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The inhibitory effect of some natural volatile oils on Cyanobacteria (*Anabaena wisconsinense*) and some Pathogenic Bacteria

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ABSTRACTS

The main objective of this study was to test the inhibitory effect of clove oil (*Syzygium aromaticum*), peppermint oil (*Mentha longifolia*), basil oil (*Ocimum basilicum*) and castor oil (*Ricinus communis*) on the growth of cyanobacteria (*Anabaena wisconsinense*) as well as four pathogenic bacteria (*Escherichia coli, Vibrio sp. Pseudemonas sp. and Aeromonas sp.*).

The algicidal activity of investigated volatile oils was tested at 3 concentrations of the crude oils (50, 100, 150 ppm) on the growth of *Anabaena wisconsinense,* and at 3 volumes (10, 20, 30 µl) on the growth rates of the tested species of pathogenic bacteria. Results showed that the investigated volatile oils have a strong antagonistic effect on *Anabaena wisconsinense.* Clove and peppermint oils were the highest inhibitory action against *A. wisconsinense*. Growth rates of all tested bacteria Also were inhibited with clove and peppermint oils while basil and castor oils had no effect against *Pseudemonas sp. and Aeromonas sp*. at any volumes.

Anabaena wisconsinense and pathogenic bacteria were more sensitive to the allelopathic and antibacterial effect of clove and peppermint oils.

Keywords: Biocontrol, Clove, Peppermint, Basil, Castor Cyanobacteria, Pathogenic Bacteria

INTRODUCTION

Toxic cyanobacteria are found worldwide coastal water environments. At least 46 species have been known to cause toxic effects in vertebrates **(Sivonen and Jones**, **1999)**. The most common toxic cyanobacteria in freshwater are *Microcystis* spp., *Cylindrospermopsis raciborskii*,

Planktothrix (*syn. Oscillatoria*) *rubescens, Synechococcus* spp., *Planktothrix (syn. Oscillatoria) agardhii, Gloeotrichia spp., Anabaena* spp., *Lyngbya* spp., *Aphanizomenon* spp., *Schizothrix spp. and Synechocystis* spp. **(Chorus and Bartram, 1999)**. *Anabaena* is one of four cyanobacteria genera that produce neurotoxins, which are harmful to local wildlife. Many of the bloom forming cyanobacteria produce toxins responsible for mass mortality of aquatic and exposed vertebrate populations **(Paerl *et al.*, 2001).** Many mechanical and physiochemical methods have been devised in attempts to manage cyanobacterial blooms with limited success. The most direct control method involves the use of chemical treatments such as algicides, including copper, Reglone A (diquat, 1, 1- ethylene-2, 2-dipyridilium dibromide), potassium permanganate, chlorine and Simazine (2-chloro-4,6- bis (ethylamino)-s-triazine **(Lam *et al.*, 1995).** Copper sulphate or organo-copper compounds have been used successfully to control harmful algal blooms in raw water supplies intended for human consumption **(Lam *et al.*, 1995)**. These chemicals induced cyanobacterial cell lysis, followed by the release of toxins into surrounding water. New and alternative approach to control the algal blooms involves the use of plant extracts exactly the essential oils. Plants and other natural sources can provide an extended range of complex and structurally diverse compounds. Plant extracts and essential oils possess antifungal, antibacterial, and antiviral properties and have been screened on a global scale as potential sources of novel antimicrobial compounds, agents promoting food preservation, and alternatives to treat infectious diseases **(Safaei-Ghomi and Ahd, 2010; Astani *et al.*, 2010).** Essential oils have been reported to possess significant antiseptic, antibacterial, antiviral, antioxidant, anti-parasitic, antifungal, and insecticidal activities **(Kaloustian *et al.*, 2008 and Burt, 2004).** Therefore, essential oils can serve as a powerful tool to reduce the bacterial resistance **(Stefanakis *et al.*, 2013)** in fish pathogens such as *E. coli, Pseudomonas sp., Vibrio sp.* and *Aeromonas hydrophila*, which cause serious diseases in aquaculture with high economic loss. The use of antibiotics in aquaculture has led to the development of the resistant strains and may be reducing the fish growth or immune response beside involving environmental hazards **(Cañada *et al.*, 2009; Chakraborty and Hancz, 2011)**. Antibiotics also can accumulate in soil or sediment and become harmfull for environment. Recently, many essential oils and plant extracts have been shown to be effective against fish pathogens **(Direkbusarakom, 2004**; **Bansemir *et al.*, 2005**; **Hindi and Chabuck, 2013**).

