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Evaluation the Antifungal Activity of the Alcoholic Extract of Carob (*Ceratonia siliqua* L) Plant Against Fungal Isolates Obtained from the Mouths of Infants in Ramadi City

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Abstract. The present study aimed to evaluate the antifungal activity of the alcoholic extract of carob (*Ceratonia siliqua* L) plant against fungal isolates obtained from the mouths of infants in Ramadi City. Between September and December 1, 2024, a hundred oral swabs were taken from newborns at the Women and Children's Hospitals in Ramadi. *Candida albicans*, *Candida krusei*, *Candida tropicalis*, and *Candida glabrata* were distinguished. In a well diffusion study, carob extract's anti-fungal activity at 2%, 5%, and 10% concentrations was contrasted with that of the gold standard antifungal medicine, Nystatin. At a 10% concentration, the carob extract showed a strong inhibitory action against every type of *Candida*, with a specific inhibition zones of 14.84 ± 0.78 mm against *Candida albicans*. Carob extract demonstrated excellent sensitivity to Nystatin-resistant *Candida glabrata*, suggesting it may be useful as an alternate treatment for such infections. Methyl esters and siloxanes were among the bioactive chemicals discovered by phytochemical GC-MS analysis; these compounds may have a role in the antifungal activity. Isolation, extraction, as well as genetic amplifying were all successful because electrophoresis revealed the presence of *Candida* species in each of the samples tested. These results indicate that the extract of *C. siliqua* has potential as an antifungal agent with a natural remedy for newborn oral candidiasis. To determine the safety and effectiveness of the active chemicals in clinical settings, as well as to identify and define them, additional research is needed.

1. Introduction

The human body is home to fungi and other microbes, particularly in damp environments like the skin, mouth, and digestive tract. One of the most prevalent health issues in newborns among the fungi connected with humans is oral candidiasis. Several species of *Candida spp.* causes this fungal infection of the mouth's mucosa [1]. This infection is most often caused by *Candida albicans*, however it can also be caused by *Candida glabrata*, *Candida tropicalis* and *Candida krusei*, and. Formula feeding, inadequate oral hygiene, and the utilization of immune-suppressive drugs or the antibiotics generally contribute to an imbalance in the mouth's natural microbiome, which in turn allows the fungus to develop and spread abnormally, making this disease more common in infants [2]. Despite the widespread use of Nystatin and other classic antifungal therapies, the rise of drug-resistant *Candida* species was a serious concern in the medical field. This has motivated scientists to look into natural remedies that are both safe and effective as potential replacements for conventional medicine [3].

One of these sources are the carob plant, scientifically known as *Ceratonia siliqua*, which has a long history of medicinal utilize in folk medicine for its many therapeutic advantages[4]. The carob plant



contains a wide variety of active compounds, including flavonoids, polyphenols, fatty acids, tannins, as well as esters, each of which have been shown to have antioxidants, antimicrobial, and anti-fungal impacts, as well as immune-boosting ones. Several studies have shown that carob extracts, especially alcoholic ones, can inhibit the growth of various microorganisms; this is because carob contains substances that are damaging to fungal cell walls, which effect the enzymes with physiological processes inside the cell, causing fungal growth and development to stop[5].

In light of the difficulties caused by fungal resistance to conventional antibiotics, investigating the direct impact of carob alcoholic extract on fungi isolated from newborns' mouths is an encouraging area of research that could lead to the creation of safe natural remedies[6]. Thus, the purpose of this research is to examine the effectiveness of nystatin, the gold standard antifungal, against fungi that have been collected from the mouths of infants suffering from fungal infections, and to draw comparisons between the two.

2. Materials and Methods

2.1 Collection of specimen

Between September and December 1, 2024, a hundred oral swabs were taken from newborns at the Women's and Children's Hospital in Ramadi. cultivating on Sabouraud Dextrose Agar (SDA) and incubating for 24-28 hrs at 37°C, the infants, ranging in age from 1 month to 2 years, were subjected to the experiment. Morphological diagnosis and biochemical analysis were used to diagnose the emerging fungal colonies based on their form, size, and color.

