

Effect of aqueous and ethanolic extract of *Eucalyptus camaldulensis* L. on food infection and intoxication microorganisms “in vitro”

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ABSTRACT

Herbs have been used for its medicinal properties from several thousand years ago. Herbs, essences and extracts, possess a variety level of biological activities and antimicrobial activities of a large number of them have been proved. Eucalyptus is one of these plants which the anti-virus effects of its extract has long been used to treat influenza and colds in most parts of the world. The aim of this study is evaluating antimicrobial effects of aqueous and alcoholic *Eucalyptus camaldulensis* leaves against some of the important food pathogens. Antimicrobial effects of extracts were evaluated on *Staphylococcus aureus* PTCC 2592, *Escherichia coli* PTCC1330 and *Penicillium digitatum* by “using the method of Collins” and “disk agar diffusion method”. The results showed that aqueous and ethanolic extract were quite effective in 2000 µg/ml concentration on *Staphylococcus aureus* and *Penicillium digitatum*, while both extracts have no certain antimicrobial effect on *Escherichia coli*. In “disk agar diffusion method” 20, 40, 60 and 80 mg/ml aqueous and ethanolic extract concentrations, was inhibition effect on *Staphylococcus aureus* and *Penicillium digitatum*, and 60 and 80 mg/ml aqueous and ethanolic extract concentrations, has deterrent effect on *Escherichia coli*, but at 20 and 40 mg/ml concentrations, no inhibitory effect on *Escherichia coli* was observed. Minimum Inhibitory Concentration (MIC) of ethanolic extract of *Eucalyptus camaldulensis* leaves and Minimal Bactericidal Concentration (MBC) for bacteria and Minimum Fungicidal Concentration (MFC) for fungi were performed. The results indicate that ethanolic and aqueous extracts of *Eucalyptus camaldulensis* leaves have the greatest effect on gram-positive bacteria *Staphylococcus aureus*. *Escherichia coli* were resistant to most of the aqueous and ethanolic *Eucalyptus camaldulensis* extracts. In conclusion, using *Eucalyptus* as a natural antimicrobial compounds in vitro have considerable antimicrobial ability over the studied strains.

Keywords: *Eucalyptus camaldulensis*; Aqueous and alcoholic extract; Antimicrobial effects

INTRODUCTION

Herbs have been used for its medicinal properties from several thousand years ago. Therefore according to the problem of bacterial resistance to antibiotics, it is essential to find antimicrobial compounds with minimal side effects. In recent years, considerable research have been concentrated to evaluate the antimicrobial effects of extracts, essence and spices which showed the power and ability these compounds inhibiting the growth of pathogenic and food spoilage microorganisms in a wide range of food. Since, these compounds are completely natural, their side effect for human health and the environment are much less than chemical

preservatives. Herbs, essences and extracts, possess a variety level of biological activities and antimicrobial activities of a large number of them have been proved [1]. Eucalyptus is one of these plants which the anti-virus effects of its extract has long been used to treat influenza and colds in most parts of the world. This plant was originated in Australia and cultivated in other parts of the world.

Eucalyptus with binomial “*Eucalyptus camaldulensis*” is a plant of the *Myrtaceae* family, and widely have grown in tropical, subtropical and temperate world, so until 2000 covered more than ten million hectares of an area [2]. *Eucalyptus* is one of the most popular medicinal plants. This plant is a rich

source of poly phenols and Terpenoids and the base composition (70 to 80 mg/ml) of the leaves are the Eucalyptol or Cineole [3]. Southwell [4] was evaluated the important survival and damaging effects of cineole on tea oil tree and evaluate the antimicrobial activity in order to determine MIC, agar dilution method was used. In this experiment 8-cineole concentration were used on growing of *Staphylococcus aureus*, *Escherichia coli* and *Candida albicans* as gram-positive, gram-negative and yeast from food pathogen microorganisms. Has been observed increasing the concentration of cineole, doesn't have significant inhibitory effect on these microorganisms. Eucalyptus has been used for the treatment of many diseases such as influenza, dysentery and skin diseases. *Eucalyptus camaldulensis* extract has many properties such as anti-cancer, anti-inflammatory, painkiller, antioxidant, anti-blood proliferation, anti-malaria, anti-mold and antiviral properties [5- 8].

