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ISOLATION, IDENTIFICATION AND TOXIN PRODUCTION FROM VAGINAL CLINICAL ISOLATES OF *CANDIDA ALBICANS*

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Abstract
Thirty two isolates of *candida albican* were sequestered from female patients with vaginal ulcer (lying in marjan hospital hillia city) .The vaginal swap were cultured on sabouraud dextrose broth and agar plate espectively . for differential identification of *C.albican* ,Hi chrome candida agar was used . Gram staining used to smear of vaginal scrapings . suspected isolates of *C.albican* then immunized on corn meal agar mediocre for chlamydospore, formation , for germ tube formation the isolates of *C.albican* was cultured in human serum .Carbohydrate assimilation and fe rmentation tests also used for further identification . four identification s trains of *C.albican* namely (CA1, CA2, CA3, CA4) were selected according to their antagonistic activity against standard strain of bacteria *E.coli* NCTC5933 and *staphylococcus aureus* NCTC6571 ; To produced extracellular toxin of glycoprotein nature in solid media and fermentation liquid media . The toxin which produced (secondary metabolites) don't effecting the producers strains of *C.albican* (CA1,CA2,CA3,CA4) ,but the produced toxin was inhibited the growth of standard strain of *candida albicans* ATcc8201(sensitive). Simple method used for toxin extraction by using ethanol 90%.And the extracted toxin from free cells supernatant . cytotoxicity of toxin against human blood cells were 6 ppm . also the stability of producing toxin was determined .
Key word *:candida albicans* , germ tube ,conventional technigues ,mycotoxin, vaginal swaps.

Introduction

It is an unscrupulous pathogen of fungi type that occurs as an innocuous commensal in the genitourinary and, gastrointestinal in about (70%) of humans and nearby (75%) of female suffering from *Candida* taint at least once in their generation [1]. However, it develops resourceful pathogen for immunocompromised, for some persons with weak immunologically, or unfluctuating for healthy persons.

The impurity triggered by *Candida albicans* is frequently recognized as candidiasis, it could be considered into 2 categories conditional upon Strictness of the disease. In the earliest category are the mucosal toxicities and the best known among these mucosal impurities is thrush which is characterized by (pale white, spots) in the infected membranes. These contaminations usually affect epithelial cells of GIT, vaginal, or mucosa of oropharyngeal . Furthermore, Volvo Vaginal Candidiasis less common among female , and approximately of them participations repeated occurrences of this infection, which is known as recurrent Vulvo Vaginal Candidiasis. However, it causes

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life-threatening, systemic infections to severely ill patients in whom humanity rate is about (30%) [2]. Total Candida impurities are shared to immunocompromised individuals, counting some of other infection such as HIV-infected patients, transplant recipients, chemotherapy patients, and low-birth weight infants [3].

Epidemiology, and Etiology and of candidiasis:

Occurrence of candidemia produced by strains that are unaffected to drug fluconazole is high (Candida spp.), responsible for long-lasting toxicities in the ICU inpatients and in hospitalized children, significant to significant over health-care [4].

C. albicans is an obviously happening being in the GIT of the human body, Candidiasis occurs ubiquitously [10].

***Candida. albicans* commensalism and virulence factors**:

The objective of the present study was attentive on the isolation and manufacture of secondary metabolites (mycotoxin) from clinical isolates of *C.albican* isolated from women suffering from candiasis (vaginal the samples from Marjan hospital hillia city).

Hyphal cells and Yeast are the two most major morphological appointments of (*Candid albicans)* that disorder its virulence [5,6].

Virulence influences of *Candida albicans* comprised morphological evolution amongst yeast and hyphal types of cell surface advent of adhesions, and invasions; biofilm formation; thigmotropism; phenotypic switching and hydrolytic enzyme secretion .There are many phase-specific -genes and hyphaspecific genes express proteins by directly or indirectly drive pathogenesis and virulence of *Candid albicans* [7].

