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In vitro study of the antifungal activity of natural products against *Candida Albicans*

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Dedication

To my beloved father, for your strength that carried me through every challenge, for your wisdom that shaped my path, and for the silent sacrifices that spoke louder than words. This work stands as a tribute to your unwavering love and belief in me.

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✎ *"On vient du fond, on veut toucher le ciel."*

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Abstract

This study aimed to evaluate the antifungal activity of three natural products: coconut oil, cinnamon oil, and propolis, against *Candida albicans* isolates from two types of human infections. The findings revealed that all tested substances exhibited inhibitory effects, with propolis being the most effective. It demonstrated the strongest antifungal activity, with minimum inhibitory concentrations (MICs) ranging from 0.625 to 2.5 mg/mL. Cinnamon oil showed significant activity with MICs between 0.5% and 1%, while coconut oil had moderate effects (MICs from 2% to 2.5%). The synergistic combination of coconut and cinnamon oil produced intermediate activity, with MICs varying between 0.5% and 2% in some cases. Biofilm formation, a key virulence and resistance factor, varied among isolates: vaginal strains were more likely to form moderate biofilms, whereas some oral strains showed weak or no biofilm production. These results confirm the antifungal potential of natural products, especially propolis, and support their possible therapeutic use, particularly in the context of rising global antifungal drug resistance.

Keywords: *Candida albicans, coconut oil, cinnamon oil, propolis, minimum inhibitory concentration (MIC)*

Resumé

Cette étude visait à évaluer l'activité antifongique de trois produits naturels : l'huile de coco, l'huile de cannelle et la propolis, contre des isolats de *Candida albicans* provenant de deux types d'infections humaines. Les résultats ont montré que les trois substances testées possédaient une activité inhibitrice contre les souches étudiées. Parmi elles, la propolis s'est révélée la plus efficace, avec des concentrations minimales inhibitrices (CMI) comprises entre 0,625 et 2,5 mg/mL. L'huile de cannelle a présenté une activité notable avec des CMI allant de 0,5 % à 1 %, tandis que l'huile de coco a montré une activité modérée (CMI de 2 % à 2,5 %). La combinaison synergique de l'huile de coco et de l'huile de cannelle a produit des effets moyens, avec des CMI comprises entre 0,5 % et 2 % selon les cas. Par ailleurs, la formation de biofilm, facteur important de virulence et de résistance, variait selon les souches : les isolats vaginaux étaient plus enclins à former des biofilms modérés, alors que certaines souches orales montraient une formation faible voire absente. Ces résultats confirment le potentiel antifongique des produits naturels, en particulier de la propolis, et soulignent leur intérêt thérapeutique face à la montée préoccupante de la résistance aux traitements antifongiques conventionnels.

Mots clés : *Candida albicans, huile de noix de coco, huile de cannelle, propolis, concentration minimale inhibitrice (CMI)*

الملخص

هدفت هذه الدراسة إلى تقييم النشاط المضاد للفطريات لثلاثة منتجات طبيعية: زيت جوز الهند، زيت القرفة، والبروبوليس ضد عزلات المبيضة البيضاء المستخلصة من نوعين من العدوى البشرية. أظهرت النتائج أن جميع المواد المختبرة أبدت تأثيرًا مثبطًا لنمو الفطريات، وكانت البروبوليس الأكثر فعالية من بينها، حيث سجلت أدنى تراكيز مثبطة (MIC) تراوحت بين 0.625 و2.5 ملغ/مل. أما زيت القرفة فقد أظهر نشاطًا ملحوظًا بتراكيز تراوحت بين 0.5% و1%، في حين أظهر زيت جوز الهند فعالية متوسطة، بتركيزات تراوحت بين 2% و2.5%. وأظهر المزج بين زيت القرفة وزيت جوز الهند تأثيرًا متوسطًا أيضًا، بتراكيز تراوحت بين 0.5% و2% في بعض الحالات.

من جهة أخرى، تباينت قدرة العزلات على تشكيل الأغشية الحيوية (البيوفيلم)، وهو عامل رئيسي في زيادة الضراوة والمقاومة، حيث كانت العزلات المهبلية أكثر ميلاً لتكوين بيوفيلم متوسط، بينما أظهرت بعض العزلات الفموية تكوينًا ضعيفًا أو غائبًا.

تؤكد هذه النتائج الفعالية المحتملة للمنتجات الطبيعية ضد المبيضة البيضاء، خاصة البروبوليس، مما يدعم إمكانية استخدامها كخيار علاجي بديل في ظل تصاعد مقاومة الأدوية التقليدية في جميع أنحاء العالم.

الكلمات المفتاحية : *المبيضة البيضاء (Candida albicans) ، زيت جوز الهند، زيت القرفة، البروبوليس (العكبر)، التركيز المثبط الأدنى (CMI)*

Table of Content :

Abstract

Figures list

Tables list

List of abreviations

Introduction 1

1. Background and Significance 1
2. Research Objectives 2
3. Thesis Structure 3

1. Candida albicans: General Characteristics and Clinical Significance. 4

1. Classification 4
2. Habitat and Distribution 5
3. Morphology and Structural Characteristics 5

3.1.Cellular Structure 6

3.2.Dimorphism 7

4. Cultural Characteristics 7

4.1.Growth Requirements 7

4.2.Colony Morphology 7

4.3.Growth Media 7

4.4.Identification Methods 8

4.5.Antifungal Susceptibility 10

4.5.1. Mechanisms of Action of Antifungal Agents. 10

4.5.2. Virulence Factors 11

5. Candida albicans-Associated Infections 15

5.1.Mucocutaneous Candidiasis 15

5.2.Invasive Candidiasis 16

5.3.Risk Factors for Candida Infections 16

2. Natural Products and Their Antifungal Activities Against Candida albicans.. 17

1. Cinnamon Oil 18

1.1 Botanical and Geographical Origin of Cinnamon 18

1.2 Historical use of cinnamon as an antifungal 18

1.3 Chemical composition and active principles of cinnamon 19

1.4 Mechanism of action of cinnamon oil against Candida albicans 19

1.5 Applications of cinnamon oil 20

1.6 Allerginicity and tolerance of cinnamon oil 20

2. Propolis 21

2.1 Botanical and geographical origin of propolis. 21

2.2 Historical use of propolis as an antifungal.. 22

2.3 Chemical composition and active principles of propolis. 22

2.4 Mechanism of action of propolis against candida albicans 23

2.5 Applications of propolis 24

2.6 Allerginicity and tolerance of propolis 24

3. Coconut Oil 25

3.1 Botanical and geographical origin of coconut oil 25

3.2 Historical use of coconut oil as an antifungal 26

3.3 Chemical composition and active principles of coconut oil. 26

3.4 Mechanism of action of coconut oil against candida albicans 27

3.5 Applications of coconut oil 27

3.6 Allerginicity and tolerance of coconut oil 28

3. Materials and Methods 29

A. Material 30

1. Vegetable material 30

2. Biological material 31

B. Methods 32

1. Isolation of candida albicans strains 32

2. Identification of candida albicans 32

3. Conservation of candida albicans isolates 33

4. Revival of candida albicans isolates 33

5. Biofilm Formation 33

6. Susceptibility tests 36

6.1 Coconut oil antifungal susceptibility assay 36

6.2 Cinnamon oil antifungal susceptibility assay 39

6.3 Propolis antifungal susceptibility assay 41

6.4 Mix of cinnamon oil+coconut oil susceptibility assay 43

Results and Discussion 46

1. Results 47

a) Identification of C.albicans 47

b) Biofilm formation and quantification assay 48

c) Antimicrobial susceptibility test results 50

2. Discussion of obtained results 58

a) Biofilm formation 58

b) Ethanolic extract of propolis (EEP) 59

c) Cinnamon oil 59

d) Coconut oil 60

e) Synergistic combination: coconut oil+cinnamon oil 60

Overall interpretation 60

Conclusion 62

References. 63

Annexes. 70

Figures List:

Figure 1 : The three different morphological states of C. albicans (Blanco, 2017).....6
Figure 2 : Structural organization of the cell wall of Candida albicans (Aroso et Al. 2021). 6
Figure 3 : Stages of Candida albicans Biofilm Development (Talapko et al., 2021) 13
Figure 4 : Cinammon quills photo example 18
Figure 5 : Propolis photo example..... 22
Figure 6 : Coconut oil photo example..... 26
Figure 7 : Commercially purchased pure cinnamon oil (Personal photo – 2025)..... 30
Figure 8 : Ethanolic extract of propolis 31
Figure 9 : PBS washing step during Candida albicans biofilm assay (Personal photo – 2025)..... 34
Figure 10 : Crystal violet staining step (Personal photo – 2025)..... 35
Figure 11 : Filling the designed wells with SDB and inoculum and oil dilutions (Personal photo – 2025)..... 38
Figure 12 : Loading of wells with cinnamon oil and C.albicans inoculum for microdilution test (Personal photo – 2025) 40
Figure 13 : Preparation of EEP dilutions (Personal photo – 2025)..... 42
Figure 14 : Preparation of Synergic dilutions of cinnamon oil + coconut oil (Personal photo – 2025)..... 44
Figure 15 : Colony morphology of Candida albicans on Sabouraud Chloramphenicol Agar (Personal photo – 2025)..... 47
Figure 16 : Methylene blue staining of Candida albicans observed under light microscope at 40× magnification (Personal photo – 2025)..... 47
Figure 17 : Positive germ tube test: filament formation by Candida albicans, observed under light microscope at 40x and 100x magnifications (Personal photo – 2025) 48
Figure 18 : Histogram of Biofilm Formation Levels among Oral and Vaginal Candida albicans Isolates..... 50
Figure 19 : Picture taken of the microplate showing the distribution of different concentrations of EEP and the CMIs 51
Figure 20 : A histogram showing EEP MICs on C.albicans..... 52
Figure 21 : Picture taken of cinnamon oil microdilution test showing the MICs..... 53
Figure 22 : Histogram showing Cinnamon oil MICs on C. albicans..... 54
Figure 23 : Picture taken of coconut oil microdilution test showing the MICs..... 55
Figure 24 : Histogram showing coconut oil MICs on C. albicans 56
Figure 25 : picture taken showing the MIC of coconut oil +cinnamon oil mix..... 57
Figure 26 : Histogram showing coconut oil +cinnamon oil's mix MICs on C.albicans 58
Figure 27 : Calibration C.albicans cell density using a densitometer (Personal photo – 2025)..... 73

Tables list:

Table 1 resistance mechanisms of C.albicans against antifungals 10
Table 2 final concentrations of coconut oil dilutions 37
Table 3 final concentrations of cinnamon oil dilutions 39
Table 4 final concentations of EEP diluitions 41
Table 5 final concentrations of the mix of cinnamon oil+coconut oil dilutions 44
Table 6 optical density results of biofilm formation assay 49
Table 7 Distribution of biofillm formation levels among oral and vaginal C.albicans isolates 49
Table 8 C.Albicans EEP MICs in mg/mL 51
Table 9 C.Albicans Cinnamon oil MICs in % 53
Table 10 C.Albicans Coconut oil MICs in % 55
Table 11 C.albicans coconut oil +cinnamon oil mix MICs in % 57

List of abreviations :

MIC/MICs Minimum Inhibitory Concentration(s)

C.albicans Candida Albicans

SDA: Saboureaud Dextrose Agar

SDB: Saboureaud Dextrose Broth

ECM: Extracellular Matrix

EEP: Ethanolic extract of propolis

PCR: Polymerase Chain Reaction

ITS: Internal Transcribed Spacer (genetic marker)

HWP1: Hyphal Wall Protein 1 gene

MALDI-TOF MS: Matrix-Assisted Laser Desorption/Ionization – Time-of-Flight Mass Spectrometry

CDR1/CDR2: Candida Drug Resistance genes (ABC efflux pumps)

MDR1: Multidrug Resistance 1 gene (MFS efflux pump)

FCY2/FCY1/FUR1: Genes related to 5-Flucytosine resistance (cytosine permease/deaminase/UMP pyrophosphorylase)

ERG11, ERG3, ERG5: Genes involved in ergosterol biosynthesis

FKS1, FKS2: Genes encoding β-1,3-glucan synthase subunits

ALS: Agglutinin-Like Sequence (adhesin gene family)

Als1/Als3: Specific adhesin proteins from the ALS family

Saps: Secreted Aspartyl Proteinases

ECM: Extracellular Matrix

ROS: Reactive Oxygen Species

CAPE: Caffeic Acid Phenethyl Ester

MCTs: Medium-Chain Triglycerides

CFU/mL: Colony Forming Units per Milliliter

Introduction

1. Background and Significance

Fungal infections are common diseases among humans, primarly affecting immunocompromised patients in which their immune system can't deal with excessive fungal proliferation all across the body **(Pfaller & Diekema, 2007; Brown et al., 2012).**

Specifically, *Candida Albicans* one of the most common opportunistic fungal pathogens which naturally resides on skin, mucosal surfaces and gastrointestinal tract but under certain conditions can have a range of pathogenicity from superficial skin issues to life threatening sytemic infections especially for hospitalized patients **(Calderone & Fonzi, 2001; Köhler et al., 2017)**.

Due to the increase of antifungal resistance of *C.albicans* against different classes of antifungal drugs, it becomes challenging to control and eradicate the infections and highlighted a need for alternative therapeutic approaches **(Pfaller et al., 2010; Perfect, 2017)**.

The pharmaceutical industry depends largely on natural products to develop new drugs, thus, the percentage of drugs originated from nature is 64% **(Newman & Cragg 2020)** So, nature had a major contribution in the development of innovative modern drugs offering a large reservoir of bioactive elements and phytochemicals. Therefore, it has become crucial to exploit all possibilities to find compounds with antifungal properties such as oils and extracts to overcome that drug resistance **(Cowan, 1999; Ahmad et al., 2011)**. This was due to the near absence of secondary effects, the rich sources, and the accessibility and the low costs especially in regions with limited access to conventional healthcare but mainly the reduced drug resistance by the fungal species. Also, people prefer natural products over synthetic ones.

Coconut oil is one of the major edible products derived from coconut fruit due to its high medium-chain fatty acids content such as lauric acid, monoglycerides, capric acid and caprylic acid **(Rajamohan, 2006 & Dayrit, 2015; Nevin)**, among others. Studies have revealed that

coconut oil possesses antifungal applications **(Arora et al., 2009)** anti-inflammatory and antioxidant **(Marina et al., 2009)**., and also antimicrobial **(Ogbolu et al., 2007)**.

