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RESEARCH ARTICLE

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Evaluation of the effect of Carvacrol and Linalool on the growth of Staphylococcus aureus and E. coli enterotoxin and production of ent A and ent B toxins in Staphylococcus aureus and ST1 and ST2 toxins in E. coli enterotoxins

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ABSTRACT

Diseases caused by the consumption of food contaminated with pathogenic bacteria are of a major threat to general health. Using natural substances as antimicrobial compounds seem effective for controlling the presence of pathogenic bacteria and increasing the shelf-life of processed foods. Among these compounds, extracts obtained from medicinal plants have antimicrobial properties and act as a source of antimicrobial substances against pathogens. Given their antimicrobial effects, herbal essences are usable as a substitute for antimicrobial chemicals in food industries. So the aim of this study was the evaluation of the effect of Carvacrol and Linalool on the growth of Staphylococcus aureus and E. coli enterotoxin and production of ent A and ent B toxins in Staphylococcus aureus and ST1 and ST2 toxins in E. coli enterotoxins. Carvacrol and linalool were prepared in dilutions of (1,500, 1/1000, 1/1500, 1/2000, 1/2500, 1/3000 ml / µg) and (1/100, 1/200, 1/400, 1/600, 1/800, 1/1000 ml/µg), respectively. The minimum inhibitory concentration was determined by the use of sterile 24 well plates and broth dilution. The minimum bactericidal concentration was obtained in a turbidity-absent culture, separately on the blood lipid agar medium in the case of Staphylococcus aureus, and Mckanaki agar in the case of Escherichia coli. Ent A and ent B toxins in Staphylococcus aureus were produced by high-performance liquid chromatography (HPLC) and STX 1 and STX 2 toxins in Escherichia coli were generated by Elisa method. The minimum inhibitory concentration of carvacrol was obtained at 1/1000 dilution on Escherichia coli, and 1/1500 dilution on Staphylococcus aureus, The minimum inhibitory concentration of linalool was obtained at 1/400 dilution on Escherichia coli and Staphylococcus aureus, the minimum bactericidal concentration of carvacrol was obtained at 1/1000 dilution on Escherichia coli and 1/2500 dilution on Staphylococcus aureus and the minimum bactericidal concentration of linalool was obtained at 1/400 dilution on Escherichia coli and Staphylococcus aureus. The effects of carvacrol and linalool on Ent A and Ent B toxins of Staphylococcus aureus and STX1 and STX2 toxins of E. coli showed the decreasing trend of toxin production at nanogram level. The results of this study showed that carvacrol and linalool had an inhibitory effect on the growth of both 2 bacteria and reduced the production of Ent A and Ent B toxins in Staphylococcus aureus and ST1 and ST2 toxins in Escherichia coli enterotoxigenic.

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INTRODUCTION

In the present century, the preservation of food safety and its quality is an interest that has attracted the attention of both the experts of the food industry and health authorities; inattention to this issue might entail irreparable damages to society. Today, the diseases caused by the consumption of contaminated foods exist even in advanced countries such as the United States (1-7), hence the necessity of providing food safety. The safety, health, and quality of foods are one of the important goals of food producers. Corrupt foods, in addition to the damage to the health of the consumer, are also economically harmful to producers. Because consumers are not sure about the food containing synthesis preservatives, they have no confidence in the use of natural food products used as preservatives (alternative chemical preservatives). The natural ingredients that can be used as preservatives in food are herbal essences and their effective compounds. Extracts, herbal essences, and their effective compounds have antibacterial, antifungal, antioxidation, and anticancer properties able to control the growth of pathogens and the production of poison by microorganisms (1, 4, 8-10).

