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Antibacterial Effects of Low Power Laser Light and Volatile Oil of Fennel (*Foeniculum vulgare* var. *dulce*) on Gram-positive and Gram-negative Bacteria

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ABSTRACT

In this study, the antibacterial effects of a toluidine blue O (TBO), light from a helium/neon (He - Ne) laser and essential oil extracted from seeds, leaves and stems of Sweet fennel plants (foliarly sprayed with nicotinamide by concentrations in a range of (zero, 20, 40, 80 & 100 mg/L) on *Escherichia coli, Serratia marcesence, Klebsiella pneumoniae* as a gram-negative and *Staphylococcus aureus* as a gram-positive bacteria was determined. Both photosensitizing dye and essential oil could be successfully inactivating the bacterial species under study. The essential oil extracted from stems and seeds foliarly sprayed with nicotinamide by 80 and 40 mg/L, respectively exhibited the strongest inhibitory effect on tested bacteria at both cuttings. Whereas, a minor activity could be detected by application of the essential oil extracted from the leaves. It could be concluded that photoactivation of touidine blue by red laser radiation at wavelength 632.8 nm and essential oils of *Foeniculum vulgare* treated with nicotinamide especially in presence of 40 and 80 mg/L in food systems to prevent the growth of food borne bacteria and extend the shelf life of the processed foods also to stop the growth of the microbes causing human diseases.

Key Word: Bacteria; Essential oils; *Foeniculum vulgare*; Laser; Nicotinamide and Toluidine blue

INTRODUCTION

The growing resistance against antibacterial agents has generated a search for alternative antimicrobial treatments. In particular, the use of topical antibiotics is under discussion since it has been suggested that such an approach induces antibiotics resistance faster than the use of oral antibiotic (Coates *et al*., 1997; Ross *et al.,* 2003). An interesting approach is to use photodynamic therapy (PDT) for treating local infections since a wide range of microorganisms including bacteria, yeast, in addition to viruses can be killed by photodynamic therapy (Wilson *et al*., 1992; Paarde kooper *et al*., 1995).

Several studies pointed to photodynamic therapy as a treatment alternative for several infections or diseases (Wilson *et al*., 1993; Nseyo *et al*., 1998; Chan & Lai, 2003). *In vitro* studies revealed that the use of laser associated with a photosensitizer is very effective against bacteria, Yeast, Viruses and parasites (Wilson & Mia, 1993; Sarkar & Wilson, 1993; WainWright, 1998). However, a great number of variables may influence the number of microorganisms affected by this technique including; type and concentration of the photosensitizer, microorganism's physiologic stage, photosensitizer incubation period before the radiation, exposure period and density of laser energy (Wilson & Mia, 1993). Photodynamic therapy is a technique based on the photosensitization of dyes by low-power laser. This technique promotes the destruction of the target cell by oxidation mechanisms that lead to cell membrane lysis and protein inactivation (Teichert *et al*., 2000). It has been demonstrated that Gram-positive, Gram-negative bacteria and fungi can alike successfully photodynamically inactivated by toluidine blue (Jackson *et al*., 1999; Komerik & Wilson, 2002). Also, some oral bacteria are susceptible to death by red light after their sensitization with toluidine blue (Soukos *et al*., 1996). The sensitization depends on the parameters related to the laser such as wavelength, power density or light intensity that arrive to the tissue and the energy density that is responsible for the desired radiation effect. These effect exhibit alternations in the mitochondrial gradient of ionic concentration, interfering in the respiratory chain, inhibiting or stimulating ATP synthesis and at the same time producing, singlet oxygen that is toxic to cell (Borotoletto *et al*., 2004). *P. gingivalis*, *F. nucleatum***,** *A. actinomycetencomitans* and *candida* species have been treated with different photosenstizers and only toluidine and methylene blue were effectively lethal for all the target-microorganisms (Wilson & Mia, 1993; De Souza *et al.,* 2006). On the other hand, a request of reducing the use of pesticides in agriculture prompts the need for the development of alternative active compounds, possibly harmless to the consumers and to the environment and useful for the protection of crops from parasites and/or methods for control of plant bacterial diseases to be used in integrated crop management (Rice *et al*., 1998) as well as in bioorganic agriculture. (Rice *et al*., 1998; Cantore *et al.,* 2004) has pointed out on the possibility of using the essential oils and/or their components in medical and plant pathology as