Candid albicans and its β-glucans of cell wall are capable to rouse Wbc monocyte reprogramming as one of the main immunological responses in the hosts [8]. The active pathogen-associated , pattern molecules masking by a *Candidias* can concession recognition of the yeast by the immune system [9].

Material and method

Samples : thirty two isolates of *candida albicans* were isolated from women clinically identified as a case of vaginal ulcer (candidiasis) .

Collection of vaginal samples . from patients ,by using sterile cotton swaps gently smeared on the ulcer region in the vaginal region then immediately . Swab cultured on sabourad dextrose broth (PH 5.6), with dextrose , peptone one percent in D.W. , chloramphenicol was added (5mg) . then incubated at 37 C for 24-48 h rs. also we used vaginal scraping for culturing for this purpose a heat sterilized platinum spatula , the scrab samples was cultured on the sabouraud dextrose agar plate .

Empathy of candida -Hichrome candida agar (pH 6.5) ,)Liofilchem) was used for candida empathy the sample swabs , and scraps were straight cultured on (Hichrome agar)) Liofilchem(, and raised at (37C)for (48)hrs.

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Gram staining

Smear of cotton swaps and vaginal scrabs were tested by using Gram stain technic the examination and observation of shape of candida below lens (100X) of type microscope called bright field (11)

Clamydospore Formation

The isolated colony of *candida* were cultured on media of corn meal with (PH 7) (**Mosca** *et al*,(2003) .

Germ tube formation

The suspected candida were cultured in to test tube containing about half ml of human serum then incubated at (37C) for 2 hrs .(13)

After incubation period a full of loop from culture was put on slide and covered to get examination for formation germination .(13)

Carbohydrate utilization

For identification of Candida to the species level .we used carbohydrate utilization test by using Carbohydrate as a foundation of C in presence of O_2 .for his test we used dextrose ,maltose, melibiose ,cellobiose, sucrose, lactose, galactose inositol, xylose, raffinose, trehalose, the test done according .(14)

Auxanographic carbohydrate assimilation method for large scale yeast identification. (14).

Toxin production

Organisms:

Four *Candida albican* isolates isolated in this study used for toxin production the selective of the isolates according to their antagonistic .Activity against standard bacteria *E.coli* (NCTC 5933) and *S.aur*. (NCTC6571).These four isolates of *C.albican* were isolated from female patient clinically suspected as a case of vaginal candidiasis (15). The cultures of the producer were maintained on sabauroud's dextrose agar medium for 2days.

Fermentation medium for toxin production (Sabauroud 's dextrose broth) was used . Disc of 3MM of the *C.albican* strains were inoculated in the 100 ml of medium containing peptone 1% ,dextrose 2% (PH6) in 500 ml conical flask shake cultured for 24 hrs at 37C˚ .then 3 ml of shaked cultured were seeded in shaking flask containing 120 ml medium of glucose 2% peptone 0.1% malt extract 2%(PH 6) culture were shaked for 3days at 30C (16).

Test organisms:

For this study we used typical pathogenic bacteria

(S.aureus NCTC6571)

(E.coli NCTC 5933)

(Candidia albican ATCC 8201)

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The standard bacteria were maintained in well stopper vials containing nutrient agar (Difco) for *C.albican* in sabaroud's dextrose agar(Himedia).

Assay methods:

Primary screening for toxin production was assayed by agar –cup method (17) against test bacteria the titer determined by measuring the diameter of inhibition zone around the cup.

Secondary screening :

By measuring the potency of fermentation cultures filtrate also by agar cup assay against test organisms (16).

Extraction and purification of mycotoxins

Isolation : the supernatant of (100ml) of fermentation culture was excreted with -1 100ml 90% ethanol. The ethanolic extraction was evaporating and contracted by rotary evaporate at room tempture . the samples dryness stored overnight in a refrigeration , the result compound was seprated in crystalline form recrystallized from 90% ethanol . the yield about 0.1mg of white crystalline substance. Them this active extract checked by agar cup method against test bacteria (18).