Plant essential oils are aromatic compounds, hydrophobe, and Condensed, which exist in secretory single or complex cells and trichome, secretory gland, secretory canal and in different parts of various organs, including leaf, flower, fruit, bud and plant branches; these compounds are also obtained by physical methods such as extracts and distillation from all or some parts of plants. Of course, steam distillation is the most common commercial method in the production of essential oils. The main mechanism behind the formation of essential oils is not clear, but these compounds are generally produced by the main processes of plant metabolism, especially under the influence of stresses which are not chemically homogeneous and are often observed in different ways with the origin of terpenes (1-3, 6, 7, 10). Given their antimicrobial effects, herbal essences are usable as a substitute for antimicrobial chemicals in food industries (1, 2). So the aim of this study was the evaluation of the effect of Carvacrol and Linalool on the growth of Staphylococcus aureus and E. coli enterotoxin and production of ent A and ent B toxins in Staphylococcus aureus and ST1 and ST2 toxins in E. coli enterotoxins.

Materials and Methods

Chemicals and enzymes: Essential oils and their effective compounds such as carvacrol and linalool were prepared from the centers of plant essential oils. Linalool C10H18O with code 818627, CAS Number: 78-70-6, and concentration of 154.25 gr/mol and carvacrol C10H14O with code 282197, CAS Number: 499-75-2 and concentration of 150.217 gr/mol were prepared from the Merck agent. The RNX-Plus solution was prepared from the sinacolon Company (RN7713C) and other chemicals from Merck or Sigma company.

Bacterial strains and culture medium: The standard strains of Enterogenic Escherichia coli were employed with ATCC25922 and PTCC1338 code, and Staphylococcus enterotoxins were used with ATCC25923 and PTCC1112 code. The strains of these bacteria were prepared from Iran's scientific and Industrial Research Organization (PTCC), the center of industrial microorganisms. The nutrient media of non-phosphating broth, blood agar, and Mcconki agar were prepared from Merck company.

Species activation of bacteria: A 24h colony of the cultivated bacteria in a suitable culture medium (Escherichia coli in Mckanaki agar and Staphylococcus aureus in Baird Parker Agar) was performed to prepare the uniformity of the bacteria of 0.5 McFarland. In this method, a standard solution was used to prepare a 0.5 McFarland solution, and the microbial correct densities of standard turbidity were compared via adsorption measurement in a spectrophotometer. The absorption rate at 625 nm wavelength should be between 0.08 and 0.13. The standard of 0.5 McFarland is the equivalent of a bacterial suspension containing the 1.5 × 108 CFU/ml.

Dilution of extracts: Effective compounds of the essential oils and their dilutions in this study contains: carvacrol (1/500, 1/1000, 1/1500, 1/2000, 1/3000 ml/µg), linalool (1/100, 1/200, 1/400, 1/600, 1/800, 1/1000 ml/µg). In this method, in order to prepare different dilutions of carvacrol, after adding 50 λ of carvacrol to 9 ml of medium sterile non-phosphyl broth containing 1 ml of DMSO (for their dissolution), a Shaker machine mixed action was completely performed. Also, in order to prepare different dilutions of linalool, after adding 10λ of linalool to 9 ml of sterilized non-phosphate medium containing 1 ml of DMSO (for their dissolution), a Shaker machine mixed action was completely performed. In this work, the subsequent dilutions were prepared from the first prepared dilution. 0.22 mm filter was used for sterilization.

The antimicrobial effect of dilution in Wells: The experiment was done to determine the effect of minimum inhibitory concentration on the growth of bacteria (MIC): This was done through determining the concentration by sterilized 24 wells plate with broth dilution method. first, different dilutions of essential oil compounds with the non-phosphating nutrient broth medium and DMSO were prepared individually. The bacteria of Escherichia coli and Staphylococcus aureus were also separately

dilutions of 0.5 McFarland. In 24 wells plate, 1 ml prepared different dilutions effective compounds of the essential oil with DMSO was added to each well and then its same volume (1 ml) of the suspension 0.5 McFarland of each turbidity is created, is considered as MIC. The tests were performed three times. After 22-24 hours of incubation at the 37 °c temperature, the last well that not observed turbidity was considered as MIC.