The aim of this study was evaluated of antimicrobial effects of aqueous and alcoholic *Eucalyptus camaldulensis* leaves against some of the important food pathogens and spoilage in foods and food products, extracts if accept at result we can increasing the storage time of fruits and vegetables by spraying *Eucalyptus camaldulensis* extract in the space industrial food storage refrigeration or treat the fruit's cover by *Eucalyptus camaldulensis* extract.

MATERIALS AND METHODS

Plant material

Eucalyptus camaldulensis fresh leaves collected from Behbahan and the species were identifying in the herbarium of Ferdowsi University of Mashhad. The leaves were dried in shadow in appropriate condition and then milled in to fine particle for extraction.

Extract preparation

Maceration method was used to prepare extracts. The amount 50 gram of *Eucalyptus* leaves powder was Added to 250 ml ethanol 96 degree or distilled water. The ethanolic extract mixture was preserved at laboratory temperature for 24 hours and was stirred every few hours with a glass rod. The aqueous mixture was boiled for 20 minutes with low flame until the cream colored liquid was obtained. The collecting supernatant was centrifuged by 3000rpm for 10 min. The

resulting extract (supernatant) volume has reached to the original with ethanol or distilled water, and then samples were stored into the dark container at refrigerator temperature after filtering by 0.45 μ Whatman filter paper [9].

Determination dry weight of extracts

At first the weight of a tube were measured, and then 1ml of aqueous and alcoholic extracts were poured in it. The contents of the tube were dried at room temperature. After drying the extract, the tubes were weighed again. Weight differences are equivalent weight of 1ml aqueous or alcohol extract. Average of three replicates, was calculated as the dry weight of the extract[10].

Source of microorganisms

The bacterial strains used were *Staphylococcus aureus* PTCC 2592 , *E. coli* PTCC 1330 and mold used was strains *Penicillium digitatum* PTCC 5251. For each test, to evaluating the antimicrobial effects, fresh medium was prepared.

Preparation of Microbial suspension

To preparing microbial suspensions, requires 24-hour culture from each microorganism. So, 24 hours before experiments, microorganisms were inoculated from storage medium to nutrient agar medium slope. After 24 hours, the cultures were washed by Ringer solution and microbial suspensions were prepared. Then some of the bacterial suspension was poured in sterile tubes containing Ringer solution and its turbidity, was measured by spectrophotometer at 530 nm wavelength. It was diluted by Ringer solution until the solution turbidity equalizes with 0.5 McFarland standard solutions. Suspension should have contains 1.5×10^8 CFU / ml [11, 12].

Antimicrobial activity

Adding extracts to the culture medium "according of the method of Collins *et al.*[13]" and "disk agar diffusion method" were done and to evaluated the antimicrobial effects of aqueous and alcoholic *Eucalyptus camaldulensis* leaves extracts. Then 0.2 gram of aqueous and ethanol extract, were added to 5 ml of sterile distilled water. Then it was stirred with vortex system to assist being steady. Then 1 ml of this solution was added to sterile plates. The final concentration of the extract was 2000 μ g / ml [14]. In the next

step, Mueller Hinton agar (Merck-Germany) medium were sterile and used for bacteria, and Sabouraud Dextrose Agar (SDA) medium for mold, were added to the plates, and placed at room temperature, so the medium was prepared. One loop of each standard strain culture media was cultured inoculums on these medium. The plates were incubated for 48 hours at 37 ° C for bacteria and 72 hours at 27 ° C for mold. The culture with extract and without bacteria and mold was used as control [14]. The disk agar diffusion method, at first a loop of each standard strain culture media was cultured on the plates, and then paper discs (from Whatman filter with 6 mm diameter) with 20, 40, 60 and 80 mg/ml extract concentrations, were prepared in distilled water and was treated with *Eucalyptus camaldulensis* extract and placed in culture medium by Sterile loop. Then it was fixed on the media with a light little pressure. After incubation time, the diameter of free zone was measured exactly by using a ruler in millimeters. All experiments were performed with 3 replicates [15].