Various chemotherapeutics have been used to treat bacterial infections in cultured fish for the last decades. However, the incidence of drug-resistant bacteria has became a major problem in fish culture **(Aoki, 1992).** The medicinal plants are currently used in commercial aquaculture as growth promoting substances, nutrients and antimicrobial agents for preventing and controlling of fish diseases. It developed as an environmentally sound supplement alternatively to the use of chemicals and for producing organic fish **(Galina *et al.*, 2009).**

The aim of this study is investigate the influence of some concentrations of clove (*Syzygium aromaticum*), peppermint (*Mentha longifolia*), Basil (*Ocimum basilicum*) and Castor (*Ricinus communis*) oils on the growth of cyanobacteria (*Anabaena wisconsinense*) as well as four different pathogens (*E. coli, Vibrio sp. Pseudemonas sp. and Aeromonas sp.*).

Materials and Methods

Cultivation of *Anabaena wisconsinense*

Strains of the Cyanobacteria *Anabaena wisconsinense* was isolated from Abbassa fish ponds **(Reham, 2012)** and cultivated in the phytoplankton laboratory belongs to limnology department, Center laboratory for aquaculture research (CLAR) according to **Venkataraman (1969**). Alga grow as single cell in BG11 liquid media which adjusted to pH 8 with NaOH and HCl **(Rippka *et al.*, 1979)**. The Cyanobacteria were incubated at 26 ± 1 ˚C under an illumination intensity of 2500 lux, with a 12 / 12 h light / dark interval. The microalga was cultivated to the exponential growth phase for use. The density was monitored every two days.

Preparation of the pure alga for inoculation

Cyanobacteria *Anabaena wisconsinense* culture in 50 ml was prepared as primary inoculum, and then 500 ml of cultured media was prepared and inoculated with it, and incubated at 26 ± 1 °C in the presence of light for 14 days.

The investigated oils:

The investigated oils Clove (*Syzygium aromaticum*), Peppermint (*Mentha longifolia*), Basil (*Ocimum basilicum*) and Castor (*Ricinus communis*) used in the present study were obtained from the local market of Zagazig city.

Activity evaluation of the investigated oils against Cyanobacteria (*Anabaena wisconsinense*)

The algicidal activity of the four investigated volatile oils were tested at 3 concentrations of the crude oils (50, 100, 150 ppm) and monitored

throughout 12 days against the *A. wisconsinense* isolate According to **(Kim *et al.*, 2002)**. The investigated oils were dissolved in equal volumes of 95% ethanol (V:V, 50%). The test results indicated that the concentrations of ethanol added had no effect on the growth of the tested alga. The tested oils were added to a flask containing 20ml of alga and 180ml of culture media (BG11). During the experiment, the culture condition (temperature, and light) were adjusted as mentioned before. The density of alga was monitored every 2 days by the estimation of chlorophyll-a content (indirect method of Cyanobacterial biomass determination), where the pigment can be completely extracted in aceton.

The estimation of chlorophyll- a:

The cyanobacterial culture was taken and centrifuged at 5000 rpm for 10 min. the pellet was washed twice in distilled water, thus the pellet was re-suspended in 4 ml of 80 % acetone and vortexes thoroughly. Tubes were incubated in a water bath at 60°C for 1 h in dark with occasional shaking. The suspension was centrifuged at 5000 rpm for 10 min and the supernatant was stored. Absorbance of the supernatant was read at 663 nm in U.V. spectrophotometer against 80% acetone as blank.

