EFFECT OF *SYZYGIUM AROMATICUM* L. ESSENTIAL OIL EXTRACT AGAINST PATHOGENIC *CANDIDA.ALBICANS IN-VITRO*

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ABSTRACT

***Candida albicans* is an universal fungi, belong to the phylum Ascomycota, the infection caused by *Candida albicans* is called candidiasis. The study aimed to evaluate the *Syzygium aromaticum* extract as antifungal activity and fungicidal against *C.albicans*. Furthermore, gas chromatography-mass spectrometry (GC-MS) analysis was performed to determine active components of clove oil which indicated the presence of eugenol (41.24%), followed by caryophyllene (18.36%) . The disc diffusion method and minimum inhibitory concentration was done to evaluate the anti-candidal activity of different *S.aromaticum* concentration. Extract with *S. aromaticum* yield percentage of 46.6 %. The *in-vitro* anti-candidal extract exhibited the highest antifungal activity against *C. albicans*, with inhibition zone diameters of 22.0 mm at 0.5mg/disc. The minimum inhibitory concentration of the extract was 1mg/ml against *C. albicans*, while the minimum fungicidal concentration was 2mg/ml.**

Keywords: *C.albicans*, clove oil, eugenol , inhibitory concentration, fungicidal.

تأثير مستخلص زيت القرنفل الاساسي ضد المبيضات المسببة للعدوى في المختبر

بان مناف علي عروبة محمد سعيد
باحث استاذ

المستخلص

المبيضات البيضاء هي فطريات عالمية, تنتمي الى شعبة Ascomycota. وتسمى العدوى التي تسببها المبيضات البيضاء داء المبيضات. هدفت الدراسة الى تقييم مستخلص *Syzygium aromaticum* كنشاط ضد الفطريات وقاتل للمبيضات البيضاء. علاوة على ذلك تم اجراء تحليل كروماتوكرافيا الغاز لقياس الطيف الكتلي (GC/MS) لتحديد المكونات الفعالة لزيت القرنفل التي دلت على وجود الايوجينول 41.24% و يليه كاريوفيلين18.36%. تم اجراء طريقة انتشار القرص والحد الادنى من التركيز المثبط لتقييم النشاط المضاد للفطريات لتراكيز مختلفة من *S.aromaticum*. نسبة حصيلة المستخلص 46.6%. اظهرالمستخلص المضاد للفطريات اعلى نشاط ضد المبيضات البيضاء, مع قطر منطقة التثبيط 22 ملم في 0.5 ملغم/قرص. اقل تركيز تثبيطي لمستخلص *S.aromaticum* كان 1 ملغم/مل ضد المبيضات البيضاء بينما اقل تركيز قاتل كان 2 ملغم/مل.

الكلمات مفتاحية: المبيضة البيضاء , زيت القرنفل, ايوجينول, التركيز المثبط, قاتل للفطريات.

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INTRODUCTION

Fungi are noticeable organism and they form a separate kingdom for purposes of classification. Fungi are unicellular or multicellular, they are divided in to either yeasts which are single cells or moulds (i.e. hyphal fungi) (1,18). Fungal infection frequently occurs after the spores either breathed in, come in to contact skin or invade the body through wound, cut or injection, the yeast *Candida albicans* can live in people without causing symptoms, and can cause superficial mild candidiasis in healthy person, such as vaginal infection or oral thrush, and sever systemic candidiasis in people with weak immune system (21). Antifungals can be classified into three groups according to their site of action: azoles, which inhibit the synthesis of ergosterol (the main fungal sterol); polyenes, which interact with fungal membrane sterols physicochemically; and 5-fluorocytosine, which inhibits macromolecular synthesis (7). Clove(Syzygium aromaticum) is an median size tree (8-12m) from myrtaceae family in east Indonesia, it is also cultured in India, Malaysia, and Sri Lanka (14).The phytochemical of clove composed of numerous classes and groups of chemical components like monoterpenes, phenolic and hydrocarbon components, The main compounds in clove oil are mainly eugenol (70-85%) followed by eugenyl acetate (15%) and β-caryophyllene (5-12%), eugenol is a phenylpropene compound which consider as the principle bioactive ingredient of clove essential oil gained from Eugenia caryophyllata buds and leaves (15). Eugenol permits the donation of an hydrogen atom and subsequent stabilization of the phenoxil radical generated forming stable compounds that do not start or propagate oxidation (9). It is also used as antifungal against different strains such as *Mucor sp, Fusarium monoliforme, Aspergillus sp, Candida albicans*, cause lysis of the spores and micelles. In addition, cause membrane disruption and deformities of macromolecules (25,26).