Determination of Minimum Inhibitory Concentration (MIC)

MIC was determined according to agar dilution method [16]. Various concentrations (20, 40, 80, 160, 320, 640, 1280, 2560 mg/ml) of extract was prepared in 10 cm experimental tubes containing SD broth for fungi and 10 cm experimental tubes containing Muller Hilton broth for bacteria. Each tube contains 9 ml of SDA for fungi and Muller Hilton for bacteria was sterilized by autoclaving. On cooling, 1 ml of each extract (watery & ethanoli) concentration were added to each tube, to make the final concentrations of 2, 4, 8, 16, 32, 64, 128, 256 mg/ml. The mixture of SDA or Muller Hilton and extract was poured into plates aseptically in a laminar flow cabinet. On solidification of the agar medium, 2 µl of adjusted spore suspension were added to each plate by micropipette and incubated at 27 ° C for fungi and 35 ° C for bacteria [17]. The SDA and Muller Hilton without any herbal extract served as control. The MIC was regarded as the lowest concentration of the extract that did not show any visible growth after 12 days of incubation (compared with control).

Determination of Minimum bactericidal concentration (MBC) and minimum fungicidal concentration (MFC)

The in vitro fungicidal based on MFC and MBC were determined for each extracts (watery & ethanoli) as previously described [18] with slight modifications. The MFC and MBC were determined by incorporating various concentrations of extracts (2-256 mg/ml) in SD broth in tubes for fungi and Muller Hilton broth for bacteria.

One milliliter adjusted spore suspension was added to each tube and incubated at 27°C for 3 days. The SD broth without incorporation of dried herbal extract and 1 ml of adjusted spore suspension served as positive control and SD broth alone served as negative control. The tubes which showed no visible growth after 3 days incubation were subculture on extract free SDA plates and incubated at 27°C for 7 days [17]. The MFC was regarded as the lowest concentration of the extract that prevented the growth of any fungal colony on the solid medium.

Statistical analysis:

All the assays were carried out in triplicates. The experimental results were expressed as mean ± standard deviation. The data were analyzed using one way analysis of variance (ANOVA) using SPSS version 17.

RESULTS

The results of the antimicrobial effects of aqueous and alcoholic extracts, by “using the method of Collins *et al.* [13]” were show on in Tables 1. The results showed 2000 µg/ml concentration of both aqueous and alcoholic extracts, were quite effective on reduce of growth *Staphylococcus aureus* and *Penicillium digitatum* and were had prevent growth over the medium. However, 2000 µg/ ml concentration aqueous and alcoholic extracts, have no significant antibacterial effect on *E. coli* and it is not able to prevent the growth of bacteria on culture. Negative sign (-) in the table shows the growth of bacteria on culture, and the lack of antimicrobial activity of aqueous and alcoholic *Eucalyptus* extracts. The results of the antimicrobial effects of aqueous and alcoholic *Eucalyptus* extracts, by “the agar diffusion method” are presented in Tables 2 and figure 1.

Table 1: Antimicrobial effects of 2000µg/ml ethanolic and aqueous *Eucalyptus camaldulensis* leaves extract concentrations, on *Staphylococcus aureus*, *E.coli* and *Penicillium digitatum* (using the method of Collins *et al.* [13])

Microorganism	Antimicrobial effects of <i>E. camaldulensis</i> leaves extract
Aqueous <i>E. coli</i>	-
Aqueous <i>S. aureus</i>	+
Aqueous <i>P. digitatum</i>	+
Ethanolic <i>E. coli</i>	-
Ethanolic <i>S. aureus</i>	+
Ethanolic <i>P. digitatum</i>	+

- (-) in Table showed the growth of bacteria on culture and the lack of antibacterial activity of aqueous Eucalyptus extract
- (+) in Table showed no bacterial growth on culture and antibacterial activity of aqueous Eucalyptus extract

Table 2: Average diameter (mm) of microbial free zone area of ethanolic and aqueous *Eucalyptus camaldulensis* leaves extract on *E. coli*, *S. aureus* and *P. digitatum* (disk agar diffusion method).

