolic substrates generating quinones, which undergo a process of autopolymerization and turn into melanin.The dark pigment retained in the cell wall of the fungus is responsible for the color shown by the colonies (Casadeval *et al*.,2000; Polacheck *et al*.,1990) .

Colonies of melanin producing *Cryptococcus* species show a display of colures varying from brown to black when grown in agar media which contain L-dopa(L-3,4- dihydroxyphenylalanin) (Chaskes and Tyndall ,1975), caffeic acid (Pulverer and Korth ,1971), bird seed(*Guizotia abyssinica*)(Staib *et al*.,1987) , sunflower seed (*Helianthus anus*)(Katiyar *et al*.,2011), tobacco (Tendolkar *et al*.,2003) , henna (Nandhakumar *et al*.,2007) ,mustard seed(Nandhakumar *et al*.,2006) and pinus halepesis seed and blackberry (Mseddi *et al.*, 2011). These media are useful in selecting colonies of *C. neoformans* from mixed cultures expected from environmental samples and clinical samples such as respiratory specimens and urine .

The cultivated apple, *Malus domestica*, belongs to the Rosaceae (Rose) Family, Apples are worldwide diffused fruit with many health beneficial effects, mostly due to the presence of phenolic compounds. Polyphenols are common secondary metabolites of plants, with a well known putative role in protection

against the infection by plant pathogens (Walker, 1994) .Apples also ranked the second for total content of phenolic compounds, including quercetin, catechin, phloridzin and chlorogenic acid(Bravo,1998; Tedesco *et al.,2001).*

Eggplant ,*Solanum melongena*, is a rare ethno medicinal herb belonging to the family Solanaceae. The plant can be found in many places and used as therapeutic agent for various diseases moreover due to the presence of phenolic compounds and other compounds (Fategbe *et al.*,2013).

The present report describes a new medium containing leaves extract of apple (*Malus domestica*) and eggplant (*Solanum melongena*) which supports growth of *C.neoformans* and allows its easy differentiation by formation of brown colored colonies. To the best of our knowledge this is the first instance of the use of in the preparation of a differential medium for *C.neoformans.*

2. Materials and Methods

2.1 Preparation of apple leaves agar and eggplant leaves agar .

The apple leaves agar and eggplant leaves agar was prepared by a similar technique which is used to prepare niger seed agar and henna agar(Staib *et al*.,1987; Nandhakumar *et al*.,2007). Freshly plucked leaves of apple and

eggplant were washed and dried in the shade (3–5 days). Followed by grinding in a domestic blender .To 50g of each type of leaves ,one liter of distilled water was added .The mixture was boiled for 30minutes. The decoction was then cooled and filtered through gauze and the volume was readjusted to 1 liter with distilled water. The pH was adjusted to 6.0, and 20 g agar-agar (Difco) was added before the mixture was autoclaved at 121°C for 15 min. The media was allowed to cool to 45–55°C and dispensed into sterile Petri dishes.

This study was carried out in the Department of biology of The sciences college /Al-mustansyria university. A total of 5 *C. neoformans* isolates which were obtained from various clinical samples, which were received in the Mycology section were included in the study. The definitive identification of *C. neoformans* was done on the basis of: Growth at 37° C, Hydrolysis of Christensen's urea agar, Inositol and nitrate assimilation, and production of brown pigment (Paliwal and Randhawa, 1977; Evans and Richardson, 1989; Staib ,1994; Mitchell and Perfect, 1995).

The pigment production of *C.neoformams* was observed on the Sunflower seed agar as positive control .Two isolates of *Candida albicans* were testes as negative control .All the test isolates were initially grown on Sabouraud's dextrose agar at 37°C for 48 h. The media (apple leaves agar and eggplant leaves agar) were inoculated with isolates of *C. neoformans* and *Candida albicans* and incubated at 37°C for 48 hours, isolates also seeded on Sunflower seed agar as positive control.

Agar plates were held for 7 days to check for any variations in colony color.