MATERIALS AND METHODS

Plant Collection: Clove *Syzygium atomaticum* buds were collected from local market in Baghdad and then disinfected using 5% sodium hypochlorite solution (NaOCl), washed three times using distilled water, and kept to dry. The plant preparation was done in veterinary medicine college/pharmacology laboratory.

Preparation of clove oil (*Syzigyum aromaticum*): Organic solvent extraction of the *S.aromaticum* bud was prepared by using *n*-hexane (95%) which is very effective in extracting the active components of the plant by using Soxhlet-apparatus depending on the method designated by Ishaq *et al*. (12).

GC-MS analysis of *S. aromaticum* Oil: Phytochemical investigation of the *S. aromaticum* extracts was achieved by GC-MS for the detection of active ingredient revealing antifungal activity. The GC-MS analysis was accomplished using the GC-MS Thermo Trace GC Ultra / TSQ Quantum GC. The phytochemical study was done using a Agelint HP- 5ms Ultra Ineit capillary column, (30 m × 0.25 μm film thickness). The Ramp rates were (ramp 1 was 60 °C hold to 3 min, ramp 2 was 60-180 °C hold to 7 min, ramp 3 was 180-280 °C hold to 8 min, ramp 4 was 280 °C hold to 3 min). The operating circumstances were as follows: helium as a carrier gas 99.99%, injector and detector temperatures were 250 °C. The chemical elements of the clove bud extracts were recognized by comparing the results of the GC-MS analysis with the reference retention time and spectral mass data of the NIST database (29).

Identification of *candida albicans: Candida albicans* isolation: Pathogenic isolate of *Candida albicans* was obtained by Department of microbiology in College of Medical Tecnology / Ashur University from mouth suffering from oral thrush, it was used as a challenge strain. The strain had a white-creamy colony on Sabrouraud dextrose agar, the challenge fungi was the same strain which was used in *in-vivo* antifungal activity assay.

Germ tube formation test: A germ tube formation test is a diagnostic method to determine if this strain is pathogenic or non and was performed according to Jawetz *et al.* (13). Small colony of the yeast was added in a test tube containing 0.5 ml of human serum, and then incubated for three hours at 37 ^{0}C. After that a drop from suspended yeast was put on the clean slide and stained with crystal violet for 1 minute for identification of germ

tube. Lastly the slide was coated with a cover slide, and examined microscopically under (40x lens) for the presence of germ tube.

Pharmacodynamic analysis: Estimation of MIC and MFC: According to Veiga *et al*. (27), the micro-dilution studies were carried out in sterile 96-well micro-plates with a U-shaped bottom. All of the wells of the plates were filled with 100 µL of broth (Sabouraud broth). 100 µL of antifungal solutions were added to the first well of each column (columns 1 to 11), (serial dilutions were done 32-0.0625 mg/ml by passing 100 µL of clove oil solutions through wells 1 to 10 of the lines. Then, by passing 100 µL of each line's wells 1 to 10, serial dilutions were done. 10 µL of the relevant standardized inoculum (1×10^6 cfu/ml of *C. albicans*) was put to each test hole (columns 1 to 11). For the negative control (column 12). The plates were incubated for 24 h at 37^{o}C. After incubation, 20 µl of 0.05 % (w/v) bromocresol purple solution was added to each well, and the plates were incubated for another 2 h. The lowest concentration that inhibited fungal growth was considered as the MIC value (22). All determinations were performed in triplicate and carried out three times to ensure the reproducibility of the results. The minimum fungicidal concentration (MFC) was determined by sub-culturing each well component that did not show visible indicator changes on fresh Sabouraud agar plates (16).