The amount of chlorophyll a in the sample was calculated according to **Kim *et al.* (2002**) using the following formula:-

$$\text{Chl.a} = \frac{\text{A663} \times 12.63 \times \text{volume of acetone}}{\text{Volume of sample}} \ \mu g.ml^{-1}$$

A663: absorbance at 663nm.

12.63: correction factor and the amount were expressed as μgml^{-1}.

Bacterial isolation

Escherichia coli, Aeromonas sp., Pseudomonas sp. and *Vibrio sp*. were isolated from polluted water source. For enumeration of coli form group and *E. coli*, MacConkey agar was used while, for *vibrio cholera* selective media, Thiosulphate-Citrate-Bile-Sucrose agar (T.C.B.S) was used. Also selective media *pseudomonas* was used for isolation *Pseudomonas sp*. the isolated bacteria were identified according to standards bacteriological methods described by **APHA (1989)**.

Bacterial culture preparation

Nutrient broth (NB) was prepared and was inoculated with the tested organisms. A loop full of microorganism was taken and inoculated in the NB and was incubated at $37^{o}C$ for 24 hrs to obtain a viscous growth. Cotton

swabs were dipped in the culture broth and were swabbed on the solidified media surface previously prepared in petri dishes.

Antibacterial activity (In vitro) of investigated oils by Paper disk assay according to (Bauer *et al.*, 1966):

Antibacterial activity of tested oils in vitro was carried out using disc diffusion method. The sterilized nutrient agar (NA) medium poured in sterilized Petri dishes. After solidification the plates were inoculated with 0.1ml of fresh bacterial suspension (24 h live). Sterilized paper discs were impregnated with (20, 40 and 60 µl) diluted tested oils, where tested oils were dissolved in equal volumes (V:V, 50 %) of 95 % ethanol and air dried. Another sterilized paper discs were impregnated with ethanol at (10, 20 and 30 µl) and used as control. Paper discs were placed over the agar surface. The plates incubated at 37ºC for 24 hrs and examined for inhibition. The diameter of the inhibition zones were measured in millimeters.

Allelopathic activity on growth inhibition: was estimated by percentage of inhibition (PI), which is defined as follow -:

Inhibition percentage (IP %) =[(diameter of inhibition zone in treated bacteria - diameter of inhibition zone in control)/diameter of normal growth ×100] .

Statistical analysis

Statistical analysis was applied according to **Steel and Torrie (1980)**, data were analyzed using the GLM procedure with two way analysis of variance **(SAS, 2002),** Differences between means were tested for significance according to Duncan's multiple rang test **(Duncan, 1955)**.

Results and Discussion

1- The evaluation of the investigated oils against Cyanobacteria (*Anabaena wisconsinense*) :

Our results in (Table 1) recorded that, in clove oil the highest anti-algal impact expressed as 0 µg / ml as chlorophyll-a concentration at 100 and 150 ppm from the 8th day till the end of the experiment and the same result was obtained in peppermint at the concentration of 100 ppm at the 12th day and at concentration 150 ppm from the 4th day till the end of the experimental period. Compared by the control group (algae free from any oils), that represented continuous increased in concentration of chlorophyll a along with time as shown in Table(1).

