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## The Efficiency of Apple Vinegar as A Solvent in Comparison to Water and Ethanol for The Extraction of Some Plants used Against *Candida Spp.* Biofilm Formation

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### Abstract

Apple vinegar has many uses that include burn and wound healing and as an antimicrobial agent against different microorganisms, but not as a solvent. Therefore, this study aimed to use commercial apple vinegar as solvent to the plants of roselle (*Hibiscus sabdariffa*), green tea (*Camellia sinensis*), and clove (*Syzygium aromaticum*). The effects of apple-vinegar extracts of these plants were compared with those of aqueous and ethanolic extracts against biofilm formation by *Candida* genus. Clove vinegar extract demonstrated antibiofilm activity against *C. albicans*, alone ( $2.4907 \pm 0.382$ ) or in combination with the antifungal agents fluconazole ( $1.689 \pm 0.33$ ), nystatine ( $1.941 \pm 0.64$ ), and clotrimazole ( $2.0353 \pm 0.71819$ ). These plant extracts possessed a variable number of antimicrobial compounds, as tested by the HPLC technique. Therefore, apple vinegar was the most efficient solvent, in comparison with the other solvents used in this study, to obtain some phytochemical compounds from the tested plants that have antibiofilm activity against *C. albicans*.

**Keywords:** Antibiofilm activity; antifungal agents; *C. albicans*; apple vinegar.

### كفاءة خل التفاح كمذيب مقارنة بالماء و الكحول الايثيلي في استخلاص بعض النباتات المستخدمة ضد تكوين الغشاء الحيوي للمبيضات

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تقي

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### الخلاصة

لخل التفاح استخدامات عديدة في التئام الجروح والحروق ومضاد فعال ضد احياء مجهرية مختلفة ولكن ليس كمذيب، لذلك هدفت الدراسة إلى استخدام خل التفاح التجاري كمذيب للنباتات التالية: كجرات (*Hibiscus sabdariffa*)، الشاي الأخضر (*Camellia sinensis*) والقرنفل (*Syzygium aromaticum*)، ومقارنة فعاليته مع المستخلص المائي والايثانولي لهذه النباتات ضد الغشاء الحيوي المكون من قبل الجنس *Candida*. اظهر مستخلص خل التفاح والقرنفل فعالية عالية مضادة لتكوين الغشاء الحيوي لهذه الخمائر بمفرده ( $2.4907 \pm 0.382$ ) أو مع مضادات الفطرية: فلوكونازول ( $1.689 \pm 0.33$ )، نيساتين ( $1.941 \pm 0.64$ )، كلوتريمازول ( $2.0353 \pm 0.71819$ ) وتمتلك هذه المستخلصات عدد من

المركبات المضادة للأحياء المجهرية كما أظهرتها تقنية كروماتوغرافيا السائل ذات الأداء العالي. لذلك يعد خل التفاح مذيبا جيدا للحصول على بعض المركبات الكيميائية من النباتات المختبرة والتي تمتلك فعالية مضادة لتكوين الغشاء الحيوي لـ *C. albicans* عند مقارنته مع المذيبات الأخرى المستخدمة في هذه الدراسة.