Sensitivity test: The anti-candidal activity of *S. aromaticum* extract was evaluated by the well diffusion method. This technique was accomplished to evaluate the anticandidal potency of the extract. Sabouraud Dextrose agar was distributed into sterile Petri dishes of each, after that spread of *C. albicans* suspension (10^6 CFU/ml) on to the surface of the agar. Sterile blank Whatman paper discs of 6 mm diameter were impregnated with clove oil concentrations of 0.5, 1, 2, 4 and 6 mg/ml, Clotrimazole 25 µg and Fluconazole 50 µg was used as the positive control, while sabouraud dextrose broth served as the negative control. The sterile blank discs were impregnated with 30 µl of the various clove oil concentrations, including the positive and negative controls. The discs were air-dried at room temperature before to placement on the agar surface. The plates were then incubated in the upright position at 37 C^o for 24 hours. Three replicates were carried out for each concentration extract and the activity of plant extract was determined by measuring the diameter of inhibition zone around each well by millimeter using (Vernier caliper) against the tested organism (20). The results and standard errors means values were tabulated and the percentage inhibition of diameter growth (PIDG) was evaluated following the antifungal sensitivity test. The sensitivity of *C. albicans* to clove oil was compared to clotrimazole and fluconazole as the positive control. PIDG values were determined according to the following equation:

$$\textbf{PIDG}\ \% = \textbf{diameter of sample}\ (\textbf{mm}) - \textbf{diameter of positive control}\ (\textbf{mm}) \div \textbf{diameterof positive control x100}$$

Effect of Clove oil concentration on release of cellular materials: To determine the influence of different concentrations of Clove oil on the release of cellular materials. Suspension of 1×10^6 cells/ml of *C. albicans* was harvested by centrifugation at 400 × *g* for 15min. The pellet was washed twice with phosphate buffer solution (pH 7.4), two group treatments were considered, control group: Cells control suspended in PBS, treated groups: Cells suspended for 2 hours in PBS containing different concentrations of Clove oil (0.25xMIC, 0.5x MIC, 1x MIC, 2x MIC, and 4x MIC). After treatment cells were centrifuged at 12,000 × *g* for 2 min. Then, the absorbance of the supernatant was determined using a UV-spectrophometer at 260 nm.

Effect of time on cell lysis Curve: Fungal suspension equivalent to ($1x10^6$ CFU/ml) was prepared from overnight fungal culture. Suspension of 1×10^6 cells/ml of *C. albicans* was harvested by centrifugation at 400 × *g* for 15min. The pellet was washed twice with phosphate buffer solution (pH 7.4). Two group treatments was considered, control group: Cells control suspended in PBS, treated groups: Cells suspended in PBS containing different concentrations of Clove oil (2x MIC, and 4x MIC) treated for different periods of time: 0, 10, 20, 30, 40, 60 and 90 min. After treatment cells were centrifuged at 12,000 × *g* for 2 min. Then, the absorbance of the

supernatant was determined using a UV-spectrophometer at 260 nm.