Toxin purification -2

This layer chromatography (Tcc) technique used to purified ethanolic extract as -3 follows. Layer of ethanol extract across origin of silica gel plates 5*20cm (0.2mm) .developed with taoluens –ethyl acetate 90% from acid (TEF)(5:4:1).

Then cut into equal parts . silica gel removed from the bands and put into Pasteur pipette columns which contain a glass wool plug retain the silica gel . the elute the column with the same extraction (90% ethanol) collect the eluent then assayed for the activity against test bacteria . the active fractions were collected and concentrated stored overnight in refrigerator .(18)

Chemical identification of toxin

Carbohydrate test (molisch test) to one ml of extracted toxin added 3-5 drops of -1 α –naphtnol mixed well ,then 3ml of concentration H_2SO_4 were added through the sides of test tube purple ring appear at the junction of two liquids the appearance of his ring indicates the presence of carbohydrate in the produced toxin .(19)

Ninhydrine reaction -2

One ml of 0.2% ninhydrine solution was added to one ml of extracted toxin in a test tube , then boiled for 2min . purple color appeared which indicate the presence of amino acids.(19)

Mycotoxin stability

Ethanol extract of toxin was adjusted to pH 2.0,7.0, 9.5 , kept at 30C and 60C in seald, test tube . after aperiod of time (specified hurs) the liquid of each solution was adjusted to PH 7. And then measured the activity against standard bacteria .(20)

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Determination of cytotoxicity :

We used human blood by making a suspension of 1ml blood in 20ml physiological saline .a solution of extracted toxin in DMSO with varying concentration (100ml each) was added to 2ml of blood interruption ,the turbidity was read after 10,30 and 60min .

(21)

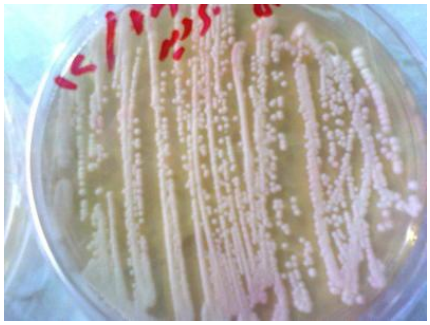
Effect of produce toxin on the producers strains

The activity of the produce toxin (ethanolic extract) was measured against the producers strain (CA1,CA2 ,CA3 ,CA4) As follows . the toxin was spotted on a TLC .plate (5*20cm 0.2mm) and developed by (TEF5:4:1) the plate then marked for individual spots . the four producers isolate (CA1,CA2,CA3,CA4) and standard strain (*C.albicans* ATCC8205) were suspended in 10ml of saline50ml of the culture suspension which were mixed with sabaurouds dextrose agar medium (SDA). Held at 45C˚ and then poured uniformly over the developed TLC (thin layer chromatography), allowed to test . the plate incubated at 35C˚ in moist environment chamber for 24-72 Hrs. the diameter of inhibition zone was determined and recorded for the respective spots of the plate .(22)

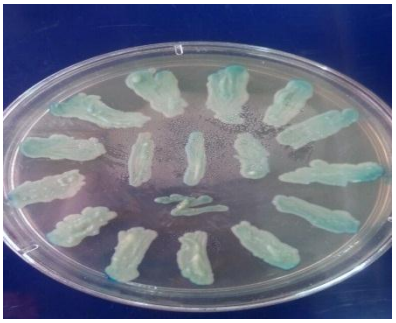
RESULTS

Samples :

32 isolates of *Candida albicans* was isolates of from women with vaginal ulcer . The isolates grow on the (SDA) appeared as white to creamy colonies smooth , butyrous texture and convex this characters with agreement with kurtzman and Fell (1998) (23), the gram stained smear of isolates performed as budding mold cells and pseudomycelium was current in most cases ,on Hichrome *candida* agar, the insulates formed glistening with green color colonies the straining of *C.albicans* . Prolonged from yeast cells.