2.2 Collection of Carob (*C. siliqua*) Samples

The carob pods were gathered from different spots at Euphrates River Basin, near Hit, in the city of Ramadi, from September 1, 2024, to October 15, 2024. Following a thorough washing in running water, the pods were spread out on a towel and allowed to air dry in a draught-free environment. They were subsequently reduced to a powdered form by use of an electric grinder and kept until they were required.

2.3 Ethanol Extract Preparation

Fifty grams of powder were mixed with 500 milliliters of 70% ethyl alcohol in a 1000 milliliter to make the carob pod alcoholic extract. After that, the mixture was left for 24 hours on a magnetic stirrer with a hot plate. The next step was to strain the mixture through many gauze layers. After that, the filtrate was transferred to a glass dish and heated in an electric oven at 40°C for 72 hours, or until the liquids had evaporated and the filtrate had transformed into a powder that was still adhered to the glass. The powder was subsequently removed by skimming and stored in airtight glass jars until needed[7].

2.4 *Ceratonia siliqua* Volatile Organic Compound detection and Identification via HS-SPME-GC-MS

Solid phase micro-extraction linked to gas chromatography-mass spectrometry (GC-MS) was a method that can be used to detect and identify volatile organic compounds (MVOCs) in plants. To identify MVOCs, we compared their mass spectrum and linear temperatures program retention indices (LTPRIs) to those of known compounds in a database. The method outlined here enabled tracking of volatile metabolite production over a defined time frame.

2.5 Sensitivity test to Antimicrobial

Alcoholic extracts and antibiotics (Nystatin) were tested for fungal isolate sensitivity using the disk diffusion method, in accordance with Clinical and Laboratory Standards Institute (CLSI, 2021) recommendations. For every *Candida spp.* sample, 0.1 ml of yeast suspension was distributed on SDA medium utilizing Swab; the turbidity of this suspension was compared with that of McFarland's 0.5% solution. The samples were then allowed to dry for 15 minutes. The next step was used a cork borer with a diameter of 6 mm to dimple the agar. The procedure involved making a central hole and four outer holes, and then added 50 ul of the extract to every well at concentrations of 10, 5, and 2 µg/ml. After that, for a whole day, the plates were kept in an incubator set at 37°C. We used a ruler to measure the diameter of the inhibitory zone in millimeters, then we compared these measurements to the antifungal [8].

2.6 Molecular study

2.6.1 DNA extraction

A kit prepared by Geneaid Biotech Ltd. (Presto™ Mini DNA Yeast Kit) was used, and the extraction was performed according to the manufacturer's instructions.

The FavorPrep™ Genomic DNA extraction Mini Kits were used to extract the DNA from fungal growth, in accordance with the company's guidelines. Eluted DNA concentrations along with quality were assessed and subsequently kept at −20 °C for future testing. The extracted DNA samples were visualized by performing electrophoresis on a 1% agarose gel.

2.6.2 Primers

Table 1. The primers used for the study

Primer	Primer sequence 5' - 3'	Tm (°C)	Size of Product (bp)
<i>ITS1 F</i>	5'-TCCGTAGGTGAACCTGCGG-3'	55TM	500-700bp
<i>ITS4R</i>	5'-TCCTCCGCTTATTGATATGC-3'	55TM	500-700bp

2.6.3 PCR Amplification

Table 2. The best possible detection conditions molecular identification

PCR step	Temp.	Time	Repeat
Initial Denaturation	95C	5min	1
Denaturation	95C	30sec.	35 cycles
Annealing	58C	30sec.	
Extension	72C	1 min.	
Final extension	72C	5min.	1
Hold	4C	Stop	-

2.7 Ethical approval

The present study was carried out in accordance with the ethical principles outlined in the Declaration of Helsinki. The study was performed following the acquisition of both verbal and written consent from the patients before collecting the samples, this study was approved by the University of Anbar (200 in December 23, 2024).