The experiment determining the minimum bactericidal concentration on the bacteria (MBC): After reviewing the MIC results of sterilized 24 wells plate, to determine the minimum bactericidal concentration (MBC) of no turbidity wells, Staphylococcus aurous was cultured on blood agar and Escherichia coli was cultured on MacKanaki Agar, separately. After 24 hours the lowest concentration of essential oils that has not grown 99.9 percent of bacteria was considered as the Minimum bactericidal concentration (MBC).

Determination of minimum inhibitory concentration in the production of toxin: A Study on the production of entA and entB toxins in Staphylococcus aureus was done by high-performance liquid chromatography and production of STX 1 and STX 2 toxins in Escherichia coli via Elisa method.

Evaluation of ent A and ent B toxins production in Staphylococcus aureus by LC/MS: Staphylococcal enterotoxin B and A protein (100 mg) Staphylococcal enterotoxin B protein (100 mg) was dissolved in 2mL of 0.1% (v/v) TFA–water solution (50 mg/mL). Further dilution (1:10) in 100mM ammonium bicarbonate (pH 8.5) was necessary to perform the trypsin digestion and generate tryptic peptides. Synthetic tryptic peptides were dissolved in a 0.1% (v/v) TFA–water solution (100 mg/mL). The peptide stock solutions (100 mg/mL) were diluted in a 0.2 M ammonium bicarbonate buffer (pH 8.5) at a concentration of 200 pmol/mL to prepare the non-isobaric tagged standards and internal standard also referred to as reference internal standard method in quantitative proteomics.

The HPLC system (Agilent 1200-6410) contained a Thermo Surveyor autosampler and a Thermo Surveyor MS pump (San Jose, CA, USA). The used quadrupole ion trap (QIT) system was a Thermo LCQ Advantage (San Jose, CA, USA).

Evaluation of the STX1 and STX2 production in Escherichia coli by HPLC: Purified toxin (55 mg ml21) was incubated at 37 °C in 0.11% glutaraldehyde in 0.1 M sodium phosphate buffer (pH 8) for 30 min. Lysine was added to a two-fold molar excess over glutaraldehyde. The resulting toxoid solution was three times dialyzed against phosphate-buffered saline (PBS) (pH 7). The toxoid solution was divided into fractions (0.5 ml) containing 10 mg of toxin protein and frozen until further use.

The sample solution (0.1–1.0 ml) was mixed with 50 ml of each antisera to Stx1 and Stx2 and was allowed to stand for 5–120 min. The mixed solution was centrifuged at 12000 rpm for 15 min, and the resulting precipitate was washed with PBS followed by distilled water. The precipitate was dissolved in acetonitrile-water (1:9)–0.2% formic acid and analyzed by HPLC.

The HPLC system consisted of an HP1100 series binary pump, a degasser, and a column compartment (Hewlett Packard, Palo Alto, CA, USA). The HPLC column was a TSK-GEL Phenyl 5PW-RP column (75 3 2.0 mm, Tosoh, Tokyo, Japan) or a PLRP-S column (150 3 2.0 mm, Michrom Bioresources, Pleasanton, CA, USA). The solvent system was a linear gradient of solvent A mixed with solvent B from 10% B to 90% B in 30 min. Solvent A was comprised of water containing 0.2% formic acid, and solvent B was acetonitrile containing 0.2% formic acid. The flow rate was 0.2 ml in 21 min and the eluent was introduced into an ESI interface.

Results

Determination Results of Minimum Inhibitory Concentration on Bacterial Growth (MIC)

Among the 6 dilutions used of Carvacrol, 1/1000 dilution was obtained on Escherichia coli, and about Staphylococcus aureus was obtained in 1/1500 dilution as MIC dilution (Minimum inhibitory and on concentration based on turbidity or Non-turbidity of culture medium).

Also among the 6 dilutions used of Linalool on Escherichia coli and Staphylococcus aureus was obtained in 1/400 dilution, and it was considered as MIC dilution (Minimum inhibitory and on concentration based on turbidity or Non-turbidity of culture medium).