Propolis is a sticky substance that bees gather from plants, composed of various active compounds like flavonoids and phenolic acids **(Bankova et al., 2000; Sforcin, 2007)**. It is known for its wide range of health benefits, including the ability to fight fungi, bacteria, reduce inflammation, and neutralize free radicals **(Marcucci, 1995; Kujumgiev et al., 1999)**. Various studies has shown the antifugual activity of propolis **(Wagh, 2013)**

(Bankova, V. et al. 2000) (Bruschi,. et al 2003) (Salomão, K. et *al.* 2005). This facts Making it a valuable natural option to help manage fungal infections and address antifungal resistance **(Salomão et al., 2004 ; Silva et al., 2012).**

Cinnamon oil is an essential oil extracted from the bark or leaves of *Cinnamomum* species, rich in bioactive compounds such as cinnamaldehyde, eugenol, and cinnamic acid **(Burt, 2004; Ranasinghe et al., 2013)**. It exhibits potent antimicrobial, antifungal, antioxidant, and anti-inflammatory properties **(Chang et al., 2001; Rao & Gan, 2014)**. Numerous studies have demonstrated the effectiveness of cinnamon oil and its components against fungal pathogens, including *C.albicans*, highlighting its potential as a natural alternative for antifungal therapy **(Hammer et al., 1999 ; Matan et al., 2006)**.

2. Research Objectives

The objectives of the present study were:

- To assess in vitro antifungal activity of propolis, cinnamon oil, and coconut oil against *C. albicans* clinical isolates through minimum inhibitory concentrations (MICs) determination of these natural compounds.
- To study potential synergistic effects when these kinds of natural products are used concurrently.
- To evaluate biofilm formation capacity

3. Thesis Structure

This thesis is organized into two main chapters:

- Chapter 1 provides a comprehensive overview of *C. albicans*, including its biological characteristics, epidemiology, pathogenicity, and clinical significance. This chapter also addresses the current challenges in managing *Candida* infections, particularly regarding antifungal resistance.
- Chapter 2 focuses on the natural products investigated in this study propolis, cinnamon oil, and coconut oil. Their origin, composition and allegation or therapeutics uses.
- Experimental Part the practical methodology used to investigate the antifungal activity of selected natural products. It includes details about the materials, sample collection, preparation of extracts, microbial strains, and the experimental procedures applied

Chapter 1 *C.Albicans:* General Characteristics and Clinical Significance

C.albicans is a microscopic unicellular yeast capable of forming filaments. Its cells are ovoid with a single bud and are generally several microns in diameter. In contrast, the filaments are elongated cells attached end to end. It is normally found in the human body in relatively limited amounts but can cause serious diseases, the frequency of which remains constant **(Pfaller et al. 2007)**.

C.albicans can transform between a variety of morphological types and phenotypes, and its colony morphology can vary from yeast-like to pseudohyphae and hyphae, realizing its transition from symbiotic to pathogenic state **(Tavanti et al., 2005).**

1. Classification

The *Candida* genus includes around 200 species, among which the most commonly associated with human pathology are: *C.albicans, C. tropicalis, C. glabrata, C. krusei, C. guilliermondii, C. parapsilosis, C. kefyr, and C. dubliniensis* **(Fitzpatrick et al., 2006)**.

From a taxonomic perspective, *Candida* species are classified among the Ascomycetes **(Krick et *al.,* 2008)**.

The taxonomic classification of *C.albicans* is as follows:

- Kingdom: *Fungi*
- Division: *Ascomycota*
- Class: *Saccharomycetes*
- Order: *Saccharomycetales*
- Family: *Saccharomycetaceae*

- Genus: *Candida*

- Species: *C.albicans*

2. Habitat and Distribution

C.albicans is a commensal yeast found in the digestive tract of humans, mammals, and birds. It is typically not found in the environment unless contamination occurs from humans or animals. This opportunistic yeast can become pathogenic under the influence of general or local predisposing factors **(Segal, 2005)**.

3. Morphology and Structural Characteristics

C.albicans is an aerobic, non-capsulated, and non-pigmented yeast. This diploid yeast reproduces asexually through multilateral budding from a mother cell known as a blastospore, leading to the formation of creamy white colonies **(Chu et al., 1993; Graser et al., 1996)**.

Three main morphological forms can be observed:

- The blastospore form (yeast), which is round or oval, measuring 2 to 4 µm, and sometimes displays a bud.
- The pseudomycelium form (pseudohyphae), composed of a chain of connected cells, measuring 500 to 600 µm in length and 3 to 5 µm in width **(Sudbery, 2004)**.
- The true mycelium (hupha), a filamentous structure specific to *C.albicans* (Gow, 2002).

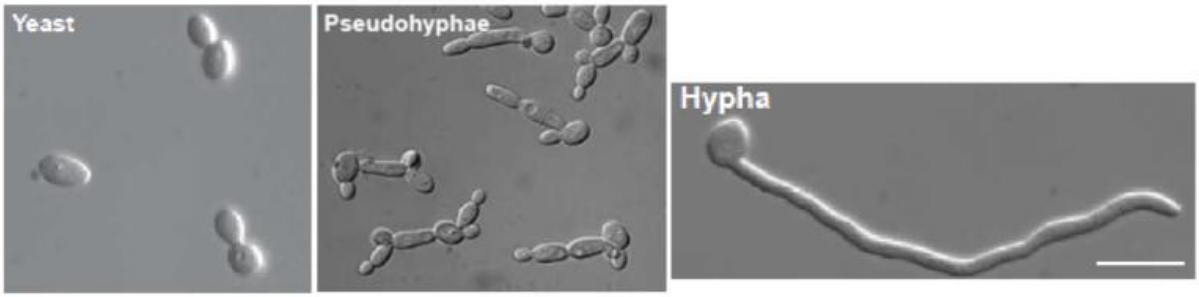


Figure 1 : The three different morphological states of *C. albicans* (Blanco, 2017).

The cell wall of *C.albicans* is a complex, multilayered structure composed primarily of:

- *β*-1,3 and *β*-1,6 glucans (approximately 60%)
- Chitin (1-2%)
- Mannoproteins (mannose-containing glycoproteins, 40%) **(Lenardon et al., 2010 ; Gow et al., 2017)**

This cell wall not only provides structural support but also plays a crucial role in host-pathogen interactions, adhesion to host tissues, and immune evasion **(Netea et al., 2008 ; Gow et al., 2017)**.

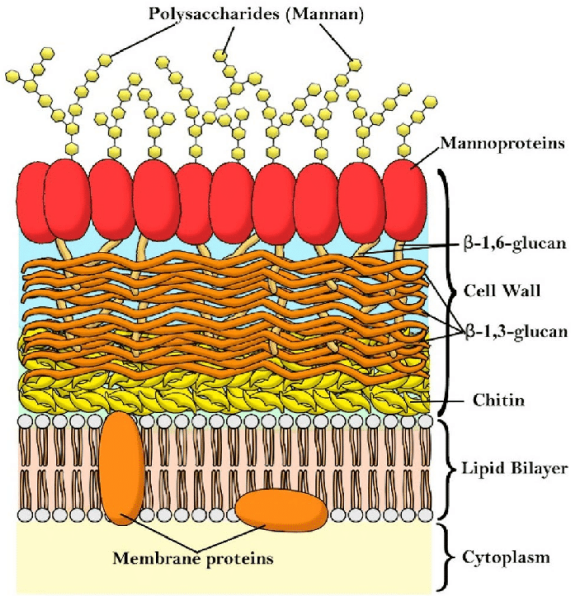


Figure 2 : Structural organization of the cell wall of *Candida albicans* (Aroso et Al. 2021).

3.1. Dimorphism

The ability of *C.albicans* to transition between yeast and hyphal forms is known as dimorphism **(Bachewich, 2007; Sudbery, 2011; Whiteway)**. This morphological switch is influenced by various environmental cues, including:

- Temperature (37°C promotes hyphal formation)
- pH (neutral to alkaline pH favors hyphal growth)
- Presence of serum or N-acetylglucosamine **(Lo et al., 1997;Sudbery, 2011).**
- Carbon dioxide concentration
- Nutrient limitation

4. Cultural Characteristics

4.1. Growth Requirements

C.albicans is a facultative anaerobe that grows optimally at 37°C **(Pappas et al., 2016)**. It can utilize various carbon sources and can grow across a wide pH range (2.0-8.0), although it prefers slightly acidic conditions (pH 5.4-6.0) **(Odds, 1988; Pfaller & Diekema, 2007)**.

4.2. Colony Morphology

On Sabouraud Dextrose Agar (SDA), *C.albicans* typically forms smooth, creamy, white to cream-colored colonies with a characteristic yeasty odor after 24-48 hours of incubation at 30-37°C **(Odds, 1988; Pappas et al., 2016)**.

4.3. Growth Media

Common media used for the isolation and cultivation of *C.albicans* include:

- Sabouraud Dextrose Agar (SDA)
- Sabouraud Dextrose Broth (SDB) (for susceptibility testing)
- Mueller Hinton Broth (for susceptibility testing)
- CHROMagar

- Sabouraud Chloramphenicol Agar: **Chloramphenicol**: An antibiotic added to suppress bacterial contaminants, making the medium selective for fungi like C.albicans **(Pappas et al., 2016)**.
- Cornmeal Agar
- Candida chromogenic medium (HaiBo) **(Odds, 1988; Pfaller & Diekema, 2007)**
- RPMI 1640 medium (for susceptibility testing)

4.4. Identification Methods

Methylene Blue Staining

- Observation of Yeast Morphology:
- This staining technique allows for the visualization of yeast cell morphology, including budding forms, under a microscope. This is valuable for assessing the growth and reproductive status of *C.albicans* populations **(Matsumoto, et al. 2022)**.
- Differential Staining of Live and Dead Cells :
- Methylene blue is commonly used to differentiate between live and dead yeast cells, including *C.albicans*. Live cells possess active reductase enzymes that reduce methylene blue to a colorless form, rendering them unstained or lightly stained. In contrast, dead cells lack this enzymatic activity and thus absorb the dye, appearing dark blue under microscopic examination **(Takagi, et al. 2000)**.

Germ Tube Test

The germ tube test is a rapid and cost-effective method for the identification of *C.albicans*, based on its ability to form germ tubes filamentous outgrowths when incubated in serum at 37°C for 2 to 3 hours. This morphological feature distinguishes *C.albicans* from most other Candida species, which do not produce germ tubes under these conditions **(Tankeshwar, A. 2015)**.

In practice, a small inoculum of yeast is suspended in 0.5 mL of human or bovine serum and incubated at 37°C. After 2 to 4 hours, a drop of the suspension is examined microscopically for

the presence of germ tubes—tube-like projections extending from the yeast cells without constriction at their origin **(Tankeshwar, A. 2015)**.

Sugar fermentation (Candida API gallery)

To identify *C.albicans* using sugar fermentation, the classical method used is the carbohydrate fermentation test, which evaluates the organism's ability to ferment specific sugars and produce acid and/or gas. **(Larone, D. H. 2011)**

Biomol identification (PCR)

PCR is a molecular biology technique used to amplify specific DNA sequences of an organism. To identify *C.albicans*, PCR targets unique genetic markers in its DNA (such as the ITS region or the HWP1 gene) (**Mirhendi, H. et al. 2006)**. Among its properties it is:

- Fast and highly specific
- Can distinguish *C.albicans* from similar species like *C. dubliniensis*
- Requires only a small DNA sample (**Romeo, O. & Criseo, G. 2009)**

Maldi-toff-ms identification

MALDI-TOF MS stands for:

- Matrix-Assisted Laser Desorption/Ionization
- Time-of-Flight Mass Spectrometry

It's a rapid, accurate, and modern technique used in microbiology labs to identify microorganisms (bacteria, yeasts, fungi) by analyzing their protein profile, mainly ribosomal proteins.

4.5. Antifungal Susceptibility

4.5.1. Mechanisms of Action of Antifungal Agents and *C. albicans* resistance mechanisms

This table presents a comprehensive overview of the primary antifungal resistance mechanisms identified in *Candida albicans*, alongside the most commonly used antifungal agents and their estimated resistance rates

Table 1 resistance mechanisms of C.albicans against antifungals

Antifungal Class	Action mechanism	Resistance Mechanisms of *Candida albicans*	Resistance Rate
Polyenes : Amphotericin	Amphotericin B binds to ergosterol in the fungal cell membrane, creating pores that lead to leakage of cellular contents and eventual cell death	- *Alterations in ergosterol biosynthesis* (e.g., mutations in *ERG3*, *ERG5*) lead to reduced ergosterol levels, the primary binding site of polyenes, reducing drug efficacy *(Cuenca-Estrella, 2004; Perlin, 2015)*	<1–2% ***(Cuenca-Estrella, 2004)***
Echinocandins : Caspofungin, Micafungin, Anidulafungin	inhibit the synthesis of β-1,3glucan, an essential component of the fungal cell wall	- *Mutations in FKS1 and FKS2* genes (encoding subunits of β-1,3-glucan synthase) reduce the binding affinity of echinocandins, impairing inhibition of fungal cell wall synthesis *(Perlin, 2015)*	1–3% ***(Perlin, 2015)***
Nucleoside analogs : 5-Flucytosine (5-FC)	Disrupt DNA and RNA synthesis by acting as antimetabolites, leading to cell cycle arrest	- *Mutations in FCY2* (cytosine permease), *FCY1* (cytosine deaminase), or *FUR1* (UMP pyrophosphorylase) prevent drug uptake or activation, leading to resistance *(Vermes et al., 2000)*	4–10%, especially with monotherapy ***(Vermes et al., 2000)***
Azoles : Fluconazole, Itraconazole, Voriconazole	inhibit the enzyme lanosterol 14-demethylase, disrupting ergosterol biosynthesis and compromising membrane integrity	- *Point mutations in ERG11* gene alter the structure of the target enzyme (lanosterol 14α-demethylase), decreasing drug binding affinity - *Overexpression of ERG11* leads to increased production of the enzyme, requiring higher drug concentrations - *Efflux pumps* (*CDR1, CDR2, MDR1*) actively export the drug out of the cell, reducing intracellular drug concentration *(Arendrup & Patterson, 2017; Perlin, 2015)*	5–10%, up to 25% in hospitals ***(Arendrup & Patterson, 2017)***

4.5.2. Virulence Factors

C.albicans has a remarkable ability to adapt and can become pathogenic when environmental conditions are favorable. The transition from a saprophytic to a pathogenic state involves the activation of various virulence factors, including adhesion to host cells, morphological polymorphism, and the ability to form biofilms.

I. Morphological Transition or Dimorphism

One of the major virulence factors of *C.albicans* is its ability to alter its morphology and grow in three distinct forms: yeast (blastospore), filamentous (hypha or pseudohypha), and chlamydospore.

This morphological transition, known as dimorphism, is considered a survival strategy that enables the fungus to spread within the host **(Nobile et al., 2017)**.

According to the literature, the hyphal form is involved in tissue penetration and immune evasion, whereas the yeast (blastospore) form is better suited for dissemination through the bloodstream and the initiation of infection.

