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Anew Medium for Isolation and Diagnosis of *Cryptococcus neoformans*

A Research

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بسم الله الرحمن الرحيم

نرفع درجات من نشاء وفوق كل ذي علم عليم

صدق الله العلي العظيم

DEDICATION

To our families

To our supervisor prof.

To our professors

ACKNOWLEDGMENTS

We would like to express our gratitude for the god of this universe who kept showing us the light till the end of the tunnel and provided us with the necessary strength to keep working till the very end of the path of this project Thanks also goes for Prof.Dr.Neeran Obied Jasim who has been guiding and supervising us during the project as well as our dean Prof.Dr Basim Irahim for his support. Special thanks for our families the ones without them we would not be where we are, and the cause of our existence to this life and our top supporters in the long life struggle In the end of this acknowledgement no words can express or describe our gratefulness for those who are standing in the frontal lines of battles to protect our homelands , those who are standing in front of the bullets and shields sacrificing their lives to save ours, the words "THANK YOU " are just not enough at all.

Summary:

Melanin produce by *Cryptococcus neoformans* yeast was used to characterize this yeast .In present study ,we used red onion extract in prepared a new medium .The isolates of yeast were cultivation . After 48 hours results show ,the isolates of *C.neoformans* were appear in dark brown colonies while the colonies on SDA not produce any pigment .Also analysis results of red onion extract by used UV-Spectometer and FT-IR appear this extract content phenolic compounds which causes to produce brown pigment .thus , this medium (RO A) useful for rapid identification of yeast *C.neoformans* .

Chapter One
Introduction

1-Introduction:

Cryptococcus neoformans is a fungal pathogen which causes Cryptococcosis .It is opportunistic yeast that mainly infected immunocompromies patients ,its morbidity and mortality yeast (Chen *et.al*.,2008).C.neoformans belongs to the class Basidiomycetes and found in aged pigeon droppings .infections are acquired by inhalation of airborne infectious propgules of basidiospores (Springer &Chaturvedi,2012).The virulence factors of this yeast include :(1)a polysaccharides capsule .(2) ability to grow at 37°c .(3) enzyme phenol oxidase that acts on phenolic substrates to produce melanin pigment (Coelho et.al.,2014).The ability of melanin production is the most criteria for identification of C.neoformans from clinical and environmental samples .Also melanin molecule act as antioxidant agent and production the yeast from host defenses (Heitman *et.al*.,2011) .Melanin production is tested in some agar media which contains a precursors of melanin such as :Niger seed agar ,Sunflower seed agar ,Mustard seed agar and Henna agar (Ruchi Katiyar *et.al*.,2011) ;Fava bean agar (Hamzia ,2015).

Thus , in this study we prepared simple medium from red onion fruit extract to isolation and identification of C.neoformans .and including the

following steps :

1- Preparation of agar based media

2- Test for Phytochemical Analyses (phenolic compound)

3- UV-Vis spectroscopy for detected of phenol

4- FT-IR for detected of phenol

Literature review

Chapter Two

2.1- Cryptococcus neoformans

Cryptococcus neoformans belongs to the basidiomycetes . and grow as like yeast. Its opportunistic yeast that affects immunosuppressed persons (Immunocompromised), which cause high rates of morbidity and mortality and also affects people who are immunosuppressed .(Chen *et al.*, 2008).

The genus Cryptococcus contains more than 100 species classified according to modern classification (Fonseca,*et.al*. 2011). However, the pathogens of humans and animals are very few, most important and most severe pathogenicity is *Cryptococcus neopormans* and *Cryptococcus gattii* as there is two species *C albidus. and C laurentii*. which rarely cause the disease (Baddley and Dismukes, 2003).

C neoformans. yeast is characterized by its spherical cells to oval shape diameter(4-6) mm and is surrounded by a thick capsule made of polysaccharides, ranging in size from 1 to 30 µm.Reproduction by Budding and consists of a single bud narrow base and do not be true hyphae and Germ tube. (Koneman et al., 1992) .

C neoformans. Yeast are characterized by being white colonies into a creamy 'smooth' color mucous on the solid media such as SDA .Also muciod of colony dependent on the size of the capsule. Yeast is grown at a rate lower than other yeast, such as: *Candida albicans and saccharomyces cerevisiae* under the same conditions (Mitchell and Perfect, 1995)

C neoformans. can grow at a temperature of 37 ° C and this is what

distinguishes them from .The other non-pathogenic species of yeast .

Yeast *C neoformans*.used lactose sugar and nitrate .Also its able to used Creatinine as the sole source of nitrogen, which explains their growth on the dropping of birds which is rich by this compound. *C Neoformans*. yeast possesses the ability to produce melanin, urease, starch and Inositol (Kwon-chung and Bennett, 1992)

The modern scientific classification of yeast is as follows (Hibbett *a.l et*, 2007).

