A study of the inhibitory effect of the Ethanolic extract of *Cyperus rotundus* , *Eugenia caryophyllus* and *Coriandrum sativum* on the *in vitro* growth of *Candida albicans* on a Sabouraud Dextrose Agar

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Abstract:

The present study was designed to evaluate the inhibitory effects of three local plant ethanolic extracts (*Cyperus rotundus* , *Eugenia caryophyllus* and *Cariandrum sativum*) against the growth of *Candida albicans* in culture media by using of agar well diffusion method. For this purpose graduate concentrates for each extract (25 , 50 , 100 , 150 , 200 , 400)mg/ml prepared and tested .The result showed that the extract of *Cyperus rotundus* was more effective than *Eugenia caryophyllus* ,while *Coriandrum sativum* has no effect in all concentrates on *Candida albicans.*

The statistical analysis by using ANOVA with LSD at level ($p<0.05$) showed that the concentrations 200 , 400 mg/ml for *Cyperus rotundus* and 200 mg/ml for *Eugenia caryophyllus* were a significantly preeminence on the effect of other concentrations and from the effect of Nystatin and Clotrimazole .

دراسة تأثير المستخلص الأيثانولي لنباتات السعد والقرنفل والكزبرة في تثبيط نمو فطريات المبيضات المنماة على وسط أكار السابرويد دكستروز

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الخلاصة:

صممت هذه الدراسة لتقييم الفعل المثبط للنمو للمستخلص الكحولي الأيثانولي لثلاثة من النباتات الطبية المحلية (السعد،القرنفل والكزبرة) ضد خميرة المبيضات البيضاء المنماة في الوسط الزرعي وبطريقة ألانتشار في الأكار.ولهذا الغرض تم تحضير سلسلة من التخافيف لكل مستخلص (25، 50، 100، 150، 200، 400) ملغم/ مل والتي تم اختيارها بناءا على التجارب المختبرية. أظهرت نتائج الدراسة أن المستخلص الأيثانولي لنبات السعد كان هو الأكثر فاعلية مقارنة بنبات القرنفل في تثبيط نمو خميرة المبيضات في الأطباق الزرعية بينما لم يظهر الكزبرة أي تأثير مثبط للمبيضات وبمختلف تراكيزه المستخدمة قيد الدراسة . تم إجراء التحليل الإحصائي باستخدام اختبار تحليل التباين مع اقل فرق معنوي تحت مستوى احتمالية (0،05) حيث أظهرت النتائج ان تراكيز (400,200) ملغم/ مل لنبات السعد وتركيز (200) ملغم/ مل لنبات القرنفل كانت متفوقة معنويا عن بقية التراكيز وعن التأثير الناتج عن الأدوية المضادة للفطريات(النستاتين والكلوترايمازول).

Introduction:

In many parts of the world , there is a rich tradition in the use of herbal medicine for the treatment of many infectious diseases (1) . Since time immemorial , man has looked to plants and their products as a source of innovative medicines . It is estimated that 66-80 % of medicines used in developing countries are based on plants and 80 % of the worlds population relies on traditional medicines. Within the developed countries about 25 % of medicines contain active principals derived from plants , and the majority of drugs in current use were developed following studies of traditional plant treatments (2). Plants are rich in a wide variety of secondary metabolites such as tannins terpenoids, alkaloids flavonoids, etc, which have been found in vitro to have antimicrobial properties.(3,4). Traditional medicine , in Iraq has a major therapeutic role , traditional healers have been using different local plants to treat patients for thousand of years , this return to the Iraqi flora are rich in various plants which possible have different biological and pharmacological properties (5, 6) .The incidence of serious infections caused by yeast , particularly species of candida , has increased dramatically during the past decade , Candidiasis is one of the diseases treated by herbalists in the different countries. It caused by a dimorphic fungus called Candida albicans. This fungus exists as oval,single yeast cells , which reproduce by buding (7,8) .Although *C.albicans* most frequently infects the skin and mucosal surfaces , it can cause systemic infections manifesting as pneumonia , septicaemia , or endocarditis in severely immuno-compromised patients , *C.albicans* also can grows vigorously in the vagina and pregnant women transmit the infection to the babies during birth (9).*Candida albicans* has become resistant to the already limited , toxic , and expensive anti-candida agents available in local markets , these factors necessitate the search for new antifungal agents so this study was investigate the antifungal activity of ethanolic extracts of each (*Cyperus rotundus* , *Eugenia caryophyllus and Cariandrum sativum*) against growth *of candida albicans in vitro*.

