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Research Article

Antioxidant, Antibacterial and Anti-Enterotoxin Activities of *Lippia citriodora, Kelussia odoratissima Mozaff* and *Allium iranicum* Essential Oils Against *Staphylococcus aureus*

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Abstract

Antibiotic resistance bacteria are increasing due to inappropriate use of antibiotics in humans and animals. Therefore, the necessity to find potentially effective natural alternative antibiotics is increased. The aim of this study was assessment of the antioxidant, antibacterial and anti-enterotoxin activities with affecting of *Lippia citriodora, Kelussia odoratissima Mozaff* and *Allium iranicum* essential oils (EOs) against *Staphylococcus aureus*.

For determining of total phenolic content Total phenolic content and antioxidant activity of three EOs was used of Folin-cocalteu and DPPH method. Antibacterial and anti-enterotoxin activities of EOs on *S. aureus* were also determined using microdilution method and enzyme linked immunosorbent assay (ELISA).

The content of total phenolics (mg of gallic acid equivalent/g) ranged from 797 ± 2.83 in *Lippia citriodora* EO to 1972 ± 2.83 *Kelussia odoratissima Mozaff* EO. All of the EOs presented antioxidant capacity and the overall antioxidant strength was in the order *Kelussia odoratissima Mozaff* EO > *Lippia Citriodora* EO > *Allium Iranicum* EO. Every one of EO was showed a strength antimicrobial activity and was coused a inhibitory action against the production of *SEA, SEB, SEC, SED* and *SEE* enterotoxins in *S. aureus* at all concentrations studied (0.781, 1.56, 3.1, 6.5, 12.5 and 25 µg/ml).

The results of this study revealed that the above EOs act as antioxidant agents due to their phenolic compounds and antibacterial activities.

Keywords: *Lippia citriodora; Kelussia odoratissima Mozaff; Allium iranicum; Staphylococcus aureus*; Antibacterial Activity

Introduction

Use of chemicals as food preservatives and preventing their corruption by food pathogens is one of today's concerns of the food and health sectors, because wrong selection of these materials can be considered a risk factor for health. Among different food phatogenic bacterial *Staphylococcus aureus* has an important role in nosocomial infections. *S. aureus* is also among the four most common causes of food borne illnesses. Staphylococcal food poisoning (SFP) results from consumption of food contaminated with staphylococcal enterotoxins (SEs) produced by *S. aureus* [1-3].

S. aureus produces 15 different type of denterotoxins one or more toxin for staphylococcal poisoning is necessary that usually classic toxins (A-E) are the reason for 95% of staphylococcal food poisoning among (A-E) toxins SEA and SED are more common than others. The one study by Dwards-Jones et al. in 2004 that was affected on EO on methicillin –resistant *S.aureus* was showed strength antibacterial activity against of MRSA that can be used for treatment of MRSA infections [4]. This study demonstrated the potential of EOs as antibacterial agents and for use in the treatment of MRSA infection.

With the advent of technology in the modern world and the effects of chemical drugs, the demand and the desire to use natural products are increasing. One of these natural products that can be used in the food and pharmaceutical industry are essential oils that, in addition to increasing the taste and taste of food, The antimicrobial effect has also been studied in many studies. Phenolic compounds in essential oils are considered as antimicrobial agents of essential oils, which are recognized as safe compounds and can therefore be used as natural antimicrobial compounds in the food industry [5-7].

Lippia citriodora is belong to *lippia* genus a plant with small trees and shrubs so that it is a member of Verbenaceae family. it is cultivated in northern Africa, southern Europe and north of Iran. Most of them are traditionally utilized as remedies for gastrointestinal and respiratory problems. Some species have shown antimalarial, antiviral, and cytostatic properties [4,5].

The genus *Lippia* (Verbenaceae) includes approximately 200 species of herbs, shrubs, and small trees. The *Lippia citriodora* a member of Verbenaceae family, is cultivated in northern Africa, southern Europe and north of Iran. Most of them are traditionally utilized as remedies for gastrointestinal and respiratory problems. Some species have shown antimalarial, antiviral, and cytostatic properties [4,5].