well as in the food industry for the control of microorganisms pathogenic to consumers and/or responsible for food spoilage. Also, a recent trend in food production and cosmetic preservation is to avoid the use of chemical agents looking for natural antimicrobial alternatives. Plant derived essential oils possess potential as natural agents for cosmetic preservation due to their versatile content of antimicrobial compounds (Nostro *et al*., 2004). Several studies that were performed with different bacterial species contribute to the development of treatment options for different types of infectious diseases that can occur in different sites of the human body and for a better compression of the transmissibility of those bacteria (Schimpff, 1993; Wilson & Henderson, 1998). Among the important species, *E. coli*, *S. marcescens, K. pneumoniae* and *S. aureus* (Wilson *et al*., 1993; Wilson & Pratten, 1994; Usacheva *et al*., 2001). The antimicrobial effects of plant material commonly used in food, drug and cosmetic products have been recognized for a long time. Antimicrobial effects of plant extracts, aromatic chemicals and especially essential oils were evaluated against bacteria and moulds in general (Kivanç & Akgűl, 1988). Systematic evaluation of the vapor activity was first reported using the petridish technique (Ayse *et al.*, 2005). The compositions of essential oils are changeable importantly depending on the plant species; climatic conditions and therefore their antimicrobial effects may very enormously (Lawrence, 1985). Essential oils distilled from members of Umbelliferae have been used both cosmetically and therapeutically for centuries with the most commonly used species being *Foeniculum vulgare*. Sweet fennel is perennial plant and one of the most widespread spice allover the world. The fresh leaves and dried fruits are used commonly as a flavoring agent in many food products and as a local mateia medica in Turkey. Essential oils extracted from the fruits of *Foeniculum vulgare Miller var. vulgare* (Miller) and *Coriandum sativum* L. exhibited a bactericidal action for the control of bacterial diseases of plants and for seed treatment, particularly, in organic agriculture. Also, the significant antibacterial activity of essential oils against the bacterial pathogens of mushrooms was recorded (Cantore *et al*., 2004).

The present study aims to contribute with the search for alternative therapies in the treatment of infections by *E. coli*, *S. marcescens, K. pneumoniae* and *S. aureus* bacteria, evaluating the effects of photosenstization of toulidine blue by laser radiation and to evaluate the antibacterial activity of the essential oils (extracted from seeds, leaves & stems) of sweet fennel plant foliarly sprayed with nicotinamide by concentrations of zero, 20, 40, 80 and 100 mg/L towards bacteria responsible for spoilage of food and human diseases using the disk diffusion assay.

MATERIALS AND METHODS

Bacterial species. Bacterial species, *Echerichia coli*, *Staphyloccocus aureus*, *Klebsiella pneumoniae* and *Serratia marcescens* were kindely provided from El-manyal Hospital, Faculty of Medicine, Cairo University. The bacterial strains were plated on nutrient agar (Difeco, USA) and incubated at 37 or 28°C for 24 h. After this period, the cultures were suspended in 5 mL of sterile 0.1 M phosphate buffer saline (PBS, pH.7.0), centrifuged at 3000 rpm for 10 min, washed twice with (PBS) and suspended in 2 mL PBS suspension. Culture from each species was divided into 4 groups. Group L^+P^+: were irradiated with laser light in presence of photosenstizer; Group L^+P^-: were treated only with laser radiation; Group L^-P^+: were treated with the photosensitizer and Group L^-P^-: were exposed to neither the laser light nor to photosensitizer.

Photosensitizer and laser. The Photosensitizer used in this experiments was toluidine blue O (TBO, sigma, UK), prepared in dissolution in bidistilled water at a concentrations of 12.5, 25 and 50 µg/mL, filtrated using Wattman 0.2 µm. Bacterial filtrates stored at 4°C in the dark before used. The light source used was helium/neon (He - Ne) gas laser (NEC corporation, Japan) with out put power 7.3 mW that emits; light in a collimated beam (diameter of 1.3 mm) with a wave length of 632.8 nm. The irradiation time was 5, 10 and 15 min, resulting in an energy dose of 2.19, 4.38 and 6.57 J for each sample.

***In vitro* photosensitization.** In a sterile 96 well flat-bottomed micro dilution plate, 100 µL of bacterial suspension and 100 µL of the photosensitizer were added. The plate containing samples was incubated for 15 min in dark before irradiation according to the previously described 4 groups. Aliquots (100 µL) were plated from each well onto nutrient agar plate and allowed to grow for 24 - 48 h at 28 - 37°C. The number of colonies were determined by direct plate enumeration and expressed as colony forming units/mL (CFU/mL).