Kingdom: Mycetae

Phylum: Basidiomycota

Subphylum: Basidio mycotina

Class: Tremellomycetes

Order: Filobasidiales

Family: Filobasidiaceae

Genus: Filobasidiella (Cryptococcus)

Species: neoformans

C neoformans. yeast contains four serotypes based on the antigenic property.: A, B, C, D (Kwon-chung and Varma, 2006). Serotype A is the most common species, and is responsible for the largest proportion of infections. (Vu *al et*.2013) Yeast has two var.: *C.neoformans* var neoformans include (A, D) and C.neoformans var gattii:. Include (C, B) serotypes (Springer and Chaturvedi, 2010) Cryptococcus contain in addition of *C.gattii and C.neoformans*

98 daignostic speciesses according of classified by modern classification (Fonseca *al et* 2011). Most of these species are you cannot live and survive within the organism because of relatively high body temperature . (Eshar et al., 2010)

C neoformans Yeast is. is widely found in various environmental sources, such as: soil and birds, especially pigeons, which is the main environmental habitat of yeast, and**.**

the genus *Columba livia* as host reservoir for this yeast while *C.gattii*. associated with trees. (Springer and Chaturvedi, 2010).

Studies suggest that yeast can be isolated from other environmental sources such as fruits and vegetables were first isolated from peach juice in 1894 as well as from decomposed wood (Junior *et al*., 2013). Yeast has also been isolated from faeces.other birds, such as Munia birds, canary , nests bads and old nests . (Lazera *et al.*, 1993)

2-2-Virulance factor of *C.neoformans*

C. neoformans. possesses a number of virulence factors that increase the yeast infection ,which includes all the mechanisms that allow the yeast to grow and reproduce within the organism (Cadieux *et al*., 2013; Coelho *et al*., 2014). Generally, risk factors include:

2-2-1-Capsule :

Many studies suggest that the capsule is one of the most important virulence factors of the .(Meara and Alspaugh, 2012; Vecchiarelli *et al.*, 2013) its found at,the cellular wall of the yeast and

sometimes up to twice the size of the yeast.

Studies have shown the size of the capsule increases during the infection stage (Zaragoza *et. al.* 2010. ; Okagaki *et al*., 2010) .

The capsule consists of a complex structure made up of several types of polysaccharides and the most important of these sugars. Glucuronoxylomannan (GXM) which is the predominant sugar in the composition of the capsule of *C neoformans*. It is an antigen that determines the serotypes of yeast.(Heiss *et al*., 2013).

In addition to this sugar, the capsule consists of two other components: Glucuronoxylomannogalactan (GalXM) and Mannoproteins, (MP) which are found in a small amount in the capsule compared to (GXM). (Jesus *et al*., 2010)

GXM accounts for 88% of the components and has a molecular weight of -700-.1000.7 kg). When studying the chemical composition of GXM for all serotypes, it was found.It consists of three components: Glucuronic acid and Xylose and Manose, as it was noted that the differences between these patterns is due to different ratios. (McFadden etal., 2006)

Also was found the GalXM consist from Galactose, Xylose and ,Mannose and forms 10% of the components of the capsule and molecular weight 572 kD (. Zaragoza, *et al*, 2009). MP is a secondary compound of the yeast wall, which acts as a gain Of the iron at the yeast surface which is made up of a polysaccharide coupled with protein and forms a 1-2 % ratio of.capsule Components. (Cadieux *et*

al., 2013)

Several studies have shown that the capsule possesses several additionalsugars such as beta-glucan as well as Chitin, (Cordero *et al*., 2011), which acts as a base for the GXM in the cell wall (Ramos *et al*., 2012)