Microorganism	concentration (mg/ml)			
	20	40	60	80
Aqueous <i>E. coli</i>	-	-	6.8 ±0/28	8 ±0/28
Aqueous <i>S. aureus</i>	15±0/57	19±0/57	21 ±0/28	24.5±0/28
Aqueous <i>P. digitatum</i>	8±0/57	11±0/57	13±0/57	15±0/57
Ethanolic <i>E. coli</i>	-	-	7.5 ±0/28	9±0/28
Ethanolic <i>S. aureus</i>	20.2±0/57	22±0/57	25.1 ±0/28	26.9±0/28
Ethanolic <i>P. digitatum</i>	9.1±0/28	11.3±0/57	14.4 ±0/76	17.1±0/28

^aValues are means ± standard deviations, n=3.

(-) in Table showed no inhibitory effects was shown

MIC results of the ethanolic extract of *Eucalyptus camaldulensis* leaves are given in Table 3 and Figure 2. The results shows that MIC of ethanolic extract of *Eucalyptus camaldulensis* leaves for *S. aureus* was 4 mg/ml, for *E. coli* was 32 mg/ml and for the mold *P. digitatum* was 8 mg/ml. The results indicate that ethanolic extract of *Eucalyptus camaldulensis* leaves mostly had been effective on *S. aureus* and has the least impact on *E. coli*. MIC results of the aqueous extract of *Eucalyptus camaldulensis* leaves are given in Table 3 and Figure 2. The results shows that MIC of aqueous extract of *Eucalyptus camaldulensis* leaves for *S. aureus* was 8 mg/ml, for *E. coli* was 64 mg/ml and for the mold *P. digitatum* was 32 mg/ml. The results

indicate that aqueous extract of *Eucalyptus camaldulensis* leaves mostly had been effective on *S. aureus* and has the least impact on *E. coli*.

Minimum Bacterial Concentration (MBC) results of the ethanolic extract of *Eucalyptus camaldulensis* leaves are given in table 4 and figure 3. The results shows that MBC of ethanolic extract of *Eucalyptus camaldulensis* leaves for *S. aureus* was 16 mg/ml, for *E. coli* was 64 mg/ml. Minimum Bacterial Concentration (MBC) results of the aqueous extract of *Eucalyptus camaldulensis* leaves are given in Table 4. The results shows that MBC of aqueous extract of *Eucalyptus camaldulensis* leaves for *S. aureus* was 32 mg/ml, for *E. coli* was 256 mg/ml.