Materials and methods:

1- plant collection and preparation:

In this experiments we used three local medicinal plants include : *Cyperus rotundus* , *Eugenia caryophyllus and Coriandrum sativum.* All these plants were obtained from the local market and identified by the national Iraqi institute for herbs, we take the flower of the first plant , rhizomes of the second plant and seeds of the third plant , then all the chosen parts of the above plants were subjected to aerial drying for two weeks, after drying of these parts we grinded it very well until it became as a fine powder . The Ethanolic extraction of the three plants were done by Harborn method (Harborn ,1975) by

using of Ethanol at a concentration (96%).

***2- Candida albicans* Isolates**:

Six isolates of *Candida anbicans* obtained from the laboratory of microbiology of Veterinary Medicine College at Al-Qadisiyia University ,these isolates were identified by (Microscopic examination , culturing on sabouraud s destrose agar , Germ tube formation test (10) activated in a nutrient broth (HIMEDIA laboratories,Mumbai,India) and after (24) hour in the incubator at (37◌ C) the isolates were became active.

3- Antifungal:

In this study we used two antifungal drugs Nystatin drops(Nystasyr) (Phamasyr, Dmascus ,SYRIA) 100 IU / ml , and the second antifungal was Clotrimazle solution (Candistan)(Arab drug company ,Cairo,A.R.E.) 30µg/ml.

4- Serial dilutions:

For each of the tested medicinal plants we had been made a serial dilution to study the effect of the plants in inhibition the growth of *Candida albicans* at a different concentrations and select the most effective concentration of the plant extract depending on the zone of inhibition of growth that been given by each concentration , we started with a concentration (400)mg/ml (prepared by add 10 ml from Ethanol 96% to 4 gm of the plant extract) and the second concentration is 200 mg/ml(prepared by taking 4ml from the first dilution and we add 4 ml of ethanol 96% to it ,the third dilution is 150 mg/ml (prepared by taking 3 ml from the second dilution and add 1 ml of ethanol 96% to it) , the fourth dilution is 100 mg/ml (prepared by taking 2ml from the second dilution and add 2 ml of ethanol 96% to it) ,the fifth dilution is 50%(made by taking 2 ml from the fourth dilution and add 2 ml of ethanol 96 % to it) and the last dilution 25% was made by taking 2 ml from the fourth dilution and add 2ml of ethanol 96% to it . These serial dilution was decided depending on clinical trails

5-Senstivity test study:

After preparation and Ethanolic extraction of the studied medicinal plants and activation of *Candida albicans* in the nutrient broth we were prepared a Sabouraud s Dextrose Agar (HIMEDIA laboratories ,Mumbai ,India) 65 mg/ 1000 ml D.W. by adding the agar powder to the distal water in a flask and after complete dissolving of the agar by using of Benzen burner we used the autoclave for antiseptic the agar at 15 IB for 15 Minutes ,then the agar were empty into Petri plates(6 Petri plates for each test agents) and then after the media is solidified in all the plates we made (6) pores in the Sabouraud Dextrose agar in the Petri plates to put the tested agents dilutions in it and a central pore for the control (Ethanol 96%) ,these pores were made by using a pasture pipette in a diameter of (5mm) and we put (0.1) ml from each concentration of the tested agents in these pores by using of micropipette . For each one of the tested agents (medicinal plants : *Cyperus rotundus, Eugenia caryophyllus ,Coriandrum sativum , and antifungal* drugs Nystatin and Clotrimazole) we made (6) plates and each plate contain (6)

pores we put in these pores the serial dilution of the tested medicinal plants and the seventh pore for the control (Ethanol 96%) *Candida albicans* were planted in the Petri plates that contain the Sabourauds dextrose agar before putting the tested agents and the we added the serial dilution of each on of the medicinal plants (25,50,100,150,200,400) and put the Petri plates in the incubator for (48) hr at 37ºC. Following overnight incubation, the culture was examined for areas of no growth around the pores (zone of inhibition) in millimeter (mm)(11).

Result and discussion:

The genus *Candida* is composed of an extremely heterogeneous group of organisms that grow as yeasts. Most members of the genus also produce a filamentous type of growth (pseudohyphae) , In addition to pseudohyphae, *Candida albicans* and *C. dubliniensis* form true hyphae (germ tubes) and thick-walled cells referred to as Chlamydospores, both of which are used by mycology diagnostic laboratories in identifying these species , *Candida* species are now emerging as major agents of hospital-acquired infections; they are ranked as the third or fourth most commonly isolated bloodstream pathogens, surpassing gram-negative bacilli in frequency . Although *C. albicans* is the predominant etiologic agent of candidiasis, other *Candida* species that tend to be less susceptible to the commonly used antifungal drugs such as *C. krusei*, *C. glabrata*, *C. lusitaniae*, and the newest *Candida* species, *C. dubliniensis*, have emerged as substantial opportunistic pathogens , *Candida dubliniensis* shares with *C. albicans* many virulence factors, such as germ tube formation, exoenzyme production, and phenotypic switching (12).*Candida albicans* is a diploid fungus (a form of yeast) and is a causal agent of opportunistic oral and genital infection .(13).

