



## ANTIMICROBIAL ACTIVITY OF ETHANOLIC EXTRACTS OF CLOVE AND THYME

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### ABSTRACT

This study was carried out to evaluate the antimicrobial activity of clove (*Syzygium aromaticum*) and thyme (*Thymus vulgaris*) ethanolic extracts. Different concentrations of ethanolic extracts were prepared and antimicrobial activity was determined against some bacterial, yeast and mold strains. The antimicrobial activity of examined extracts at different concentrations was determined against different cell population of tested strains and expressed as diameter of inhibition zone. Results showed that all clove and thyme ethanolic extracts were markedly inhibited the growth of all tested strains, however, the inhibition effect was differed with regard to the concentration of ethanolic extract as well as the type of tested microorganism. Generally, clove ethanolic extract had stronger antimicrobial activity rather than that of thyme ethanolic extract. Subsequently, clove and thyme are an extremely strong antimicrobial agent with usefull potential applications in food processing as a natural safe unsted of synthetic antimicrobial agents.

**Keywords:** Clove, Thyme, Extraction and Antimicrobial activity.

### INTRODUCTION

The use of antimicrobial agents is a common practice for preserving foods; incorporation in a food recipe of chemical antimicrobial agents towards inhibition of spoilage and pathogenic microorganisms resulted in compositional modification of food environment. This application or treatment is nowadays undesirable for the consumer, who likes natural products, and minimally processed foods of

high quality, with a fresh appearance, easy to eat, tasty, prepared without chemical preservatives, safe and possessing long shelf-life (**Lucers et al 2012**).

Spices and medicinal plants are considered as natural antimicrobial and antioxidant. Spices and some medicinal plants have been used in traditional medicines from many thousand years ago. (**Tajkarimi et al 2010; Pandit et al 2011 and Rani et al 2012**

Most of food borne bacterial pathogens are sensitive to extracted plants such as garlic, turmeric, cinnamon, clove, thyme and oregano. Clove (*Syzygium aromaticum*) is one of the major spices containing natural antimicrobial and antioxidant components. It commonly consumed as most popular medicinal herbs around the world and it is originates from India, Southeast of Asia and Indonesia. Generally, the powder produced from this natural product has been commercially used as a spice, flavorent, preservative and additive. Besides, it is also utilized as a therapeutic agent used in several foods; because of its low side effects (**Zhang et al 2006; Rani et al 2012 and Koul et al 2013**).

The natural phytochemical components in thyme such as thymol was reported to possess numerous medicinal properties and wide spectrum of biological actions which include; antioxidant, anticarcinogenic, antiinflammatory, antimutagenic, anticoagulant, antidiabetic, antifertility and antimicrobial activities (**Nikolić et al 2014 and Sandhir et al 2015**); also reported therapeutic property against a variety of diseases ranging from cancer to cystic fibrosis and this provides a basis for exploring its endodontic applications. Thymol are is a natural monoterpenoid phenol component known

to have strong antimicrobial and antioxidant activities (Prasanna et al 2011 and Nikolić et al 2014). This compound due to a variety of biological activities has been gained significant attention of researches all over the world.

The aim of study was to evaluate antimicrobial activity of prepared ethanolic powder extracts of clove and thyme that used as natural antimicrobial in food products.

## MATERIALS AND METHODS

### Materials

Clove (*Syzygium aromaticum*) and thyme (*Thymus vulgaris*) powders were purchased during 2017 from local market, Cairo, Egypt. All chemicals and reagents were analytical grade produced by Sigma-Aldrich, Inc. (St. Louis, M., USA); also all media used in determination of antimicrobial activity were produced by Oxoid Limited (England) and purchased from EL. Gomhouria Trading Chemicals and Drugs Company.

The tested microorganisms are: bacterial strains included; *Bacillus cereus* DSMZ 345, *Staphylococcus aureus* ATCC 6528, *Escherichia coli* ATCC 25922, and *Salmonella typhimurium* ATCC 14028; while yeast strains included: *Candida albicans* ATCC 10231 and *Geotricum candidum* NRRL Y-552 and finally mold strains included; *Aspergillus niger* ATCC 102 and *Aspergillus flavus*. The aforementioned strains were obtained from the Egyptian Microbial Culture Collection (EMCC) at the Microbial Resource Center (Cairo, MIRCEN), Faculty of Agriculture, Ain Shams University, Cairo, Egypt.