## Introduction

Biofilms are sessile microbial cell populations composed of exopolysaccharide, proteins, and DNA. Biofilms provide nutrients and stable adherence to the cells, along with their roles in conferring resistance to host immune responses and acting as a barrier to treatment with antimicrobial agents [1]. Therefore, biofilm formation on biotic and abiotic surfaces is associated with high rates of morbidity and mortality among hospitalized patients [2]. *Candida* species are the most popular human pathogenic fungi and can infect various locations in the body, causing severe morbidity worldwide due to their colonization of medical devices, such as catheters, by forming biofilms [3,4]. The formation of biofilms is one of the important virulence factors of *Candida* species, via limiting the penetration of antifungal agents through the matrix and protecting cells from immune responses of the host [5]. Resistance to many antifungal drugs has led to the need to finding new therapies for medicinal uses [6]. Therefore, several studies supported the use of medical and traditional plants which possess good antifungal compounds that can be used for the treatment of fungal disease [7,8]. In addition, many studies used different solvents, such as ethanol methanol, hexane, acetone, chloroform, distilled water, and ethyl acetate, for the extraction of bioactive components from plants that show antifungal activities [9–12]. A previous study found that clove extract has the ability to destroy cell walls and membranes or enter the cells to inhibit DNA and proteins synthesis. Also, it could inhibit the production of some enzymes in the microorganisms [13]. Other studies found that roselle had the strongest antibacterial activity among other plants extracts. In addition, green tea revealed remarkable antifungal activities against *Candida albicans* and *Cryptococcus neoformans* [12,14]. Apple vinegar has been used for burn and wound healing and as an antimicrobial agent against different microorganisms [15–18], but not as a solvent. Therefore, more studies are needed to study the effects of vinegar plant extract on mature biofilm formation of *Candida* spp. and, hence, this research was conducted.

## Materials and Methods

### Isolation and identification of *Candida* species

Nineteen clinical isolates of *Candida* strains, including twelve *C. albicans*, six *C. krusei*, and one *C. tropicalis* were obtained from mouth swabs of tuberculosis patients attending Al- Yarmouk teaching hospital during the period from September 2018 to January 2019. The swabs were streaked directly on Sabouraud dextrose agar and incubated at 37°C for 72 hrs. The yeasts were identified according to their morphology and chemical characters [19].

### Fungal susceptibility testing

The disk diffusion method on PDA agar was used to study the susceptibility of *Candida* isolates against three antifungal disks, namely fluconazole (Flu 10 µg), clotrimazole (Clo10 µg), and nystatin (Nys 10 µg) disks, according to a previously described method mentioned [20].

### Extracts preparation

Roselle, green tea, and Clove were purchased from local markets. Aqueous, ethanolic, and apple vinegar extracts of these plants were prepared according to an earlier mentioned method [21]. The final concentration for all extracts was 50mg/ml.

### Antifungal agent solution preparation

Stock solutions of the antifungal agents of nystatine, fluconazole, and clotrimazole were prepared by dissolving 50 mg of the antifungal agent in 1ml of sterile distilled water [22].

### Detection of biofilm formation

Biofilm formation was detected by using the microtiter plate assay [23] with some modifications. *Candida* isolates were grown in brain heart broth and incubated at 37 °C for 48 hrs. The broth cultures were diluted to 1/100 by adding 10 µl of yeast suspension to 1000 µl of brain heart broth with 0.1 % glucose. Of this suspension, 200 µl was added into each well of a sterile 96-well. The control column was filled only with brain heart broth, then all plates were incubated for 72 hrs at 37 °C. The supernatant was discarded and the microtiter plates were washed for three times with distilled water to remove the plankton cells and left to dry for 15 minutes at room temperature. After that, 200µl of crystal violet (0.1%) was added in each well and left for 20 min. Then, the stain was washed three

times by distilled water and the plates were left to dry. In the last step, 200µl of ethanol was added to the plates which were left for 10 min and read for their optical density values by using an ELIZA reader at 630nm.

### Antibiofilm detection

The antibiofilm activities of plant extracts and antifungal agents were studied on mature biofilms by adding 50 mg of plant extracts or antifungal agents (100µl) in each well as triplicate. Then, the plate was incubated at 37°C for 72 hrs. After that, all the remaining steps of the reference method were followed [24].