2.8 Statistical Analysis

The Statistical Package for the Social Sciences (SPSS) 2019 software was employed to analyze the data and examine the impact of various factors on the features under investigation. Substantial differences between means were analyzed using the least significant difference (LSD) test, while significant differences between percentages were assessed using the chi-square test.

3. Results and Discussion

The isolated *Candida spp.* were identified based on the morphological characteristics of the colonies growing on SDA medium, where the colonies showed a convex circular shape, a smooth, shiny surface, and a creamy color [9], as shown in Figure (1).



Fig 1. *Candida spp.* colonies on SDA medium.

While microscopic examination after taking a part of the colony growing on SDA medium and placing it on a glass slide and then staining it with a drop of Lactophenol-cotton blue dye passed over the flame and the slide cover was placed and then examined under the microscope showed that all isolated species were colored blue in a spherical shape [10, 11]. Figure (2).

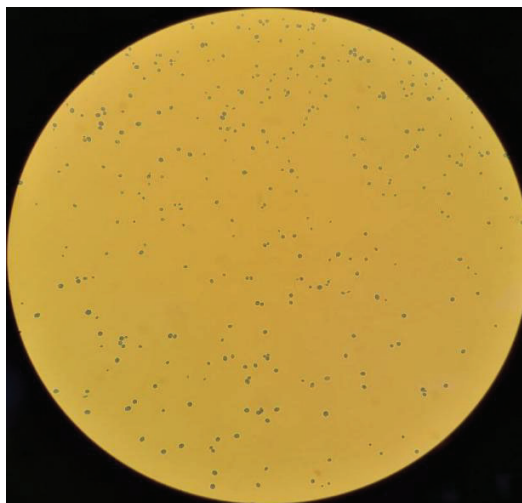


Fig 2. *Candida* under the microscope stained with Lactophenol-cotton blue.

The present study revealed the presence of 13 different compounds have been identified in **carob** extract, using GC-MS analysis, representing 95.46% of the total constituents. These compounds belong to several chemical families: cyclosiloxanes, fatty acids and their derivatives, alkenes and hydrocarbons. The chemical components of MFHAL are presented in Table 3, which includes information about their retention time (RT), molecular formula, molecular weight (MW), and concentration (%). The amount of methyl ester content that was derived from hexadecanoic acid was 18.96%.

Table 3. Volatile compounds of carob by GC-Mass

80206-1					
Number	RT (min)	Area%	Name	Quality	CAS Number
1	11.169	1.92	Cyclopentasiloxane, decamethyl-	90	000541-02-6
2	15.185	9.62	Cyclohexasiloxane, dodecamethyl-	93	000540-97-6
3	18.828	9.58	Cycloheptasiloxane, tetradecamethyl-	47	015089-22-2
4	22.097	2.31	Cyclooctasiloxane, hexadecamethyl-	38	073420-30-1
5	24.93	0.74	Cyclononasiloxane, octadecamethyl-	87	000556-71-8
6	25.869	18.96	Hexadecanoic acid, methyl ester	98	000112-39-0
7	26.963	5.79	Hexadecanoic acid, ethyl ester	97	000628-97-7
8	27.467	17.81	Cyclononasiloxane, octadecamethyl-	47	038147-00-1
9	28.416	1.72	1,3-Butadiene, 2,3-dimethyl-	22	000513-81-5
10	28.546	2.10	6-Octadecenoic acid	53	1000336-66-8
11	28.987	11.65	Methyl isostearate	99	005129-61-3
12	29.542	11.26	Ethyl Oleate	99	000111-62-6
13	37.72	2.01	Heptasiloxane, hexadecamethyl-	64	019095-24-0
95.46					

The result obtain in present study agreement with other study conducted by Gregoriou et al., 2021[12] on *C. siliqua* polyphenol content in extracts. Ydjedd et al. (2017) also found that the phenolic and flavonoid concentrations were highest in the *C. siliqua* ethyl acetate extracts [13]. Research conducted by Avallone et al. (1997) found that carob contains significant amounts of condensed tannins [14].