Additionally, hyphae are inherently invasive and capable of detecting and following surface irregularities such as grooves or pores, allowing directed growth along solid substrates or mucosal surfaces — a phenomenon known as thigmotropism **[(Noble et al., 2010); (Pande et al., 2017)].**

The hyphal and yeast (blastospore) forms are for the virulence of *C.albicans*. The yeast-to-hyphal transition is regulated by multiple signaling pathways, including the cAMP-PKA and MAPK cascades **(Hazan et al., 2002; Bahn et al., 2007).**

In contrast, the role of chlamydospores remains unclear and appears to be of little or no significance in the pathogenicity of this yeast **(Kumamoto and Vinces, 2005).**

II. Adhesin Production on the Cell Surface

Adherence to biotic surfaces (such as tissues and mucous membranes) and abiotic surfaces (like catheters and probes) is a critical step in the colonization process by any pathogen.

In *C.albicans*, this key step involves both non-specific adhesion mechanisms — including electrostatic forces and Van der Waals interactions — and specific ligand–receptor interactions with mannoproteins present in the yeast cell wall.

These specific interactions are mediated by adhesins, notably those encoded by the ALS (Agglutinin-Like Sequence) gene family, which code for surface glycoproteins **(Jacobsen et al., 2012).**

Among the most studied adhesins are Als1 and Als3, which enable *C.albicans* to adhere to endothelial and epithelial cells. It is noteworthy that Als1 expression is influenced by the composition of the surrounding medium, whereas Als3 expression is specific to the pseudohyphal and hyphal forms and is absent in the yeast form **(Liu and Filler, 2011).**

III. Secretion of Hydrolytic Enzymes

The secretion of hydrolytic enzymes by *C.albicans* during infection significantly enhances its virulence by degrading host mucosal surfaces and compromising immune defenses.

Three main types of hydrolases are secreted by this yeast: aspartyl proteinases (Saps), phospholipases, and lipases **(Wächtler et al., 2012).**

IV. Biofilm Formation

The concept of biofilm was introduced in 1978 to describe a structured community of microorganisms embedded within a hydrated matrix composed mainly of extracellular polymers, and attached to a surface [1]. Today, the term refers to various microbial assemblies that exhibit distinct biological behaviors compared to free-floating, individual cells in suspension **(Lebeaux, & Ghigo, 2012).**

Our knowledge of the ultrastructure of *C.albicans* biofilms was first established through scanning electron microscopy, which revealed the presence of yeast cells, pseudohyphae, and hyphae within the biofilm matrix **(Hawser and Douglas, 1994)**. Subsequent investigations **(Hawser and Douglas, 1994; Baillie and Douglas, 1999; Chandra et al., 2001; Ramage et al., 2001; Douglas, 2003)** have further demonstrated that in vitro biofilm development follows three fundamental stages:

1. initial adhesion and colonization of yeast cells onto a surface,
2. proliferation and expansion of these cells to form a basal layer of anchoring cells,
3. the emergence of pseudohyphae and extensive hyphal growth, accompanied by the production of extracellular matrix components **(Finkel, & Mitchell. 2011)**.

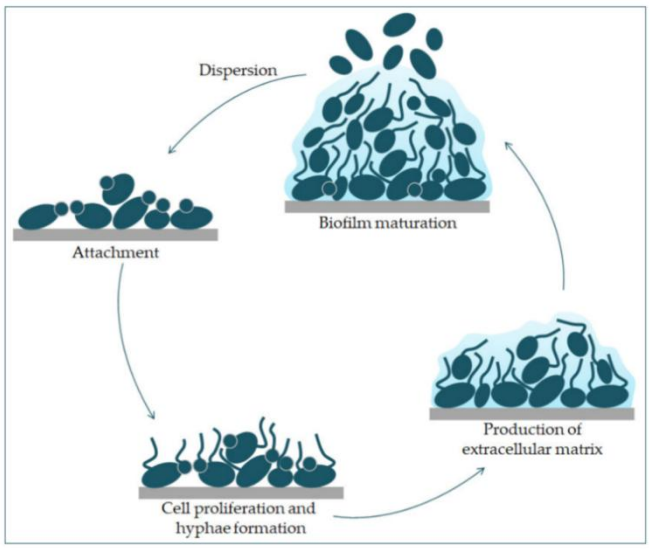


Figure 3 : *Stages of Candida albicans Biofilm Development (Talapko et al., 2021)*

Biofilms resistance mecanisms :

C.albicans cells in their sessile, biofilm-associated form exhibit significantly greater resistance to antifungal agents compared to their planktonic counterparts. This multifactorial resistance is attributed to both fundamental physical barriers and intricate regulatory mechanisms **(Ramage et al., 2012)**.

A. Extracellular Matrix (ECM) as a Physical Barrier:

The extracellular matrix (ECM) of *C.albicans* biofilms is primarily composed of β-1,3-glucans, mannans, proteins, lipids, and extracellular DNA. It serves as a crucial physical barrier that impedes the penetration of antifungal agents toward fungal cells. Additionally, the glucans within the matrix can sequester antifungals, thereby reducing their availability to effectively reach and act on their cellular targets **(Gulati, M., & Nobile, C. J. 2016).**

B. Overexpression of efflux pumps:

Efflux pump genes, such as CDR1, CDR2 (from the ABC family), and MDR1 (from the MFS family), are highly overexpressed in *C.albicans* biofilms **(Imran et al., 2023)**. These pumps actively expel antifungal agents out of the cells, thereby lowering their intracellular concentration and contributing to antifungal resistance, especially to azoles **(Cavalheiro & Teixeira, 2021).**

C. Persister Cells:

Within biofilms, a subpopulation of cells called persister cells enter a metabolically dormant state. Due to their significantly reduced metabolic activity, these cells exhibit a high level of tolerance to antifungal treatments, as they are less affected by the mechanisms through which these drugs act **(LaFleur et al., 2006).**

D. Alterations in ergosterol biosynthesis:

Changes in the ergosterol biosynthesis pathway, including mutations in the ERG11 and FKS1 genes, contribute to reduced susceptibility of biofilms to membrane-targeting antifungal drugs like azoles and echinocandins. By lowering the ergosterol content in the cell membrane, these genetic alterations impair the effectiveness of these antifungal agents **(Kelly et al., 1995; Perlin, 2015).**

E. Cell density:

The high cell density of a mature biofilm can act as a physical barrier, protecting the cellular community from environmental stresses **(Ramage et al., 2012).** This increased density leads

to nutrient limitation and a slower growth rate of cells within the biofilm. Sessile cells, in particular, exhibit very slow growth, which reduces their susceptibility to antifungal agents (**Mah and O'Toole, 2001).**

F. Specific Genetic Regulation in Biofilms:

Biofilm formation triggers specific genetic regulation, activating genes associated with antifungal resistance. For instance, the increased expression of the FKS1 gene is involved in the synthesis of β-1,3-glucans, which strengthens the extracellular matrix and contributes to biofilm resistance **(Nobile et al., 2006).**

5. *albicans* -Associated Infections

5.1. Mucocutaneous Candidiasis

- **Oropharyngeal Candidiasis (Thrush)**

Characterized by white, curd-like lesions on the oral mucosa, tongue, and pharynx. Common in neonates, the elderly, and immunocompromised individuals **(Pappas et al., 2016).**

- **Vulvovaginal Candidiasis**

Affects approximately 75% of women at least once in their lifetime, presenting with pruritus, irritation, and characteristic white discharge **(Pappas et al., 2016).**

- **Cutaneous Candidiasis**

Includes intertrigo, paronychia, and onychomycosis, typically occurring in warm, moist areas of the skin. Transmission from mother to infant at birth may occur when the mother is colonized, contributing to early immune system education **(Willinger et al., 1994).**

5.2. Invasive Candidiasis

- **Candidemia**

The presence of *Candida* species in the bloodstream, associated with significant morbidity and mortality, especially in critically ill patients **(Pappas et al., 2016).**

- **Disseminated Candidiasis**

Involves deep-tissue invasion, potentially affecting multiple organs including the kidneys, liver, spleen, and brain **(Pappas et al., 2016).**

5.3. Risk Factors for Candida Infections

- Immunosuppression (HIV/AIDS, chemotherapy, organ transplantation)
- Broad-spectrum antibiotic use
- Diabetes mellitus
- Indwelling medical devices (catheters, prosthetic joints)
- Extended hospitalization, especially in intensive care units
- Extremes of age (neonates and elderly)
- Pregnancy
- Disruption of normal microbiota

Chapter 2: Natural Products and Their Antifungal Activities Against *C.albicans*

Introduction

Natural products have been integral to traditional medicine systems worldwide for millennia. In many African regions, particularly in West Africa's Fouta and Fouladou areas, communities have long relied on empirical knowledge passed down through generations to manage a variety of ailments, including fungal infections. Traditional healers have historically utilized plant extracts, resins, and vegetable oils as therapeutic agents to treat conditions linked to opportunistic pathogens, reflecting a rich pharmacopeia based on local biodiversity and cultural heritage **(Diallo et al., 1999 ; Abo et al., 2008).**

The global rise of antifungal resistance among pathogenic yeasts has become a significant public health concern, especially due to the limited arsenal of effective antifungal drugs and the adverse effects associated with their prolonged use **(Arendrup & Patterson, 2017)**. This challenge has renewed scientific interest in identifying natural compounds with antifungal potential that could serve as alternatives or adjuncts to conventional therapies.

Among these natural products, coconut oil, cinnamon oil, and propolis have garnered considerable attention due to their documented antimicrobial activities in various in vitro studies. Their bioactive constituents have demonstrated inhibitory effects against a range of microorganisms, including fungal pathogens **(Kujumgiev et al., 1999 ; Ogbolu et al., 2007; Tabassum & Vidyasagar, 2013)**. Nonetheless, systematic evaluation of their antifungal efficacy specifically against *C.albicans* remains crucial to substantiate their therapeutic applicability.

This chapter focuses on the in vitro assessment of the antifungal activity of coconut oil, cinnamon oil, and propolis extract against *C.albicans*.

1. Cinnamon Oil

1.1.Botanical and Geographical Origin of Cinnamon

Cinnamon is a spice derived from the inner bark of several tree species belonging to the genus Cinnamomum, part of the Lauraceae family. The most renowned variety is Ceylon cinnamon (Cinnamomum verum or C. zeylanicum), which originates mainly from Sri Lanka (formerly Ceylon), southern India, Bangladesh, and Myanmar. Other species such as Cinnamomum cassia (China), C. burmannii (Indonesia), and C. loureiroi (Vietnam) are also cultivated and sold under the name cinnamon, although they are generally considered of lower quality compared to Ceylon cinnamon **(Ravindran et *al*., 2004)**.



Figure 4 : *Cinammon quills photo example*

1.2.Historical Use of Cinnamon

The medicinal use of cinnamon dates back to ancient times, where it was employed in traditional Chinese and Ayurvedic medicine to treat various diseases, including skin and digestive infections **(Al-Hatamleh et al., 2021)**. However, it was only in the 20th century that modern science began rigorously studying its antifungal properties **(Pinto et al., 2009)**. Interest in cinnamon oil notably grew during the 1990s as resistance to conventional antifungals

increased **(Al-Hatamleh et al., 2021)**. Studies revealed that bioactive compounds in cinnamon, especially cinnamaldehyde and eugenol, inhibit the growth of pathogenic fungi **(Singh et al., 2007)**. Since then, numerous scientific publications have confirmed the effectiveness of cinnamon oil in preventing biofilm formation and disrupting yeast cell membranes **(Pinto et al., 2009)**. This has paved the way for natural therapeutic applications against fungal infections **(Al-Hatamleh et al., 2021).**

1.3. Chemical Composition and Active Principles of Cinnamon

Cinnamon oil is particularly rich in cinnamaldehyde, the main compound extracted primarily from the bark, making up 50 to 75% of the oil's composition. This compound gives cinnamon its distinctive aroma and exhibits strong antimicrobial and antifungal activity. Eugenol, predominantly found in the oil extracted from leaves, is another major component with antiseptic, antifungal, and anti-inflammatory properties **(Al-Hatamleh et al., 2021).**

The oil also contains smaller amounts of other compounds contributing to its therapeutic effects, including linalool, β-caryophyllene, benzaldehyde, coumarin, methyleugenol (estragole), and various terpenes, phenols, alcohols, and esters. These substances often act synergistically to enhance the antifungal, antibacterial, and antioxidant properties of the essential oil **(Singh et al., 2007).**

1.4. Mechanism of Action of Cinnamon Oil Against *C.albicans*

Cinnamon oil targets *C.albicans* mainly through its bioactive compounds, notably cinnamaldehyde and eugenol, which affect the fungal cell membrane. Cinnamaldehyde disrupts membrane integrity by altering its permeability, causing leakage of essential intracellular components such as ions, amino acids, and proteins. This osmotic imbalance leads to cell lysis and fungal death **(Pinto et al., 2009).**

Beyond membrane disruption, cinnamon oil interferes with the yeast's energy metabolism. Cinnamaldehyde inhibits key enzymes involved in mitochondrial respiration, reducing ATP production and causing intracellular oxidative stress. This stress is further intensified by the

generation of reactive oxygen species (ROS), which damage fungal proteins, lipids, and DNA **(Shreaz et al., 2016).**

Additionally, the essential oil inhibits biofilm formation—a critical virulence factor of *C.albicans*. Cinnamaldehyde and eugenol downregulate genes responsible for adhesion and morphogenesis, such as ALS3 and HWP1, thereby preventing the morphological transition to the hyphal form necessary for tissue invasion. This limits the fungus's ability to adhere firmly to surfaces and establish persistent colonies **(Jafri et al., 2020).**

Moreover, cinnamon oil has been shown to enhance the effectiveness of conventional antifungals like fluconazole. By disrupting the fungal membrane, it facilitates drug penetration into the fungal cell, reducing required doses and lowering the risk of resistance development **(Matan et al., 2006).**

1.5. Applications of Cinnamon Oil

- **Medicinal Use:** Cinnamon oil is applied as a natural antifungal agent against various pathogenic strains, especially *C.albicans*. Its active compounds disrupt fungal membranes, inhibit biofilm formation, and cause cytoplasmic leakage **(Pinto et al., 2009).** The oil also demonstrates antibacterial and antiviral activities against pathogens such as Staphylococcus aureus, E. coli, and certain viruses due to its lipophilic properties which damage microbial membranes **(Lopez et al., 2005).**
- **Food Industry:** It serves as a natural preservative to inhibit mold and bacterial growth in foods, thereby extending shelf life **(Matan et al., 2006).**
- **Cosmetics and Hygiene:** The oil is incorporated into soaps, toothpastes, and lotions for its antiseptic, aromatic, and toning qualities, contributing to oral hygiene and prevention of skin infections **(Burt, 2004).**

1.6. Allergenicity and Tolerance of Cinnamon Oil

- **Skin sensitivity**: Cinnamon oil contains *cinnamaldehyde*, a known skin irritant that can cause contact dermatitis or allergic reactions if applied undiluted **(Goins et al., 2015).**

- **Respiratory irritation**: Inhalation of concentrated cinnamon oil vapors may trigger coughing, bronchial irritation, or asthma attacks in sensitive individuals **(Kimbarowski & Mokrow, 1963).**
- **Potential hepatotoxicity**: High intake of cinnamon oil, particularly *Cinnamomum cassia*, may cause liver damage due to its coumarin content **(Abraham et al., 2010).**
- **Contraindications**: Not recommended for use during pregnancy, in young children, or in individuals with liver conditions without medical supervision.