RESULTS AND DISCUSSION

Extraction of clove oil and: GC-MS) analysis: Extraction of clove bud with 95% n-hexane revealed a bright yellow color extract with typical clove oil smell and bud powder yield percentage of 46.6%. The GC-MS analysis of active compounds in clove oil showed the presence of 16 individual compounds. Eugenol was the major compound that represented 41.24% of the total compounds, followed by Caryophyllene, which comprised 18.63%, phenol 17.73%. Additionally, other compound were presented such as, Humulene 5.56%, Trimethoxyacetophenone 3.49%, Octadecanoic acid 2.64%, 1,2,-benzenedicarboxylic acid 1.66%, Tetracyclo [6.3.2.0.(2,5).0(1,8)] tridecan-9-Oi, 4,4-dimethyl-Adamantane 1.54%, Hexadecanoic acid 0.86% Gamma-Muurolene 0.66% , Bis (2-ethylhexy) phthalate 0.67%, Isoaromadendrene epoxide 0.66%, and Longifolene 0.6%. (Figure 1).\

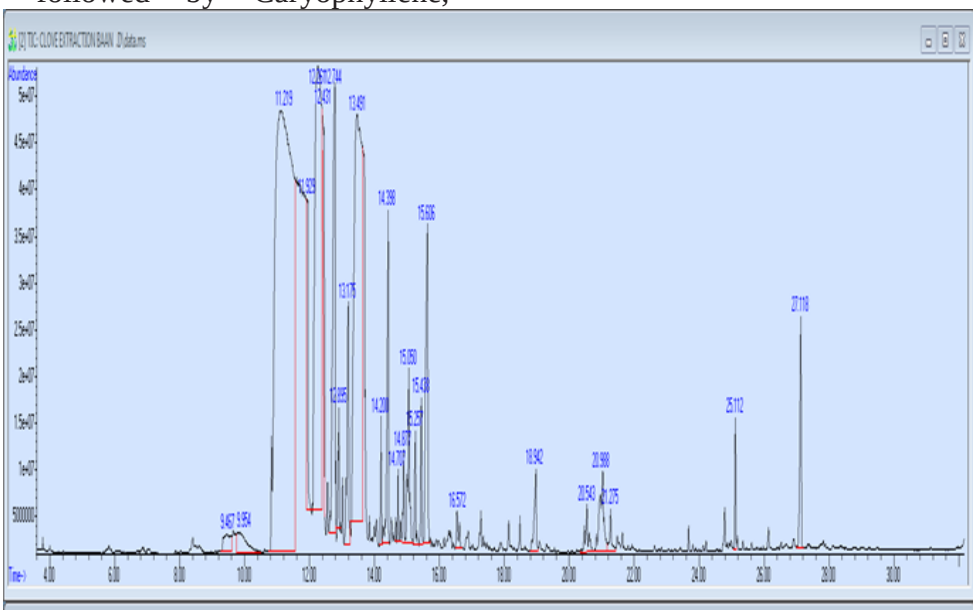

Figure 1. GC-MS of phytochemical compounds of *Syzygium aromaticum*

Solvent extraction is a best method to extract essential oils from plant constituents as a result of its less energy consumption and high extraction efficiency. Hexane has a great ability to extract oil and this is attributed to the presence of several isomers. It is favored for solvent extraction as a result of its high volatility and low sensible heat. Besides, it can be simply detached from oil or extract with low energy consumption. This result is almost comply with the results of Ishaq *et al.* (12) who discovered that the percentage yield of hexane extract was 48.84% from clove bud powder which was extracted by using a soxhlet apparatus. The high percentages for clove oil extract by hexane can be described mostly because in the plant, essential oils are deposited or located in conduits, glands, sacs, or glandular hairs or in reservoirs in the plant; thus, the exposure of these reservoirs to the action of the distillation vapor permits the vapor to soften or break the walls of the oily glands, releasing the oils, favoring extraction yield as confirmed by Selles *et al.* (24). Phytochemical analysis of the *S. aromaticum* n-hexane extract revealed that eugenol was the most abundant active ingredient (41.24%), followed by Caryophyllene (18.36%) and phenol (17.73). This is in agreement with the previous studies results that showed similar components in the essential clove oil (11). Khalil *et al*. (15) who mentioned that the range of the percentage of eugenol was between 75.22% and these dissimilarities may be due to the effect of harvesting and environmental factors and also to obtain high quality of clove oil with increase in elevation (28).