Disk diffusion assay. Thirty essential oils, hydro-distilled from seeds, leaves and stems of sweet fennel plant foliarly sprayed with nicotinamide in concentrations of zero, 20, 40, 80 and 100 mg/L during two cuttings were analysed by GC/MS (Gharib, 2002). Antibacterial activity of the fennel essential oil obtained from different treatments of nicotinamide against *Escherichia coli, Staphloccoccus aureus*, *Klebsiella pneumoniae* and *Serratia marcescens* was determined by agar-diffusion technique using filter paper discs, 10 mL of nutrient agar medium were seeded with aliquots of target bacterial suspension, maintained at 45°C, to obtain a final population of about 10^7 CFU/mL (Cantore *et al.*, 2004). The medium was left to cool at room temperature. The sterile 6 mm diameter Whatman No. 1 filter paper discs were completely moistened by 10 µL of stock solution of thirty fennel essential oil samples, placed upon the surface of the inoculated plates and then incubated at 37˚C for 24 h. The assays were performed twice with three replicates and the diameter of the inhibition zone was measured in mm.

Statistical analysis. The experimental data was performed using ANOVA and student-t-test. $P < 0.05$ was considered statistical significant.

RESULTS AND DISCUSSION

As can be seen data in Table I revealed that the growth in presence of photosensitizer (L^+P^+) presented the lowest mean CFU/mL value for all the bacterial species under study, indicating that the irradiation with laser in presence of toluidine blue was able to reduce the viability of these bacterial species. Moreover, *S. marcesence* species was sensitive to laser even in the absence of photosensitizer (Group L^+P^-) and exhibited reduction in CFU/mL number in relation to other treated groups. *In vitro* studies revealed that the use of laser associated with a photosensitizer is very effective against bacteria, Yeast, Viruses and parasites (Wilson & Mia, 1993; Sarkar & Wilson, 1993; WainWright, 1998).

The percentage of reduction of bacterial species viability exposed to laser in presence of photosensitizer (L^+P^+) was higher for *E. coli*, followed by *S. aureus, K. pneumoniae* and *S. marcesence*, respectively in relation to the group treated with neither the laser nor with the photosensitizer (L^-P^-) (Table I). Data in (Fig. 1 & Fig. 2) indicated that as the concentration of TBO or light energy dose increased, there was a significant gradual reduction of CFU/mL of all studied species. Similarly, photo-activation of toludine blue presented lethal effect on several Gram-positive and Gram-negative bacteria (Wilson & Mia, 1993; Wilson *et al*., 1993). Also, (Nitzan *et al.*, 1983) have demonstrated that *S. aureus* can be killed by using a white light source and hematoporphyrin as a photosensitizer. O'Neill *et al.* (2002) observed a reduction of 97.4% of the microorganisms present in the dental bio-film when toludine blue and He - Ne laser (632.8 nm) was employed. Sarkar and Wilson (1993) also verified significant reduction in the viability of both aerobic bacteria (91.6%) and anaerobic ones (96.6%) present in sub-gingival bio-film, after use of toulidine blue and He - Ne Laser.

Antibacterial activity of *Foeniculum vulgare* oil. The results listed in Tables II, III and IV indicate that the *in vitro* antibacterial activities of the essential oil extracted from various parts of *Foeniculum vulgare* (foliarly sprayed with nicotinamide) display remarkable antibacterial activity against *E. coli, S. marcescens, K. pneumoniae* and *S. aureus*, where the diameter of inhibition zone ranging from (6 - 20 mm). By the agar diffusion method, the most sensitive bacteria were *S. marcescens* and the most resistant one was *K. pneumoniae* during first and second cuttings. Several investigations have demonstrated considerable inhibition of the growth of *E. coli, S. aureus* and *K. pneumoniae* by fennel essential oil (Singh *et al*., 2002; Ozkan *et al*., 2003; Dadalioglu & Evrendilek, 2004).