2. Propolis

2.1 Botanical and Geographical Origin of Propolis

Propolis is a natural resinous substance produced by bees from exudates (resins, gums, and balsams) collected from the buds, bark, or other parts of specific plants. Bees mix these plant materials with wax, enzymes, and pollen to form a sticky compound that is used to seal cracks in the hive, sterilize the internal environment, and protect against pathogens **(Sforcin & Bankova, 2011).**

The botanical origin of propolis depends heavily on the surrounding vegetation. In temperate regions such as Europe, the primary plant sources include poplars (Populus spp.), birches (Betula spp.), and elms (Ulmus spp.). In tropical regions like Brazil, the main sources are plants such as Baccharis dracunculifolia, Clusia spp., and Dalbergia ecastaphyllum **(Bankova et al., 2014).**

From a geographical perspective, the chemical composition of propolis varies considerably depending on the collection region. For example, South American propolis (from Brazil and Venezuela) is rich in artepillin C and prenylated acids, while European propolis is dominated by flavonoids such as pinocembrin and galangin. This botanical diversity results in significant differences in the chemical makeup and biological activity of propolis based on its geographical origin **(Salatino et al., 2011).**



Figure 5 *: Propolis photo example*

2.2 Historical Use of Propolis as an Antifungal

Propolis has been used since ancient times for its antifungal properties. The ancient Egyptians employed it in the mummification process due to its preservative effects, which helped prevent tissue degradation caused by microorganisms. This practice suggests an empirical understanding of its antifungal and antiseptic properties **(Wagh, 2013**). Later, the Greeks and Romans applied propolis to wounds and skin infections, which likely included fungal infections caused by organisms such as Candida spp. **(Sforcin & Bankova, 2011)**. In traditional Chinese medicine, it was used to treat oral and throat infections—areas often affected by pathogenic yeasts like *C.albicans* **(Huang et al., 2014)**. In Eastern Europe, rural populations used propolis topically for foot fungus and nail fungal infections, long before the development of modern antifungal treatments **(Kuropatnicki et al., 2013)**. These traditional uses are now supported by scientific research, which attributes the antifungal activity of propolis to active compounds such as flavonoids, phenolic acids, and CAPE, which disrupt fungal cell membranes and inhibit their growth **(Sforcin & Bankova, 2011; Huang et al., 2014).**

2.3 Chemical Composition and Active Principles of Propolis

Propolis is a naturally complex substance composed of more than 300 identified chemical constituents to date. Its exact composition varies depending on the plant species available around the hive, as well as geographic and seasonal conditions. It contains a high proportion of

flavonoids (such as pinocembrin, galangin, kaempferol, quercetin, and apigenin), which are widely present in temperate-region propolis. It also contains phenolic acids (such as caffeic acid, ferulic acid, p-coumaric acid, and cinnamic acid) and their esters (notably CAPE – caffeic acid phenethyl ester), which are very characteristic phenolic compounds found in propolis.

Propolis also contains terpenes (monoterpenes, sesquiterpenes, and diterpenes), aliphatic and aromatic hydrocarbons, fatty acids, steroids, alcohols, amino acids, vitamins (notably B1, B2, B6, C, and E), and trace elements such as iron, zinc, copper, manganese, and selenium. In addition to these major components, it may also contain waxes, pollen, and volatile compounds (essential oils) that contribute to its organoleptic properties (smell and taste). The richness in flavonoids is typical of European propolis, whereas Brazilian green propolis is particularly rich in artepillin C and hydroxycinnamic acids derived from Baccharis dracunculifolia.

This great biochemical diversity gives propolis highly variable chemical profiles depending on its origin, but also makes it a versatile natural resource in pharmacology **(Lotfy, 2006 ; Salatino et al., 2011 ; Bankova et al., 2014; Huang et al., 2014).**

2.4 Mechanism of Action of Propolis Against *C.albicans*

Propolis exhibits antifungal activity against *C.albicans* through multiple synergistic mechanisms, primarily involving its active compounds such as flavonoids, phenolic acids, and derivatives like caffeic acid phenethyl ester (CAPE). These components disrupt the fungal cell

membrane integrity, causing leakage of intracellular contents and compromising membrane stability **(Lotfy, 2006 ; Sforcin & Bankova, 2011).**

Moreover, propolis interferes with the synthesis of ergosterol, a critical element of the fungal membrane, leading to further destabilization. It also effectively inhibits biofilm formation, which is a protective mode of growth that makes *C.albicans* more resistant to conventional antifungal agents. By preventing the adhesion of fungal cells to surfaces and blocking the development of hyphae (filamentous structures involved in tissue invasion), propolis reduces fungal virulence **(Koru et al., 2007).**

Additionally, CAPE and other polyphenols in propolis contribute to the inhibition of virulence-related enzymes, such as proteinase and phospholipase, which are crucial for *C.albicans* pathogenicity **(Silici et al., 2005)**. Propolis also displays immunomodulatory properties, enhancing the host's immune response by activating macrophages and modulating cytokine production **(Sforcin, 2007).**

2.5 Applications of Propolis:

- Propolis has long been used in traditional medicine due to its antimicrobial, antifungal, antiviral, anti-inflammatory, and wound-healing properties **(Sforcin & Bankova, 2011)**.
- It is commonly applied to treat oral conditions such as gingivitis, stomatitis, oral candidiasis, and aphthous ulcers **(Wagh, 2013).**
- Propolis is also incorporated into cosmetic and dermatological products, including creams and ointments designed for healing burns and wounds **(Lotfy, 2006)**.
- In dentistry, it serves as a disinfectant for root canals and helps reduce the formation of bacterial biofilms **(Sforcin & Bankova, 2011).**
- It is found in dietary supplements aimed at enhancing the immune system **(Wagh, 2013).**
- Additionally, propolis has been investigated for its potential anticancer effects and immunomodulatory benefits, particularly in immunocompromised **individuals (Lotfy, 2006).**
- Various pharmaceutical formulations use propolis, such as mouthwashes, eye drops, syrups, and capsules **(Sforcin & Bankova, 2011).**

2.6 Allergenicity and tolerance of Propolis

- **Allergic reactions**: Propolis is rich in flavonoids and plant resins and may cause contact allergies, especially in individuals sensitive to bee products **(Castaldo & Capasso, 2002)**.
- **Oral irritation**: Frequent use of oral propolis preparations may lead to mouth ulcers, itching, or mucosal irritation in some users **(Kędzia, 2008).**
- **Lack of standardization**: The chemical composition varies greatly depending on geography and local flora, making dosage and safety guidelines difficult to establish **(Bankova et al., 2014).**
- **Drug interactions**: Propolis may interact with anticoagulants or immunosuppressants, requiring caution in patients taking such medications **(Sforcin, 2007).**

- **Limited clinical evidence**: Despite promising in vitro data, several of its medical applications lack strong clinical validation.

3. Coconut oil

3.1.Botanical and Geographical Origin of Coconut oil

Coconut oil is extracted from the fruit of the coconut palm tree (Cocos nucifera), which belongs to the Arecaceae family **(Lebrun & Laude, 1985).**

The coconut palm is native to tropical coastal regions, with its botanical origin believed to be in Southeast Asia, particularly the Malay Archipelago, Indonesia, the Philippines, Malaysia, and possibly New Guinea **(Liyanage et al., 2016).**

Some older theories suggested a South American origin; however, recent genetic and botanical studies favor an Indo-Pacific origin **(Ravindran, 2008 ; Liyanage et al., 2016)**.

Geographically, coconut palms grow primarily in tropical coastal areas around the world, especially in countries such as the Philippines, Indonesia, India, Sri Lanka, and Pacific Islands, where coconut oil production is a traditional practice **(Ravindran, 2008).**

The coconut fruit's natural buoyancy allows it to disperse via ocean currents, aiding the spread of coconut palms before human trade further extended its geographical range **(Lebrun & Laude, 1985; Ravindran, 2008).**



Figure 6 : *Coconut oil photo example*

3.2.Historical Use of Coconut oil

Coconut oil has been used for generations in traditional medicine systems throughout tropical regions, including Southeast Asia, the Pacific Islands, and parts of India, where it was applied to treat a variety of skin and mucosal infections believed to be of fungal origin **(Verallo-Rowell et al., 2008)**. Its use was common in rural communities that relied on plant-based remedies, where coconut oil served not only for skin care but also in managing infections affecting the mouth, scalp, feet, and genital areas. With the advancement of scientific research, interest in its antifungal properties has grown, particularly concerning its application in health care and alternative medicine. In modern studies, coconut oil has been evaluated for its ability to combat various fungal strains, including its documented effectiveness against *C.albicans* in both laboratory and clinical contexts. It has been reported to reduce fungal growth and is being explored as a supportive treatment in managing fungal overgrowth in the oral cavity, vaginal tract, and on the skin **(Hilmarsson et al., 2007; Shilling et al., 2013; Muthukumaran et al., 2016).** Its appeal lies in its natural origin, accessibility, safety profile, and growing evidence supporting its role in antifungal therapy, making it a subject of increasing attention in integrative medical approaches **(Sweeney et al., 2002 ; Dayrit, 2015).**

3.3.Chemical Composition and Active Principles of Coconut oil

The coconut (Cocos nucifera) has a rich and varied chemical composition, which depends on the part of the fruit used (pulp, water, oil, milk). The dried pulp (copra), used to produce coconut

oil, contains about 60 to 70% lipids, mainly in the form of medium-chain triglycerides (MCTs). The most abundant fatty acids include lauric, myristic, capric, caprylic, and palmitic acids **(Dayrit, 2015)**. Lauric acid, which accounts for approximately 45 to 53% of the total fatty acids in coconut oil, is considered the main active compound, known for its antimicrobial, antifungal, and antiviral properties. In addition to fatty acids, coconut oil also contains polyphenols, tocopherols (vitamin E), phytosterols, and antioxidant compounds, which contribute to its health benefits and oxidative stability **(Nevin & Rajamohan, 2004)**. Coconut water, on the other hand, is rich in electrolytes (potassium, sodium, calcium, magnesium), amino acids, natural sugars (glucose, fructose, sucrose), and antioxidants, making it a naturally hydrating and nutritious beverage **(Campbell-Falck et al., 2000).** These elements make coconut a valuable functional resource in food, traditional medicine, and cosmetics.

3.4. Mechanism of Action of Coconut oil Against *C.albicans*

Coconut oil exhibits strong antifungal properties against *C.albicans*, primarily due to its high content of medium-chain fatty acids—particularly lauric, capric, and caprylic acids. These fatty acids disrupt the fungal cell membrane by altering its lipid composition, which compromises membrane integrity, leads to intracellular leakage, and ultimately causes fungal cell death **(Ogbolu et al., 2007; Muthukumaran et al., 2016)**. Additionally, research has shown that coconut oil can inhibit the growth of *C.albicans* biofilms, which are critical for the fungus's resistance and persistence in infections **(Shilling et al., 2013)**. The oil also reduces the fungus's ability to adhere to host cell surfaces, thereby limiting its tissue invasion. Together, these mechanisms support the growing use of coconut oil as a promising natural alternative or supplement to conventional antifungal treatments.

3.5.Applications of Coconut Oil:

- Skin care: Used as a natural moisturizer, it improves skin elasticity and helps treat eczema, dermatitis, and skin irritations **(Verallo-Rowell et al., 2008).**
- Hair care: Helps reduce protein loss and nourishes dry or damaged hair **(Rele & Mohile, 2003).**
- Dietary use: Rich in medium-chain triglycerides (MCTs), it is used in cooking and may help boost energy metabolism **(Dayrit, 2015).**

- Antimicrobial properties: Effective against various bacteria, viruses, and fungi, including *C.albicans* **(Ogbolu et al., 2007).**
- Traditional medicine: Used in many cultures to treat skin infections, wounds, and to support the immune system **(DebMandal & Mandal, 2011).**
- While it has moisturizing and antifungal properties, topical coconut oil may have limited penetration into deeper skin layers **(Toba et al., 2013).**

3.6.Allergenicity and Tolerance of coconut oil

- **Rare allergic reactions**: Coconut oil is generally well tolerated, but some individuals may develop mild allergic skin reactions, such as itching or rash **(Agero & Verallo-Rowell, 2004).**
- **Food allergy risk**: Though rare, true coconut protein allergy has been reported in children and adults, especially with ingestion **(Kabir et al., 2019).**
- **Limited systemic absorption**: While it has moisturizing and antifungal properties, topical coconut oil may have limited penetration into deeper skin layers **(Toba et al., 2013).**
- **Caution in special populations**: Though generally safe, care is advised when using in infants or individuals with atopic dermatitis due to possible sensitivity.

Material And Methods

A. Material

1. Vegetable material:

- **Coconut oil:** was purchased from a local commercial market in Medea, Algeria.
  It was labeled for cosmetic/culinary use and stored at room temperature in a sealed container until use.
- **Cinnamon oil:** was obtained from a local herbal market in Ain Bassam, Bouira, Algeria.
  The product was natural and used as received, without further purification.



Figure 7 : *Commercially purchased pure cinnamon oil (Personal photo – 2025)*

- **Ethanolic extract of propolis:** the extract was kindly provided by our supervisor, Mr. Fodhil Mohamed.
  Briefly, crude propolis was collected from an apiary in Djelfa Province, Algeria. The ethanolic extraction was performed by macerating the crude propolis in 80% ethanol for 7 days at room temperature, with daily shaking. The mixture was then filtered, and the solvent was evaporated under reduced pressure using a rotary evaporator (Rotavap) at 40°C. The resulting extract was stored in a dark container at 4°C until use.

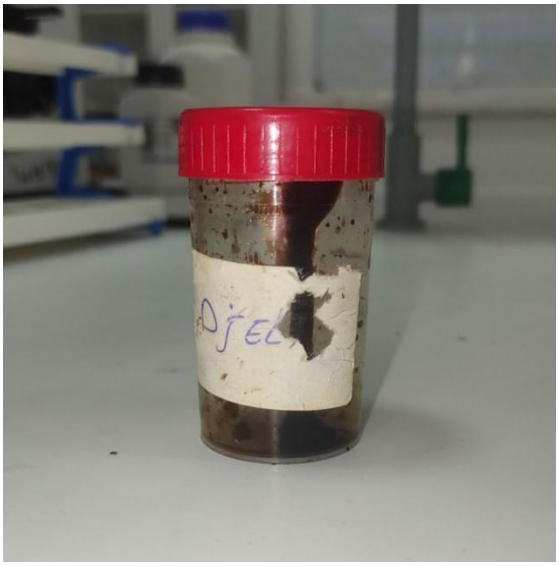


Figure 8 : *Ethanolic extract of propolis*

2. Biological Material

A total of seven (7) isolates of *C.albicans* were used in this study.