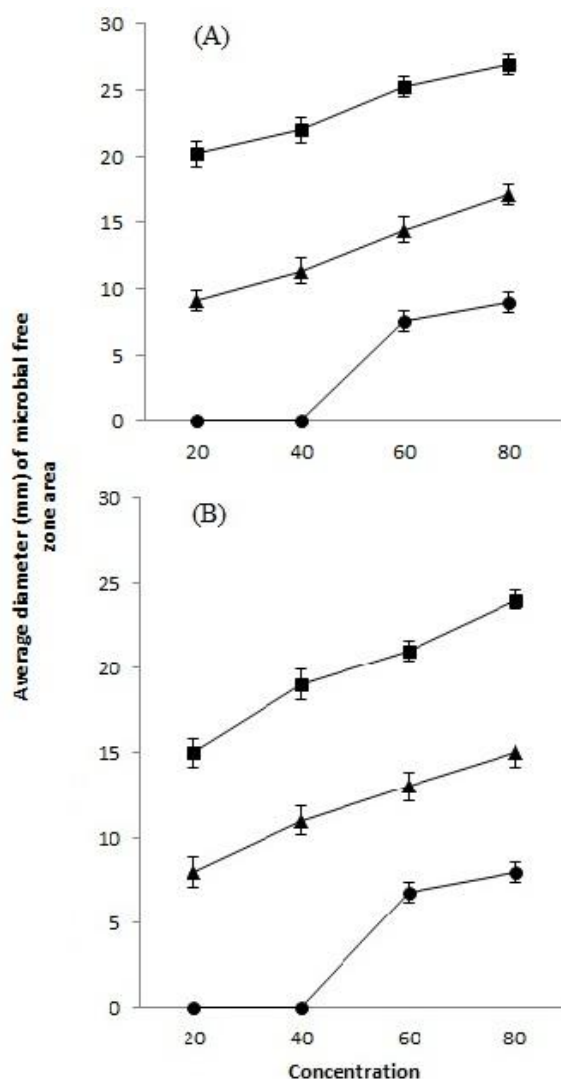


Fig. 1: Antimicrobial activity of ethanolic (A) and aqueous (B) at different concentration of *Eucalyptus camaldulensis* leaves extract on *E. coli*, *S. aureus* and *P. digitatum*. ●, *E. coli*; ■, *S. aureus*; ▲, *P. digitatum* (Measurements were carried out in triplicate).

Table 3: Minimum Inhibitory Concentration (MIC) of ethanolic and aqueous *Eucalyptus camaldulensis* leaves extract on *E. coli*, *S. aureus* and *P. digitatum*.

Microorganism	concentration (mg/ml)						
	64	32	16	8	4	2	control
Ethanolic <i>E. coli</i>	+	+	-	-	-	-	-
Ethanolic <i>S. aureus</i>	+	+	+	+	+	-	-
Ethanolic <i>P. digitatum</i>	+	+	+	+	-	-	-
Aqueous <i>E. coli</i>	+	-	-	-	-	-	-
Aqueous <i>S. aureus</i>	+	+	+	+	-	-	-
Aqueous <i>P. digitatum</i>	+	+	-	-	-	-	-

+: Positive inhibition
- : Negative inhibition

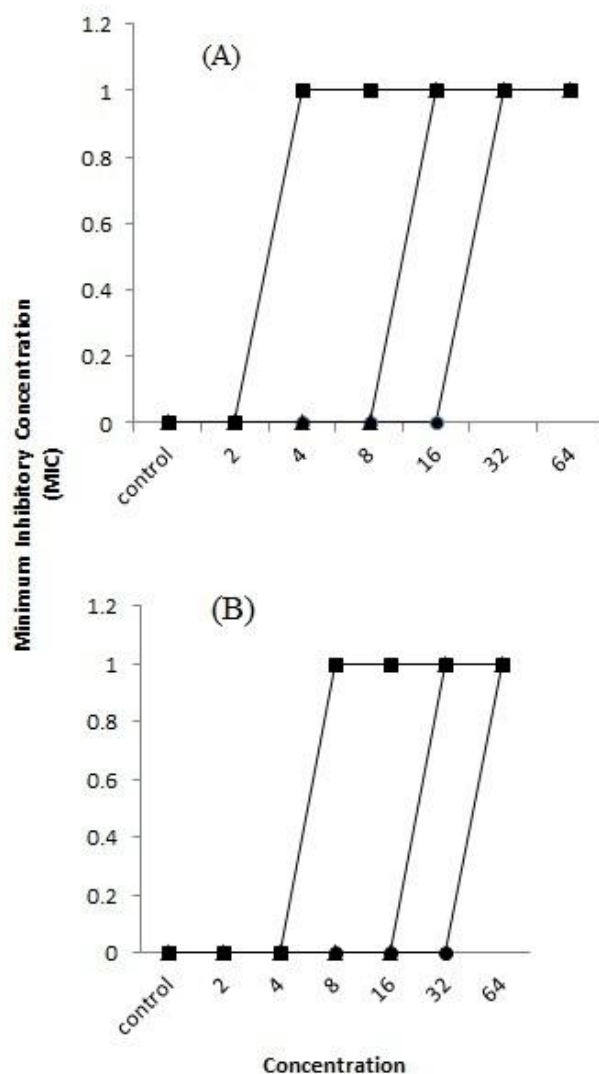


Fig. 2: Minimum Inhibitory Concentration (MIC) of ethanolic (A) and aqueous (B) at different concentration of *Eucalyptus camaldulensis* leaves extract on *E. coli*, *S. aureus* and *P. digitatum*. ●, *E. coli*; ■, *S. aureus*; ▲, *P. digitatum* (Measurements were carried out in triplicate).