### Methods

#### Preparation of clove and thyme ethanolic extracts

Plant materials were ground and macerated for extracting. Dried clove and thyme powder samples (5 g) were homogenized for 1 min in 50 mL of 80% ethanol and 2 ml dimethyl sulfoxid (DMSO), extraction was carried out by shaking overnight at 1,500 rpm at room temperature. Then, ethanolic clove and thyme extracts were centrifuged at 3,000 rpm for 15 min and filtered through 0.22 µm poly-tetrafluoroethylene (PTFE) filters, the residue was re-extracted twice, (Carvalho et al 2015).

#### Determination of antimicrobial activity of clove and thyme ethanolic extracts

##### Preparation of inoculates

Stock cultures of tested bacterial strains were activated by loopful of stock culture into nutrient broth medium and incubated at 30 or 37°C for 24 h. then the incubated suspensions of tested strains were enumerated using plate count method. The abovementioned procedure was repeated for tested yeast strains using potato dextrose broth medium and incubated at 28-30°C for 48-72 h with regular shaking. They were counted using plate count method on potato dextrose agar medium. Finally, the tested mold strains which were intended to achieve the antifungal assay were prepared as follows: stock cultures were activated on potato dextrose agar medium after acidification which was immediately poured into Petri dishes (90 mm). Stock cultures were activated by seeding the plates in the center with 5 mm agar disc of 7 days old culture of tested fungi which were grown on the same medium, and then the plates were incubated for 7-10 days at 28°C.

##### Screening of the antimicrobial activity against bacterial and yeast strains

The paper disc diffusion technique was used as screening method to determine the antimicrobial activity of clove and thyme ethanolic extracts against bacterial and yeast strains, the method of Lai et al (2009) was used with some modifications as follows: About 3 ml of soft nutrient agar (0.75% agar) containing 50 µl inoculums of tested bacterial strain was layered over 20 ml of hard nutrient agar containing (2.0% agar), while about 3 ml of soft potato dextrose agar (0.75% agar) containing 50 µl inoculums of tested yeast strain was layered over 20 ml of hard potato dextrose agar containing (2.0% agar). Sterilized filter paper discs (6 mm of diameter) were injected with 15 µl of different concentrations 2.5-25 (mg/ml) of clove and thyme ethanolic extracts. The injected discs were put in the middle of plates, and then plates were incubated at 30 or 37 °C for 28 h for bacteria and at 28-30 °C for 48 h for yeasts.

The principle behind the assay technique is fairly simple; when an antibiotic injected disc is placed on agar previously inoculated with the rest organism and on moistening, the antibiotic diffuses rapidly outwards through the agar producing antibiotic concentration gradient. A clear zone or ring

will present if the agent inhibit microbial growth. The wider zones surrounding the disc indicated that substance is more active. The zone diameter of inhibition around each of the discs (disc diameter included) was taken as measure of antimicrobial activity. All tests were performed in duplicates. Negative control was prepared using 15 µl of ethanol with DMSO solution.

#### Determination of the minimal inhibitory concentrations (MICs), bacteriostatic and bactericidal effects

The antimicrobial activity of clove and thyme ethanolic extracts against both Gram-positive, Gram-negative bacteria and yeast strains were examined by detecting the minimum inhibitory concentrations (MICs). The appropriate concentrations of clove and thyme ethanolic extracts; which selected as MICs were added to 25 ml of nutrient broth for bacterial strains, and potato dextrose broth for yeast strains. The broth media were inoculated with 50 µl of tested strains. Different flasks were incubated at 37 °C (*St. aureus*, *E. coli* and *S. typhimurium*) and at 30°C (*B. cereus*, *C. albicans* and *G. candidum*) with regular shaking for 24 h. Every 4 hours, growth was compared with the control broth visually and through the measurement of the log number of colony forming units (log cfu/ ml) by plate count procedure (Rachana and Venugopalan, 2014).

#### Determination of antifungal activity

The antifungal activity of clove and thyme ethanolic extracts was estimated using a growth inhibition assay technique (agar dilution method), described by (Guo et al 2007). Fungal spores were grown on petri dishes filled with potato dextrose agar medium. After 7 days of incubation at 28 °C, the fungal plates were stored in a refrigerator at 5 °C. To test the antifungal effect; different prepared of clove and thyme extracts were added aseptically to sterile melted PDA media to prepare the concentrations (10, 20, 30, 40, and 50 mg/ml) and divided into two groups (duplicate for each was carried out). To each plate in the experimental group (E), an appropriate amount of the clove and thyme extracts was added. To those of control group (C), an equal amount ethanol with DMSO solution was added. After plates were cooled, the fungal inoculums were placed onto the agar surface. Both groups were incubated for 3-5 days at 28°C. The diameters of the largest and smallest

fungal colonies were recorded and the averages were calculated.