### Phytochemical screening by high performance liquid chromatography (HPLC)

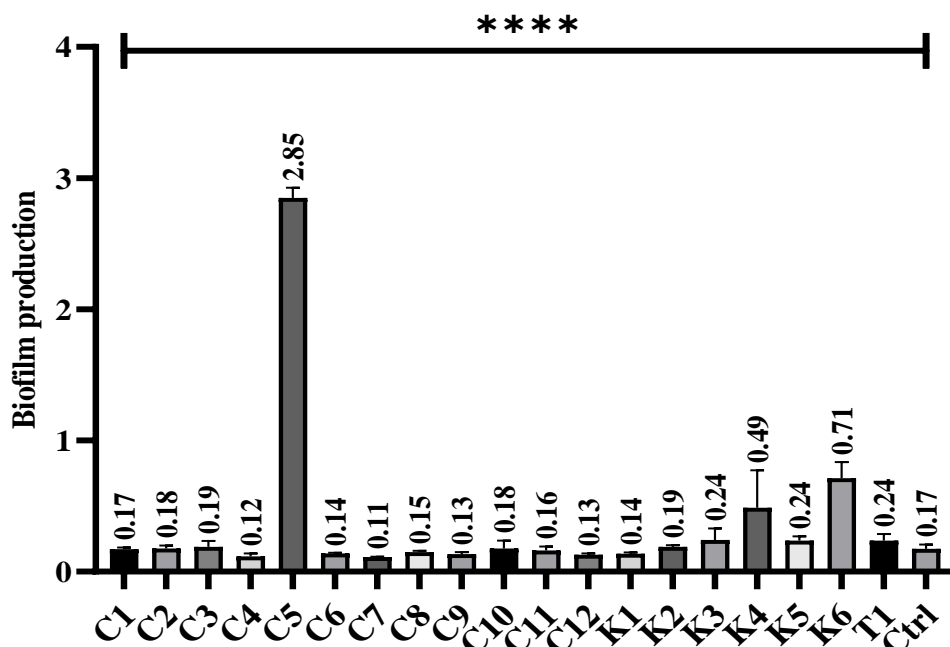
One mg of powdered aqueous, ethanolic, and vinegar extracts of clove, roselle, and green tea were dissolved in one ml of methanol to obtain 1mg/ml and then the solutions were filtered by a Millipore filter (0.45µm) for sterilization. The standards were prepared by dissolving 1 mg of each standard stock in 1ml of methanol and sterilized by filtering through a Millipore filter (0.45 µm) before being subjected to the HPLC analysis according to a previously described method mentioned [25].

### Statistical analysis

The data were given as mean ± standard deviation (n=3) and analyzed by using the analysis of variance (ANOVA) test performed with GraphPad Prism version 8.0.0 for Windows [26]

### Results and Discussion

The results of biofilm production revealed that, from nineteen *Candida* spp. isolates, there were two isolates that gave strong biofilm production, which are *C. albicans* (C5) ( $2.850 \pm 0.077$ ) and *C. krusei* (K6) ( $0.712 \pm 0.124$ ). Also, two isolates showed moderate biofilm production, namely those of *C. krusei* (K3) ( $0.241 \pm 0.087$ ) and *C. krusei* (K4) ( $0.487 \pm 0.285$ ), while fifteen isolates showed weak production of biofilm, as shown in Figure-1. According to these results, the C5 isolate was selected for further experiments in this study, since it gave the highest productivity and appeared to have resistance to all antifungal disks (Flu, Clo, and Nys), as shown in Table-1. This finding agrees with that previously reported [21]. *C. albicans* is a human pathogen that has several virulence factors, such as adhesion, proteinases secretion, biofilm formation, hyphal formation, and phenotypic switching. Therefore, it can colonize different body sites including the skin, oral cavity, gastrointestinal tract, and vagina [22,23].