Table 4: Minimum Bacterial Concentration (MBC) of ethanolic and aqueous *Eucalyptus camaldulensis* leaves extract on *E. coli*, *S. aureus* and *P. digitatum*.

Microorganism	concentration (mg/ml)								control
	256	128	64	32	16	8	4	2	
Ethanolic <i>E. coli</i>	+	+	+	-	-	-	-	-	-
Ethanolic <i>S. aureus</i>	+	+	+	+	+	-	-	-	-
Aqueous <i>E. coli</i>	+	-	-	-	-	-	-	-	-
Aqueous <i>S. aureus</i>	+	+	+	+	-	-	-	-	-

+: Positive inhibition

- : Negative inhibition

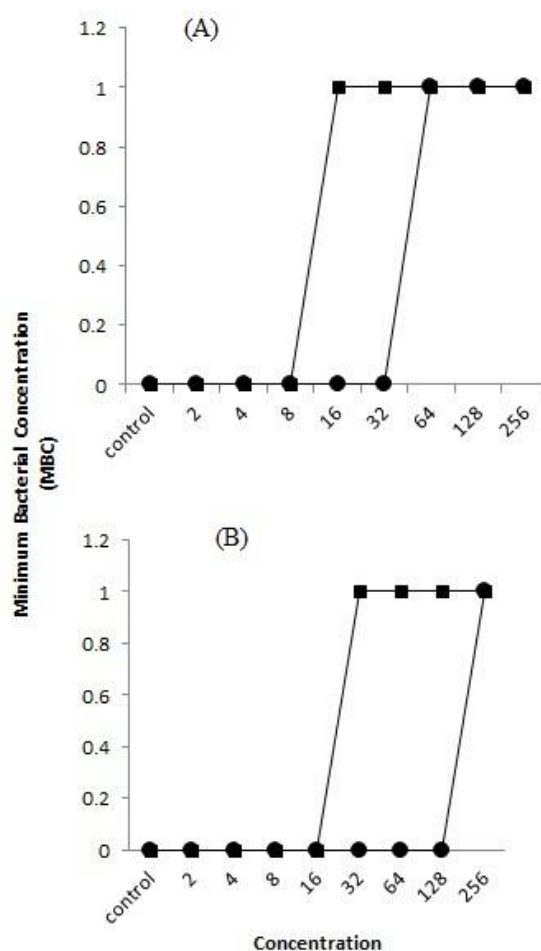


Fig. 3: Minimum Bacterial Concentration (MBC) of ethanolic (A) and aqueous (B) at different concentration of *Eucalyptus camaldulensis* leaves extract on *E. coli*, *S. aureus*. ●, *E. coli*; ■, *S. aureus*; (Measurements were carried out in triplicate).

Minimum Fungicidal Concentration (MFC) results of the ethanolic extract of *Eucalyptus camaldulensis* leaves are given in table 5 and figure 4. The results show that MFC of ethanolic extract of *Eucalyptus camaldulensis* leaves for *Penicillium digitatum* was 16 mg/ml.

Minimum Fungicidal Concentration (MFC) results of the aqueous extract of *Eucalyptus camaldulensis* leaves are given in Table 5. The results show that MFC of aqueous extract of *Eucalyptus camaldulensis* leaves for *Penicillium digitatum* was 64 mg/ml.

Table 5: Minimum Fungicidal Concentration (MFC) of ethanolic and aqueous *Eucalyptus camaldulensis* leaves extract on *Penicillium digitatum*

Fungi species	concentration (mg/ml)								
	256	128	64	32	16	8	4	2	control
Ethanolic <i>Penicillium digitatum</i>	+	+	+	+	+	-	-	-	-
Aqueous <i>Penicillium digitatum</i>	+	+	+	-	-	-	-	-	-