Table 4. Comparison of fungal isolate type with the average diameter of inhibition (mm) of the antifungal and the concentrations of *C. silique*.

Isolates	Nyststin	Concentration of <i>C. silique</i>			LSD
		%2	%5	%10	
<i>C.albicans</i>	12.60 ±0.68 b	7.368 ±0.37 c	11.60 ±0.57 b	14.84 ±0.78 a	2.073 *
<i>C.krusei</i>	10.95 ±0.57 b	8.136 ±0.49 b	10.09 ±0.54 b	13.50 ±0.67 a	2.189 *
<i>C.Tropicalis</i>	10.72 ±0.62 b	8.36 ±.42 c	12.56 ±0.67 ab	14.81 ±0.72 a	2.086 *
<i>C.glabrata</i>	0 ±0.00 d	6.66 ±0.29 c	10.30 ±0.46 b	13.83 ±0.72 a	3.176 *

Means with different letters within a row are significantly different from each other.* (P≤0.05).

Table 4, compares the antifungal effect of Nystatin and different concentrations of **carob** extract (2%, 5%, and 10%) on four *Candida* species: *Candida albicans* (*C. albicans*), *Candida krusei* (*C. krusei*), *Candida tropicalis* (*C. tropicalis*), and *Candida glabrata* (*C. glabrata*). A standard antifungal (Nystatin) was used as a reference. Efficacy was evaluated by measuring the mean diameter of inhibition (Mean ± standard deviation).

Nystatin demonstrated a favorable effect on *C. albicans*, *C. krusei*, and *C. tropicalis*, with diameters of inhibition ranging from 10.72 ±0.62 to 12.60 ±0.68 mm. However, it failed completely against *C. glabrata* (0 mm), indicating this isolate's resistance to Nystatin.

Increasing the concentration of **carob** extract led to an increase in the diameter of inhibition in all isolates, confirming a direct relationship between concentration and antifungal activity. The highest activity was recorded at a concentration of 10% in all species was recorded (14.84 ±0.78, 13.50 ±0.67, 14.81 ±0.72, 13.83 ±0.72) mm against *C.albicans*, *C.krusei*, *C.Tropicalis*, *C.glabrata* respectively, indicating that **carob** at a concentration of 10% was more potent than Nystatin against this isolates. All

values with different letters (a, b, c, d) indicate significant differences ($P \leq 0.05$). Figure 1, show effect of **carob** extract and Nystatin against isolates