3.Results and Discussion

Five isolates of *Cryptococcus neoformans* and three isolates of *Candida albicans* as negative control, were inoculated in apple leaves agar and eggplant leaves agar . At 48 hours post-inoculation ,all five isolates of *C neoformans* showed brown colonies on both media [figures 1 and2],while those of *C. albicans* remained white up to maximum period of incubation 7 days in both media [figures 3 and4], moreover all five isolates produced a brown pigment on sunflower seed agar(positive control).

Katiyar *et al*.(2011) reported the appearance of brown pigmented colonies of *C.neoformans* on Sunflower seed agar ,in our study also reported the same finding ,but the isolates of *C.neoformans* produced dark brown pigmented colonies within 48 hours post inoculation on apple leaves agar and eggplant leaves agar.

Melanin production by species of *Cryptococcus* is widely used to characterize *C. neoformans*. An agar medium which contains a precursor of melanin is used to test the pigment production by *C. neoformans* (Franics Xavier *et al.*,2013). Pigment production in *C.neoformans* is dependent upon presence of exogenous substrate unlike some other fungi which can make pigments endogenously(Chaskes and Tyndall, 1975) .The pigment of *C. neoformans* is cell-associated and is a melanin like pigment, formation of which is catalyzed by a laccase enzyme(Wang *et al.*, 1995; Williamson, 1997).

The ability of *C. neoformans* to produce brown colures effect (BCE),in media containing phenolic or polyphenolic substrates ,used for the rapid and accurate identification of *C. neoformans* in the clinical laboratories .A number of selective media were used for isolation and identification of *C. neoformans* from different material ,but the main problems was appear in unavailable of this material in the market , elevated costs and complex media preparation ,therefore we used leaves of apple and eggplant for preparing simple media that contains only leaves extract of apple and eggplant and does not require any additives only agar-agar ,therefore its inexpensive and widely available ,on the other hand apple and eggplant cultivated over the countries ,tropic and subtropics regions.

Shaw and Kapica (1972) showed that *C.neoformans* produces brown pigmented colonies when grown on agar media made from an extract of potatoes and carrots, broad beans(*Vicia faba*), or *Guizotia abyssinica* seeds. Similar specific pigment was produced by *C. neoformans* on chemically defined agar media which contained different substrates of phenoloxidase

Previous studies reported that o-diphenol substrates occur in potato, carrot (Craft and Audia ,1962), *Vicia faba* seed (Guggenheim, 1913), and *G. abyssinica* seed (Strachan *et al* .,1971), on the other hand many studies reported that phenol compounds occur in leaves and fruits of *Malus domestica* and *Solanum melongena* (Fratianni *et al*.,2007;Mikulic *et al*.,2008;Papadaki *et al*.,2008;Fategbe *et al*.,2013) while we did not study the exact component of apple leaves and eggplant leaves that is responsible for BCE, because of this is the first report on the usage leaves of apple and eggplant in the identification of *C. neoformans* ,therefore we are need further analysis for the elucidation of the exact mechanism of pigment production on this new media.

4. Conclusions

The need for rapid and accurate identification of *Cryptococcus neoformans* in the clinical laboratory has led to the development of many identification tests based on the phenoloxidase enzyme activity of the organism. Two

media were developed for the rapid identification of *C. neoformans* based on pigment produced by the organism's phenoloxidase activity.

C. neoformans produces brown pigmented colonies, within 48 hours post inoculation, when grown on agar media made from a leaves extract of apple and eggplant ,while *Candida albicans* maintained their normal morphology and did not produce the reaction product. Therefore apple leaves agar and eggplant leaves agar are used for rapid identification of *C. neoformans.* Further studies are planned to deeply investigate the production of pigment from *C. neoformans* when grown on agar media made from an extract of apple and eggplant leaves.