The antifungal in terms of inhibition percent of mycelia growth (antifungal index) was calculated after mycelium of fungi was reached the edges of control plates as follows:

$$\text{Antifungal index (\%)} = [(C - E) / C] \times 100$$

Where C: the average diameter of the largest and smallest colonies of control groups.

E: the average diameter of the largest and smallest colonies of the experimental groups.

If the inhibition ratio as antifungal index (%) was greater than 20%, the test strains would be considered inhibited and the minimal inhibitory concentration (MIC) of these strains were then determined.

## RESULTS AND DISCUSSION

### Antimicrobial activity of prepared clove and thyme ethanolic extracts

#### Preliminary screening of antimicrobial activity

In vitro, preliminary screening of the antimicrobial activities of clove and thyme ethanolic extracts at different concentrations against four strains of pathogenic bacteria and two yeast strains, were determined and the results are given in **Tables (1 and 2)**.

The antibacterial activity values of clove and thyme ethanolic extracts at different concentrations against pathogenic bacteria were assayed and the measured inhibition zone diameters (mm) are represented in **Table (1)**. The control plates for all bacterial strains which treated with ethanol and DMSO did not show any inhibitory effect against all tested bacterial strains. Where, the clove and thyme crude ethanolic extracts were able to inhibit markedly the growth of all tested bacterial strains; however, the effects differed with regard to the concentration of ethanolic extracts and tested bacterial strain.

The antimicrobial activity of clove and thyme ethanolic extracts was exhibited obviously with the increasing of the extract concentration. Additionally, clove ethanolic extracts significantly demonstrated stronger antimicrobial activity compared to thyme ethanolic extracts (Xie et al 2015). Clove and thyme ethanolic extracts generally showed stronger antibacterial activity against examined Gram-positive bacterial strains than those of Gram-

negative bacterial strains; it was ranged between (10 – 15 mg/ml) for clove ethanolic extracts; and also ranged between (15-20 mg/ml) for thyme ethanolic extracts. *B. cereus* and *St. aureus* strains observed more sensitivity in presence of both clove and thyme ethanolic extracts compared to *E. coli* and

*S. typhimurium*. Concerning the obtained inhibition zones diameter were 29, 32, 31, and 32 mm, respectively for clove ethanolic extracts; when thyme ethanolic extracts were examined, whereas their measured diameter were 25, 28, 28 and 24 mm, respectively.

**Table 1.** Antimicrobial activity of clove ethanolic extract against bacterial and yeast strains

Strains	Conc. (mg/ml)	Diameter of inhibition zones (mm.) <sup>*</sup>										(mg/ml)
		2.5	5	7.5	10	12.5	15	17.5	20	22.5	25	
<i>B. cereus</i>		9	15	24	29	29	29	30	29	30	30	10
<i>St. aureus</i>		10	19	26	32	32	32	32	33	32	31	10
<i>E. coli</i>		8	14	19	22	27	31	31	30	31	32	15
<i>S. typhimurium</i>		8	17	24	26	28	32	32	31	30	31	15
<i>C. albicans</i>		12	19	25	32	32	32	31	33	23	32	10
<i>G. candidum</i>		11	17	24	28	33	34	34	33	34	34	15

\* Including disc diameter of 6 mm.

**Table 2.** Antimicrobial activity of thyme ethanolic extract on bacterial and yeast strains

Strains	Conc. (mg/ml)	Diameter of Inhibition Zones (mm.) <sup>*</sup>										MICs <sup>**</sup> (mg/ml)
		2.5	5	7.5	10	12.5	15	17.5	20	22.5	25	
<i>B. cereus</i>		10	14	17	22	24	25	25	25	26	26	15
<i>St. aureus</i>		9	15	19	26	27	28	28	29	28	28	15
<i>E. coli</i>		8	13	18	19	23	25	26	28	28	27	20
<i>S. typhimurium</i>		9	12	14	17	19	21	23	24	24	23	20
<i>C. albicans</i>		11	14	17	24	25	26	26	27	26	26	25
<i>G. candidum</i>		12	16	21	25	27	29	29	28	29	30	15

\* Including disc diameter of 6 mm.