Identification of *Candida albicans:* Results of basic culturing on Sabouraud dextrose agar, at 37 ^{o}C, for 24 hrs, characterized by smooth colonies, small, white- creamy, white creamy opaque colonies. Germ tube test is a diagnostic method to make sure that the strain of *C. albicans* is pathogenic Figure (2,3).

Figure 2. *C.albicans* colony on Sabouraud dextrose agar

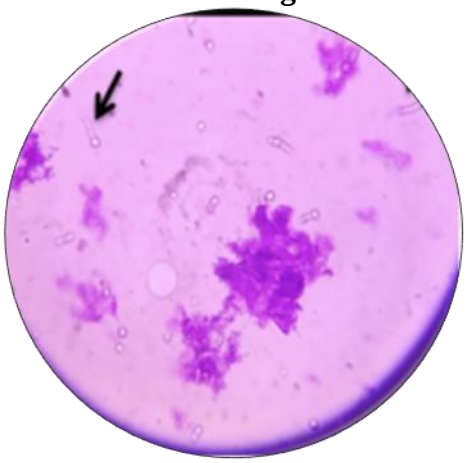

Figure 3. Microscopic appearance of germ tube of *C.albicans*

The most commonly medium used for isolation candida isolation is SDA, although allowing growth of candida, inhibiting the growth of many species of bacteria due to its low pH. Combination of antibiotics into SDA will improve its selectivity (17). Typically SDA is incubated aerobically for 24–48 hrs at 37°C *Candida* grows as cream, smooth, pasty convex colonies on SDA and differentiation among species is rarely probable and this result was supported by the result of (5).We considered as actual and positive germ tube structure the elongated daughter cells originated from the round mother cell without any constriction at their origin, and the constriction of the hyphae at the round mother cell was referred to as pseudo hyphae and this is similar to the result of (23).

Determination of the MIC and MFC

The results showed that the concentration 1.0 mg/ml of clove oil was active against *C. albicans*, this concentration of clove oil was shown to have a positive value that inhibit the growth and 2.0 mg/ml killed *C. albicans* so it was considered as the (MFC) that tested in micro-dilution assay as shown in the Figure (4).

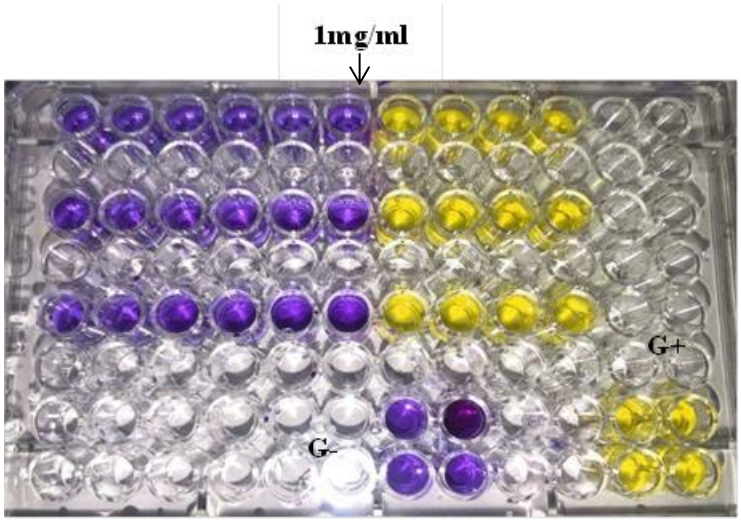

Figure 4. Minimum inhibitory concentration of Clove oil, against *C. albicans* {purple (no growth) yellow (growth)}.