Determination of minimum bactericidal concentration (MBC)

Out of the six dilutions used for carvacrol on Escherichia coli 1/1000 and on Staphylococcus aureus 1/2500 was obtained as MBC dilution (the minimum inhibitory concentration of growth based on growth or non-growth of bacteria). Also, among the six dilutions used for Linalool on Escherichia coli and Staphylococcus aureus, 1/400 was achieved as MBC dilution (the minimum inhibitory concentration of growth based on growth or non-growth of bacteria).

Determination of Monoterpenes Inhibition on Toxin Production

The effects of Carvacrol and Linalool on EntA and EntB Toxins of Staphylococcus aureus and STX1 and STX2 Toxins of Escherichia coli were determined, where the falling trend of toxin production was observed at nanogram levels. The results showed

that toxin production was affected by both monoterpenes and the effect of carvacrol was greater than linalool in reducing the toxin production.

A positive control sample was used for Escherichia coli and Staphylococcus aureus without treatment with carvacrol or linalool. The results showed that the lower dilutions of carvacrol and linalool monoterpens further reduced the production of bacterial toxins. At 1/2500 carvacrol dilution, toxin production decreased from 10.84 ± 0.06 ng /ml to 2.51 ± 0.06 ng /ml, whereas at lower dilution, 1/3000, the production of the toxin was reduced from 10.84 ± 0.06 ng/ml to 0.06 ± 1.94 ng/ml.

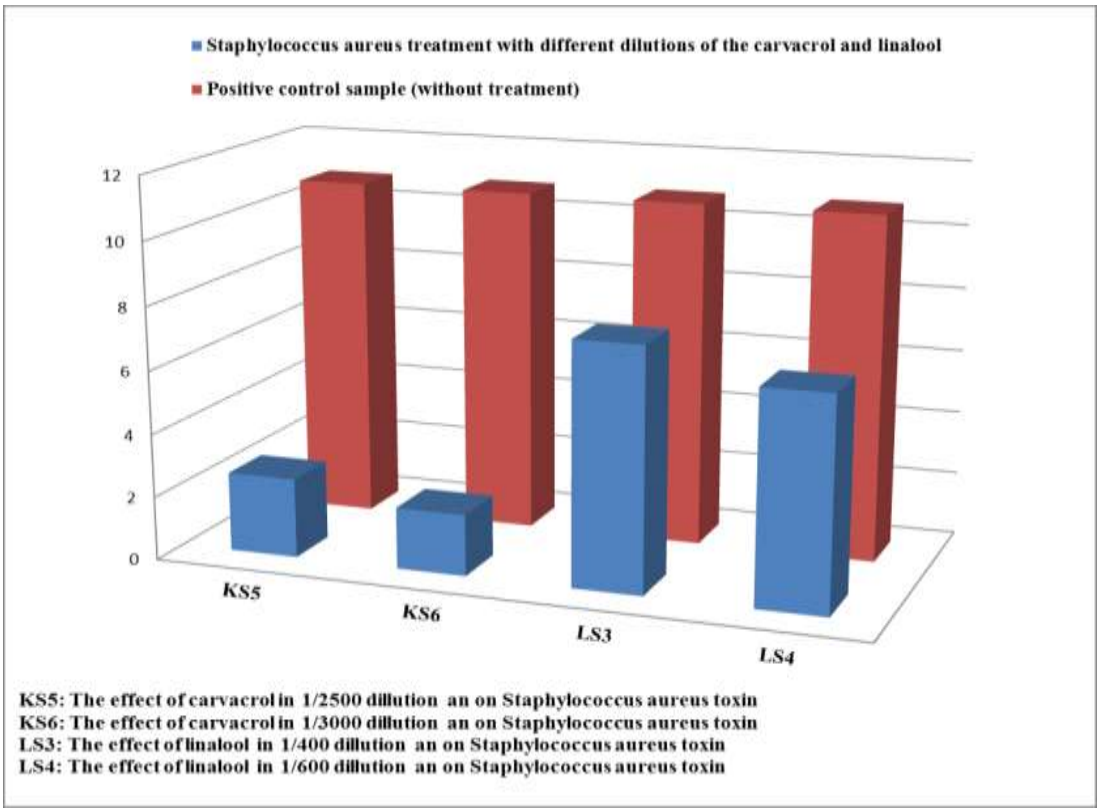

Figure 1: Comparison of carvacrol and linalool inhibitory effects on the production of Staphylococcus aureus toxins

