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RESEARCH ARTICLE

New culture medium ,fava bean(*Vicia faba*) agar,for cultivation and identification of *Cryptococcus neoformans.*

Hamzia Ali Ajah
Department of Biology, College of Science, Mustansiryia University, Baghdad, Iraq

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Corresponding Author

Hamzia Ali Ajah

Abstract

Melanin production by *Cryptococcus neoformans* is widely used to characterize *C. neoformans*in mycology laboratories.In this study, we used new medium prepared from fava bean leaves extract.The isolates of *C.neoformans* and *Candida albicans* ,as negative control ,were cultured on fava bean agar, results showed that at 48hours 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 fresh fava bean leaves by high performance liquid chromatography(HPLC)showed that the fava bean leaves content :Gallic acid ,Caffeic acid ,Chlorgenic acid ,Protocatechic acid, Coumaric acid and Quinicacid.The present phenolic compound confirms fava bean agar useful as media for the rapid identification of *C. neoformans.*

INTRODUCTION

Cryptoccus neoformans is a major opportunistic fungal human pathogen found worldwide which causes life-threatening meningoencephalitisin immune compromised people including patients of acquired immunodeficiency syndrome (AIDS) [1].*C. neoformans* is the most common species in temperate climates throughout the world .The most serious cryptococcal infections develop in patients with AIDS or reticuloendothelial malignancy ,organ transplantation and corticosteroid treatment [2].*C. neoformans* has three major virulence factors include :the ability to grow at 37ºC, production of a polysaccharide capsule (composed of galactoxylomannan , glucuronoxylomanan and mannoproteins) and melanin formation [3].Melanins are family of pigments with shared properties, including dark colour , insolubility resistance to acid hydrolysis and susceptibility to degradation by strong oxidizing agents [4],[5].Unlike other melanotic microbes , *C.neoformans* melanizes only in the presence of exogenous substrates sub as L- DOP[6].Melanization in *C.neoformans* is catalyzed by laccase, a cell-wall associated diphenoloxidase that catalyses the oxidation of diphenolic compounds to their respedive quinones [7].

The laboratory identification of medical important *Cryptococcus* sp takes into account the particular characteristics of this genus the majority are yeasts that produce capsules, able to grow at37º C ,and produce enzymes urease and laccase . When cultured in media containing phenolic or polyphenolic substrates ,they from a pigment called melanin [8].Enzyme laccase present in yeast acts on these phenolic substrates generating quinones, which undergo a process of autopolimerization and turn into melanin ,the dark pigment relined in the cell wall of the fungus is responsible for the color shown by the colony [9],[10].Colonies of melanin –producing Cryptococcus species shown a display of colors varying from brown to black when grown in agar media such as sun flower seed agar (*Helianthus annus*) ,niger seed agar (*Guizotia abyssinica*) potato –carrot agar ,and other chemically defined media such us L- dopa and caffic acid agar [11].

Some recent studies have shown the production of pigment in mustard seed and chilli pepper agar , *pinus halepnesis* seed and blackberry agar, henna agar, tobacco agar,apple leaves agar and eggplant leaves agar ,cowitch seed agar, and in media containing substrates methyldopa , epinephrine , and norepinephrin [12-21].

Fava bean (*Vicia faba* L.), belongs to the Fabaceae Family, is a grain legume and it is among the oldest crops in the world. Globally, it is third most important feed grain legume. Currently, 58 countries produce this bean on large scale. Probably fava beans are one of the best performing crops under global warming and climate change scenario because of its unique ability to excel under all most all type of climatic and soil conditions , Fava bean is a good source of lysine rich protein and good source of *leva dopa* (*L-dopa*), a precursor of dopamine, can be potentially used as medicine for the treatment of Parkinson's disease [22], [23].while the media prepared with chemically resources are widely used by some authors ,they undergo a complex preparation and expensive ,so we proposed this study to prepare simple media from fava bean leaves to identification and cultivation of *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 /Al-mustansyria university. This isolates was maintained on slants of sabourauds dextrose agar until used.