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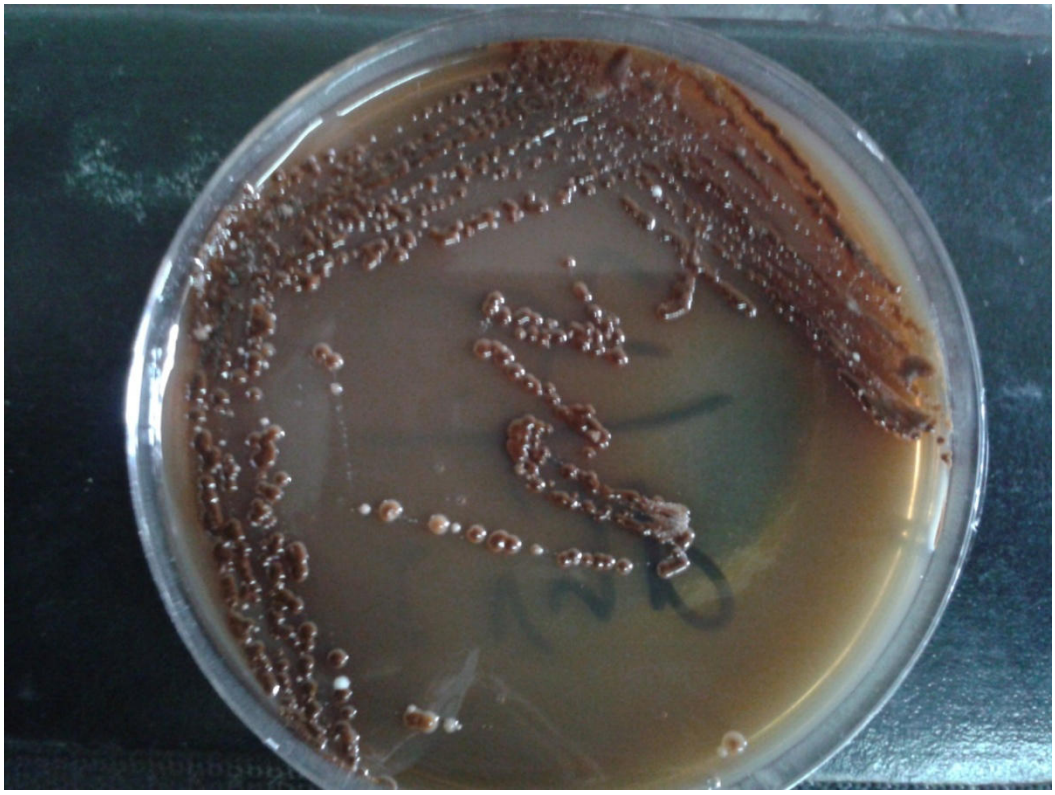
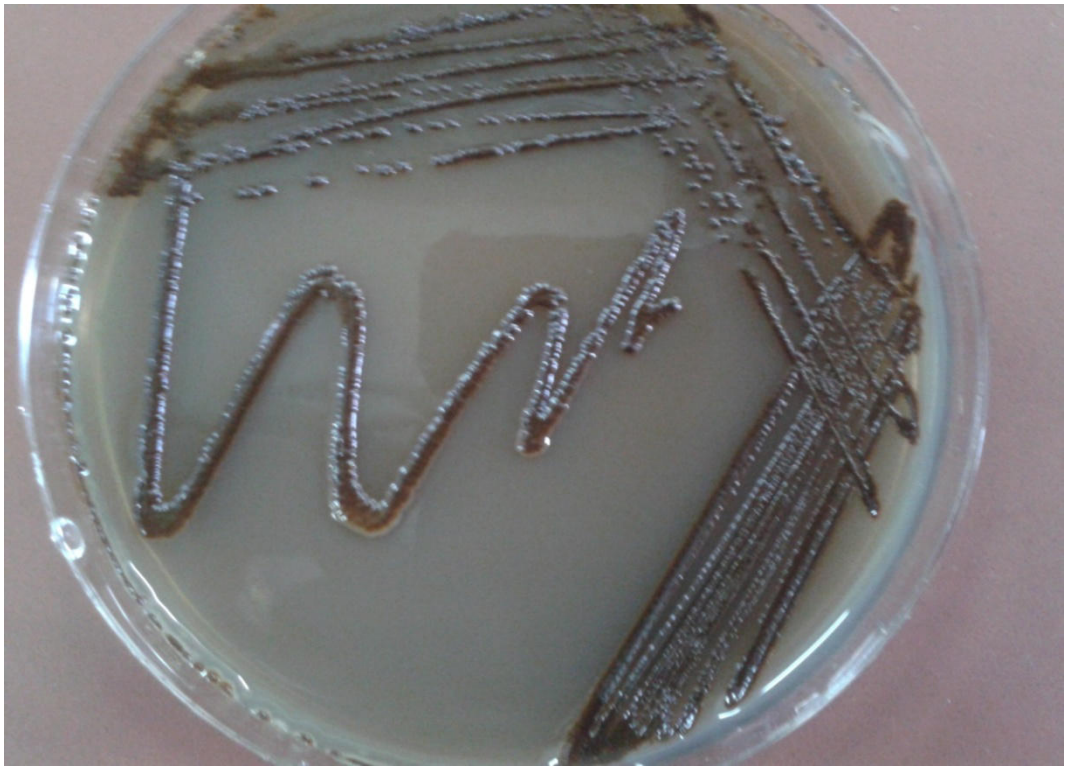
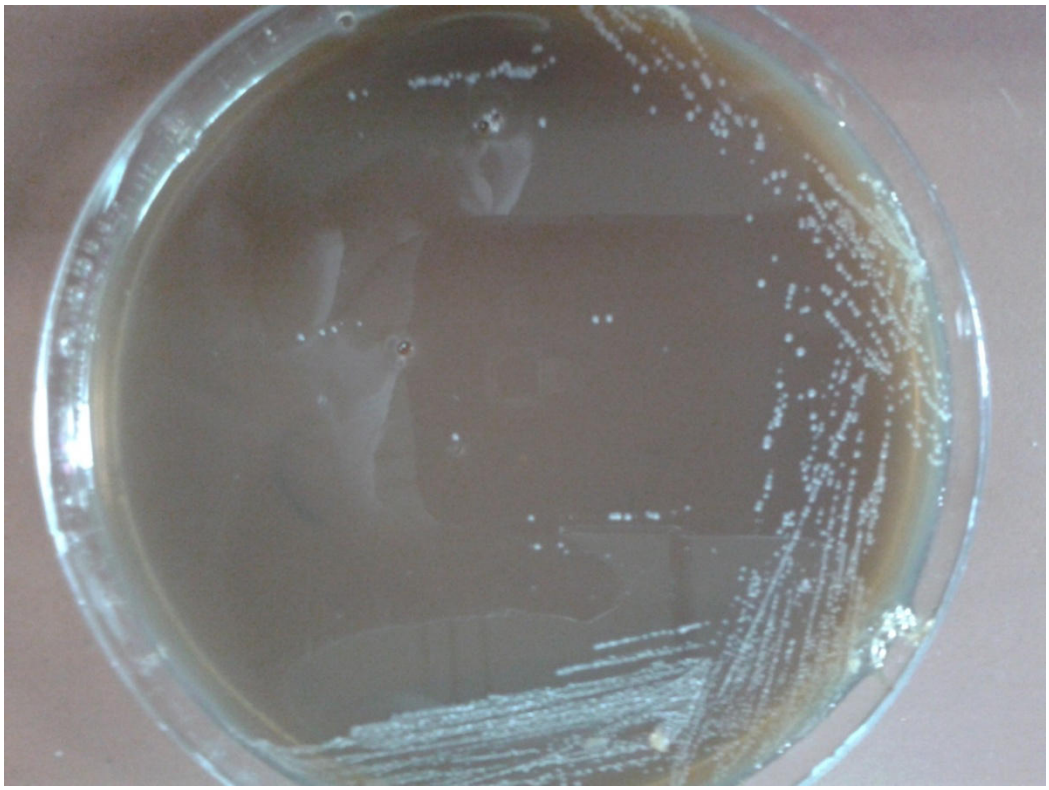


[Figure 1]:Brown pigment of *Cryptococcus neoformans* on apple leaves agar



[Figure 2]:Brown pigment of *Cryptococcus neoformans* on eggplant leaves agar.



[Figure 3]:White colonies of *Cndida albicans* on apple leaves agar.

[Figure 4]:White colonies of *Cndida albicans* on eggplant leaves agar.