Candida has been known to infect every organ of the body ,but it is ability to cause infection depend upon the presence of a sufficient amount of fungal organism or generally reduced resistance or both.(14) . In our study we try to experiment the effect of selected medicinal plants in comparison to antifungal drugs on *Candida albicans* growing *in vitro* in a Sabouraud Dextrose Agar by using of agar diffusion method .

Agar -based methods are attractive because of their simplicity and low cost ,in addition to that it may help to detect if their any resistance from the yeast or the bacteria to any drug , medicinal plants or agents that may be used to study it is effect(15).

The fungal infections due to *Candida* species are an important cause of morbidity and mortality especially in immunocompromised patients. The use of available treatment options for invasive mycoses is limited due to limited spectrum of activity, drug resistance, toxicity and drug-drug interactions (16) In view of this, there is a need to develop more effective and less toxic agents for the treatment of common, as well as drug resistant fungal infections.. This has led to a search

for therapeutic alternatives, particularly among medicinal plants and compounds isolated from them used for their empirically antifungal properties. In these natural sources, a series of molecules with antifungal activity against different strains of fungus have been found, which are of great importance to humans and plants(17).

In vitro *anticandida albicans* effect of three local plants Ethanolic extract (*Cyperus rotundus, Eugenia caryophyllus and cariandrum sativum)* and two standard antifungal drugs (Nystatin , Clotrimazole) were studied in this experiment (table 1 and 2). The active ingredient that are present in *Eugenia c aryophyllus include :* volatile oil (14-21%) ,tannins (10-13%) and caryophyllin(18a,b)While the active constituents that are present in *Cyperus rotundus* include : rotundines (A,B,C) (19) , sesquiterp-enes hydrocarbons-isorotundene , cyperadiene , norrotundene , cyperadione(19),triterpene and oleanolic acid (20) , and *Coriandrum sativum* active ingredient include:(0.5-1%) volatile oil,Linalol (45-60%), Pinen, tripinen and Geraniol (18).The Ethanolic extract of the three medicinal plants showed a various antifungal activities against the isolates of *Candida albicans* according to the type of the medicinal plants and the concentration which had been used. The most active extract were that obtained from *Cyperus rotundus* (CR)(picture1) that gave a zone of inhibition with Standard Error (SE) as following (11.83± 0.25,15.08±0.14,15.83±0.52,19.75±0 .13,20.5±0.15 and 20.75±0.13 mm) at concentration 25,50,100,150,200, 400 mg /ml respectively ,while Ethanolic extract of *Eugenia caryophyllus* (EC)(Picture 2) showed a moderate activity and gave an inhibition zone with Standard Error (SE) as follow : (0±0,9.5±0.15,12.58 ± 0.14, 14.83 ± 0.11, 16.33 ± 0.14 ,20.33 ± 0.22) at concentration 25,50,100,150,200,400 mg/ml respectively ,while *Coriandrum sativum(CS)* had no effect on all *Candida albicans* isolates at all studied concentration (figure 1).Our study showed that there was a proportional relationship between the concentrations of the plants extract that had been used and the zone of inhibition of *Candida albicans* growth in the media of Sabouraud Dextrose Agar at the Petri plates at a confidence interval ($P<0.05$), when the concentration increase this lead to increase the zone of inhibition of the growth.

The antifungal drugs produce an inhibition zones as follow : Nystatin (100IU/ml) (15.5±0.26 mm) Clotrimazole (30 µg /ml) (18.8± 0.61 mm) and Ethanol 96% (0±0).

Clotrimazole (30 µg / ml) had a significant differences ($P <0.05$) in the zone of inhibition than that for Nystatin (100 IU) and Ethanol 96% (15.5± 0.26 versus 18.8±0.61 and 0±0) respectively. The result of (EC) in this study was close to those obtained by the oil of the (EC), the yeast *Candida albicans* was sensitive to essential oil of *(*ER*)*, the tested essential oil exhibited inhibitory activity against *Candida albicans* at

low concentrations (21) , these result also supported by(22,23) who reported that *Eugenia aromatica* posses antimicrobial properties. This anti fungal effect of (EC) was also supported by (24) that showes that *Eugenia* oleoresin had a fast killing effect on yeast cells(*Candida albicans*) and the lethal effect was higher at 37ºC.