\*\* MIC: Minimal inhibitory concentration.

It could be noticed that, *B. cereus* and *St. aureus* were the most sensitive tested strains, with the MICs value (10 mg/ml) in the presence of the clove ethanolic extract and with MICs value (15 mg/ml) in the presence of the thyme ethanolic extract.

**Figures (1 and 2)** illustrated that, the antibacterial effects of both determined clove and thyme ethanolic extract concentrations; which were recorded as MICs values showed bactericidal effect.

Generally, ethanolic extracts of clove had a stronger antimicrobial effect on Gram-positive bacteria rather than Gram-negative ones by components present as active ingredients (phenylpropanoids) such as carvacrol, thymol, eugenol and cinnamaldehyde. These compounds inducing leakage of ions and cell contents, leading to cell death (Amrita et al 2009; Tongnuanchan and Benjakul, 2014).

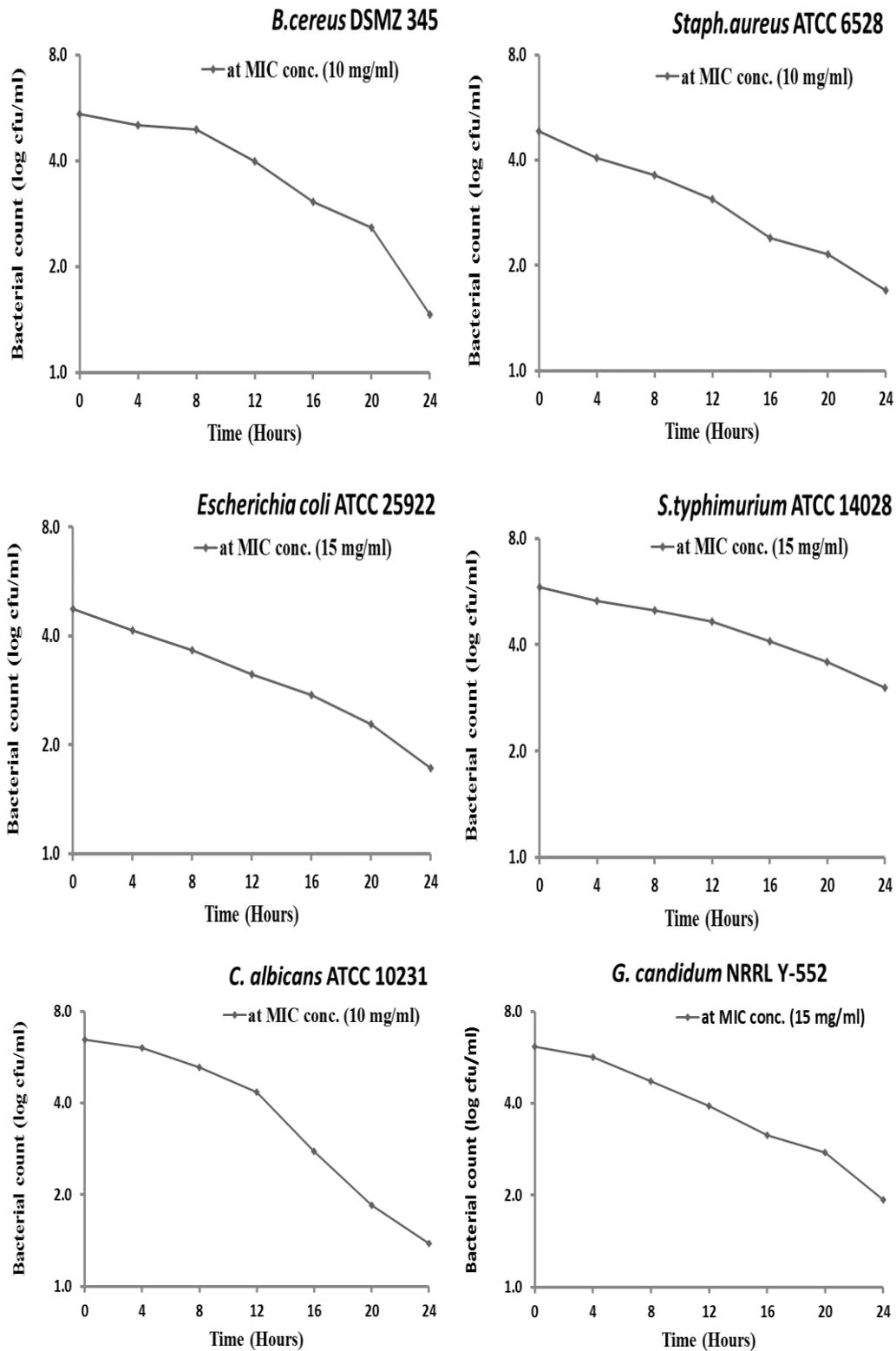


Fig. 1. Growth behavior of examined strains in presence of detected MICs values (mg/ml) of clove ethanolic extract.