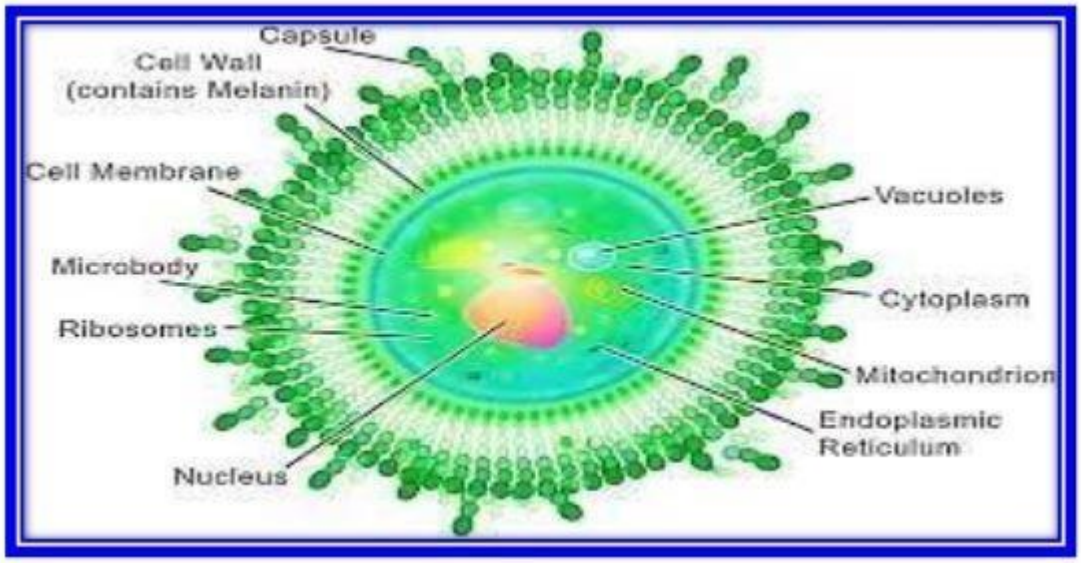


Figure (1): Illustration of the composition of the yeast cell with capsule

(Bose.*al et*., 2003)

.2.2 - Melanin and Laccase :

The presence of melanin is one of the most important factors of virulence characterized by yeast and contribute to the increase yeast Infection in addition of Capsule (Casadevall and Eisenman , 2012).Laccase is the enzyme responsible for the produced of melanin from catecholamines and dysfunction in laccase leading to malfunction in the production of melanin and dysfunction of yeast virulence (Liu. And Nizet ,2009).The melanin molecule acts as a free radical stabilizer, and antioxidant very well .The function is very important for yeast as it works to protect the yeast from the free radicals it produces as such (. Dadachova *et al*., 2008; Heitman *et al*. , 2011)

Yeast have two types of laccase: lac1, which is found frequently in the cell wall and lac2 is found frequently. In cytoplasm, but both are responsible for darkening the cell wall of yeast. (Missall *et al*., 2005).

2.2. 3.- The ability to grow at 37 ° C :

C .neoformans grow well at a temperature of 37 ° C is characteristic of this type from the rest of non-pathogenic species that do not have the capacity to grow in such a manner to be able to live at 37 ° C, the yeast must possess an effective gene containing a unit. Cloning for the production of Calcineurin A . (Brown *et al*., 2007) .

For threonine-serine is a special phosphatase enzyme that stimulates phosphatase .By calcium ions Ca^{+2} has been found that mutant isolates that do not contain calcineurin from yeast can not live at 37 ° C. This is evident from the fact that Calcineurin is a key element in the survival of yeast within the body of the host and then it is a factorVery necessary in yeast infections (Perfect.,2006).

2.2-4-Analysis enzymes:

First: Proteinase:

All isolates of *C .neoformans*. that were isolated

from clinical and environmental samples were found contains proteinase enzyme..(Rodrigues *et al.*, 2003)

Yeast showed ability on the decomposition of host proteins, such as: elastin, collagen, immunoglobulins , fibrinogen. (Chen *et al.*, 1996). Both Casadevall and Tucker (2002) hypothesized that yeast multiplication inside the phagocyte cells is linked to the production of enzymes containing both proteinases and Phospholipases to break down the membrane of the phagocyte cells, so the yeast has the ability to decomposehost tissues are destroyed and used as food, also helps in yeast migration to the nervous system . (Vu *et al.*, 2014).

Second: Phospholipase :

Is the enzyme that analyzes phospholipids by smashing one or more of the esters found in glycerophospholipids are one of its forms Phospholipase B (PLB), which is produced by the yeast during the infection of the host, which works to survive yeast from the phagocyte cells (Cox, *al et* ., 2001), and that the phospholipids are analyzed for the host by (PLB) the membranes of the thrombocytes that enable the yeast to pass will malfunction easily into the components of cytobalum and this plays a key role and directly in the acquisition of nutrients (Chrisman *al et.*

2011) .Recent studies have shown that PLB produced by yeast is easy to use endothelial adhesion to the surface of the endothelial cells of the lung and glial cells of of the brain. (Lev *et al*., 2013)

Third: Urease :

The enzyme urease plays an important role in *C neoformans* yeast. which is a secondary enzyme eliminates the toxicity of urea from the alkaloids found in the medium by hydrolysis of the urea into ammonia and causing a local increase in the pH ratio, making it easier of the process of taking nitrogen (Maruvada. *al et* , 2012) It has been found that the enzyme urease works.to support the yeast traversal of the visible barriers and also helps to sweep the brain(Shi *et .al* , 2010; Singh *et al*., 2013)