Other study that supported our result was the study that indicates that (EC) oil have a considerable antifungal activity against clinically relevant fungi, and it is deserving further investigation for its clinical applications on the treatment of fungal infections(25).(26)showed in his study that the volatile oil of the medicinal plant *Cymbopogon nervatus* have antifungal effect against *Candida albicans* and he refer to the content of this volatile oil that it it is composed mainly of oxygenated monoterpenes and as we noted from the review of the active ingredient of *Cyperus rotundus* that it contain triterpenes in it is content so this may lead us to the possibility that the antifungal effect of (CR) may be relate

to this active ingredient(terpenes). Another plant from the genus *Cyperus is C.articulatus* which had been documented as anti –yeast actions against *Candida*(27). Either Nystatin suspension or the Clotrimazole douches is the drug of choice in *Candidiasis* for non-immunosuppressed adults.(28). The *AntiCandidal albicans* effect of Nystatin had been approved in many researches (29,31) also (30) had been proved the fungicidal effect of Nystatin The antifungal effect of Clotrimazole was also supported by invivo study on the *anticandidal* effect of this drug in treatment of *candid*a vaginitis (30), another invitro study (31) indicate the inhibition effect of this drug against *Candida albicans* , and against other fungal infection than *Candida albicans* Nystatin is less stronger than clotrimazole (31) which was also proved in our study We could conclude in this study that there is a required to study the effect of the medicinal plants from many other sided than the known effect of it , as we noted in this study that there is very rare references that study the antifungal effect of *Cyperus rotundus and Eugenia caryophyllus* against *Candida albicans* although we approved in this study that there is a clear and very strong effect of these medicinal plants in inhibition the growth of *Candida albicans* that planted in Sabouraud Dextrose agar while no effect is actually gain from *Coriandrum sativum* against the studied yeast.

Table (1): anticandida albicans activity of some local plants extracts.

name of plant	Extract concentrations (mg / ml)					
	25	50	100	150	200	400
Cyperus rotundus	11.83± 0.25 aA	15.08± 0.14 bA	15.83± 0.11 bA	19.75± 0.13 cA	20.5± 0.15 cA	20.75± 0.13 cA
Eugenia caryphyllus	0±0 aB	9.5± 0.15 bB	12.58± 0.14 cB	14.83± 0.11 dB	16.33± 0.14 eB	20.33± 0.22 fA
Cariandrum satirum	0±0 aB	0±0 aC	0±0 aC	0±0 aC	0±0 aC	0±0 aC

- Values were expressed as means ± standard error
- Values with different capital letters are significant differences vertically at ($p < 0.05$).
- Values with different small letters are significant differences horizontally at ($p < 0.05$).

Table (2): anticandida albicans activity of some antifungal drugs (positive control) and ethanol (negative control).

Name of Antifungal drug	Inhibition zone (mm)
Nystatin (100 IU)	15.5±0.26
Clotrimazole (30 µg / ml)	18.8±0.61
Ethanol (96%)	0 ± 0