2.2 Preparation of fava bean leaves agar

The fava bean leaves agar was prepared by a similar technique in [17],[24]. Freshly plucked leaves of fava bean were washed and dried in the shade (3–5 days). Followed by grinding in a domestic blender for the preparation of this medium 5g of powder from leaves of *Vicia faba* was added to 100ml of sterile distilled water in conical flasks and boiled for 10 minutes with occasional shaking . The extract was then filtered through muslin cloth for coarse residue and finally filtered through WhatmanNo.1. The final volume adjusted to 100ml. The pH was adjusted to 6.0, Agar-Agar powder (2g) was then added to it as solidifying agent ,then sterilized by autoclaving at 121ºC for 15 minutes at 15lb pressure. The medium was allowed to cool to 45-55ºC then 15-20ml was poured into sterile Petri dishes.

2.3 Inoculation of yeast isolates on fava bean agar

All the test isolates were initially grown on Sabouraud's dextrose agar at 37 ℃ for 48 h. The fava bean leaves agar were inoculated with isolates of *C.neoformans* and *Candida albicans* as negative control, also the isolates seeded on Sunflower seed agar as positive control. Agar plates were held for 7 days to check for any variations in colony color.

2.4 Extraction of phenolic compounds from fava bean leaves

A 0.2-g fresh leaf of fava bean was homogenized with 15 mL of methanol with an Ultraturrax
extracted with 15 mL of acetone and centrifuged again. A third extraction of solid residue was developed with other 15 mL of methanol and centrifuged as described. The three extracts were combined, filtered through a membrane filter 0.22µm, and concentrated in a little volume. The extracts were stored at –4℃ until HPLC analysis. Before chromatographic analysis, extracts were completely evaporated under N2, dissolved with 1 mL of methanol–water 1:1 (v/v), and filtered through a 0.22 µm membrane filter[25].

2.5 High Performance Liquid chromatography (HPLC)

The separation occurred on liquid chromatograph Shimadzu 10AV-LC equipped with binary delivery pump model LC-10A Shimadzu,the eluted peaks were monitored by UV-Vis10-SPD .The main compound were separated on fast liquid chromatographic (FLC) Colum , phenomenex C-18 ,3µm particle size (5o×2.0mm I.D) column ,a flow rate of 1.5ml/min was used, and detection was performed at270nm. Mobile phase, linear gradient of,solventA:0.1%formic acid solvent B:was(6:3:1,v/v)of acetonitrile:methanol:0.1%formic acid, gradient program from0%Bto100%Bfor10 minutes .Identification of compounds was a achieved by comparing their spectra and retention times of standards when available, each standard was 25µg/ml. The concentration of phenolic compound calculation as follow:[26]

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

3-Results and discussion

The isolates of *Cryptococcus neoformans* and *Candida albicans* were inoculated in fava bean leaves agar ,all isolates of *C. neoformans* showed dark brown colonies on fava bean agar at 48 hours post –inoculation (figure 1)

,while those of *C.albicans* remained white up to maximum period of incubation (7days) figure 2 , moreover on sunflower seed agar ,as positive control ,all isolates of *C.neoformans* produced a brown pigment (figure3). This is the first study conducted in Iraq and the world, which used fava bean leaves to prepare medium that use for identification of *C.neoformans* .

The human fungal pathogen *C.neoformans* produces melanin in the presence of various substrates of phenolic compounds .The enzyme laccase (phenoloxidase) catalyses the formation of melanin by oxidizing phenolic compounds like L-DOPA ,initiating series of presumably spontaneous reactions that ultimately leads to the polymerization of the pigment in the yeast cell wall [27],[28].

In the clinical laboratories, the ability of *C.neoformans* to produce brown colonies in media containing phenolic compounds ,used for rapid identification of it. Many studies have been performed to formulate a culture medium that is effective for the presumptive isolation and identification of isolates of the *C.neoformans*. Previous studies grown *C. neoformans* in agar media which contain chemical material such as L-dopa (L-3-4-dihydroxyphenlalanin),caffic acid and methyldopa[21],[29],[30].Moreover media prepared from natural resources such as niger seed agar,sunflower seed agar ,mustard seed agar, henna agar and tobacco agar[31].