The MIC and MFC of clove oil towards *C. albicans* were both 1mg/ml and 2mg/ml. The MIC and MFC values obtained in the study explain that clove oil acts as a fungicidal agent against *C. albicans*. Exposure of *C. albicans* to increasing clove oil concentrations resulted in drastic reductions in fungal growth. The results of this study showed that clove oil exhibited antifungal properties towards *C. albicans* and was concentration dependent, which is in agreement with a study by Himratul-Aznita *et al*. (10). It is reasonable to assume that the activity of this oil can be related to the presence of a high concentration

(41.24%) of eugenol. Eugenol was found to be an active constituent of clove oil, with MIC values ranging from 1 to 2 mg/ ml. The importance of the phenolic hydroxyl groups for the antimicrobial activity of clove oil has been previously reported (3).

Sensitivity test

Different concentrations of oil extract caused different degrees of zones of inhibition against *C. albicans*. The size of inhibition zones were different according to concentration of extract, the size of inhibition zone were proportionally increased with increasing of concentration of clove oil extract.The results showed that *C albicans* was more sensitive to oil extract of *S. aromaticum* than clotrimazole and fluconazole in the concentrations using in this study. The results obtained for the inhibition zones were incorporated into the formula for PIDG. These results are reflected in Figure (5,6), which shows that the PIDG values of clove oil concentrations of (0.5 and 1mg/ml) were negative in reference to the PIDG of the positive control. In contrast, at 2, 4 and 6mg/ml, the concentration of clove oil was shown to have a positive value, indicating that at this concentration the antifungal activity was stronger than the positive control in preventing the growth of *C. albicans*.

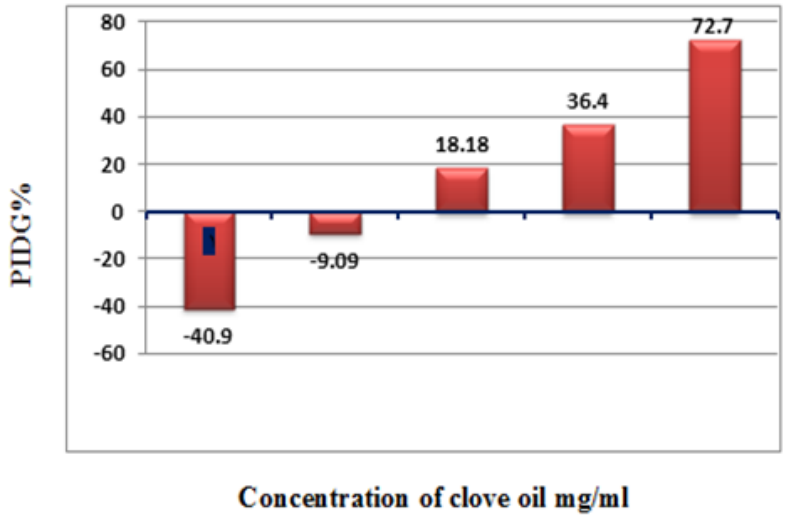

Figure 5. Percentage inhibition of diameter growth (PIDG) versus concentration of clove oil on *C.albicans* compared with fluconazole.