+: Positive inhibition
- : Negative inhibition

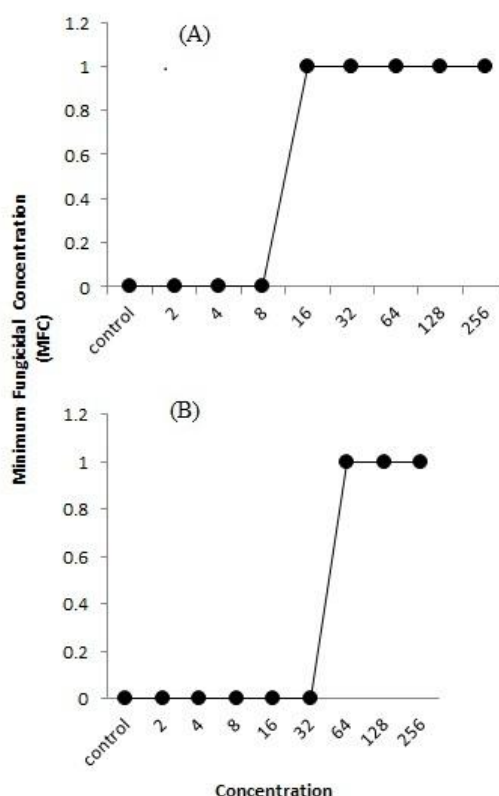


Fig. 4: Minimum Fungicidal Concentration (MFC) of ethanolic (A) and aqueous (B) at different concentration of *Eucalyptus camaldulensis* leaves extract on *Penicillium digitatum*. ●, on *Penicillium digitatum* (Measurements were carried out in triplicate).

DISCUSSION

Base on the results, aqueous and ethanolic leaf extract of *Eucalyptus camaldulensis* in this study have significant antimicrobial activity on the studied microorganisms. Antimicrobial effect of the extracts was different, depending on the type of microorganisms, thus, the gram-positive bacterium *Staphylococcus aureus*, is higher sensitivity compare to gram-negative bacteria *E. coli* (Figure 2), and showed inhibitory effects at lower concentrations of *Eucalyptus camaldulensis* leaves extracts. Gram-positive bacteria are more sensitive than gram-negative bacteria to volatile oils of *Eucalyptus*. Due to differences in cell structure of gram-negative and gram-positive bacteria, because gram-positive bacteria have more mucopeptide in their cell wall composition while gram-negative bacteria have only a thin layer of mucopeptide and most of their cell structure is lipoprotein and lip-polysaccharides. Thus, gram-negative bacteria are more resistant [19, 20]. These points are consistent with the results obtained in this study.

The results shows all aqueous and alcoholic extract concentrations have inhibition effect on growth of *Staphylococcus aureus* and *Penicillium digitatum*. However, aqueous and alcoholic extract of *Eucalyptus* leaves are able to inhibitory of growth the *E. coli* only in 60 and 80 mg/ml concentrations and no antimicrobial activity observe at 20 and 40 concentrations. Oyedele *et al.* [21] examined antimicrobial properties five species *Eucalyptus* essential oils. At 5 mg/ml concentration of five species *Eucalyptus* leaves volatile oil, have been significant antimicrobial properties, against gram-positive bacteria, gram-negative bacteria and molds. Also, alcoholic extract compared to the aqueous extract was more effective and has a greater deterrent. The reason of these phenomena may be extracting more effective materials extracted by ethanol from *Eucalyptus*. These results are consistent with the findings of a study by Mahasneh [22] on Qataris mangrove species and it is found the aqueous mangrove extract, don't have a significant antimicrobial effect, and the butanol extract, is able to inhibit

Pseudomonas aeruginosa. Sattari *et al.* [10] showed 3.2 µg / ml concentration of alcoholic *Eucalyptus* extract and 17.5 µg / ml concentration of aqueous *Eucalyptus* extract, can be good to prevent the growth of *Pseudomonas aeruginosa* standard isolates. Tian *et al.*, [23] investigates the antibacterial effects of “*Galla chinensis*” (a medicinal plant native to China) reported that the juice extracted by the solvent ethyl acetate, ethanol and water are the highest antibacterial effect. In this study the plant extracts are show that gram-positive bacteria (*Bacillus cereus*, *Staphylococcus aureus*, *Bacillus Subtilis*) are more sensitive than gram negative bacteria (*Escherichia coli*, *Shigella Dysentery*). This result is consistent with the findings of this study [23]. In another study researchers study the antibacterial effects *Triticum sativum* Lam, and experiments are done to study the antibacterial effect of aqueous, ethanol, ethyl ether and hexane extract of *Wheat* on some microorganisms, and they find the antimicrobial properties of the plant [24].