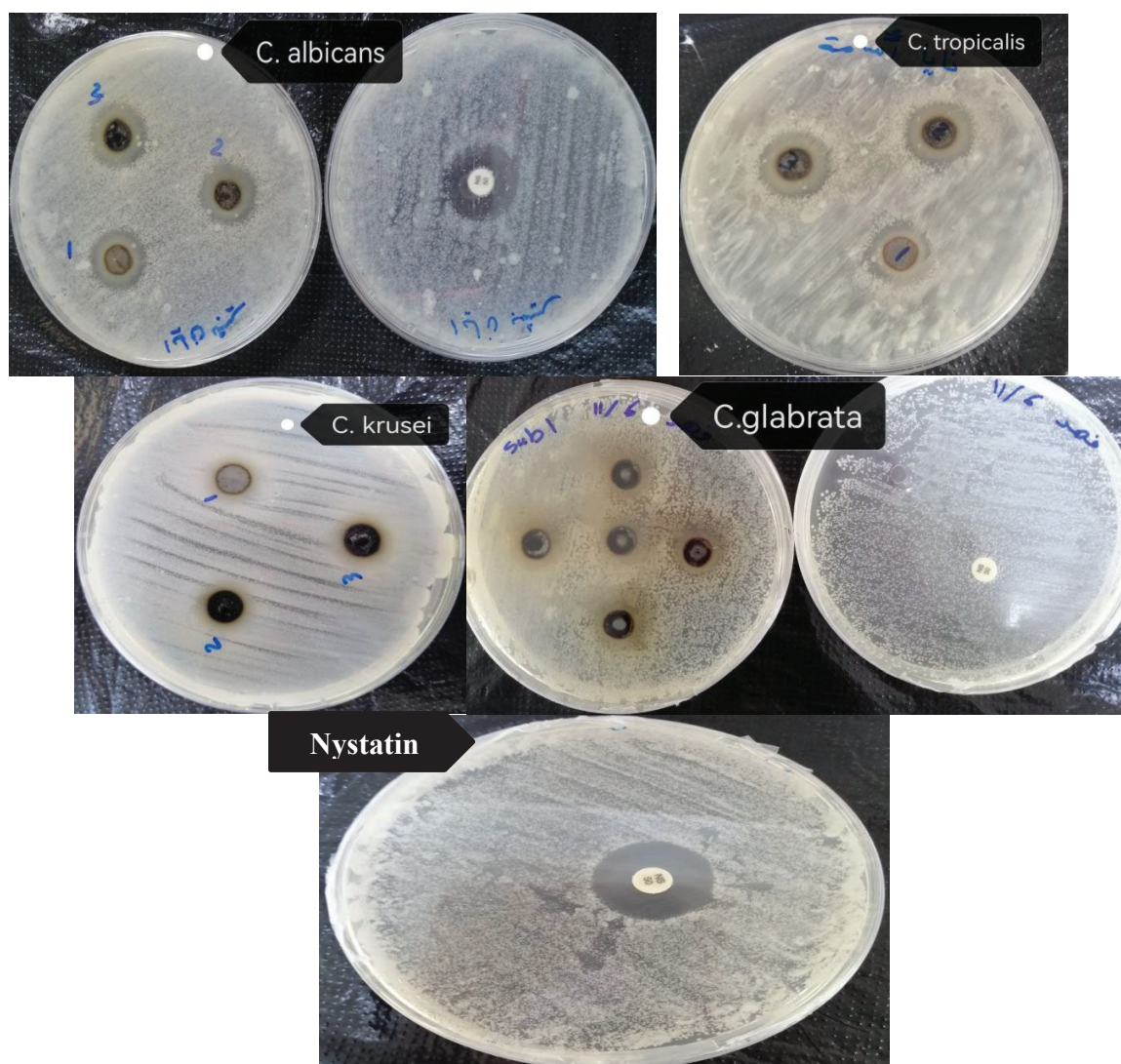


Fig 3. Effect of **carob** extract and Nystatin against isolates.

These results support the idea of using medicinal plants as sources of new antifungals, especially with the increasing resistance of fungi to traditional medicines. The potential for developing natural medicinal products from carob (such as topical creams or ointments) to treat skin and mucosal fungi. The findings align with those reported by, who established that carob extract exhibits antifungal properties attributable to its elevated levels of polyphenols and tannins, which impact on cell wall and membranes.

The result aligns with the findings indicated that plant extracts can be more effective than traditional antibiotics, particularly against resistant strains [15]. Conversely, In a study was demonstrated that the enhanced efficacy of the extract with rising concentration signifies a direct correlation between the quantity of active chemicals and the inhibitory impact [16].

3.1 DNA Extraction

In order to confirm the presence of DNA in samples of fungi collected from newborn mouths, the researchers in this study utilized gel electrophoresis for amplifying ITS region products of *Candida* sp. utilizing primers *ITS1* and *ITS4*. A molecular ladder was used to assess fragment size (100-1000 bp) in 14 samples of *Candida* spp. A distinct band measuring around 540 bp was visible in all of the samples (Figure 4), by utilizing PCR.