- Four isolates were obtained from Chifaa Laboratory in Médéa specialized in medical laboratory testing, including routine blood tests, biochemistry, hematology, and serology.
- Three isolates were collected in form of oral swabs of pediatric patients during immediate medical consultations conducted by Dr. MEMMI, a pediatrician in Medea, Algeria.

3. Reagents and Chemicals

- DMSO (Dimethyl sulfoxide) — solvent for oil dispersion
- TTC (2,3,5-triphenyltetrazolium chloride) — for cell viability staining
- Sterile physiological saline (0.9% NaCl)
- Ethanol 80%
- Tween 80

4. Culture Media

- Sabouraud Dextrose Broth (SDB), pre-boiled and sterilized
- Mueller Hinton Broth (MHB)

5. Glassware and Laboratory Supplies

- 96-well flat-bottomed sterile microtiter plates (polystyrene)
- 96-well round-bottomed sterile microplates
- Sterile pipette tips and micropipettes

B. Methods:

1. Isolation of Candida albicans Strains

Origin:

- Pre-isolated isolates were obtained from El-chifaa laboratory in Medea, all of them were vaginal swabs.
- Buccal swabs were obtained using sterile cotton swabs.

Each sample was directly inoculated onto Sabouraud agar supplemented with chloramphenicol to suppress bacterial contamination.

The plates were incubated at 37°C for 24 to 48 hours. If no visible growth was observed, incubation was extended up to 48 hours. Colonies showing typical yeast morphology (creamy, smooth, and white to off-white) were subcultured to obtain pure isolates. The purified strains were maintained on fresh Sabouraud-Chloramphenicol agar and stored at 4°C for further analysis.

2. Identification of *C.albicans*

The isolates were identified based on microscopic and biochemical characteristics, using **methylene blue staining** and the **germ tube test**.

I. Methylene Blue Staining (Read Annex1)

Methylene Blue staining is a simple technique used to identify *Candida albicans* by enhancing the visibility of its oval shape and budding structures under the microscope.Leave the stain to act for 10 to 15 minutes.

II. Germ Tube Test (Read Annex2)

The Germ Tube Test is a rapid and reliable technique for identifying *Candida albicans*. When incubated in serum at 37°C, *C. albicans* forms germ tubes that can be observed microscopically after 2–3 hours.

3. Conservation of Candida albicans Isolates

The *C.albicans* isolates were conserved for long-term storage using a cryoprotective solution. Briefly, fresh yeast colonies were suspended in a mixture of sterile physiological saline and 25% glycerol in equal volumes. The suspension was transferred into sterile cryotubes and stored at –20°C until further use.

(Larone, 2011)

4. Revival of Candida albicans Isolates

To revive *C.albicans* isolates after long-term storage, frozen samples preserved in physiological saline and 25% glycerol were first thawed at room temperature. A small aliquot of each suspension was aseptically inoculated onto **Sabouraud Dextrose Agar (SDA)** using a sterile inoculating loop. The plates were incubated at **37°C for 24 to 48 hours,** allowing for sufficient yeast recovery and colony growth.

(Larone, 2011)

5. Biofilm formation :

a) Preparation of the Inoculum

Fresh colonies of *C.albicans* were suspended in sterile physiological saline. The turbidity of the suspension was adjusted to a 0.5 McFarland standard using a densitometer, corresponding

to approximately 1×10^8CFU/mL. This suspension was then diluted 1:10 in sterile Sabouraud Dextrose Broth (SDB) to prepare the working inoculum.

b) Initial Adhesion Phase

A volume of 100 µL of the work inoculum was dispensed into the wells of a sterile, flat-bottomed 96-well polystyrene microtiter plate. The plate was incubated at 30°C for 2 hours under gentle agitation (moderate speed) to promote yeast adhesion to the plastic surfaces.

c) Biofilm Development Phase

Following the adhesion period, 100 µL of sterile Sabouraud Dextrose Broth (SDB) was added to each well, bringing the final volume to 200 µL per well.

In parallel, control wells were filled with 200 µL of sterile SDB only (without inoculum) to serve as negative controls. These controls were used compare with background absorbance.

All plates were incubated at 25–30°C for 48 hours under static conditions to allow biofilm maturation.

d) Washing and Fixation

After incubation, wells were carefully washed three times with phosphate-buffered saline (PBS, pH 7.2) to remove non-adherent cells. The adhered biofilms were then fixed with 100 µL of absolute ethanol for 15 minutes at room temperature.

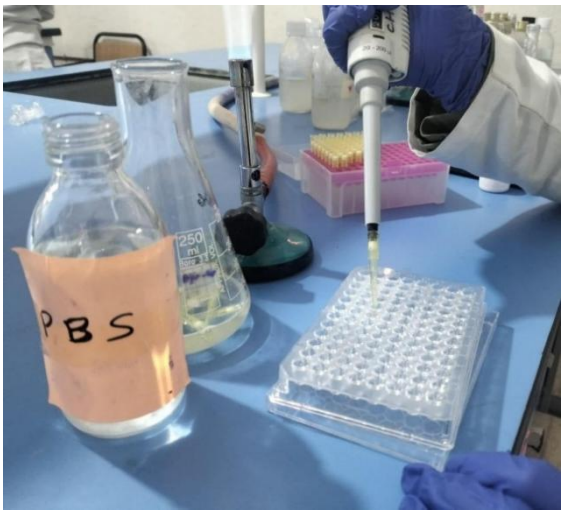


Figure 9 : *PBS washing step during Candida albicans biofilm assay (Personal photo – 2025)*

e) Staining of Biofilms

Biofilms were stained using a 0.1% crystal violet solution. The dye was added to each well and incubated for 15–20 minutes at room temperature to allow sufficient penetration into the biofilm matrix. After staining, the wells were gently rinsed three to four times with sterile distilled water to remove any unbound dye. The microplate was then left to air-dry completely at room temperature.

(Ramage et al., 2001)

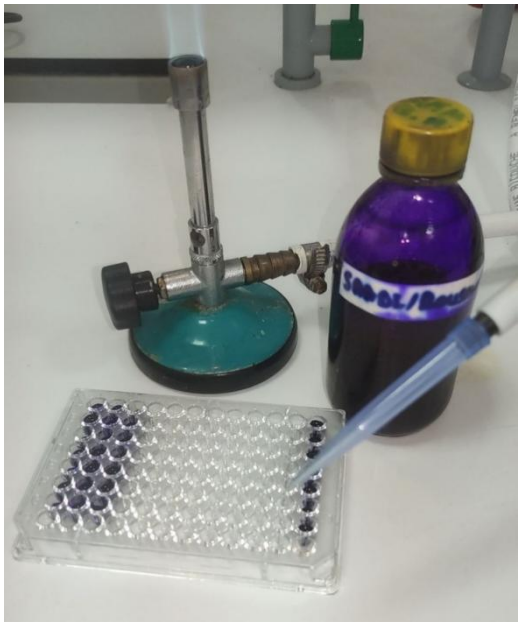


Figure 10 : *Crystal violet staining step (Personal photo – 2025)*

f) Quantification of Biofilm Biomass

Following drying, the bound crystal violet was solubilized using either 95%. The optical density (OD) of the solubilized stain, which correlates with the amount of biofilm formed, was then measured at 570 nm using a microplate reader.

(Ramage et al., 2001)

The biofilm-forming ability of each *C.albicans* isolate was determined based on the optical density (OD) values measured at 570 nm after crystal violet staining. A strain was considered non-adherent if its OD was equal to or less than the cut-off value (ODc), calculated as the mean

OD of the negative control wells. Strains were classified as weak, moderate, or strong biofilm producers depending on whether their OD was:

- between 1–2× ODc: **Weak**
- 2–4× ODc: **Moderate**
- greater than 4× ODc: **Strong** (Stepanović et al., 2000).

6. Antifungal Susceptibility tests:

The investigation of the antifungual susceptibility test of chosen natural products was carried out using microdilution method (CLSI, 2010). The MICs were determined.

Preparation of the Inoculum (Read annex3)

The inoculum was prepared and diluted in Sabouraud Dextrose Broth (SDB) to reach required cell density

6.1 Coconut Oil Antifungal Susceptibility Assay Against Candida albicans

To evaluate antifungual activity of cocnut oil, the brouth microdilution susceptibility test was used. Minimum inhibitory concentration (MIC) and Minimum bactercidial concentration (MBC) values were assessed for each C. albicans isoltaes.

Preparation of Coconut Oil Dilutions

- Stock Solution (10%):

  Mix 1 mL of coconut oil with 9 mL of DMSO to prepare a 10% (v/v) stock solution.

  Vortex or stir gently to ensure proper dispersion.

- Working Dilutions
  Prepare serial dilutions from the stock in SDB to achieve the following concentrations (check table n°01)

Table 2 final concentrations of coconut oil dilutions

N°	source	Volume	Volume of Medium (mL)	Final concentrations (%)
1	SS	1	1	5
2	SS	1	3	2,5
3	SS	1	4	2
4	SS	1	9	1
5	2%	1	3	0,5
6	1%	1	3	0,25
7	0,25%	1	1	0,125

Microdilution Assay protocol :

- ❖ Dispense 100 µL of each coconut oil dilution into designated wells of a sterile 96-well microplate.
- ❖ Add 100 µL of the diluted *C.albicans* inoculum to each test well (final volume = 200 µL).

- Controls :
  - ❖ Positive control: 100 µL of SDB + 100 µL of inoculum (no oil).
  - ❖ Negative control: 200 µL of sterile SDB only (no inoculum or oil).



Figure 11 : *Filling the designed wells with SDB and inoculum and oil dilutions (Personal photo – 2025)*

- ❖ Incubate the plate at 37°C for 24 hours under static conditions.

Viability Assessment Using TTC :

- ❖ After incubation, add 20 µL of 1% TTC to each well. The TTC was added to facilitate the observation of bacterial growth
- ❖ Incubate an additional 2–3 hours at 37°C.
- ❖ Observe color change :
  - Red color: indicates viable, metabolically active cells (TTC is reduced to formazan).
  - No color: indicates growth inhibition.
- ❖ The Minimum Inhibitory Concentration (MIC) is defined as the lowest concentration of coconut oil where visible growth is observed so no red color.

(Hammer et al., 1999 ; Tepe et al., 2005)

6.2 Cinnamon oil Antifungal Susceptibility Assay Against Candida albicans:

Preparation of Cinnamon Oil Dilutions

- Prepare a stock solution by mixing:

- 1 mL of cinnamon oil
- 9 mL of DMSO (Dimethyl sulfoxide)

Preparation of dilutions:

- From the stock solution, prepare a series of dilutions in Sabouraud Dextrose Broth (SDB) to obtain the following final concentrations of cinnamon oil:

Table 3 final concentrations of cinnamon oil dilutions

N°	source	Volume	Volume of medium (ml)	Final concentrations (%)
1	Stock	1	1	5
2	Stock	1	3	2,5
3	Stock	1	4	2
4	Stock	1	9	1
5	2%	1	3	0,5
6	1%	1	3	0,25
7	0,25%	1	1	0,125

Determination of antimicrobial activity :

- Add 100 µL of each cinnamon oil dilution to designated wells.
- Add 100 µL of the prepared *C.albicans* inoculum to each test well.

- Add controls as follows:
    - Positive Control: 100 µL SDB + 100 µL inoculum (no oil)
    - Negative Control: 200 µL SDB only (no inoculum or oil)

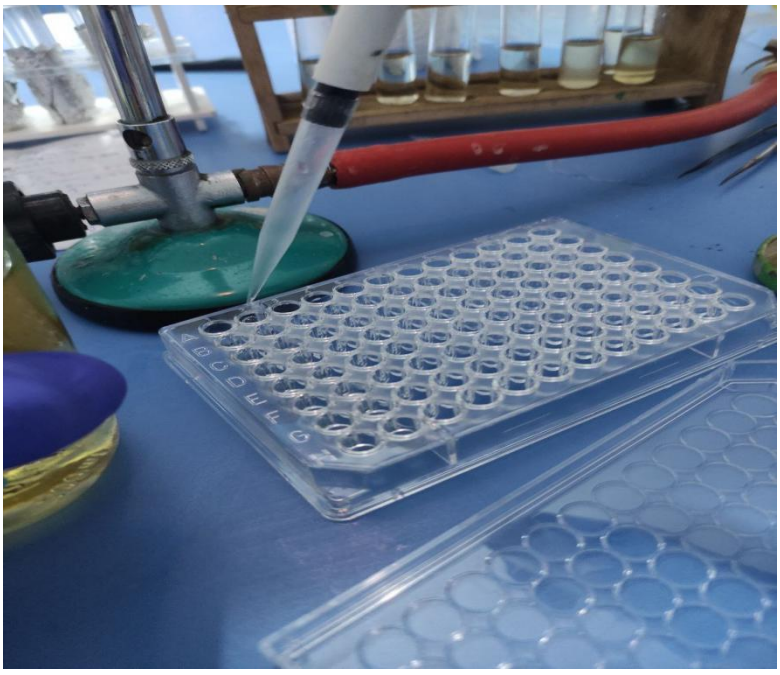


Figure 12 : *Loading of wells with cinnamon oil and C.albicans inoculum for microdilution test (Personal photo – 2025)*

- Incubate the microplate at 37°C for 24–48 hours under static conditions.

Assessment of Growth Using TTC:

- After incubation, add 20 µL of 1% TTC (triphenyl tetrazolium chloride) to each well.
- Incubate an additional 2–3 hours.
- Red color indicates metabolically active (viable) cells.
- The MIC is the lowest concentration of cinnamon oil with no red color, indicating growth inhibition.