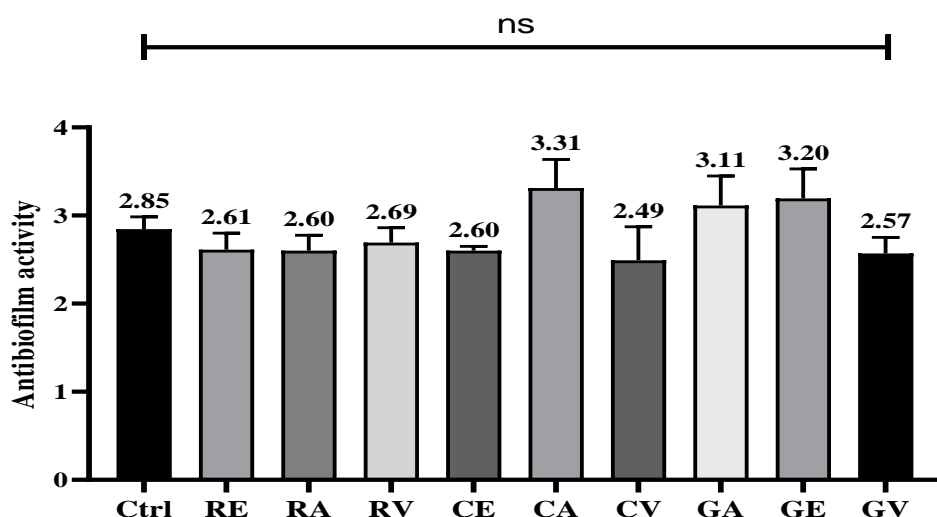
**Figure 1-** Biofilm detection of *Candida* isolates (C1-C12: *C. albicans*, K1-k6: *C. krusei*, T1: *C. tropicalis*; \*\*\*\*:  $P < 0.0001$ ).

**Table 1-** Antifungal sensitivity patterns of *Candida* isolates.

Antifungal agents	<i>C. albicans</i>												<i>C. krusei</i>						<i>C. tropicalis</i>
	1	2	3	4	5	6	7	8	9	10	11	12	1	2	3	4	5	6	1
Clotrimazole (10µg)	R	R	R	R	R	I	R	I	I	S	I	R	R	I	S	I	R	S	S
Nystatine (10µg)	R	R	R	I	R	S	I	I	R	S	S	R	I	R	S	S	R	S	S
Fluconazole (10 µg)	R	R	R	R	R	R	R	R	R	S	R	R	R	I	S	R	R	S	S

\*S: sensitive, R: resistance, I: intermediate

Figure-2 shows the effects of aqueous, ethanolic, and apple vinegar extracts of the following plants: *H. sabdariffa* (Roselle), *C. sinensis* (Green tea) and *S. aromaticum* (Clove) on mature *C. albicans* biofilm. The results revealed that apple vinegar extract of clove (2.4907± 0.382) has significant effects against *C. albicans* biofilm when compared with control group (untreated) (2.85± 0.138) and other extracts. There was no significant difference between plant extracts against *C. albicans* as compared to the control (untreated). Table-2 shows that β-Caryophyllene and p-cymene were the major compounds in all extracts. These compounds have antibiofilm activity against *C. albicans* due to their synergistic actions [26–30].



**Figure 2-** Antibiofilm formation effects of plant extracts against *C. albicans* as compared to the control (untreated). (R: roselle, C: clove, G: green tea, E: ethanol, A: aqueous, V: vinegar, ns: non-significant).

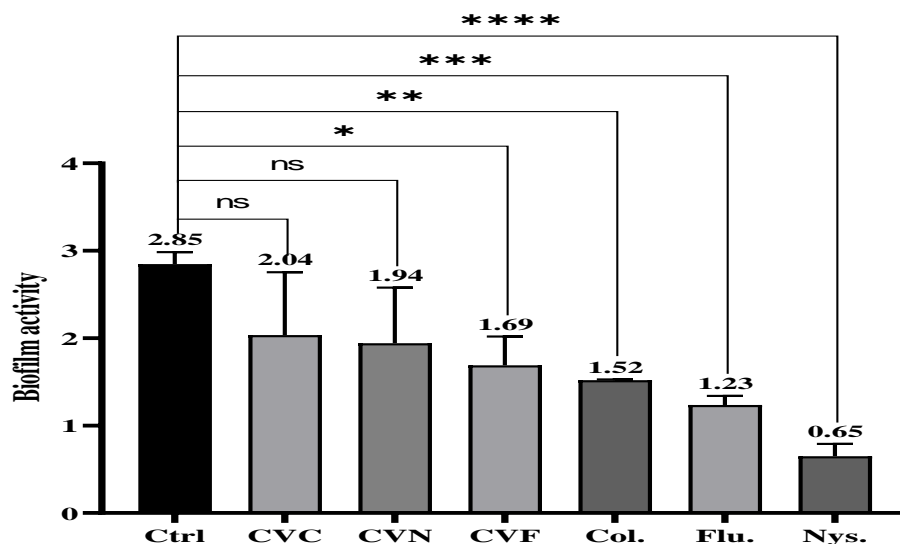
**Table 2-** Phytochemical screening of the components of roselle, clove and green tea extracts by HPLC.