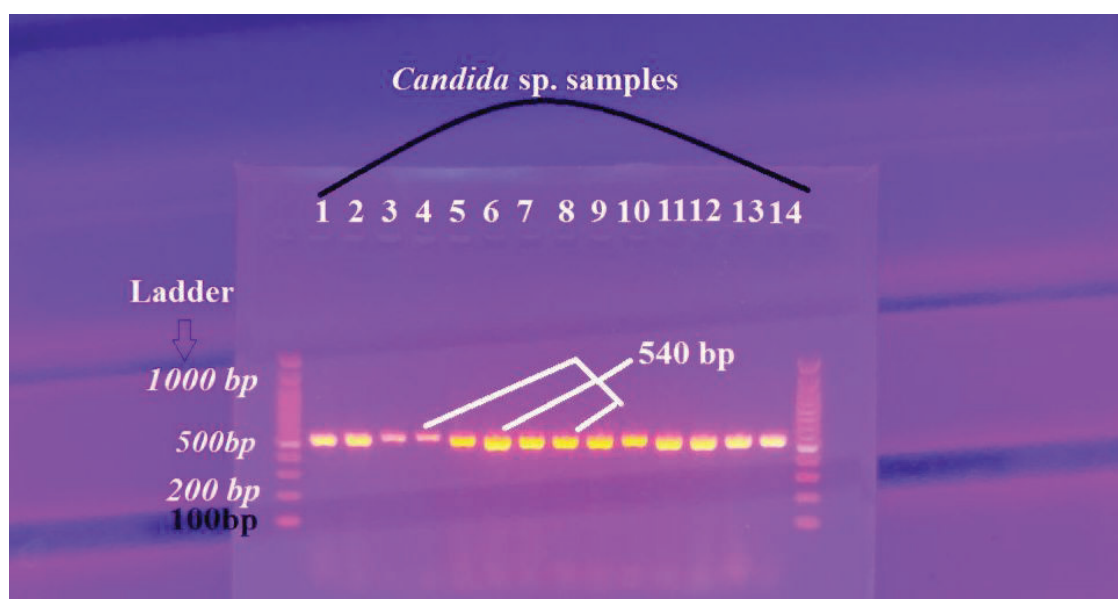


Fig 4. Gel electrophoresis of pcr product(ITS gene), 1% agarose gel, 5 vol/cm, 1:15 hours. M: DNA ladder

A plethora of studies have shown that rapid fungal diagnosis is now possible using diagnostic equipment. PCR based procedures are important because they preserve specificity while efficiently addressing the declining sensitivity of current laboratory methods [17]. Consistent with other prior investigations employing the identical primers, this work showed that all *Candida* spp. successfully amplified an ITS region of about 540 bp. Bands spanning 500 to 600 bp for different *Candida* species were seen in a study Ghahri *et al.*, 2013[18] that used *ITS1* and *ITS4* primers. After ethidium bromide staining, the bands were clearly visible under UV light. One of the most reliable regions for differentiating *Candida albicans* from related species like *C. glabrata* and *C. tropicalis* is the intergenic space (ITS) region amplified by *ITS1* and *ITS4*, according to a study (Sugita *et al.*, 2004 [19]. Consistent with the present findings (540 bp), it showed that the fragment size varied between 520 and 560 bp. This primer set has shown to be an invaluable tool for amplifying the ITS region of many different types of fungi, not limited to *Candida* [20]. The predicted fragment sizes in *Candida*, according to their research, are around 540-560 bp.

4. Conclusion

The results obtained in this study indicated the effectiveness of carob extract against fungi isolated from children, as the higher the dose, the greater the inhibition efficiency. This will open the filed to creating natural therapeutic options that are both safe and effective. When it comes to children's complementary and preventative healthcare, this represents a huge step forward.

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