As can seen from Table II, the inhibitory effect of the essential oil extracted from the seeds of *Foeniculum vulgare* plant exhibited a very strong antibacterial activity against *S. marcescens*, where the diameter of inhibition zone ranging from (7 - 20 mm) during two cutting. Nicotinamide treatments increased the antibacterial activity of seed oil towards tested bacteria. The most effective treatment was 40 mg/L nicotinamide recording 20 and 13 mm inhibition during two cuttings. This inhibitory effect may be due to nicotinamide treatments increased the percentage of limonene (28.11), α-pinene (2.972) and fenchone (4.394) but markedly decreased *trans*-anethole (35.556) and estragole (1.658) at 40 mg/L in comarsion with (21.52%) limonene, (0.96%) α-pinene, (1.35%) fenchone, (59.31%) *trans*-anethole and (2.16%) estragole for control sweet fennel seed oils. However, the opposite trend was obtained in presence of 80 and 100 mg/L nicotinamide, where *trans*-anethole and estragole were increased but α-pinene, limonene and fenchone markedly decreased in comparsion with the seed oil of control plants (Gharib, 2002). Similarly, the most frequently occuring constituents in 49 essential oils showing high antifungal activity against *Botrytis cinerea* were D- limonene, α-pinene, β-pinene and camphor (Wilson *et al*., 1997).

Fig. 1. Reduction curves of *E. coli, S. aureus, K. Pneumoniae* and *S. marcesence* exposed to different concentrations of TBO (12.5, 25 and 50 µg/ml) and a light dose of 4.38 J each point is the mean of at least three experiments ± SD, samples kept in the dark for 15 min, P< 0.05 compared with untreated control

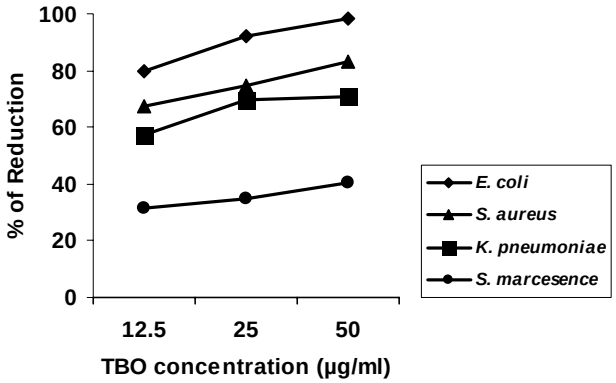

Fig. 2. Reduction curves of *E. coli, S. aureus, K. Pneumoniae* and *S. marcesence* exposed to 25 µg/ml TBO and different exposure time (5, 10 & 15 min), each point is the mean of three experiments ± SD

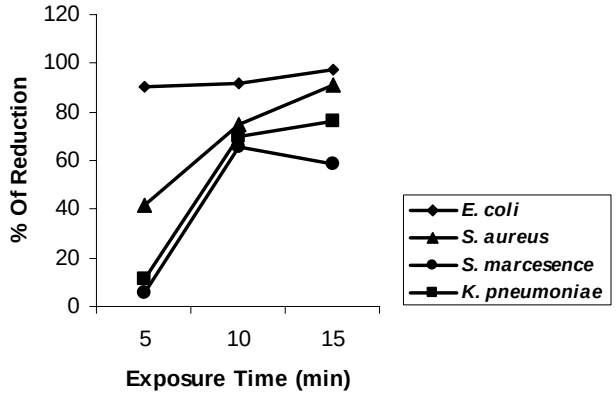

Table I. The logarithm of colony forming units/ml (CFU/ml) for L^+P^+: group irradiated with laser in the presence of photosensitizer; L^+P^-: group treated only with laser; L^-P^+: group treated only with photosensitizer and L^-P^-: group treated neither with laser nor with photosensitizer. Mean values ± standard deviation

Genus	CFU (Log)/ml			
	L^+P^+	L^+P^-	L^-P^+	L^-P^-
E. Coli	1.38 ± 0.20	2.91 ± 0.10	2.96 ± 0.07	2.94 ± 0.08
S. marcesence	2.22 ± 0.29	2.83 ± 0.18	2.85 ± 0.05	2.88 ± 0.15
K. Pneumoniae	2.45 ± 0.31	2.52 ± 0.05	2.83 ± 0.06	2.80 ± 0.08
S. aureus	1.30 ± 0.38	2.50 ± 0.12	2.49 ± 0.10	2.52 ± 0.11

Table II. Inhibitory effect of *Foeniculum vulgare var. dulce* (foliarly sprayed with nicotinamide) essential oil (extractedfrom seed) on the growth of bacteria