The results show that aqueous extract have no antimicrobial effect, although, organic extracts have the better effects on gram-positive bacteria in compare to gram-negative. This result is consistent with finding of this study (Table 2, Figure 1).

MIC concentrations of ethanolic extract of *Eucalyptus camaldulensis* leaves for *Staphylococcus aureus* is 4 mg/ml, and for *E. coli* is 32 mg / ml, while the MIC concentrations of aqueous extract of *Eucalyptus camaldulensis* leaves for *Staphylococcus aureus* is 8 mg/ml, and for *E. coli* is 64 mg/ml. Several mechanisms are discussed to explain the antimicrobial effect. Kotzekidou *et al.*, [25] find that the antimicrobial compounds in the plant extract, have interaction with the phospholipids’ two layers membrane, and affect the permeability of the bacterial cell membrane, and released the intracellular components. In many studies, the mechanism of the cell wall is considered. They have report that cell wall and cell membrane affected and changed their permeability cause Release of intracellular contents, which can be associated with impaired membrane function, such as electron transfer, enzyme activity or nutrient uptake.

Delaquis *et al.* [26] evaluated the antimicrobial activity of essential oils and fractions of dill, parsley, coriander and *Eucalyptus* tested alone

or in mixed. Essential oils of this plant was analyzed by fractional distillation and evaluated by gas chromatography and spectroscopy. Then *Salmonella typhimurium*, *Listeria monocytogenes*, *Pseudomonas fragi*, *Serratia*, *Enterobacter agglomerans*, *Yersinia enterocolitica*, *Bacillus cereus*, *Saccharomyces cerevisiae* and *Streptococcus* were tested. The minimum inhibitory concentration (MIC) was determined for each of fractions against gram-positive bacteria, gram-negative and yeast. Results showed mixture fraction, increasing, synergistic or antagonistic effects against microorganisms. Eucalyptol or alpha-cineole are highest materials identified in the *Eucalyptus* leaves and have more diverse biological effects, including antimicrobial agent. 12.5 µm/ ml and 6.25 µm/ ml MIC reports for *Streptococcus mutans* and *Streptococcus sobrinus* respectively [27]. MBC of ethanolic extract of *Eucalyptus camaldulensis* on *Staphylococcus aureus* is 16 mg/ml, and for *E. coli* is 64 mg/ml. While MBC aqueous extract of *Eucalyptus camaldulensis* on *Staphylococcus aureus* is 32 mg/ml, and for *E. coli* is 256 mg/ml. Burt *et al.*, [28] reported that a hydrophilic outer membrane, composed of lipids and LPS, with the property of selective permeability, is an important factor in the resistance of gram-negative bacteria to antimicrobial compounds.

According to the results, and the impacts of antimicrobial effects of aqueous and alcoholic *Eucalyptus camaldulensis* leaves extract, recommended spray the extract in the industrial refrigeration preservation environment, or dipped coating fruit such as oranges and tangerines, to be avoided and prevented from spoilage by the mold such as *Penicillium digitatum* (citrus green mold) and inhabits growing on the fruit. The results indicate that alcoholic extract of *Eucalyptus camaldulensis* leaves has a greater impact on all strains in compare to aqueous extract, probably because of more efficient extraction by ethanol.

In conclusion, it can suggest that *Eucalyptus camaldulensis* leaf extract “*in vitro*”, have considerable antimicrobial ability over the studied strains. In addition, more studies are needed “*in Situ*” be done, to identifying the effective dose of the extract on the microorganisms, and finally introduce the extract as a natural and novel antimicrobial

compound. Therefore, using *Eucalyptus* as a natural antimicrobial compounds in vitro requires further research on mechanism of the pharmacy plant on the microorganisms.

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