Other virulence factors are *C .neoformans*. produce other substances including mannitol, which plays an important role in increasing yeast resistance to heat stress factors and Osmotic stress . (Chaturvedi *et al*., 1996)

. 2. 3.- Clinical forms of infection :

Clinical forms of yeast infection depend heavily on the immune status of the host .The severity of the symptomless infection varies from the formation of an

occasional pulmonary nodule to the spread yeast infection, and human yeast infection, especially those with immune disorders cellular cells, especially cell-T cells that have less than 50 cells / ml, infected persons .Malignant leukemia, chronic diseases, and organ transplants and people. They regularly take corticosteroids, and other diseases that cause immune inhibition are the forms of clinical injury (Yu *et al*., 2012; Harris *et al*., 2011)

.2. 3.1- Pulmonary cryptococcosis :

Lung is the first target member of the yeast as the main entry point for empty yeast (Visnegarwala. *al et* , 1998) Symptoms This is what happens in most people with mild to moderate symptoms including shortness of breath , Cough, nausea, chest pain, night sweats, and sometimes hemoptysis (Chang *et al*., 2006). Acute symptoms of the infection, which include fever and night sweats.Weight loss is less common in people who are not immunologically inhibited unless the infection is widespread and other areas of the body (Chang, *et. al* 2006). When comparing the case of infection in people Immunosuppressants, especially people with AIDS, with people who are not discouraged, find a stage.The onset of symptoms is faster and the mortality

rate is greater than that of people who are not discouraged. (Meyohas, 1995).

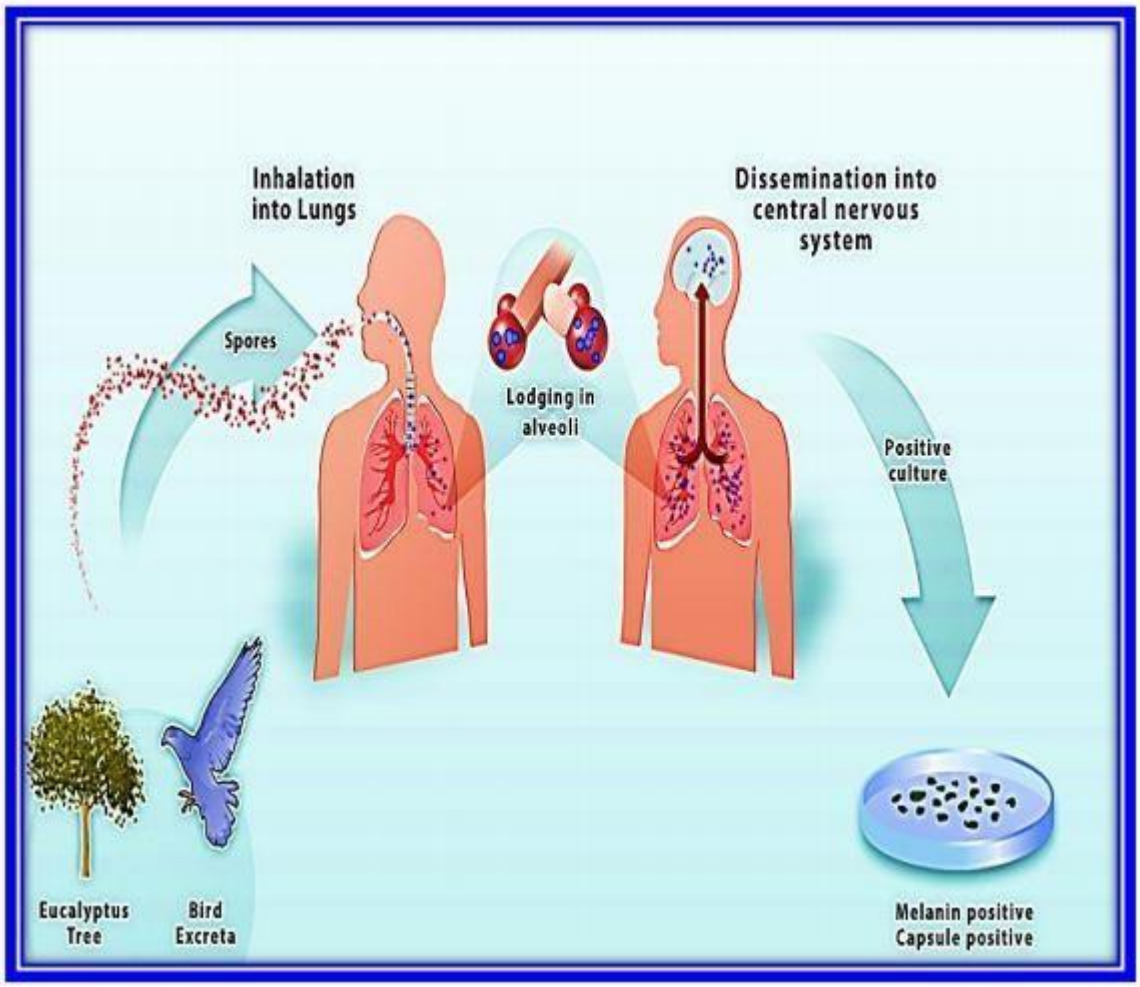


Figure (2): Lung infection and central nervous system (2013, King).