Table (1). Effect of different concentrations of different investigated oils on *A. wisconsinense* density expressed as chlorophyll a concentrations ($\mu g.ml^{-1}$)

Treatments	Concentrations ppm.	Concentration of chlorophyll a (µg / ml)					
		2nd day	4th day	6th day	8th day	10th day	12th day
Control	0	$0.05^{a} \pm 0.01$	$0.11^{d} \pm 0.02$	$0.5^{b} \pm 0.3$	$0.9^{a} \pm 0.02$	$3.4^{a} \pm 0.03$	$6.6^{a} \pm 0.01$
Clove Oil	50	$0.08^{b} \pm 0.02$	$0.03^{e} \pm 0.03$	$0.03^{e} \pm 0.2$	$0.03^{e} \pm 0.06$	$0.02^{e} \pm 0.1$	$0.01^{d} \pm 0.5$
	100	$0.01^{f} \pm 0.01$	$0.005^{g} \pm 0.1$	$0.003^{f} \pm 0.2$	$0^{g} \pm 0.01$	$0^{g} \pm 0.5$	$0^{f} \pm 0.03$
	150	$0.02^{f} \pm 0.1$	$0.005^{g} \pm 0.2$	$0.003^{f} \pm 0.1$	$0^{g} \pm 0.1$	$0^{g} \pm 0.1$	$0^{f} \pm 0.01$
Peppermint Oil	50	$0.05^{d} \pm 0.03$	$0.1^{d} \pm 0.5$	$0.07^{d} \pm 0.04$	$0.03^{e} \pm 0.02$	$0.02^{e} \pm 0.2$	$0.005^{e} \pm 0.1$
	100	$0.04^{e} \pm 0.03$	$0.02^{f} \pm 0.2$	$0.02^{e} \pm 0.05$	$0.01^{f} \pm 0.04$	$0.005^{f} \pm 0.2$	$0^{f} \pm 0.02$
	150	$0.02^{f} \pm 0.01$	$0^{h} \pm 0.1$	$0^{g} \pm 0.02$	$0^{g} \pm 0.03$	$0^{g} \pm 0.1$	$0^{f} \pm 0.03$
Basil Oil	50	$0.09^{b} \pm 0.4$	$0.6^{a} \pm 0.4$	$0.7^{a} \pm 0.1$	$0.7^{c} \pm 0.1$	$0.8^{d} \pm 0.03$	$2.1^{b} \pm 0.2$
	100	$0.07^{c} \pm 0.2$	$0.6^{a} \pm 0.3$	$0.7^{a} \pm 0.07$	$0.7^{c} \pm 0.1$	$0.8^{d} \pm 0.1$	$2.2^{b} \pm 0.5$
	150	$0.14^{a} \pm 0.06$	$0.21^{c} \pm 0.02$	$0.026^{e} \pm 0.1$	$0.3^{d} \pm 0.3$	$0.91^{c} \pm 0.03$	$1.4^{c} \pm 0.4$
Castor Oil	50	$0.05^{d} \pm 0.2$	$0.2^{c} \pm 0.03$	$0.4^{c} \pm 0.04$	$0.6^{c} \pm 0.1$	$0.9^{c} \pm 0.01$	$2.6^{b} \pm 0.1$
	100	$0.08^{b} \pm 0.03$	$0.4^{b} \pm 0.11$	$0.55^{b} \pm 0.1$	$0.7^{c} \pm 0.02$	$1.3^{b} \pm 0.01$	$2.09^{b} \pm 0.3$
	150	$0.1^{a} \pm 0.9$	$0.6^{a} \pm 0.01$	$0.7^{a} \pm 0.3$	$0.8^{b} \pm 0.2$	$1.2^{b} \pm 0.01$	$2.2^{b} \pm 0.1$

Means ± standard error. Values in the same column having the same superscript letters are not significantly different ($P < 0.05$).

There were also inhibition effect in alga growth with both basil and castor oils at the three tested concentrations but less than clove and peppermint oils. The clove and peppermint oils were the highest inhibitory action with *A. wisconsinense*, where the inhibition effect was started in

basil oil from 8th comparing with control. There was significant difference in chlorophyll-a concentrations (0.9 µg / ml) in control at 8th day and chlorophyll-a concentrations (0.7,0.7 and 0.3 µg/ml) in basil oil at (50,100 and 150ppm) respectively. In castor oil the algicidal activity started from 6th day where chlo-a concentration was (0.4 µg/ml) at 50ppm but increased at 150ppm to reach 0.7 while was 0.5 µg/ml in control.