Allium genus that is relevant to the Alliaceae family consists of hundreds of medicinal species in the world, and it is one of the most imperative source of life supporting drugs. *Allium ampeloprasum* and *Allium iranicum* species are utilized as medicinal herbs in local and traditional medicine in Iran [8].

Kelussia odoratissima Mozaff. is an indigenous, perennial and medicinal herb belonging to Apiaceae. This plant is found only in restricted areas of the Central Zagros Mountains in Iran. This plant has traditionally been used as a folk medicine to treat inflammation, hypertension, ulcer, and cardiovascular diseases [9].

Previous studies demonstrated antibacterial activity of *Lippia Citriodora, Kelussia odoratissima Mozaff* and *Allium Iranicum* plants against *E. coli, S. typhi, B. subtilis, S. enterica, P. aeruginosa* and *B. cereus* [10-12].

Therefore, this study was designed to evaluate the antioxidant, antibacterial and anti-enterotoxin activities of the *Lippia Citriodora, Kelussia odoratissima Mozaff* and *Allium Iranicum* essential oils.

Materials and Methods

Plant material

Samples of *Lippia Citriodora, Kelussia odoratissima Mozaff* and *Allium Ampeloprasum* L. var. *Allium Iranicum* were buyed of a grocery shopping in chaharmahal va bakhtiari provience of Iran plants were identified by medical plant reaserch center in Shahrekord. with a mixervevery plant was mixed after cleaning and drieng inshadow .. Dried plants (100 g each) were hydro-distilled for 3-4 h using a Clevenger type apparatus. The EOs were then dehydrated over anhydrous sodium sulphate and kept in sealed vials at 4°C. For the antibacterial properties, several dilutions of the EOs were done using 5% (v/v) aqueous dimethyl sulfoxide (DMSO) (Merck Co., Darmstadt, Germany) [13].

Determination of total phenolic content

The level of total phenols in the crude extracts were determined using Folin-Ciocalteu reagent and external calibration with gallic acid. Briefly, 0.2 mL of EOs solution and 0.2 mL of Folin-Ciocalteu reagent were added, and the content was mixed thoroughly. After 4 min, 1 mL of 15% Na_2CO_3 was added, and then the mixture was allowed to be for 2 h at normal temperature. The absorbance was measured at 760 nm using a spectrophotometer (Thermo Fisher Scientific, model 4001/4). The concentration of the total phenolics was calculated as mg of gallic acid equivalent/g using an equation obtained from gallic acid calibration curve [14].

DPPH assay

The capacity to scavenge the 2,2-diphenyl-1-picrylhydrazyl (DPPH) (Sigma-Aldrich Co, St. Louis, USA) free radical was monitored according to a method reported before. Various concentrations of sample EOs were mixed with methanol and 1 ml of methanolic solution containing DPPH radicals (0.1mM) added to the mixture. The mixture was shaken vigorously and left to stand in the dark until stable absorption values were obtained. The reduction of the DPPH radical was measured by monitoring continuously the decrease of absorption at nm. DPPH scavenging effect was calculated as percentage of DPPH discoloration using the equation: % scavenging effect = [(ADPPH -AS)/ADPPH] x100, where AS is the absorbance of the solution when the sample essential oil was added at a particular level and ADPPH is the absorbance of the DPPH solution. Scavenging activity in this assay was expressed as IC50, which represents the concentration of the essential oil (mg/mL) required

to inhibit 50% of the free radical-scavenging activity. Butylated hydroxytoluene (BHT) was used as a positive control [15].

Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) assay

The *S. aureus* (Persian Type Culture Collection:1337) was obtained from the Iranian Research Organization for Science and Technology (IROST) and cultured at 37°C in tryptic soy broth (TSB, Merck Ink, Darmstadt, Germany). In this study, for microdilution, the MIC and MBC were used. In this method, a 96-well plate was used. For this purpose, 95 µl of MHB (Merck Co., Darmstadt, Germany) medium and 5 µl of microbial suspension, equivalent to 0.5 McFarland's opacity, were used in each well. Finally, 100 µL of consecutive dilution of (serial two-fold dilutions) each EO (0.39-100 µg/mL) were added to each well. Positive and negative controls were considered as follows: Positive control of 195 µl containing DMSO and 5 µl of microbial suspension without EO was used for negative control of 200 µl of MHB medium containing DMSO without microbial suspension. At the end, the samples were mixed with shakers (300 rpm and for 30 seconds) and screwed for 24-24 hs; hk; Fahaj, and Fahsh-Nehlod-37. After the incubation, the wells were evaluated by assessing the presence or absence of Turbidity. Dilution plate of the well containing the lowest concentration of EOs that inhibited growth of bacteria (lack of turbidity) was determined as the MIC. Furthermore, the lowest concentration that showed no visible growth on MHA was determined as the MBC [16].