No.	Components	Roselle			Clove			Green tea		
		A*	E*	V*	A	E	V	A	E	V
1	α-pinene	0.027	0.003	8.94	0.8	0.02	1.15	1.43	0.41	1.04
2	β-Caryophyllene	48.71	14.28	1.35	4.56	27.54	2.45	30.02	10.67	11.09
3	chamazulene	ND	0.01	0.1	ND	ND	ND	ND	ND	1.85
4	p-cymene	73.88	16.1	ND	38.92	4.51	26.35	17.03	0.46	0.76
5	α-bisabdoxide A	ND	ND	ND	ND	ND	ND	1.91	ND	ND
6	Limonene	0.001	ND	0.03	1.16	ND	0.013	0.01	0.01	0.002
7	Emodin	ND	ND	0.01	ND	ND	ND	0.001	ND	0.001

\*A: aqueous extract, E: ethanolic extract, V: Vinegar extract, (ND): not detected

Based on the abovementioned results, apple vinegar extract of clove was selected to study the

combined effects with antifungal agents (Nystatin, Fluconazole, Clotrimazole), as shown in Figure -3. The results revealed that the mixture of clove apple vinegar extract+fluconazole (CVF) (v/v) ( $1.69 \pm 0.33$ ) was more effective than the mixture of clove apple vinegar extract+nystatin (CVN) ( $1.94 \pm 0.64$ ), clove apple vinegar extract+clotrimazole mixture (CVC) ( $2.04 \pm 0.72$ ), and the control ( $2.85 \pm 0.14$ ) against *C. albicans* biofilm formation. There was a significant difference between the control and antifungal agents alone and between the control and CVF ( $P < 0.05$ ), whereas no significant difference was seen between the control and the other mixtures (CVC and CVN).



**Figure 3-** Effects of apple vinegar extract of Clove (*Syzygium aromaticum*) and antifungal agents (500  $\mu\text{g/ml}$ ) on *C. albicans* biofilm compared to the control. (CVC: clove vinegar extract + Clotrimazole, CVF: Clove vinegar extract + Fluconazole, CVN: Clove vinegar extract + Nystatin; \*,  $P < 0.05$ , \*\*:  $P < 0.01$ , \*\*\*:  $P < 0.001$ , \*\*\*\*:  $P < 0.0001$ , ns: non-significant).

Antimicrobial resistance is a critical problem in fungal and bacterial infections, so that research in new antimicrobial drugs and targets is needed. Also, combination therapy could increase the activity of antimicrobial drugs against different fungal and bacterial infections [31–33]. Therefore, many researchers studied the effects of various traditional and medicinal plant extracts on most pathogenic microorganisms that cause different diseases in the human population [34–36]. In addition, due to the non-toxic effects of these medicinal plants to human cells, they can be used as a new treatment method to fungal infections [37].

### Conclusions

The results found that apple vinegar was the most efficient solvent to obtain some phytochemical compounds from the tested plants that have antibiofilm activity against *C. albicans* in comparison with other solvents used in this study. However, more studies are required to verify other bioactive compounds that can be extracted from medicinal plants by using apple vinegar as a solvent to inhibit the biofilm formation of pathogenic microorganisms.

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