The inhibition in *Anabaena* growth may be due to the allelochemical effect of the investigated oils. our results agrees with **Wang *et al.*, 2015** who reported an antagonestic activity of essential oils extracted from six submerged plants *Potamogeton cristatus, P. maackianus, P. lucens, Vallisneria spiulosal, Ceratophyllum demersum and Hydrilla verticellata,* showed high potency on *M. aeruginosa*. **Kobaisy *et al*. (2001)** found slightly inhibitory effect of *Hibiscus cannabinus* leaves oil extract against *Oscillatoria perornata* and no effect against one type of green algae. There was no algicidal activity observed for the oil major components when tested separately under the same conditions as the essential oil. *Rosmarinus officinalis* essential oil has high antagonistic effects on the growth rate of both *Microcystis aeruginosa* and *Chroococcus* measuring expressed as chlorophyll-a of each alga. Generally, growth rates of both algae severely decreased as the period and the concentrations of the essential oil increased comparing with which exponentially increased along with time. *M. aeruginosa* was more sensitive to the allelopathic effect of the oil **(Najem *et al.*, 2016)**. The possible explanation for the better activity of the essential oil could be due to easier absorption of the essential oil and lipophilic extracts into the cell body of the algae (**Yi *et al.*, 2011). (Barani *et al.*, 2014)** found that essential oils of three plants *Satureja khuzistanica, Satureja rechingeri, and Zataria multiflora,* strongly inhibited the growth of the dinoflagellate *Cochlodinium polykrikoides* that cause red tide.

2- Evaluation the activity of the investigated oils against some pathogenic bacteria:

In Table 2, Percentage of inhibition (IP%) pathogenic bacterial growth by investigated oils, were recorded according to the size of inhibition zone formed on the agar plates by disc diffusion method. The maximum percentages of inhibition (17,16.67 and 16.42 %) were recorded at concentrations (60, 20 and 20 µl.) against *Pseudomonas sp. and E coli* with clove, basil and castor crude oils respectively. Clove and Peppermint crude oils were recorded positive inhibitions with all tested bacteria but basil and castor oils had no effect on *Pseudomonas sp.* and *Aeromonas sp.* comparing by control so were recorded negative percentages of inhibition bacterial growth with all concentrations . From fig 2 examined clove oil

showed comparatively good effect on all pathogenic strains of bacteria comparing with control (ethanol only). The maximum inhibition were recorded at diluted concentration 60 and 40 µl. respectively against *Pseudomonas sp.* and the lowest were at conc. 60 and10 µl. respectively, against *Vibrio sp.*. **Pandey and Singh (2011)** mentioned that clove oil revealed inhibition zones against food borne pathogens (*Staphylococcus aureus, Pseudomonas aeruginosa*, *Escherichia coli*),using agar diffusion susceptibility test. Compare to ethanolic extract, methanolic extract was showing best result against gram positive culture *Staphylococcus aureus* (MTCC 2940) and two gram negative cultures *Pseudomonas aeruginosa* (MTCC 2453) and *E. coli* (MTCC 739). **Meeker and Linke (1988) and Walsh and Pease (2002)** proved that clove oil is rich in eugenol, isoeugenol and methyleugenol which have antifungal, antibacterial, antiviral, anti-inflammatory, antioxidant and anesthetic properties.