  (Hammer et al., 1999 ; Tepe et al., 2005)

6.3 Propolis Antifungal Susceptibility Assay Against *Candida albicans*:

Preparation of propolis dilutions :

- The stock solution is prepared by solving 800mg of Propolis in 4ml of Ethanol 80%, obtaining a final concentration of 200mg/ml of propolis per one ml of ethanol
- From the stock solution we prepared a series of intermediate dilutions.
- Each prepared dilution was further diluted at a 1:10 ratio in MHB taking 1ml from each and added to 9ml of the diluent giving the following final concentrations **(CLSI 2010).**

Table 4 final concentations of EEP diluitions

Step	Source	Propolis solution volume	Ethanol Volume	Intermediate concentration	Final concentration
1	Stock	1ml	0ml	200mg/ml	20mg/ml
2	Stock	1ml	1ml	100mg/ml	10mg/ml
3	Stock	0.5ml	1.5ml	50mg/ml	5mg/ml
4	Stock	0.5ml	3.5ml	25mg/ml	2.5mg/ml
5	Step 4	1ml	1ml	12.5mg/ml	1.25mg/ml
6	Step 4	0.5ml	1.5ml	6.25mg/ml	0.625mg/ml
7	Step 4	0.5ml	3.5ml	3.12mg/ml	0.312mg/ml
8	Step 7	1ml	1ml	1.56mg/ml	0.156mg/ml
9	Step 7	0.5ml	1.5ml	0.78mg/ml	0.078mg/ml

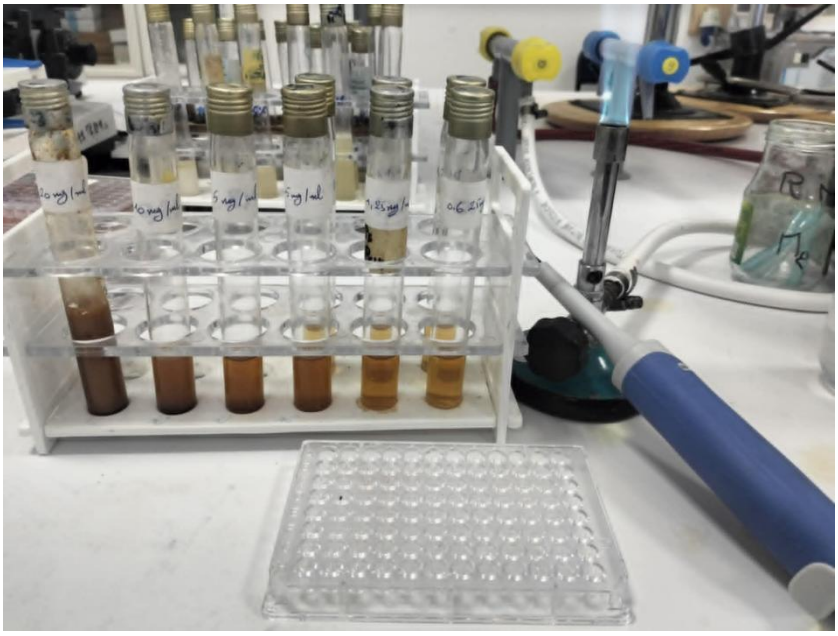


Figure 13 : *Preparation of EEP dilutions (Personal photo – 2025)*

- **Determination of antimicrobial activity :**

❖ Distribute 100 µL of each previously prepared innoculums of *C.albicans* in the wells from A to G in horizontal lines

❖ Adding 100 µL of MHB in the positive control well (100 µL of inoculum + 100 µL of MHB)

❖ Each well contains: 100 µL of inoculum (diluted)+100 µL of propolis dilutions. An aliquot of 20 µL of tween 80 was added in every well.

❖ In the 11th well 100 µLof inoculum was added to 100 µL of 10% ethanol 80%; the final concentration of ethanol in the well was 5%. This control well, was employed to show if ethanol at that concentration has an effect on *C.albicans*.

❖ Incubating the microplate at 37°C for 24Hours under static conditions

❖ Adding 20 µL of TTC 1% to each well and incubating for 2 more hours to assess and detect fungal growth

After incubating, the observation of red color indicates the life of the fungi and existence of metabolic activity

- The MIC is the lowest concentration of propolis with no red color indicating growth inhibition

6.4 Synergy of Coconut oil and cinnamon oil Antifungal Susceptibility Assay Against *Candida albicans*:

Materials :

- *C.albicans* clinical isolates (n = 7)
- Cinnamon oil + coconut oil (natural, unrefined)
- DMSO (Dimethyl sulfoxide) – used as oil solvent
- Mueller Hinton broth (MHB), previously boiled and sterilized
- Sterile physiological saline (0.9% NaCl)
- 96-well flat-bottomed sterile microtiter plates
- Densitometer or McFarland turbidity standard
- TTC (2,3,5-triphenyltetrazolium chloride) – for viability staining
- Sterile pipette tips and micropipettes

Preparation of the synergy dilution:

- Prepare a stock solution by mixing:
  - 1 mL of cinnamon oil mixing it with 1mL of Coconut oil
  - 18 mL of DMSO (Dimethyl sulfoxide) to dilute at 1:10 ratio
- From the stock solution, prepare a series of dilutions in Mueller hinton broth (MHB) to obtain the following final concentrations of cinnamon oil

Table 5 final concentrations of the mix of cinnamon oil+coconut oil dilutions

N°	Source	Volume	Volume of medium (ml)	Final concentrations (%)
2	Stock	1	1	5
3	Stock	1	3	2,5
4	Stock	1	4	2
5	Stock	1	9	1
6	2%	1	3	0,5
7	1%	1	3	0,25
8	0.25%	1	1	0,125

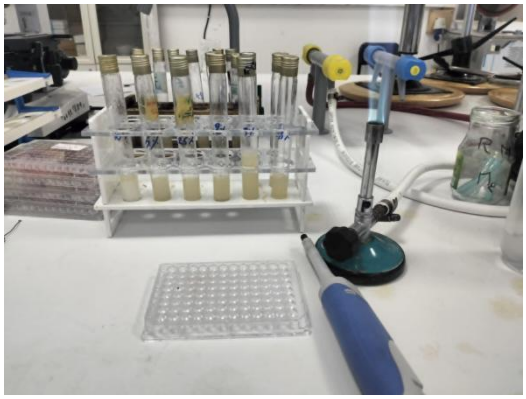


Figure 14 : *Preparation of Synergic dilutions of cinnamon oil + coconut oil (Personal photo – 2025)*

Determination of antimicrobial activity :

- Distribute 100 µL of each previously prepared innoculums of *C.albicans* in their dedicated lines from A to G in horizontal lines
- Adding 100 µL of MHB Vertically In column + 100 µL of inoculum (no oils)
- Distributing the synergic dilutions from column (2) to (8) vertically meeting with the inoculum (100 µL of the dilution + 100 µL of the inoculum)
- In column (9) ,100 µL of a previously prepared tube of ethanol 80% diluted in MHB at 1/10 ratio was added to 100µL of the inoculum + tween80, to show that ethanol at that concentration has no effect on *C.albicans*.
- Incubating the microplate at 37°C for 24Hours under static conditions
- Adding 20 µL of TTC 1% to each well and incubating for 2 more hours to assess and detect fungal growth

  After incubating, the observation of red color indicates the life of the fungi and existence of metabolic activity

  - The MIC is the lowest concentration of propolis with no red color indicating growth inhibition

Results And Discussion

1. Results

A. Identification of *C. albicans:*

I. Culture on Saboureaud dextrose chloramphenicol :

The medium was used as a primary identification method to suppress any bacterial growth and obtaining a pure fungi colony. The picture below shows creamy white budding form of the studied specie.



Figure 15 : *Colony morphology of Candida albicans on Sabouraud Chloramphenicol Agar (Personal photo – 2025)*

II. Methylene blue staining: (Read annex 1)

The technique allowed to the phenotypic identification of *C. albicans* observed under light microscope at 40x magnification

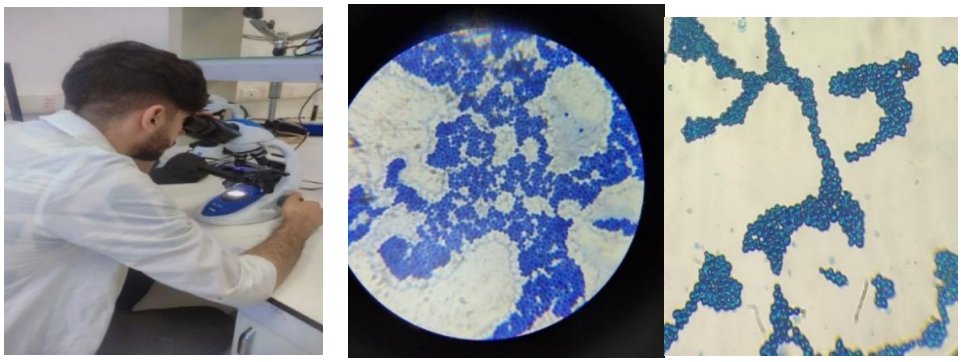


Figure 16 : *Methylene blue staining of Candida albicans observed under light microscope at 40× magnification (Personal photo – 2025)*

III. Germ tube test: (Read Annex2)

The technique is used due to its rapidity and cost-effectivity for the identification of *C.albicans*, based on its ability to form germ tubes filamentous outgrowths when incubated in serum. The picture below shows the elongation and the formation of filaments putting the light on the yeast to hyphae transitiong characterizing *C.albicans*

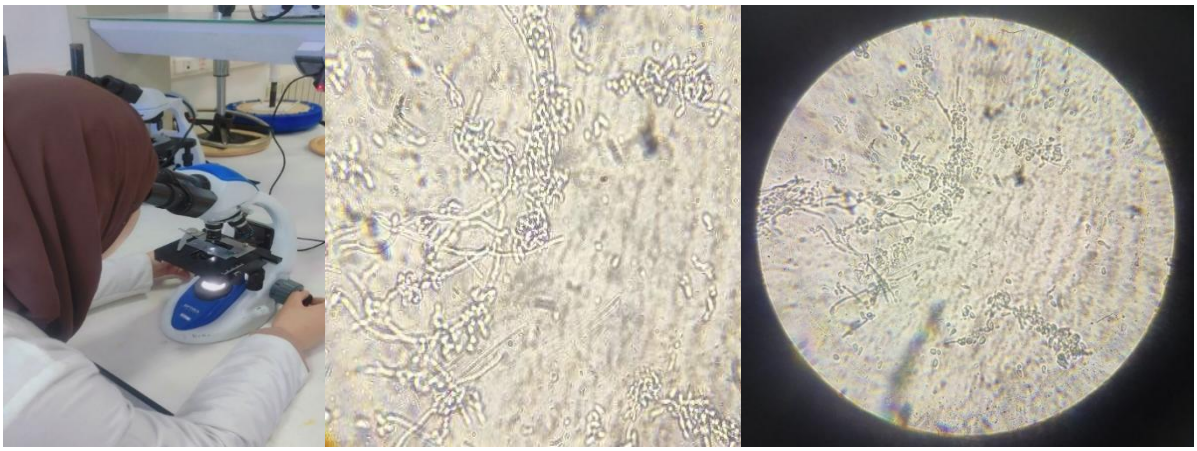


Figure 17 : *Positive germ tube test: filament formation by Candida albicans, observed under light microscope at 40x and 100x magnifications (Personal photo – 2025)*

B. Biofilm Formation and Quantification Assay

- To evaluate the biofilm-forming ability of Candida albicans isolates, the crystal violet assay was employed, allowing for quantification of biofilm biomass by measuring optical density (OD) at 590 nm. Each isolate was cultured in triplicate, and the mean OD values were calculated. The biofilm formation level was assessed by subtracting the average OD of the negative control wells from the mean OD of each isolate (ΔOD). Based on these ΔOD values, and according to the standard classification proposed by (Stepanović et al.2000), isolates were categorized into one of four groups: non-biofilm producers, weak, moderate, or strong biofilm formers.
- Table n°05 presents the optical density (OD Mean±SD) results. the average control OD, the resulting ΔOD (difference between mean OD and control), and the corresponding classification of biofilm formation:

Table 6 optical density results of biofilm formation assay

Isolate	Replicate 1	Replicate 2	Replicate 3	OD Mean±SD	Biofilm Formation
VSCA1	0.916	0.387	1.8	1.034±0,713	Strong
BSCA2	0.583	0.398	0.441	0.474±0,096	Moderate
VSCA3	0.597	0.516	0.797	0.637±0,144	Strong
VSCA4	0.826	0.66	0.643	0.71±0,101	Strong
VSCA5	0.458	0.576	1.559	0.864±0,604	Strong
BSCA6	1.042	0.668	1.375	1.028±0,353	Strong
BSCA7	0.435	0.692	0.289	0.472±0, 204	Moderate

- The negative control OD= 0,150
- VSCA : Vaginal strain candida albicans.
- BSCA : buccal strain candida albicans.

➢ This classification allows for a comparative analysis of biofilm forming ability between vaginal and oral *C.albicans* isolates, as summarized in the following table:

Table 7 Distribution of biofillm formation levels among oral and vaginal C.albicans isolates

Biofilm formation level	Oral Isolates (n = 3)	Vaginal Isolates (n = 4)
Strong	1 isolate (33,3%)	4 isolates (100%)
Moderate	2 isolates (66.7%)	0 isolate (0%)
Weak	0 isolate (0%)	0 isolate (0%)
No biofilm	0 isolate (0%)	0 isolate (0%)

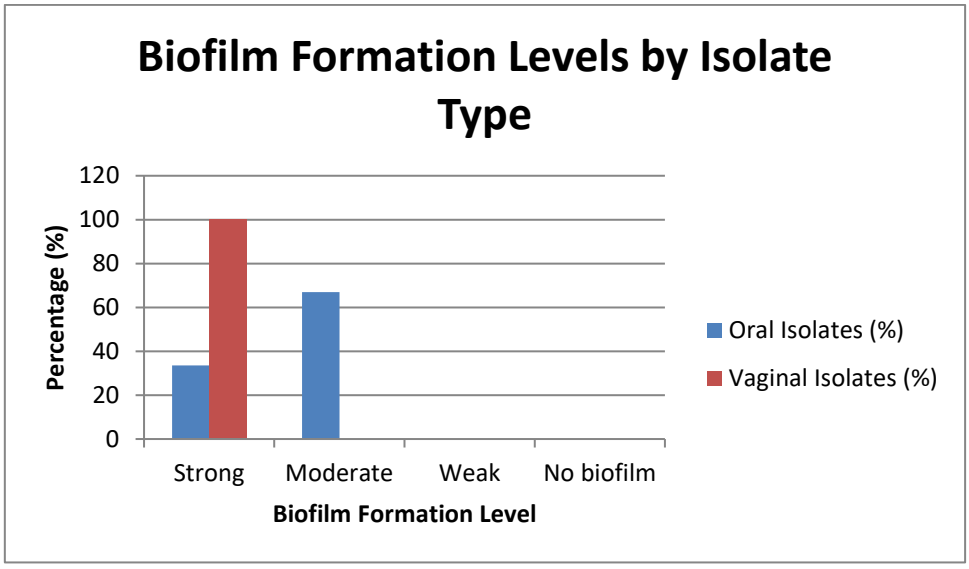


Figure 18 : *Histogram of Biofilm Formation Levels among Oral and Vaginal Candida albicans Isolates*

C. Antimicrobial susceptibility test results:

- The results are summarized in the following tables for each assay
- The Minimum Inhibitory Concentration (MIC) is defined as the lowest concentration of used natural product where no red color is observed.