2.3.2. Cryptococcal meningitis :

Encephalitis and yeast infection in the spinal cord fluid is the most common form of infection. The person with the yeast infection ranges from under severe to chronic and often leads to death in the case .Failure to take the appropriate treatment and death after a short

period of injury are in a few weeks or months of the onset of symptoms and this is often associated with the immune status of the casualty (Terada.,2010) Similar Clinical symptoms in people who are inhibited and not inhibited are immune, but in inhibitors they are more Because of the low response and the frequent presence of yeast in these people. These include: headache, vomiting and nausea (, Saag. *et al* 1992)

Nearly 30% of patients suffer from cranial nerve weakness resulting from increased pressure. Inside the cranium, invasion of the yeast to the cranium and injury to brain tissue. It is the most important symptom that indicates.To the cranial nerve infection is a lack of visual acuity and double vision and sometimes causes loss of sight, Hearing loss, facial weakness, water pooling in the head (Hydrocephalus) and nerve attacks Occur in the final stage of infection. (McHugh and Makadzange 2014) Intracranial pressure is due to the presence of yeast, which works to block the flow of fluid in the spinal cord and the accumulation of polysaccharides made up of capsules Yeast in the web works to alter the fluid flow of the spinal cord (Loyse , *et , al..*, 2010).

3.2. 3.- Ocular infection:

Eye infection occurs by entering minutes of dust contaminated with yeast cells into the eye which sometimes leads to blindness (Rippon, 1988). Almost 45% of people Meningitis patients suffer from eye diseases and many cases of meningitis have been identified after the appearance of eye diseases (Crump al et, 1992.)

Visual symptoms include double vision (Diplopia), visual atrophy (Opticatrophy), Chorioretinitis, Scotoma.(Bennett and Chung-Kwon, 1992). About20% of people who are not immunosuppressed suffer from eye infection , and loss of vision may occur within a few hours, weeks or months Due to optic nerve inflammation, or perhaps due to increased intracranial pressure . (Graybill *et al.*, 2000).

2.3.4. Cutaneous Cryptococcosis :

Skin infection is very low in people who are inhibited and not immunologically inhibited and about15% % Ofpeoplewith AIDSsufferfromskininjuries(Perfect&Mitchell 1995)

The clinical symptoms of the skin infection are in various forms such as sores, pimples, pills and inflammation Cell tissue and superficial tumors (Dismukes and Baddley, 2003) Myheartinpeople

with AIDS is evidence of the yeast's dominant form in people Non-depressant skin injury is the only site of yeast infection that results from contact Person directly with the bathroom (Sampaio**.** ***et.*** ***al.***, 1999)

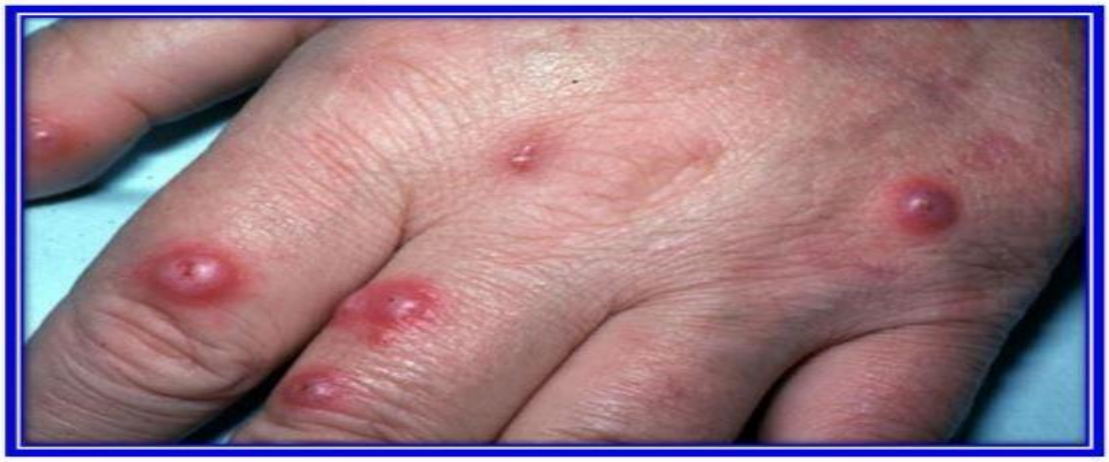


Figure 3: Skin infection (King.,2013)