Detect Staphylococcal Enterotoxins of *S. aureus*

To distinc of staphylococcal enterotoxins, *S. aureus* isolate was cultured aerobically in 10 mL of nutrient broth at 37°C overnight. The MIC of previous concentration of everyone essential oil was used for this purpose. Culture supernatants of the isolated bacteria were then used for detection of *S. aureus* enterotoxins. Staphylococcal entrotoxins *SEA, SEB, SEC, SED* and *SEE* were detected by enzyme linked immunosorbent assay (ELISA) detection kit (RIDASCREEN® SET A, B, C, D, E; R-Biopharm AG, Darmstadt, Germany) according to the manufacturer's instructions. The detection limit was 0.1 mg/mL. *S. aureus* and *S. epidermis* strains were used as positive and negative controls for each test, respectively [17].

Statistical analysis

The analyses were performed running SPSS 16.0 statistical software (SPSS Inc., Chicago, IL, USA). The results of all experiments were expressed as the mean ± standard deviation (SD) of triplicates. The differences were also considered significant at values of $P < 0.05$.

Results

The mean concentrations of total phenolic content, antioxidant and antibacterial activities of the *Lippia Citriodora, Kelussia odoratissima Mozaff* and *Allium Iranicum* EOs are presented in Table 1.

Essential oil	Total phenolic content	DPPH scavenging activity (IC50 mg/mL)	Antibacterial activities	
			MIC (µg/ml)	MBC (µg/ml)
Allium Iranicum	916 ± 1.42[a]	194.5 ± 2.12[a]	12.5	25
Lippa citriodoora	797 ± 2.83[b]	51 ± 2.83[b]	0.781	1.56
Kelussia ordoratissma Mozaff	1972 ± 2.83[c]	2.35 ± 0.1[c]	3.125	6.25

Table 1: Total phenolic content, antioxidant and antibacterial activities of the *Lippia Citriodora, Kelussia odoratissima Mozaff and Allium Iranicum* essential oils.

*Different letters a, b and c in the column indicate significant differences ($p < 0.05$).

The results of the antioxidant study showed that the most antioxidant property was related to *Kelussia odoratissima Mozaff* EO and the least property was related to the EO of *Allium Iranicum*. Overall, the total antioxidant activity ranked as follows: *Kelussia odoratissima Mozaff* EO > *Lippia Citriodora* EO > *Allium Iranicum* EO.

The results of antibacterial examination from three essential oils on of the *S. aureus* showed considerable effect of these essential oils in preventing the growth and death of this bacterium. The lowest concentration was related to MIC of *Lippia Citriodora* EO (0.781 µg/mL) on *S. aureus*, and the highest concentration in relation to MIC was related to *Allium Iranicum* EO (12.5 µg/mL). Overall, the antibacterial activity ranked as follows: *Lippia Citriodora* EO > *Kelussia odoratissima Mozaff* EO > *Allium Iranicum* EO ($p < 0.05$).

Based on the results, the use of concentrations higher than MIC in all EOs prevented the production of different types of A, B, C, D and E *S. aureus* toxins (Table 2). Also, by increasing the concentrations of the EOs, a greater inhibitory effect was observed on the production of *S. aureus* enterotoxins.