(Hammer et al., 1999 ; Tepe et al., 2005)

• Ethanolic extract of propolis (EEP):

The results show different MICs from 0.625mg/mL to 2.5mg/mL, 5/7 (≈70%) of strains have an MIC of 0.625mg/mL; 1/7 (≈15%) have and MIC of 1.25mg/mL and 1/7 (≈15%) of strains have a relatively high MIC of 2.5mg/mL

- Concentrations vary starting from 0.078 to 20mg/mL so the values obtained are considered low.
- All strains showed sensitivity to EEP

Table 8 C.Albicans EEP MICs in mg/mL

Isolate	MIC
VSCA1	2.5
BSCA2	0.625
VSCA3	0.625
VSCA4	0.625
VSCA5	0.625
BSCA6	0.625
BSCA7	1.25

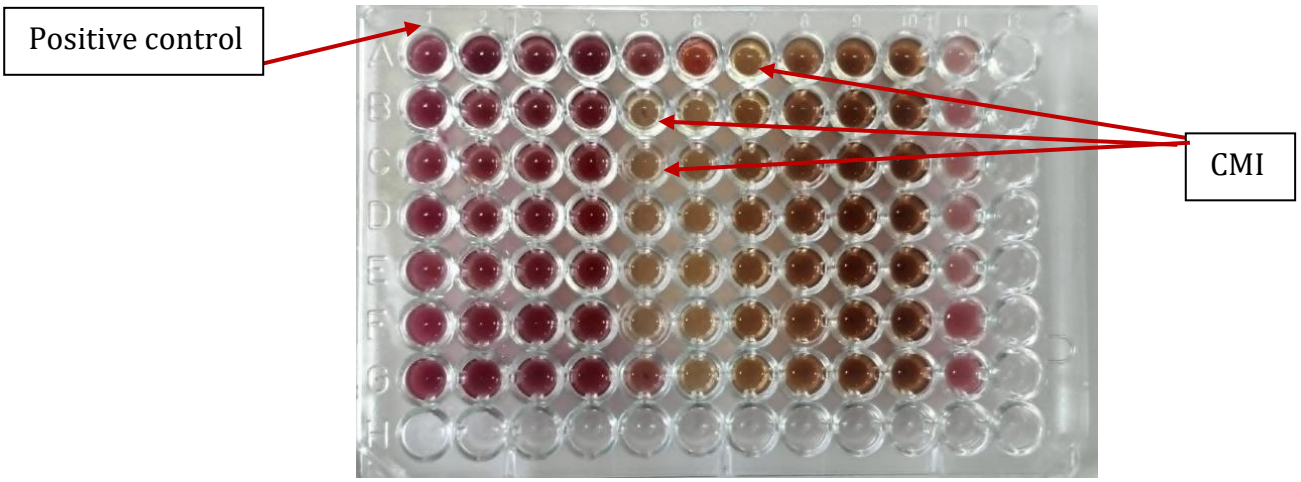


Figure 19 : *Picture taken of the microplate showing the distribution of different concentrations of EEP and the CMIs*

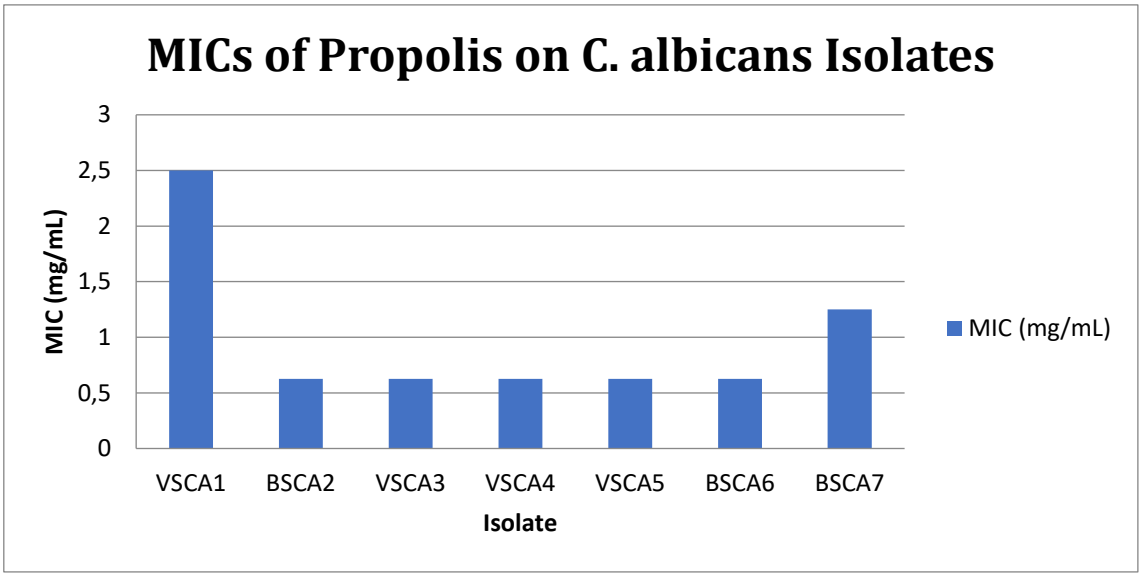


Figure 20 : *A histogram showing EEP MICs on C.albicans*

- **MIC_{50}** (Minimum Inhibitory Concentration 50%)

The MIC_{50} is defined as the minimum concentration of an antimicrobial agent required to inhibit the visible growth of 50% of the tested microbial isolates. It provides an estimate of the central tendency of the antimicrobial activity within a microbial population (CLSI, 2012).

MIC_{50} of EEP

The MIC_{50} of propolis, calculated from the MIC values obtained for seven clinical isolates of *C.albicans* (0.625, 0.625, 0.625, 0.625, 0.625, 1.25, and 2.5 mg/mL), corresponded to the fourth value (50%) of inhibition which was 0.625 mg/mL. This indicates that 50% of the tested strains were inhibited at this concentration.

- **Cinammon Oil:**

The results show different MICs from 0.5% to 1%, 5/7 (≈70%) of strains have an MIC of 1% while 2/7 (≈30) of strains have an MIC of 0.5%

- The concentrations vary from 0.125% to 10%
- All strains showed sensitivity to Cinnamon oil.

Table 9 C.Albicans Cinnamon oil MICs in %

Isolate	MIC
VSCA1	**0.5%**
BSCA2	**1%**
VSCA3	**0.5%**
VSCA4	**1%**
VSCA5	**1%**
BSCA6	**1%**
BSCA7	**1%**

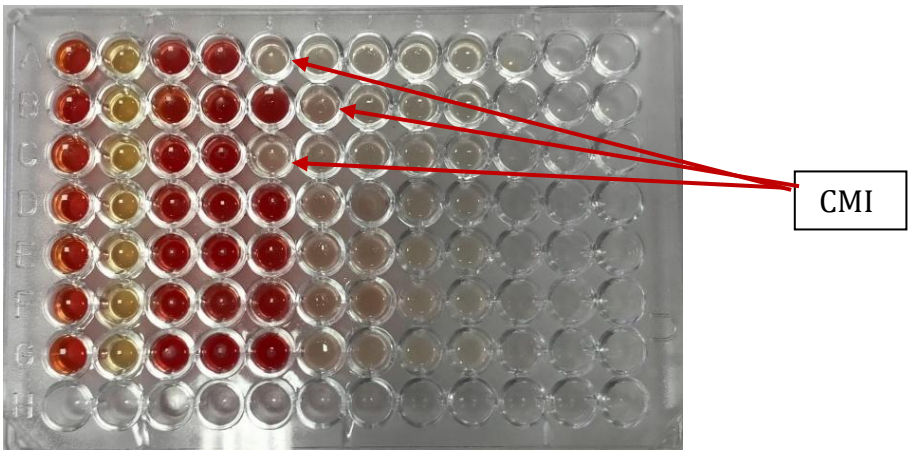


Figure 21 : *Picture taken of cinnamon oil microdilution test showing the MICs*

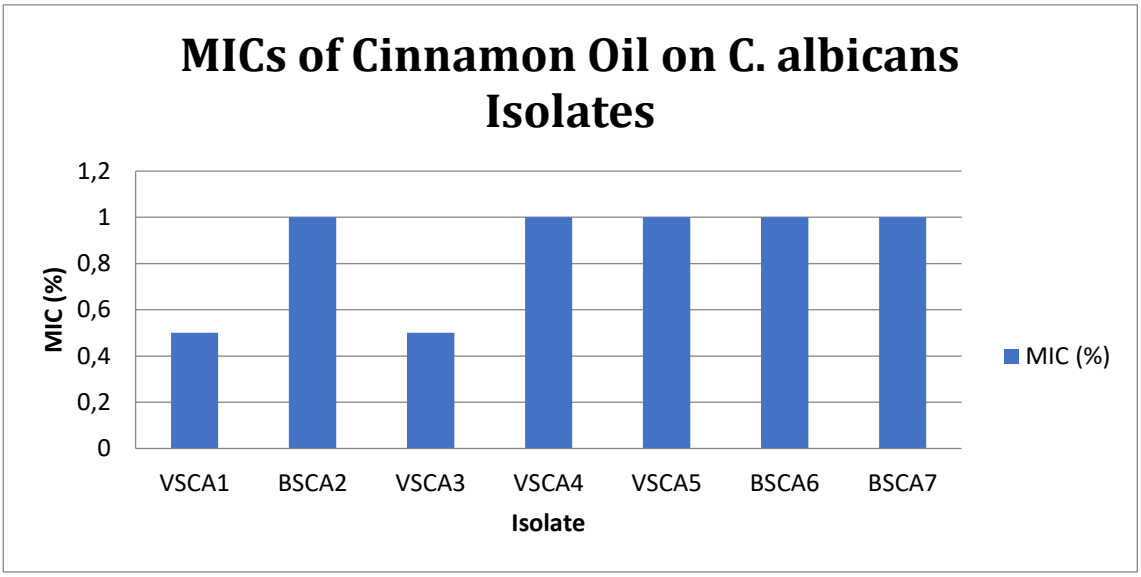


Figure 22 : *Histogram showing Cinnamon oil MICs on C. albicans*

MIC_{50} of Cinnamon Oil

The MIC_{50} of cinnamon oil, calculated from the MIC values obtained for seven clinical isolates of C.albicans (0.5%, 0.5%, 1%, 1%, 1%, 1%, and 1%), MIC 50 was 1%. This indicates that 50% of the tested isolates were inhibited at this concentration, reflecting the High sensitivity level of C.albicans strains to cinnamon oil.

- **Coconut oil:**

The results show different MICs from 2 to 2.5%, 5/7(≈70%) of strains have an MIC of 2.5% and 2/7(≈30%) of strains have an MIC of 2%

- The concentrations vary from 0.125% to 10%
- All strains showed sensitivity to coconut oil

Table 10 C.Albicans Coconut oil MICs in %

Isolate	MIC
VSCA1	2.5%
BSCA2	2%
VSCA3	2%
VSCA4	2.5%
VSCA5	2.5%
BSCA6	2.5%
BSCA7	2.5%

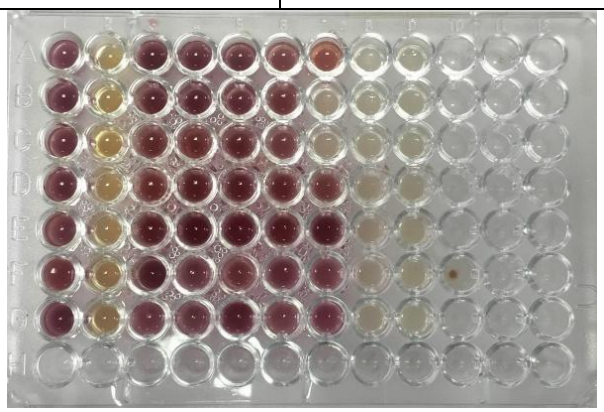


Figure 23 : *Picture taken of coconut oil microdilution test showing the MICs*

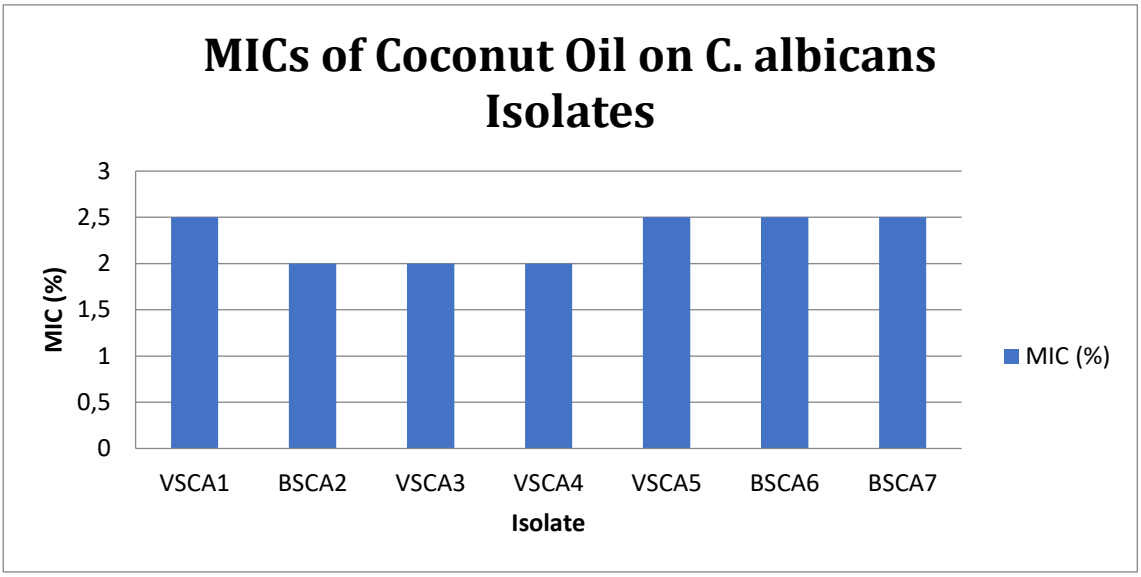


Figure 24 : *Histogram showing coconut oil MICs on C. albicans*

MIC_{50} of coconut oil

The MIC_{50} of coconut oil, calculated from the MIC values obtained for seven clinical isolates of *C.albicans* (2%,2%, 2.5%, 2.5%, 2.5%, 2.5%, and 2.5%), MIC was **2.5%**. This indicates that 50% of the tested isolates were inhibited at this concentration, reflecting the high sensitivity level of of *C.albicans* strains to coconut oil.