Treatments (gm/l)	Inhibition zone (diameter in mm)							
	Test organism Gm-ve						Test organism Gm +ve	
	E. coli		*S. marcesence*		*K. pneumoniae*		*Staph. aureus*	
	1st cut	2nd cut	1st cut	2nd cut	1st cut	2nd cut	1st cut	2nd cut
Control	8	6	10	7	7	6	7	6
20	8	6	12	8	7	7	8	6
40	12	11	20	13	10	8	13	10
80	9	8	13	10	8	9	10	9
100	8	7	13	8	7	8	8	8

Table III. Inhibitory effect of *Foeniculum vulgare* (foliarly sprayed with nicotinamide) essential oil (extractedfrom leaves) on the growth of bacteria

Treatments (gm/l)	Inhibition zone (diameter in mm)							
	Test organism Gm-ve						Test organism Gm +ve	
	E. coli		*S. marcesence*		*K. pneumoniae*		*Staph. aureus*	
	1st cut	2nd cut	1st cut	2nd cut	1st cut	2nd cut	1st cut	2nd cut
Control	6	6	6	7	7	6	6	6
20	6	6	7	8	7	7	6	6
40	11	10	13	13	10	9	10	9
80	7	8	8	10	8	7	10	7
100	7	8	8	8	6	7	8	7

Table IV. Inhibitory effect of *Foeniculum vulgare* (foliarly sprayed with nicotinamide) essential oil (extracted from stem) on the growth of bacteria

Treatments (gm/l)	Inhibition zone (diameter in mm)							
	Test organism Gm-ve						Test organism Gm +ve	
	E. coli		*S. marcesence*		*K. pneumoniae*		*Staph. aureus*	
	1st cut	2nd cut	1st cut	2nd cut	1st cut	2nd cut	1st cut	2nd cut
Control	6	6	10	7	7	7	6	6
20	14	6	15	7	8	7	12	7
40	15	8	18	13	10	8	13	9
80	17	11	20	15	11	10	20	14
100	7	8	13	8	7	7	12	10

As listed in Table III, fennel leaf oils had minor antibacterial activity against the microorganisms tested. Generally, nicotinamide treatment increased the inhibitory effect of leaf oils especilly in presence of 40 followed by 80 mg/L during two cuttings. Gharib (2002) observed that control fennel leaf oils contained 18.25% limonene and 17.32% *trans*-anethole and that nicotinamide treatment of 40 mg/L increased the percentage of limonene (29.69%) and trans-anethole (48.06%) in leaf oils. Moreover, nicotinamide treatments gradually increases the percentage of limonene in the leaves up to (80.91 - 91.48%) at 80 and 100 mg/L treatment but accompanied by parallel decrease in *trans*-anethole to 15.24 and 5.45%, respectively at the same concentrations. In this connection (Dadalioglu & Evrendilek, 2004) reported that *Foeniculum vulgare* Mill exhibited a very strong antibacterial activity against *Escherichia coli* O157: H7 and *Staphylococcus aureus* and that *trans*-anethole (85.63%) were the predominant constituents in fennel essential oils.

Furthermore, data in Table IV revealed that the oils extracted from stems treated with 80 followed by 40 gm/L nicotinamide have higher antibacterial activity towards human pathogenic bacteria belonging to either Gram-negative strains such as *E. coli* or Gram-positive strain as *S. aureus* than the control and other treatments. *K. pneumoniae* was the most resistant strain. Gharib (2002)

found that nicotinamide at 80 mgL^{-1} increased the percentage of limonene, *trans*-anethole, α-pinene and camphene in the stem up to 65.19, 27.35, 0.1 and 0.10% compared to 31.48, 20.96, 0.07 and 0.03%, respectivelly for control fennel stem oils. Singh *et al*. (2002) found that fennel seed oil is effective against *E. coli* and *S. aureus* causing infection in the human body and this oil is equally or more effective when compared with standard antibiotics at very low concentration. The hydrosols of fennel, anise, cumin and thyme showed a stronger inhibitory effect on mycelial growth of *Aspergillus parasiticus* (Özcan, 2005).

The present results revealed that all studied bacterial species were susceptible to the tested PDT protocol and fennel essential oil. The antibacterial activity of various parts of fennel oil decreased in the following order in the disk diffusion assay: Stem > Seeds > Leaves. Stem oil was most effective in presence of 80 mg/L, while Seeds and Leaves oils were effective in presence of 40 mg/L nicotinamide. These findings encourage further *in vivo* studies, may be using animal model, to explore the potential application of this protocol for bacterial pathogen treatment in immune compromised patients and also in preservation of food, pharmaceutical and cosmetic formulations to protect product from microbial activity.

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