Discussion

In recent decades, medicinal plants have been considered as natural reservoirs owing to their drug resistance. Many herbs are used daily in human and animal diets and experimentally proven to have no adverse effects. The amount and type of metabolites in different organs of the plant varies based on ecological conditions. Therefore, it seems necessary to evaluate the active ingredients of medicinal plants according to the geographical areas under their cultivation, such as the species of thyme that vary under different climatic conditions. Therefore, the correct use of medicinal plants requires accurate scientific information and knowledge of their chemical compounds which actually induce therapeutic effects in the plant (1-3, 11, 12).

This study showed that the inhibitory effect of carvacrol on the growth of Escherichia coli and Staphylococcus aureus was significantly more than linalool. In the case of toxin production, the carvacrol effect on the toxin production of Escherichia coli and Staphylococcus aureus bacteria was more than linalool.

Various studies on the biological activity of effective compounds in medicinal plants have provided a variety of results, depending on their chemical structure and effects; it is very difficult to determine the molecular mechanisms involved in the activity of these compounds. Accordingly, it is shown that each monoterpene has a separate effect mechanism. Due to the extent of the profiles and components of these compounds, their antimicrobial properties are not mechanism dependent, but different approaches and mechanisms at the molecular level play a role in this issue. Hence, it is assumed that different mechanisms are involved in the antimicrobial activity of effective herbal compounds (6, 7, 10).

The acidic nature of the hydroxyl group in thymol and its participation in the formation of hydrogen bands is considered a significant antimicrobial activity. In general, the antimicrobial performance of plant essential oils is due to the reaction between their active groups such as hydroxyl group and microorganism cell components. The effect mechanism of phenolic compounds includes

disruption of the performance of the cytoplasmic membrane, disruption of the proton move energy, and the electric flow and cell content coagulation. Inhibition of toxin production and toxin activity by plant essential oils can be indirectly caused by the disturbance in a number of factors such as fraternal transcription and translation or direct inactivation of toxins. These natural properties of plant's essential oils are preferred to many of the common antimicrobial substances that affect only a place of purpose. On the other hand, the natural essence of herbal essences has made it possible for consumers to prefer them to chemical and organic antimicrobial substances (1, 2, 13-15).