- **Synergy of Coconut oil and Cinnamon oil:**

The results show different MICs from 0.5% to 2%, 4/7 (≈57) of strains have an MIC of 2%, 2/7 (≈28%) of strains have an MIC of 1%, and 1/7 (≈14%) of strains have an MIC of 0.5%

- The concentrations vary from 0.125% to 5%
- All strains showed sensitivity to this synergy of coconut oil and cinnamon oil

Table 11 C.albicans coconut oil +cinnamon oil mix MICs in %

Isolate	MIC
VSCA1	**2%**
BSCA2	**1%**
VSCA3	**2%**
VSCA4	**2%**
VSCA5	**0.5%**
BSCA6	**1%**
BSCA7	**2%**

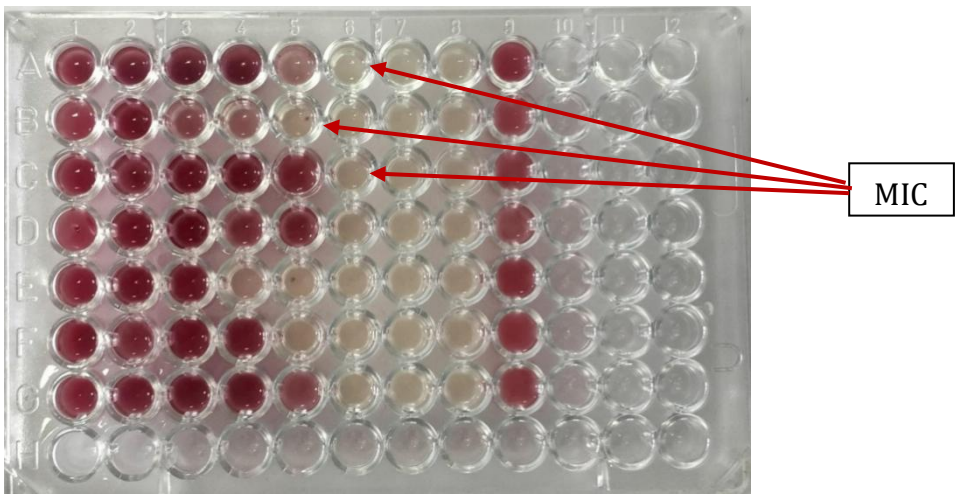


Figure 25 : *picture taken showing the MIC of coconut oil +cinnamon oil mix*

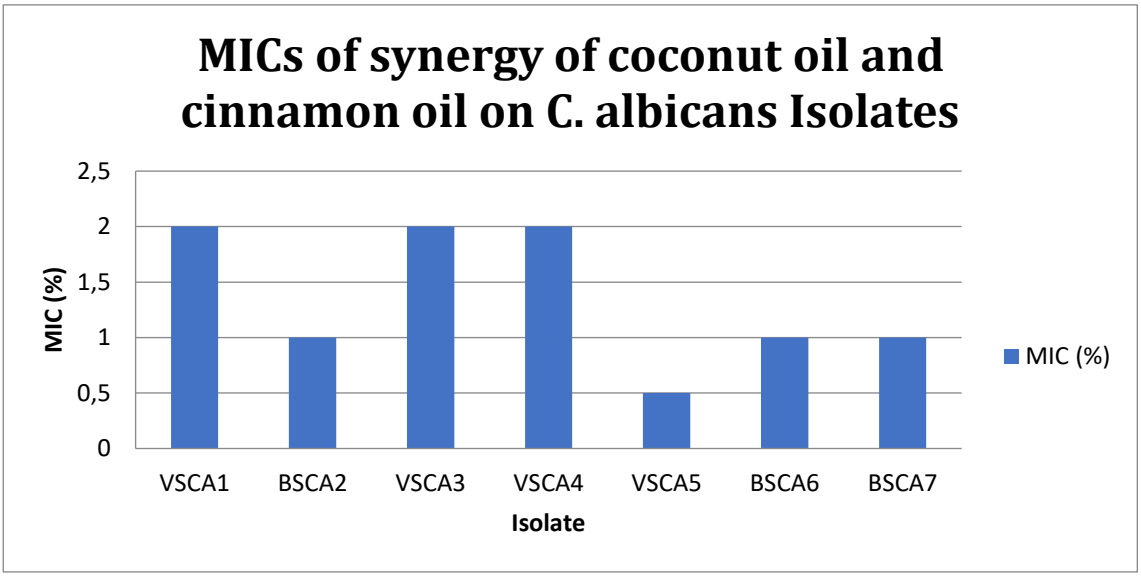


Figure 26 : *Histogram showing coconut oil +cinnamon oil's mix MICs on C.albicans*

MIC_{50} of the Combination of Coconut Oil and Cinnamon Oil

The MIC_{50} of the combination of coconut oil and cinnamon oil, calculated from the MIC values obtained for seven clinical isolates of *C.albicans* (0.5%, 1%, 1%, 2%, 2%, 2%, and 2%), corresponded to the fourth value in the ordered dataset (since 50% of 7 = 3.5, rounded up), which was **2%**. This result indicates that half of the tested *C.albicans* isolates were inhibited at a concentration of 2% of the combined oils, suggesting a moderate synergistic antifungal activity.

2. Discussion of obtained results :

All tested *C.albicans* isolates showed susceptibility to the evaluated natural products. The antifungal effects varied depending on the product and concentration, with some isolates responding more strongly to specific agents.

a) Biofilm Formation and Inhibition

The biofilm-forming capacity varied among isolates. Vaginal isolates were more frequently moderate biofilm producers, while among oral isolates, one strain was a non-producer. This

variability in biofilm formation corresponds with previous reports that different *C. albicans* strains have different capacities to form biofilms depending on their origin and clinical context (Ramage et al., 2006).

b) Ethanolic extract of propolis (EEP)

The ethanolic extract of propolis exhibited the most potent antifungal activity among the tested substances. The Minimum Inhibitory Concentrations (MICs) ranged from 0.625 mg/mL to 2.5 mg/mL. Notably, 70% of the isolates were inhibited at a low concentration of 0.625 mg/mL, indicating a high sensitivity to propolis. These results align with findings from Silva et al. (2020) and Melliou & Chinou (2004), which both highlighted the strong antifungal properties of propolis against *C. albicans*.

Propolis's activity can be attributed to its rich composition in flavonoids and phenolic compounds, such as pinocembrin and galangin. (Drago et al. 2007). While Drago's study did not directly assess antifungal activity, the presence of galangin and other flavonoids supports the idea that similar mechanisms contribute to antifungal effects. Their emphasis on formulation (i.e., hydrophilic vs. hydroalcoholic) also supports our findings—ethanolic propolis showed strong effects, though activity may further increase with solubility enhancements.

While in other suscueptibility test methods such as agar diffusion method (Chieb & Berrahal, 2021) reported inhibition zones from 8mm to ≈12mm on other Candida species using the same ethanol 80% as our study comparing to other percentages of ethanol wether higher or lower that showed lower values, indicating that propolis components are more soluble with ethanol 80%.

c) Cinnamon Oil

Cinnamon oil also demonstrated significant antifungal activity. MICs ranged from 0.5% to 1%, with the majority of isolates (70%) inhibited at 1%. This supports previous studies like those of Kaskatepe et al. (2016) and Singh et al. (2011), which identified cinnamaldehyde—the major compound in cinnamon oil as a powerful antifungal agent. Cinnamaldehyde disrupts fungal cell membranes and interferes with enzyme systems critical to fungal survival. Our results confirm its consistent performance against *C. albicans* isolates.

Importantly, essential oils, due to their volatile and lipophilic nature, penetrate fungal membranes effectively.

d) Coconut Oil

Coconut oil exhibited moderate antifungal activity, with MICs ranging from 2% to 2.5%. While less potent than propolis and cinnamon oil, it still inhibited all isolates tested. These results are consistent with findings by Ogbolu et *al.* (2007), who reported that the medium-chain fatty acids in coconut oil particularly lauric acid can disrupt the lipid structure of fungal membranes.

The higher MICs suggest a lower efficacy, potentially due to the less concentrated or slower-acting nature of the fatty acids. However, coconut oil remains a viable natural product for antifungal use, especially when considering its safety profile and compatibility with other oils.

e) Synergistic Combination: Coconut + Cinnamon Oil

The combination of coconut and cinnamon oil showed medium antifungal effects between cinnamon and coconut oil (cinnamon oil was more effective 0.5-1%). MICs ranged from 0.5% to 2%, indicating that the synergy between the fatty acids in coconut oil and the essential components of cinnamon oil improved the efficacy. One isolate showed inhibition at 0.5%, demonstrating that the blend was sometimes more effective than the individual components.

This supports previous findings by Naidu et al. (2019), who noted that combining essential oils could potentiate antifungal activity while lowering required concentrations. Synergy may occur due to membrane destabilization by fatty acids, followed by intracellular targeting by essential oil compounds.

Overall Interpretation

Among all products tested, propolis (EEP) was the most effective antifungal agent, followed by cinnamon oil and the synergistic mix. Coconut oil, while effective, had the highest MICs. These findings reinforce the antifungal potential of natural products, particularly when used in optimized formulations or combinations.

Comparing our results to existing literature validates the reproducibility of antifungal properties of natural substances. Moreover, the synergistic trends observed here echo previous work supporting the use of natural combinations to combat drug-resistant pathogens. Future work should focus on identifying and isolating the active compounds in each extract, evaluating their toxicity, and testing them in clinical settings to confirm their therapeutic potential.

Furthermore, Krol et al. (1993) and Stepanovic et al. (2003) demonstrated synergistic interactions between propolis and antifungal drugs, suggesting that the integration of propolis with conventional therapies could enhance outcomes—a direction that aligns with the combinatorial results observed in our study.

Conclusion:

The present study demonstrated the promising antifungal potential of natural products specifically coconut oil, cinnamon oil, and propolis against Candida albicans isolates. The in vitro assays confirmed that all three substances exhibited varying degrees of antifungal activity, with cinnamon oil showing the most potent inhibitory effect, followed by propolis, and then coconut oil. Moreover, synergistic interactions between these natural agents, particularly the combination of cinnamon oil and coconut oil, enhanced their antifungal efficacy, suggesting their potential use in combination therapies to combat Candida infections.

Given the increasing severity of infections caused by *C. albicans* ranging from superficial mucosal infections to life-threatening systemic candidiasis, especially in immunocompromised patients—the development of effective antifungal strategies is of critical importance. The growing problem of antifungal resistance and the limitations of conventional antifungal drugs further highlight the need for alternative or complementary antifungal agents.

In this context, the natural products studied here offer a promising solution. Their bioactive compounds, such as cinnamaldehyde in cinnamon oil, flavonoids and phenolic acids in propolis, and lauric acid in coconut oil, are likely responsible for disrupting fungal membranes and inhibiting vital metabolic processes in Candida albicans.

Future research should focus on the in vivo efficacy and formulation development of these natural agents to assess their suitability for clinical applications. These results contribute to the ongoing search for safe, effective, and accessible antifungal treatments derived from natural sources.

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Annexes :

1. Identification of isolates :

III. Methylene Blue Staining (annex 1)

Materials:

- Clean glass slides
- Inoculating loop
- Sterile distilled water
- Methylene blue solution (0.1%)
- Bunsen burner
- Wash bottle with sterile distilled water
- Light microscope
- Coverslips (optional)

Protocol:

1. Place a drop of sterile distilled water on the center of a clean glass slide.
2. Using a sterile inoculating loop, transfer a small amount of the *C.albicans* colony into the drop and mix gently to form a uniform smear.
3. Allow the smear to air dry completely at room temperature (about 5–10 minutes).
4. Once dry, fix the smear by passing the slide briefly through the flame of a Bunsen burner 2–3 times, smear side up.
5. Apply 0.1% methylene blue solution to cover the smear.
6. Leave the stain to act for 10 to 15 minutes.
7. Rinse the slide gently with sterile distilled water using a wash bottle to remove excess stain.
8. Allow the slide to air dry completely at room temperature.
9. Observe under a light microscope at 40× and 100× magnifications.

(Cappuccino & Welsh, 2019)

IV. Germ Tube Test (annex 2)

Materials :

- Fresh *C.albicans* culture (18–24 h)
- Sterile human (or equivelnt)
- Sterile Eppendorf or test tubes
- Water bath at 37°C
- Light microscope

Protocol :

1. 0.5 mL of sterile serum was placed in a sterile tube.
2. A small amount of the yeast colony was inoculated into the serum.
3. The mixture was incubated at 37°C for 2 to 3 hours.
4. After incubation, a drop was placed on a slide and examined under the microscope at 40× and 100× magnifications

(Cheesbrough, 2006).

2. Antifungal Susceptibility tests:

Preparation of the Inoculum (annex 3)

The inoculum was prepared and diluted in Sabouraud Dextrose Broth (SDB) to reach required cell density

- Preparation of the Initial Suspension (CLSI, 2008)

Materials :

- Fresh colonies of *C.albicans* (18–24 h old on Sabouraud Dextrose Agar)
- Sterile physiological saline (0.9% NaCl)
- Sterile tubes

- Densitometer
- 0.5 McFarland standard (for calibration)

Procedure :

- Using a sterile loop or swab, pick 3–5 fresh colonies of *C.albicans* from a Sabouraud Dextrose Agar plate.
- Suspend the colonies in 5 mL of sterile physiological saline.
- Mix thoroughly to create a homogenous suspension.
- Measure the turbidity using a densitometer and adjust to 0.5 McFarland, which corresponds to approximately $1,5x10^8$ CFU/mL.
    - If the turbidity is too high, dilute with more saline.
    - If too low, add more colony material.

➢ Dilution in Sabouraud Dextrose Broth (SDB)

- Purpose: To obtain a working inoculum suitable for susceptibility testing.

Procedure :

I. Prepare a 1:100 dilution by adding 100 µL of the 0.5 McFarland suspension into 9.9 mL of sterile Sabouraud Dextrose Broth.

II. Mix gently but thoroughly to ensure even distribution of cells.

(Capistano, J. J., & Welsh, C. 2019)

The final cell density was 10^6 ufc

Note : The diluted inoculum was used immediately for the susceptibility tests.

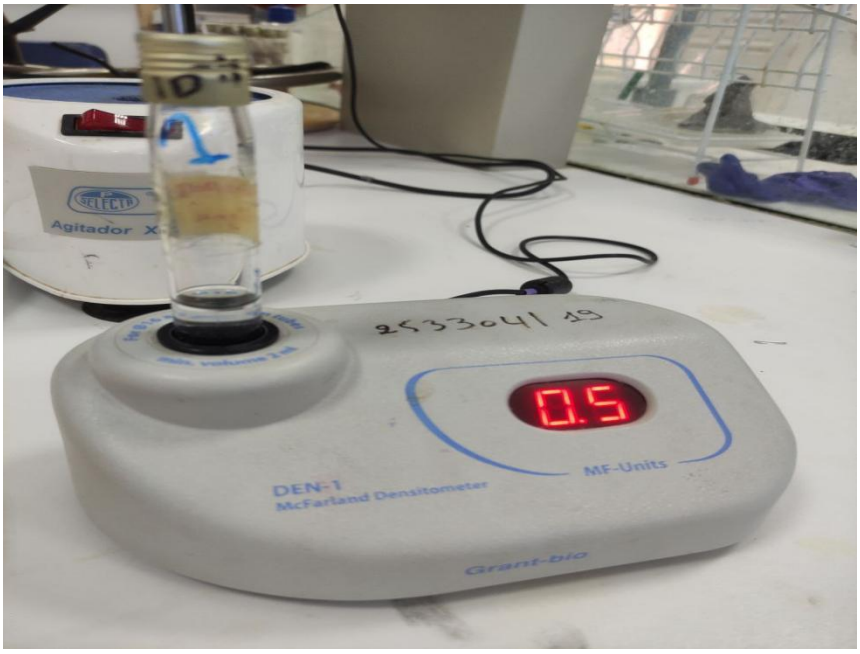


Figure 27 : *Calibration C.albicans cell density using a densitometer (Personal photo – 2025)*