Table (2). Inhibition percentage (IP%) of pathogenic bacterial growth by investigated oils:

Treatments	Oils crude concentration (µl)	Percentage of inhibition (PI%) Pathogenic bacterial growth			
		E.coli	***Vibrio sp.***	***Pseudomonas sp.***	***Aeromonas sp.***
Clove Oil	20	10.25	1.67	8.58	9.17
	40	12.25	6.42	15.00	5.83
	60	5.25	2.25	17.83	4.75
Peppermint Oil	20	5.83	-1.08	2.17	9.42
	40	7.75	2.75	13.83	4.75
	60	4.67	4.42	7.25	8.08
Basil Oil	20	16.67	13.33	-13.92	-16.67
	40	12.75	7.50	-12.25	-14.42
	60	6.92	10.83	-12.75	-14.42
Castor Oil	20	16.42	8.08	-13.92	-16.67
	40	14.25	7.75	-12.25	-14.42
	60	4.67	5.00	-12.75	-14.42

In Fig. 2, Peppermint oil effectiveness was observed against all examined bacterial pathogens. except *Vibrio* sp. where there was no significant different in inhibition zone at conc. 40 µl compared with that of control. The highest antibacterial effect were obtained against *Pseudomonas sp.* and *Aeromonas sp.* at concentrations (40 and 20 µl.) respectively comparing with control. That agree with **(Nikoli´c *et al*., 2014)**

who found potential antimicrobial activity of essential oils extracted from five plants namely, *Mentha piperita* (Peppermint),

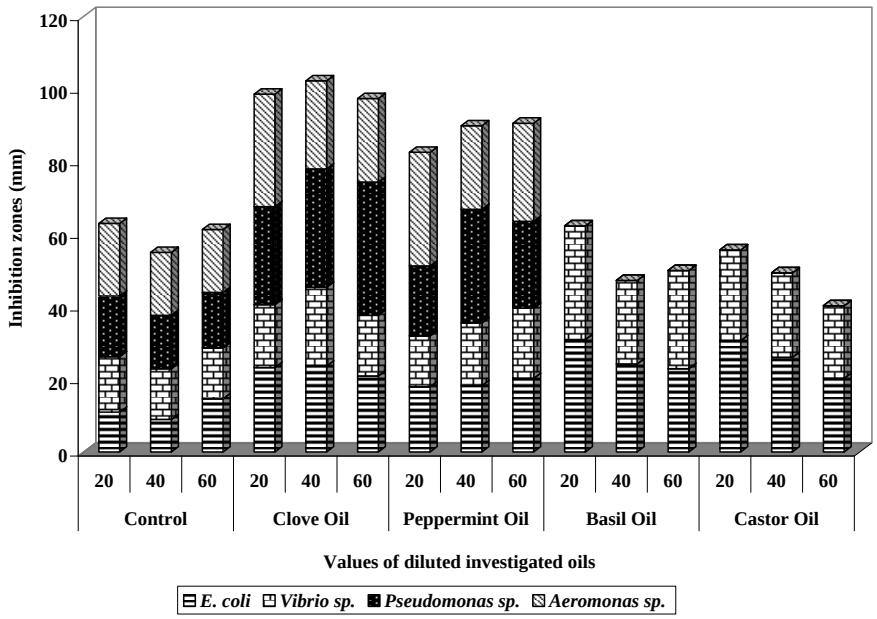

Figure (1). The effect of (Clove, Peppermint, Basil and Castor oils) on the growth of some pathogenic bacteria (inhibition zone measured as mm):

Photo (1). The effect of clove oil on *Pseudomonas sp.* growth at 60 µl (measured as diameter of inhibition zone (mm)).

Photo (2). The effect of Peppermint oil on *Aeromonas sp.* at 60 µl (measured as diameter of inhibition zone (mm)).

Photo (3). The effect of clove oil on *Aeromonas sp.* growth at 40 µl (measured as diameter of inhibition zone (mm)).

Photo (4). The effect of clove oil on *E coli* growth at 40 µl (measured as diameter of inhibition zone (mm)).