2-3-5. Osteoarticular infection:

Sickle skeletal infection occurs less than 10% in people with meningitis (2001, King). Bone infection is observed by swelling of soft tissue and soft bones It is broken and is characterized by osteoporosis. Yeast affects the bones of the skull and joints Pelvic joints and joints. The infection of the vertebrae to the yeast may lead to pressure the vertebrae on spinal cord . (Ramkillawan *et al*., 2013; Zhou *et al*., 2013) . Yeast also affects many other organs of the body as yeast passes from the lung as it is The first target targeted by the yeast through blood and lymph

vessels to reach the organs The other body, such as the heart, leads to myocarditis, which causes inflammation Kidney and ovulation (Pyelonephritis, a prostate that acts as a storehouse for yeast making it Is responsible for the relapse of AIDS patients (King ,2001), as well as, liver, and spleen Adrenal, thyroid, and esophagus (Chayakulkeeree and Perfect , 2006).

Chapter Three
Material & Methods

3--Materials and Methods

3-1-Equipments & Chemical materials:

Table (3-1): Equipment's & Chemical materials used in the laboratory experiments

No.	Equipment	Company	source
1	Incubator	Gallenkamp	(England)
2	Sensitive balance	Gallenkamp	(England)
3	autoclave	Gallenkamp	(England)
4	Hot plate with magnetic stirrer	Gallenkamp	(England)
5	Vortex	Electrothermal	(England)
6	UV Spectrophotometer	Memmert	germany
6	Whitman(No.1)filter paper	Gallenkamp	(England)
7	Agar	Gallenkamp	(England)
8	Red onion friut	Local market	
9	FT-IR	Memmert	germany
11	Sabuobaud's Dextrose Agar	BDH	(England)

3-2-Methods:

3-2-1- Isolate

Cryptococcus neoformans strain was obtained from laboratory of microbiology of science college /university of AL-Qadisiyah This strain was maintained on slants of Sabouraud's Dextrose Agar, and preserved in 10% glycerol and was subcultured on regular intervals so as to maintain fresh cultures.

3-2-2- **Plant Materials**:

Fruit of red onion was utilized for preparation of plant agar medium.

3-2-3- **Preparation of agar based media**:

For the preparation of this media, A medium-sized fruit of red onions has been crushed and the juice produced has been collected in a flask. The extract was then filtered through muslin cloth for coarse residue and finally filtered through Whatman No.1. The final volume adjusted to 100ml. Agar-Agar powder (3g) was then added to it as solidifying agent and sterilized by autoclaving The media (15-20ml) was poured into sterile Petri dishes to a thickness of 4mm.

3-2-4- **Prepration of Sabouraud dextrose Agar (SDA):**

Culture media were prepared according to the instructions of the manufacture companies, the culture media were sterilized by autoclave at 121C under pressure 15 pound /cubic inch for 20 minutes .

3-2-5- **inoculation of *C. neoformans* on plant agar**:

Inoculum of *Cryptococcus neoformans* was separately and spread with sterile L- shaped spreader. The plates were incubated at 30^{o}C for 72 hours. The growth of the fungal cells and characteristics of fungal colonies developed in the plates were recorded .

3-2-6- **Test for Phenols**

One ml of plant extract was added to 5 ml distilled water in a test tube. A few drops of neutral ferric chloride were then added to it. The development of dark green color was suggestive of presence of phenols.(Adetuyi &, Popoola 2001)

3-2-7- FTIR analysis:

dry surface of the extract was analyzed by Fourier Transform infrared spectroscopy (FTIR) .The infrared radiation is propagated through the Sample to obtain the corresponding spectrum, which was averaged from several data acquisitions. FTIR spectra were acquired in the wavenumber range of 400–4000 cm-1.

3-2-8-UV-Vis spectroscopy :