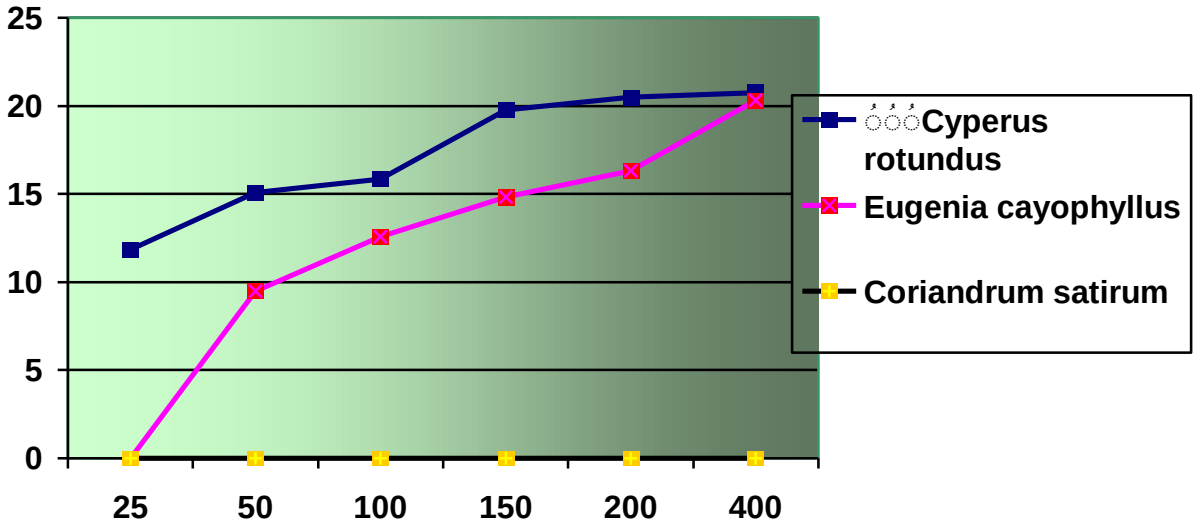
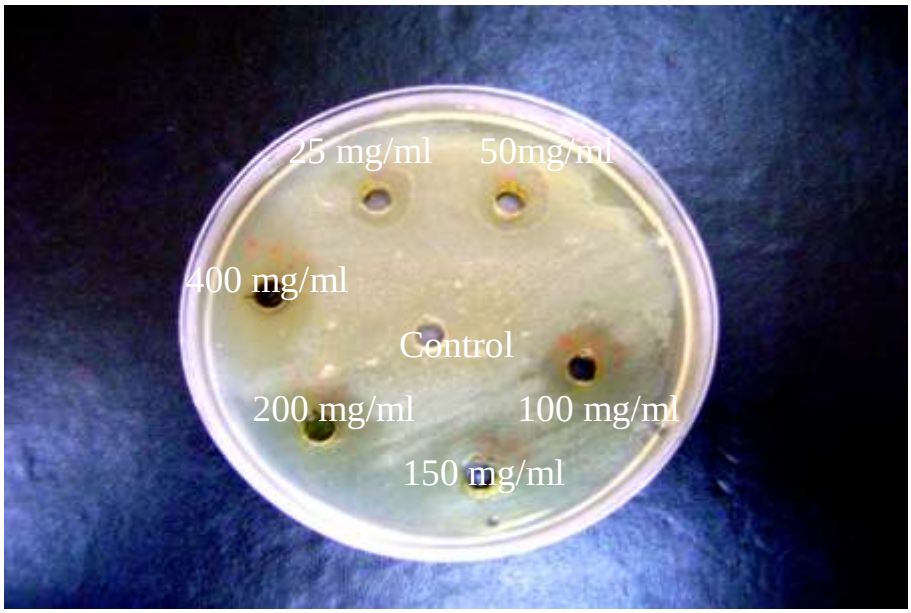
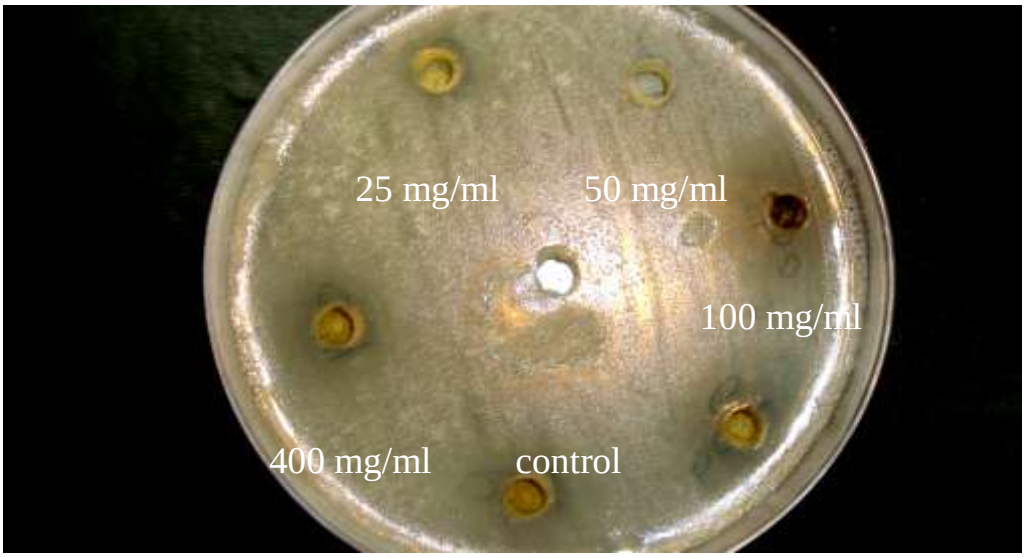


Figure (1):Anticandida albicans activity of some local plants extracts



Picture (1): *Anticandida albicans* effect of *Cyperus rotundus* in culture media.



Picture(2):*Anticandida albicans* effect of *Eugenia caryophyllus* in culture media.

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