Lavandula angustifolia, Mentha pulegium (Peppermint), *Salvia lavandulifolia and Satureja montana* against *Pseudomonas aeruginosa*, *Streptococcus pyogenes, Streptococcus mutans, Streptococcus sanguis, Streptococcus salivarius, Enterecoccus feacalis* and *Lactobacillus acidophilus pathogenic bacteria.* From Table 2 and Fig. 1, basil and castor oils showed no effect on *Pseudomonas sp.* and *Aeromonas sp.* While strong effect was recorded against *E. coli* and *Vibrio sp*. The maximum inhibition zones recorded of basil oil were at concentration 20 µl for *E. coli* and *Vibrio sp.* respectively. In castor oil the maximum inhibition was in *E. coli* at conc. 20 and 40 µl respectively. The minimum inhibition was recorded in *Vibrio sp.* and *E. coli* respectively at conc. 60 µl with castor oil and at the same concentration in basil oil the inhibition was the lowest in *E.coli*. These results agrees with **El Ashram *et al.*, (2017)** where there significant decreased in total bacterial count of fish muscle in compared with the control that may be due to the antimicrobial effect of basil oil. **Suppakula *et al.*, (2003)** reported that basil essential oils exhibited good antimicrobial activity against a wide range of microorganism. In general, the clove and Peppermint oils recorded highly effect on all pathogenic bacteria. In many cases the maximum inhibition zone was recorded at low concentration (10 µl.) On the other hand, castor and basil oils cannot effect on *pseudomonas sp.* or *Aeromonas sp.* These results provide evidence for the antimicrobial effects of the tested essential oils. This may be due to the presence of

phenolic compounds. These compounds can degrade the cell wall protein, disrupt the cytoplasmic membrane and interfere with membrane-integrated enzymes (**Shan *et al*., 2007**).

Conclusion

It could be concluded that the clove and peppermint oils had the highest inhibitory action against *A. wisconsinense*. It's obtained also that both basil and castor oils showed an inhibition effect against alga growth at the three tested concentrations but less than clove and peppermint oils. Also the tested oils (Clove, Peppermint, Basil and Castor) had great antimicrobial effect against *E. coli, Vibrio sp., Pseudemonas sp. and Aeromonas sp*.. Clove and Peppermint oils effectiveness was observed against all examined bacterial pathogens while basil and castor oils had no effect against *Pseudemonas sp. and Aeromonas sp*. at any concentrations. Further studies are required to evaluate the different plants extracts against the tested microorganisms under laboratory and field conditions.

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التأثير المثبط لبعض الزيوت الطبيعية الطيارة على طحلب البكتيريا الخضراء المزرقة (*Anabaena wisconsinense*) وبعض البكتيريا الممرضة

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الملخص العربى

تهدف هذه الدراسة إلي إختبار التأثير المثبط لزيت القرنفل (*Syzygium aromaticum*) وزيت النعناع (*Mentha longifolia*) وزيت الريحان (*Ocimum basilicum*) وزيت الخروع (*Ricinus communis*)على نمو البكتيريا الزرقاء (*Anabaena wisconsinense*) وكذلك أربعة أنواع من البكتيريا المسببة للأمراض (*Escherichia coli & Vibrio sp& Aeromonas sp Pseudemonas sp.*).

تم اختبار النشاط المضاد ل٣ تركيزات (٥٠ ، ١٠٠ ، ١٥٠ جزء في المليون) من الزيوت المختبرة على نمو الطحلب و (٢٠ ، ٤٠ ، ٦٠ ميكرولتر) على معدلات نمو الأنواع المختبرة من البكتيريا الممرضة. أظهرت النتائج أن الزيوت الطيارة لها تأثير قوي على تثبيط نمو طحلب *Anabaena wisconsinense* ، لكن زيوت القرنفل والنعناع كانت أكثر فعالية ضد طحلب *A. wisconsinens*. أيضا كان هناك تأثير مثبط لنمو جميع البكتيريا المختبرة مع زيوت القرنفل والنعناع ، لكن زيوت الريحان والخروع لم يكن لهم أى تأثير مع بكتريا *Pseudemonas sp.* و *Aeromonas sp.* عند أي من الكميات المختبرة.
سجلت النتائج أن الطحلب والبكتيريا الممرضة أكثر حساسية للتأثير المثبط لزيت القرنفل وزيت النعناع.