analyzing the UV-Vis specter using spectrophotometer at room temperature

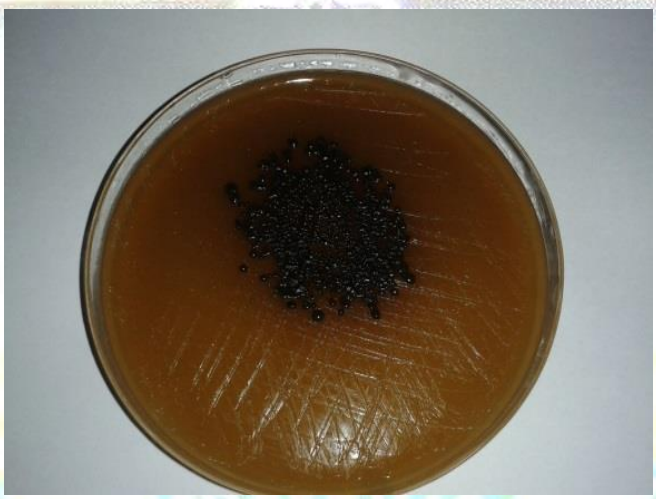
Chapter Four

Results & Discussion

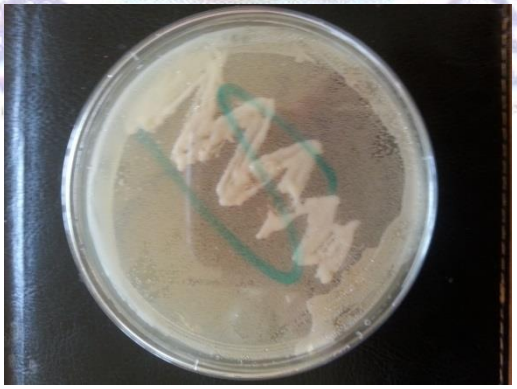
4- Results &Discussion:

4-1- The results of growth on the red onion medium :

Figure (1) appear dark brown colonies of *C.neoformans* , Due to its possession of phenoloxidase, which oxidizes the phenolic compounds found in the plant medium, depositing the melanin pigment on the yeast wall, giving them colony-like colonies distinct from other species of yeasts . These results are accordant with (Katiyar *et al* ., 2011; Minhas *et al* .,2013 ; Ajah , 2015) In terms of their use of a medium containing phenolic compounds.While Fig.(2) appear white circular colonies into a creamy, smooth muciod.



Fig(1) Dark brown colonies of *C. neoformans* on red onion agar



Fig(2) White colonies of *C. neoformans* on SDA agar

4-2- Test for Phenols:

Compounds with phenol group will form green, or red and brown on the addition of ferric chloride solution . This interaction can be used as a test for group phenols (Lecture Demonstration Manual General Chemistry/University of Colorado Boulder,1992) **.Fig.**(3) show that convert white color of red onion extract to green color . This is evidence that red onions contain phenolic compounds .

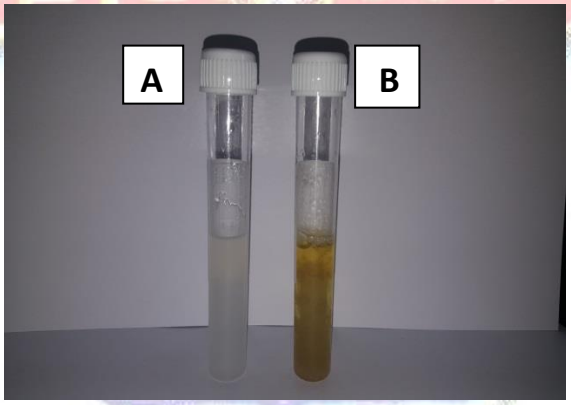


Fig.(3) Changes in color during phenol oxidation by ferric chloride .A:red onion extract ,B:red onion extract after add ferric chloride

4-3- FTIR analysis:

Fig (4) show FT-IR of red onion extract . broad band at 3250 cm-1 belongs of phenolic hydroxyl group(-OH). whereas the appearance of two bands at 2768 cm-1and 2887 cm-1 stretching vibration of aromatic (C-H) group. Also band between 1000-1500cm-1representedC=Cgroup .