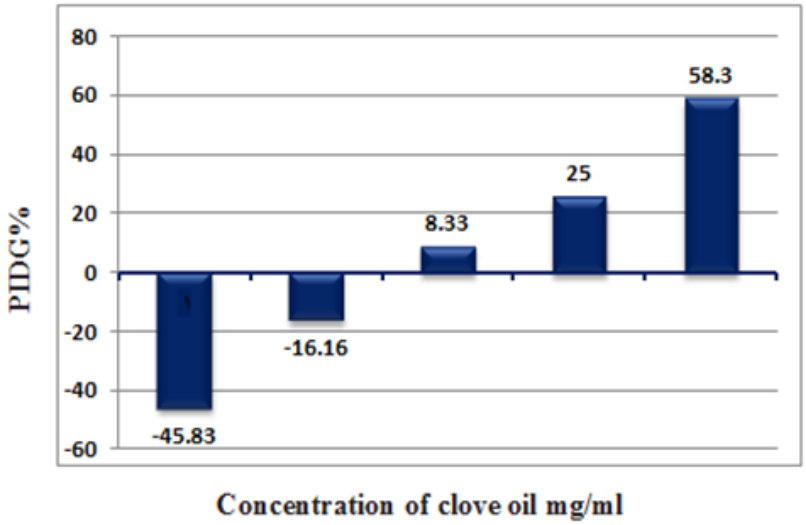

Figure 6. Percentage inhibition of diameter growth(PIDG) versus concentration of clove oil on *C.albicans* compared with clotrimazole.

The results of inhibitory zone diameter indicated the sensitivity of C albicans, towards different tested extract concentrations. All antifungal activities were observed to be concentration dependent that is in agreement with Andes (2). In addition the PIDG percentage of clove oil concentrations of (0.5 and 1mg/ml) was negative, in contrast, at 2, 4

and 6 mg/ml, positive in reference to the PIDG of the positive control. This confirm the antifungal activity of clove oil was stronger than the positive control in preventing the growth of *C. albicans* this finding is in agreement with the findings reported elsewhere that eugenol, a medicinal plant extract, exhibits antimicrobial activity towards several strains of pathogenic microorganisms(8).

Effect of clove oil concentration on cell lysis

The results showed that the release of cellular material by UV-absorbing was accompanied by total cellular mortality when *C. albicans* was exposed to 4mg/ml clove oil (Table 1).

The time-kill curve: The result showed that the 4xMIC concentrations of clove oil need 10 min. to kill 50% of the *C. albicans* population, while 2xMIC and 1xMIC concentrations of clove oil needed 20 min and 60min. to kill 50% of the *C. albicans* population respectively. These three different concentrations of clove oil exhibited a time dependent effect. The time taken to kill the *C. albicans* cells may decrease as the concentration of clove increases (Table 2).

Table 1. Effect of clove concentration on cellular mortality and the release of 260 nm absorbing material from *C.albicans*

Concentration of clove oil	Cellular material contents released at OD260 (%)
0	0
0.25xMIC	20.6
0.5xMIC	35.8
1.0xMIC	54.1
2.0xMIC	98.9
4.0xMIC	100

Table 2. Effect of time on cellular material release from *C.albicans* treated by different concentration of clove oil

Time/ min.	Absorbing of cellular material release (%)		
	4x MIC	2x MIC	1X MIC
0	0	0	0
10	52.2 ±0.2	35.9 ±0.2	24.3 ± 0.8
20	86.8 ± 0.4	54.8 ± 1.3	37.9 ± 0.6
40	98.2 ± 1.1	82.6 ±0.7	43.8 ± 1.4
60	100	94.8 ±0.6	59.7 ± 0.4
90	100	100	89.7 ± 1.5

The released cellular material was assessed by two mean variables, namely concentration-dependent and kills time-curve lysis. Our study showed that the release of cellular content increased relative to the concentration of clove oil used. Furthermore, this release was accompanied by total cellular mortality after the incubation period. Cellular material content from this assay is suggested to provide evidence of the cell damage caused by chemical and physical agents. The cytoplasmic membrane has also been recognized as a target for the action of eugenol (4,19). A complete loss of viability was observed with prolonged exposure to clove oil, indicating that the antifungal activity of clove oil is time dependent, the exposure time for *C. albicans* to be completely lysed in the presence of clove oil could elucidate the fungicidal mechanism of the antifungal due to the damaging or rupture of the fungal cell outer membrane lead to cell death (6).

CONCLUSION

In this work, the activity of *Syzygium. aromaticum* oil extract as antifungal against *Candida. albicans* has been revealed. The essential oil of clove extract rich with eugenol demonstrated great antifungal power. This extract can be suggested as antifungal to treat candidiasis and reduce the incidence of mortality rate due to systemic candidiasis.

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