The analysis of fresh fava bean leaves by high performance liquid chromatography(HPLC)showed that the fava bean leaves content ;gallic acid ,caffeic acid ,chlorgenic acid ,protocatechic acid coumaric acid and quinic acidwith concentrations 45.66µg/ml,29.67µg/ml,46.94µg/ml,62.45µg/ml,57.14µg/ml,39.99µg/ml respectively, moreover the protocalechic acid had the highest content of total phenolics compound while caffeic acid had lowest content of total phenolics compound in fava bean leaves. Previous studies showed that polyphenols in fava bean are located in several parts of the plant ,leaves ,roots and seeds [32],[33].Present high concentration of phenolic compounds in fava bean leaves confirms the possibility of using this medium for identification of *C. neoformans*.

Fig. 1. Dark Brown pigment of *Cryptococcus neoformans* on fava bean leaves agar

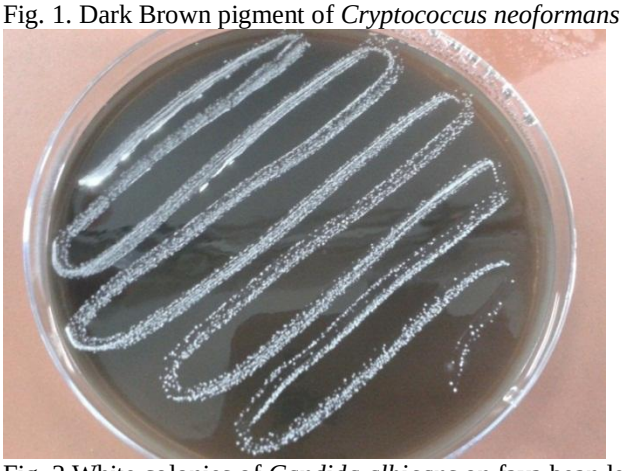

Fig. 2.White colonies of *Candida albicans* on fava bean leaves agar.

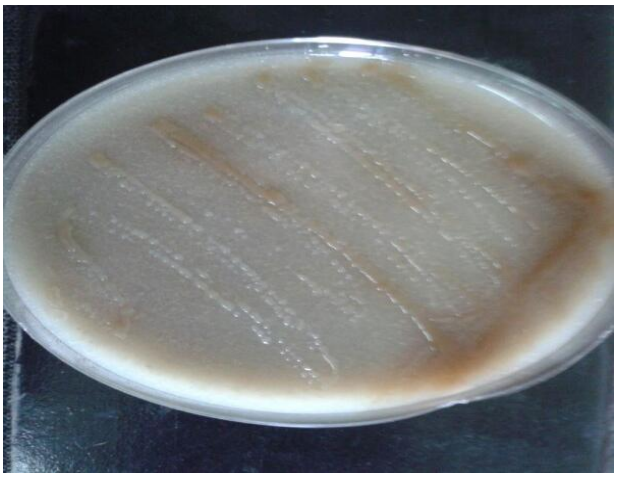

Fig. 3.Brown pigment of *Cryptococcus neoformans* on sunflower seed agar

This study prepared new medium, fava bean agar, composed by a few components ,which were of low cost ,easy to prepare, and able to show production of melanin by *C.neoformans*. Fava bean leaves used for the preparation of a simple medium contain only the leaves of plants and does not need to other compound only agar –agar for hardening of the medium , On the other hand the fava bean leaves is used which do not affect the economy as well as to the possibility of securities save for long periods and use when needed because they do not damage in the storage

Table (1): Concentration of phenolic compounds in fava bean(*Vicia faba*) leaves.

sequences	Compound	Concentration(µg/ml)
1	Gallic acid	45.66
2	Caffeic acid	29.67
3	Chlorgenic acid	46.94
4	Protocatechic acid	62.45
5	Coumaric acid	57.14
6	Quinic acid	39.99