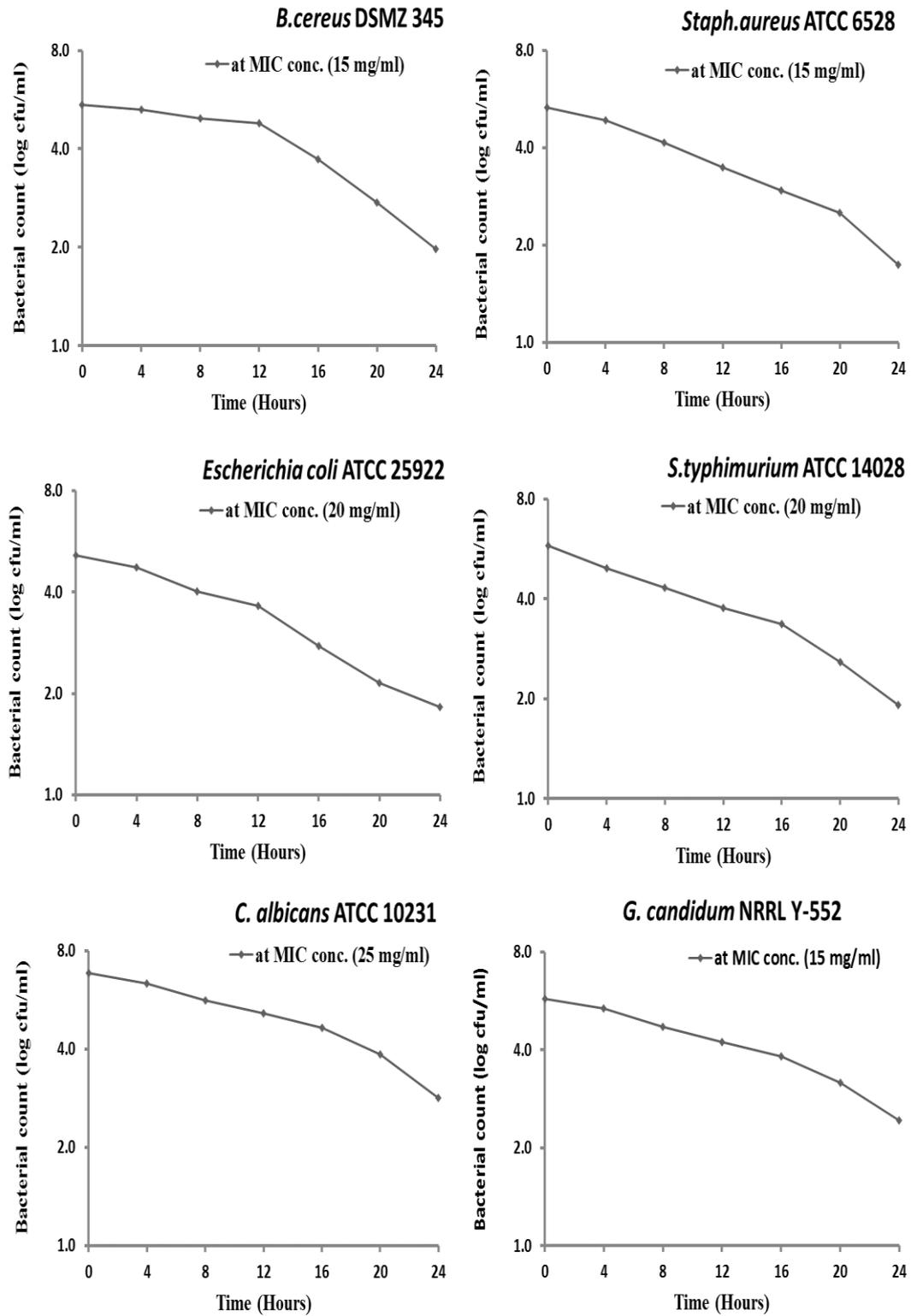


Fig. 2. Growth behavior of examined strains in presence of detected MICs values (mg/ml) of thyme ethanolic extract