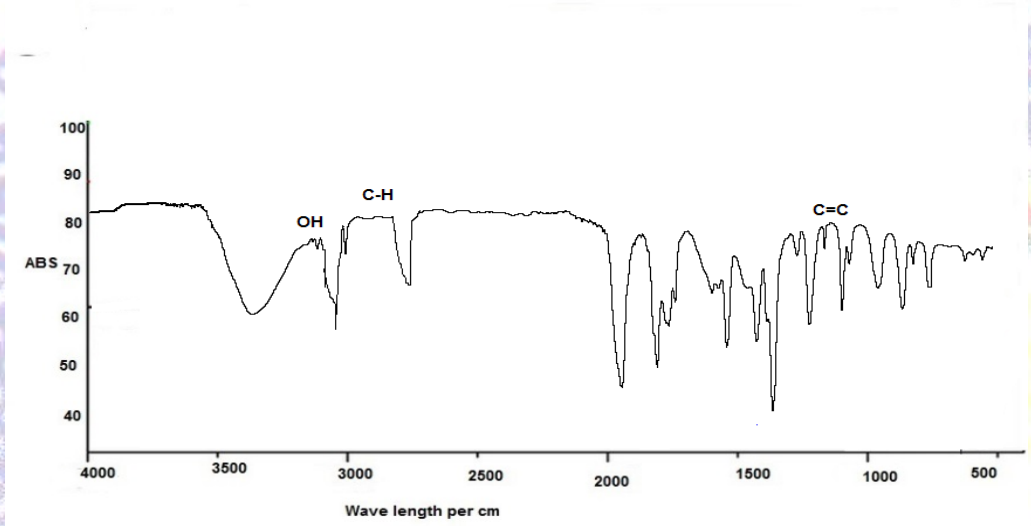


Fig.(4) FTIR spectra of red onion extract

4-4-UV-Vis spectroscopy:

Fig (5) show UV-Vis spectra of red onion extract ,the peak at 260 nm belongs of phenolic compound according of Richard Koplík in advanced strategies in food analysis which reported that λ max of phenol 211-270 nm.

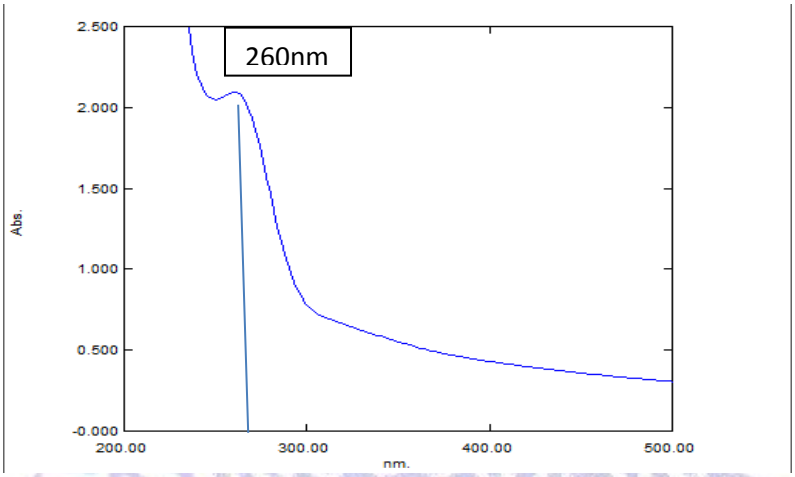


Fig.(5) UV-vis spectra of red onion extract

These results emphasis that red onion extract have phenolic compounds and this compounds used by the yeast therefore its colony appear as a dark brown color .thus the medium that prepare by using red onion extract was useful to identification of *C.neoformans* yeast and this medium was prepare at first time in this study

Conclusion & Recommendation

Conclusions :

1- red onion agar prepared for the rapid identification of *C. neoformans* based on brown pigment produced by the yeast phenol oxidase activity.

2- The primary analysis of red onion extract by ferric chloride showed that the red onion contain phenolic compound

3-UV spectrometer and FT-IR emphasis the primary analysis that red onion extract content of phenolic compound that confirms red onion agar as culture medium for rapid identification of *C. neoformans*

Recommendations:

1- The possibility of using the medium of the red onion Agar as a diagnostic medium for yeast *C.neoformans* .

2- FTIR technique could be used to quantify total phenols using multivariate analysis.

3- Analysis of red onion by other tool such as high performance liquid chromatography (HPLC) to determinate the type and concentration of phenolic compounds that it content .

4- Study other plant extract that contain phenolic compound for prepare such medium which were of low cost ,easy to prepare.

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الخلاصة :

انتاج صبغة الميلانين من قبل الخميرة الخبيئة *C.neoformans* تستخدم لتوصيف هذه الخميرة .في الدراسة الحالية استخدمنا مستخلص البصل الأحمر في تحضير وسط جديد. زرعت عزلات الخميرة على الوسط المحضر و بعد ٤٨ ساعة اظهرت النتائج ان عزلات *C.neoformans* كانت تظهر في مستعمرات بنية داكنة بينما المستعمرات على وسط السا بروبد دكستروز اكار لم تنتج اي صبغة. كما اظهرت نتائج تحليل استخلاص البصل الاحمر باستخدام الأشعة فوق البنفسجية الطيفية و FT IR احتواء المستخلص على المركبات الفينولية مما يؤدي إلى إنتاج الصباغ البني . لذلك ،يعتبر الوسط المحضر وسيلة مفيدة لتعرف والتشخيص السريع على الخميرة *C.neoformans*.

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كلية الصيدلة

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بـــــــحث مقدم
الى مجلس كلية الصيدلة /جامعة القادسية
وهو من متطلبات نيل شهادة بكالوريوس صيدلة
من قبل

اياد علي
و
آيات محسن
بأشراف
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١٤٣٩ه ٢٠١٨ م