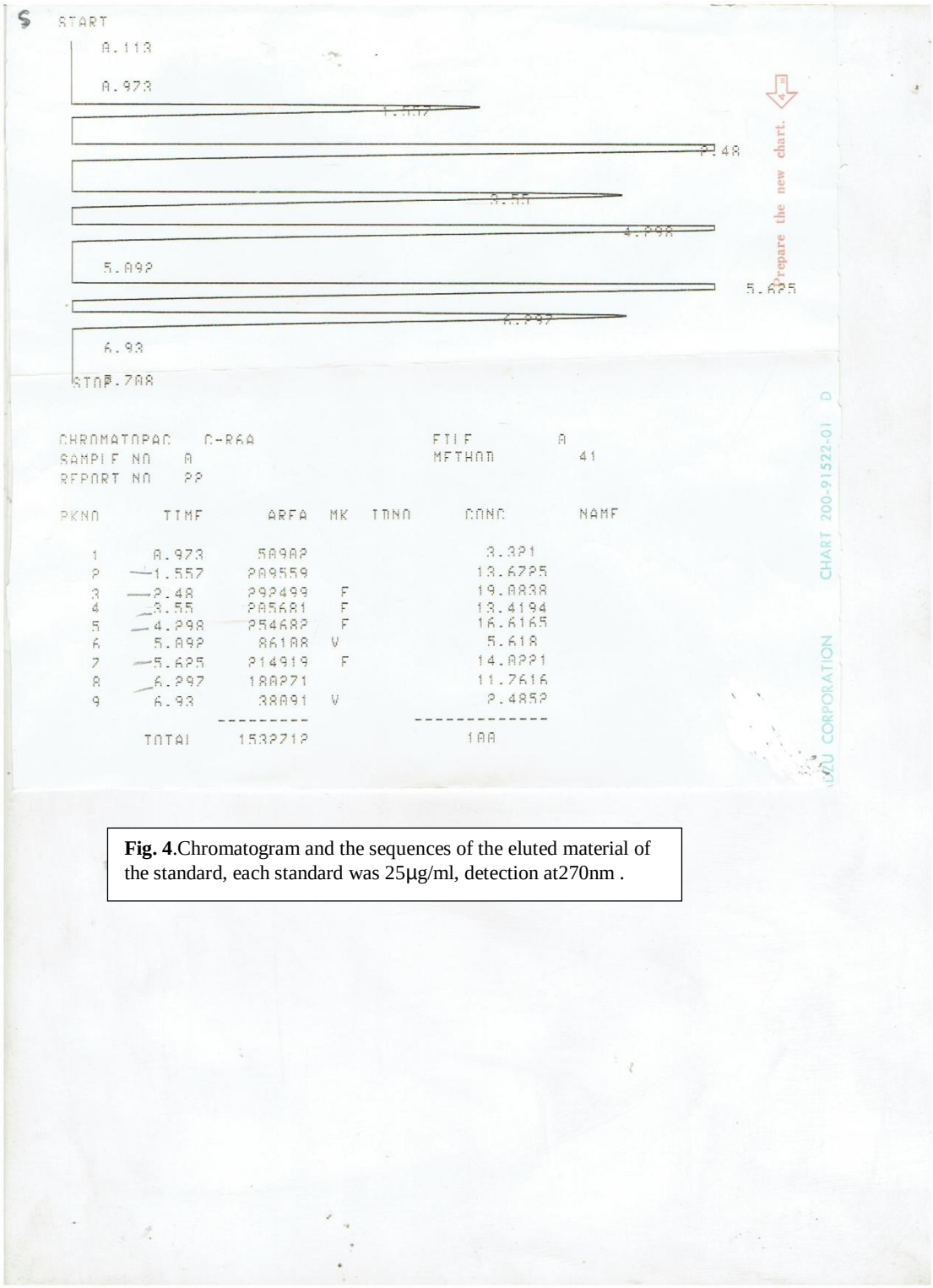

Fig. 4.Chromatogram and the sequences of the eluted material of the standard, each standard was 25µg/ml, detection at270nm .