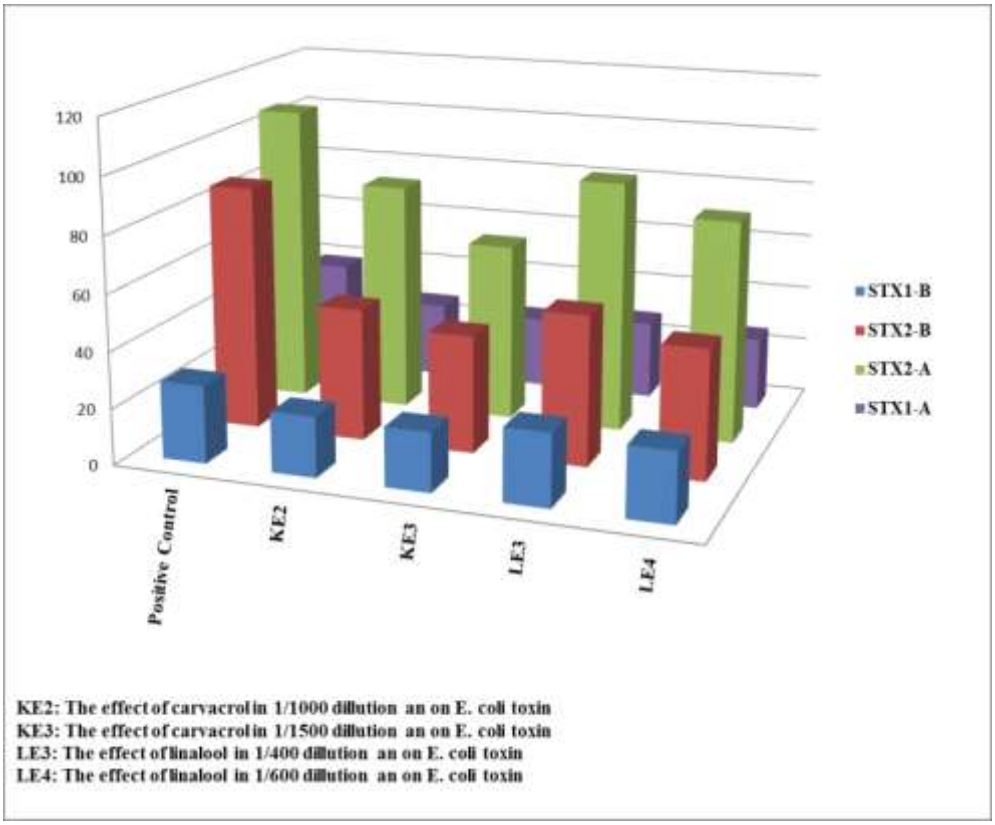

Figure 2: Comparison of carvacrol and linalool inhibitory effects on Escherichia coli toxin production

Nouri et al. (2012) investigated the antimicrobial effect of Zataria multiflora essential oil on the growth of Escherichia coli O157: H7 in minced beef during storage at refrigerated temperatures of 4 and 10 º C for 14 days in order to replace with chemical preservatives. Their results showed that as long as the minced beef was kept at 4 ° C under the influence of the highest concentration of thyme essential oil in this study (0.03%), the bacterial growth was significantly reduced ($p < 0.01$). Therefore, Zataria multiflora essential oil can be recommended as a natural preservative in meat products. In this study, 0.04% concentration of Zataria multiflora essential oil prevented the growth of Escherichia coli and caused bacterial death at 0.08% concentration. Also, 0.02% of essential oil had a significant effect on the inhibition of Shiga-toxin production(16). In a 2006 study by Razwiller, Akhundzadeh et al. regarding the effects of essential oils, thyme essential oils, temperature, and storage time on the growth of Salmonella typhimurium in cardiac and brain extracts, it was observed that Zataria multiflora Boiss can be used as a growth inhibitor of Salmonella in food products (17).

The results of this study showed that both monoterpenes, carvacrol, and linalool, had very good antimicrobial effects on Escherichia coli, Staphylococcus aureus, and many other bacteria; moreover, in general, carvacrol had higher antimicrobial activity compared to linalool against the growth of bacteria and the production of their toxins. Similar studies have replaced these compounds with synthetic antimicrobial substances (18-23).

According to scientific research, it can be concluded that effective herbal compounds such as monoterpenes have many applications in the pharmaceutical and food industries and have specific biological, pharmacological, and therapeutic activities. For instance, effective plant compounds interfere with microbial activity and, in many cases, destroy microorganisms without undesirable effects on consumer health (1-3, 20, 22).

Plant compounds have broad-spectrum antiviral activity and are also used as natural products. It seems that due to the different applications of these compounds, especially as medicinal compounds, the use of synthetic antibiotics is more and more reduced. In addition, various methods are nowadays used to control foodborne pathogens, one of which is to add certain medicines; however, due to the genetic diversity created in microbial pathogens and the appearance of resistant strains and side effects caused by many of these drugs, it is highly important to replace them by other methods and search for herbal drugs without side effects (6, 18, 19).

Food health is an important issue for consumers and food producers; also, there are reports of several cases associated with infections and poisoning due to contaminated food, hence the necessity of providing strategies to maintaining food health in society. A brief review of the literature indicates that few studies have been performed to evaluate the effects of these compounds on growth inhibition or degradation of cellular enzymatic system, especially the enzymes involved in energy production and the synthesis disruption in the constituents of these bacteria. Different regions of Iran have their own cultures and customs associated with the use of medicinal herbs; therefore, to gather valuable information on medicinal herbs more research and investigation are needed among these ethnicities (1-4, 17, 22).

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