On the other side, the observed more resistance of tested Gram-negative bacterial strains could be due to extrinsic membrane proteins and lipopolysaccharides. The minimum inhibitory concentrations (MICs) of clove ethanolic extracts at concentration of 2.5 – 25 (mg/ml) were presented in **Tables (1 and 2)**, and the different MICs values ranged from 10 to 15 (mg/ml) for clove ethanolic extracts; and 15 to 20 (mg/ml) for thyme ethanolic extracts.

Antimicrobial of clove and thyme ethanolic extracts against examined yeast strains are summarized in **Tables (1 and 2)**. Clove and thyme ethanolic extracts inhibited the growth of two tested yeast strains. Moreover, the antimicrobial effect against examined yeast strains was strengthened as the concentration of ethanolic extract increased. Clove ethanolic extracts generally showed stronger

antimicrobial activity compare to thyme ethanolic extracts (**Tongnuanchan and Benjakul, 2014**).

#### Antifungal activity of clove and thyme ethanolic extracts

Data presented in **Tables (3 and 4)** showed the antifungal activity of clove ethanolic extracts. The different concentrations of clove ethanolic extract were able to inhibit the mycelia growth of two examined fungal strains. Antifungal effect was noticed when concentration of clove extracts were increased from 10 to 50 (mg/ml). The MICs values of clove ethanolic extracts was 10 (mg/ml), whereas it was 30 (mg/ml) for thyme ethanolic extracts. The highest antifungal index (%) was found at concentration of 50 mg/ml.

**Table 3.** Antifungal activity of clove ethanolic extract against fungal strains

Fungal Strains	Conc. (mg/ml)	Antifungal index (%)					MIC (mg/ml)
		10	20	30	40	50	
<i>A. niger</i>		24	39	42	48	51	10
<i>A. flavus</i>		25	32	37	43	59	10

**Table 4.** Antifungal activity of thyme ethanolic extract against fungal strains

Fungal Strains	Conc. (mg/ml)	Antifungal index (%)					MIC (mg/ml)
		10	20	30	40	50	
<i>A. niger</i>		9.3	14.6	21.5	27.0	28.3	30
<i>A. flavus</i>		11.5	15.7	26.3	28.3	32.5	30

#### CONCLUSION

The obtained results of antimicrobial activities showed that clove and thyme ethanolic extracts inhibited the growth of all tested strains; in general clove ethanolic extracts exhibited significantly stronger antimicrobial activity compared to thyme ethanolic extracts. It could be considered that clove and thyme are natural material rich in active ingredients content with extremely strong antimicrobial efficiency.

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## النشاط المضاد للميكروبات لمستخلصات القرنفل والزعر الإيثانولية

[45]

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التأثير التثبيطي بإختلاف تركيز المستخلص الإيثانولي ونوع الميكروب المختبر. ودلت النتائج أن المستخلص الإيثانولي لمساحيق القرنفل أظهرت نشاطاً مضاداً للميكروبات بصورة معنوية بالمقارنة بمستخلصات الإيثانول لمسحوق الزعر، وبالتالي أعتبرت مساحيق القرنفل والزعر عاملاً قوياً كمضاد للميكروبات مما يفيد فى إستخدامهما فى تطبيقات محتملة فى التصنيع الغذائى كبديل طبيعى آمن للمركبات التخليقية المضادة للميكروبات.

الكلمات الدالة: القرنفل، الزعر، مستخلصات والنشاط الميكروبي

### الموجز

أجريت هذه الدراسة بهدف تقييم ومقارنة النشاط المضاد للميكروبات لمستخلصات القرنفل والزعر الإيثانولية. حيث تم تحضير تركيزات مختلفة من مستخلصات الإيثانول وتقدير النشاط المضاد للميكروبات ضد بعض سلالات البكتيريا والخمائر والفطريات. وتم التعبير عن النشاط المضاد للميكروبات لمختلف التركيزات ضد السلالات المختبرة بتقدير قطر مساحة التثبيط. وأظهرت النتائج أن جميع تركيزات المستخلص الإيثانولي للقرنفل والزعر أدت الى تثبيط ملحوظ لجميع السلالات المختبرة. وبصفة عامة إختلف