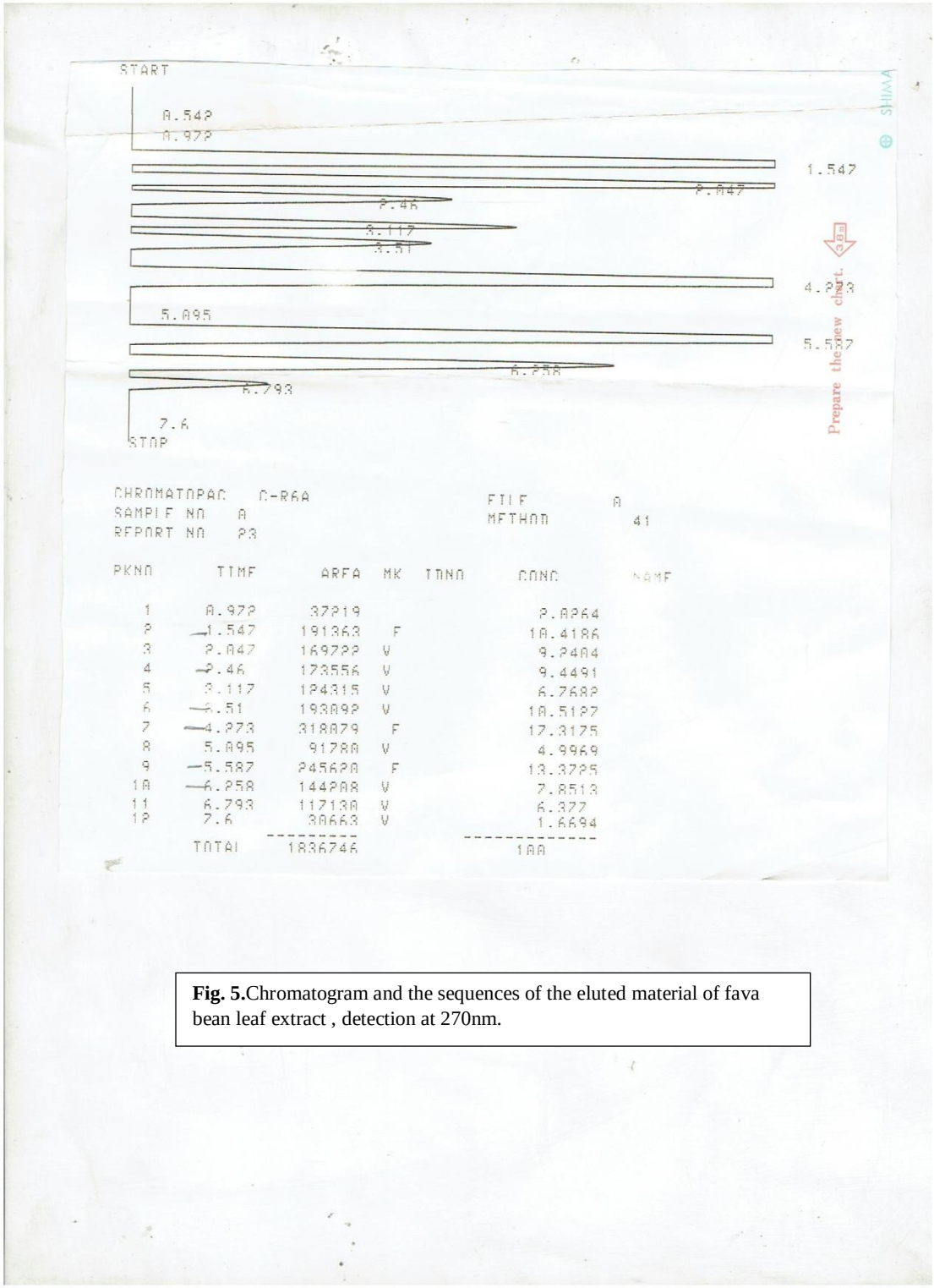

Fig. 5. Chromatogram and the sequences of the eluted material of fava bean leaf extract , detection at 270nm.

4-Conclusion

Fava bean (*Vicia faba*) leaves agar 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 fava bean ,while *Candida albicans* did not produce the reaction product. The analysis of fresh fava bean leaves by high performance liquid chromatography (HPLC) showed that the fava bean leaves content high concentration of phenolic compound that confirms fava bean agar as cuture medium for rapid identification of *C. neoformans.*

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