The culture on the sabouraud s dextrose agar plate (Fig1



The culture on Hichrome agar

(Fig 2)

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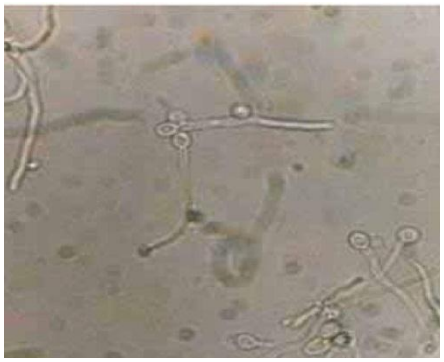
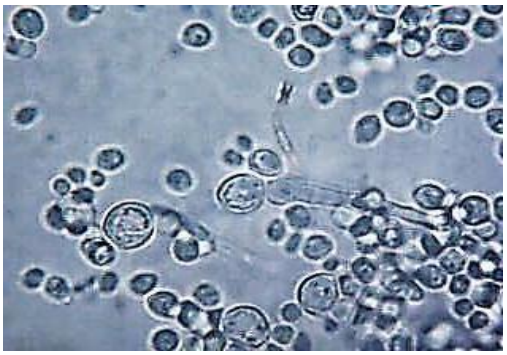


The formation of germ tube

Fig(3)



Clamydospore Formation

Fig(4)

The carbs adjustment trials for (*C.albicans*) identification are showed in table (1) . The test was positive for dextrose , maltose ,sucrose , galactose , xylose and trehalose and the test showed adverse result for lactase, melibiose, cellobiose, inositol , raffinose and dulcitol . the carbohydrate fermentation tests for isolated stains of *C.albicans* are showed in table (2). The results in table (2) showed that the fermentation of different types of carbs.

Table (1) carbohydrate utilization tests for *C.albicans*

Isolates	Substance	Result
CA1-CA32	dextrose	+
	maltose	+
	Sucrose	+
	Lactose	-
	Galactose	+
	Melibiose	-
	Cellobiose	-
	Inositol	-
	Xylose	+
	raffinose	-
	Trehalose	+
	Dulcitol	-

Not: CA1-CA4 isolates of *candida*

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Table (2) carbs utilization test for *C.albicans*

Isolates	Substance	Result
CA1-CA32	Dextrose	+
	Maltose	+
	Sucrose	-
	Lactose	-
	Galactose	+
	Trehalose	+

Primary screening of toxin

Four isolates of isolated *C.albicans* were given symbol (CA1, CA2, C3, CA4) were selected for toxin production the selection according to their antagonism activity against standard gram negative and gram positive bacteria .the identification tests showed no significant difference was recorded among the isolates. All of 4 isolates produce toxin which showed antimicrobial activity against standard bacteria .

Table (3) effect of isolated *C.albicans* (CA1,CA2,CA3,CA4) against standard bacteria in soild media .

distance of inhibition zone (mm)		
Strains	*S.aureus* NCTC 6571	*E.coli* NCTC 5933
Ca1	28	20
Ca2	28.5	20
Ca3	28	20
Ca4	28.5	20

Also the activity of toxin produced by four isolated was determined after 3days incubation in fermented medium . the results showed that the activity of toxin against standard bacteria in fermentation medium gave optimum value in comparison with solid medium.

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Table (4) the antimicrobial activity of fermentation medium of four isolates of C.albicans against standard bacteria

	Diameter of inhibition zone (mm)	
Strain	*S.aureus*	*E.coli*
CA1	<30	21
CA2	<30	25
CA3	<30	21
CA4	<30	22.5

Toxin production

The toxin is extracted from supernatant of fermentation medium dy using 90% ethanol, the crude extract was analyzed by (TLC) Plate .the RF value of extracted compound (respective spots) is 0.17. further more the biological activity of the active spots were assayed by determination of the diameter of inhibition against standard bacteria the result of this tests indicate that the respective spots are very active with diameter of inhibition zone range from 20-28mm beside both gram positive and negative bacteria (table 3).

Stability

The results of stability experiment showed that the titer of toxin is still active in acidic solution and neutral solution but showed unstable activity in alkaline solution and remained slightly active (table 5).

Table (6) showed that the toxin producers (CA1, CA2, CA3, CA4). did not effected by toxin produced by them in comparison with standard strain *C.albicans* ATcc8205 which was very sensitive to the produced toxin.

Cytotoxicity

The cytotoxicity of produced toxin against RBC cells was showed in table (7) .The results showed that the produced toxin was highly toxin compound because it induced lysis of RBC at 4.5 PPM.

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Table (5) stability of produce toxin by four isolates of *C.albicans*

Temp. C°	PH	Activity remaining %		
		0hrs	3hr	5hr
30	2.0	100	100	100
	7	100	100	100
	9.5	90	60	20
60	2.0	100	100	100
	7	100	100	100
	9.5	90	60	20

Table (6) effect of toxin on producer strains in comparison with standard *C.albicans* A TCC8205

Strains	Diameter of inhibition zone (mm)
CA1	Good growth
CA2	Good growth
CA3	Good growth
CA4	Good growth
C.albicans ATCC8205	15-19.5

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Table (7) cytotoxicity of produced toxin against human RBC

Compound	Conc.(pmm)	RBCtoxicity at hr.
DMSO	-	NT
Etracted toxin	1	NT
	1.5	NT
	2	NT
	3	NT
	3.5	NT
	4	NT
	4.5	T
	5	T

NT: not toxic

T : toxic

Discussion

Clinical infection by certain species of pathogenic candida species . such as *candida albicans* . suggest the possibility to produce many secondary metabolites (toxin)in patient tissue .(24)

This study was focused on the isolation and production of toxin from clinical isolates of *C.albicans* isolated from woman with vaginal candidiasis . the isolates of *C.albicans* used in this study were identified by various conventional techniques .some of these techniques done prior isolation . these technique are based on morphological and physical characteristic (11). *Candida albicans* produce germ tubes (17).

About ninety percent of quarantined from clinical specimens *Candida albicans* have germ tube Fig (3) .

Also chlamydo spore formation by protecting candida from primary ethos on corn meal agar .Chlamydospore (Fig 4) one of the important characters for *Candida* identification (21).

In the present study we used CHROM agar (Fig 2) as discriminating and discrepancy media for separation and identification of (*Candida* spp.) This media encompasses chromogenic material that can react with specific enzyme secreted by yeast resulting in developing special colored colonies (11) .

Many carbohydrate assimilation technique used (table 1, 2) for candida identification (14). The isolates to be identified was growth in asset of defined liquid media containing indication supplemented with different sugars a change in color indicator used as to indicate the assimilation of sugar test (14) .

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The result in table (3) and table (4) showed that the toxin (secondary metabolites) produced by clinical isolates of *C.albicans* (CA1, CA2, CA3, CA4) in solid and fermentation media wata *et al* have isolated and characterized twice sessions of great mw of Candida toxins known as Canditoxin and glycoprotein toxins, as well by way of low molecular weight toxin (25).

The ninhydrine reaction and molish test give positive results which indicating that the produced toxin active component of chemical glycol protein structure (25). The toxin were obtained from supernatant of fermentation cultures of (CA1, CA2, CA3, CA4) isolates ,the toxin fractions were apparently glycol proteins as proved by positive reaction of ninhydrine and molish tests .(25)

The toxins produced by (CA1, CA2, CA3, CA4) are extracellular toxin which inhibited the growth or the standard strain of *C.albicans* (ATCC8205) but not the producer strains (table 6).(22)

Kandel and stern (26) showed that the clinical strains of *Candida* could produce or be sensitive to killer toxins .

Further chemical and biological studies must be done on the chemical structure of shaped toxins to characterize its chemical and biological structure also to determination its activity in vivo against human health.

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