Entero-toxins	*Allium Iranicum* EO					*Kelussia odoratissima Mozaff* EO				*Lippia Citriodora* EO		
	0.781 (µg/ml)	1.56 (µg/ml)	3.1 (µg/ml)	6.5 (µg/ml)	12.5 (µg/ml)	0.390 (µg/ml)	0.781 (µg/ml)	1.56 (µg/ml)	3.1 (µg/ml)	0.390 (µg/ml)	0.781 (µg/ml)	1.56 (µg/ml)
A	0.020	0.019	0.016	0.018	0.017	0.021	0.019	0.016	0.018	0.018	0.014	0.013
B	0.026	0.022	0.015	0.020	0.019	0.020	0.017	0.020	0.016	0.022	0.015	0.013
C	0.022	0.024	0.023	0.018	0.021	0.020	0.019	0.019	0.019	0.017	0.016	0.015
D	0.021	0.026	0.024	0.023	0.025	0.020	0.021	0.028	0.021	0.020	0.021	0.019
E	0.018	0.017	0.016	0.017	0.018	0.017	0.018	0.017	0.016	0.015	0.015	0.018
Negative	0.013	0.017	0.016	0.017	0.017	0.017	0.017	0.016	0.014	0.015	0.014	0.013
Positive	1.585	1.594	1.214	1.619	1.547	1.515	1.717	1.863	1.903	2.160	2.066	2.301

Table 2: Effect of *Allium Iranicum, Kelussia odoratissima Mozaff and Lippia Citriodora* essential oils at different concentrations on enterotoxins production by *S. aureus*.

Discussion

The results of this study concerning the antioxidant properties of the three plants showed high levels of plant *Kelussia odoratissima Mozaff* antioxidant properties equaled to the other two plants.

Other studies on antioxidant properties of *Lippia Citriodora, Kelussia odoratissima Mozaff* and *Allium Ampeloprasum* L. var. *Allium Iranicum* including the study by Alavi *et al*. on two plants, *Lippia citriodora* and Thymus *daenensis*, showed that the antioxidant activity of the thymus *daenensisi* is greater than the *lippia citriodora* [18].

Generally, Gram-negative bacteria are more resistant to EOs than Gram-positive bacteria. Before tentative the effects of EOs on bacteria, we should briefly consider the differing structures of the cell walls of Gram-positive and Gram-negative bacteria.

The results regarding the antimicrobial effect of three essential oils against *S. aureus* showed that the best antimicrobial activity was associated to *Allium Iranicum* EO on *S. aureus*. Essential oil of *Lippa citriodoora* showed the least antimicrobial activity compared to other two essential oils.

Despite recent developments in food production and hygiene methods, safety of food is a crucial public health factor. About 30% of people in industrialized countries suffer foodborne diseases. Hence, novel procedures of production of safe foods with a natural or green image have emerged [19].

Food preservation method scan prepares food with high nutritional quality and microbial stability through monitoring the foodborne microorganisms and growth/survival of spoilage-associated.

Parsaeimehr., *et al*. studied on impact of Zataria multiflora Boiss essential oil, Nisin, and their mixtures on the generation of enterotoxin C and a-Hemolysin by *Saphylococcus aureus*. EO considerably ($p<0.05$) hindered production of SEC via *S. aureus* across the manufacturing process of a traditional Iranian white brined cheese (as a food model) at its minimum concentration (5 mL=100mL) [20].

Azizkhania., *et al*. in 2015 investigated on influences of essential oils of Cinna momumzeylanicum and Ocimum basilicum over the development of *Staphylococcus aureus* ATCC 29213 and enterotoxins A, C, and E gene expression where preventive influence of basil EO versus *S. aureus*, inenterotoxin secretion and growth, was larger compared with cinnamon [21].

In a study the bacterial foodborne pathogens sensitivity to Croton *blanchetianus* Baill essential oil was studied. They investigated a bactericidal influence on *Listeria monocytogenes* and *Aeromonashy drophilaand* bacteriostatic action on *Salmonella* Enteritidis. A bacteriostatic influence overmeat contaminated with *L. monocytogenes* was detected for whole tested essential oils' concentrations. Essential oil of the *C. blanchetianus* Baill leaves showed another source of potentially natural antimicrobial agents employed as a food preservative [22].

Conclusion

Taking into account the antioxidant and antibacterial features of herbal essential oils such as *Lippia Citriodora*, *Kelussia odoratissima Mozaff*, and *Allium Iranicum* and usage of these local blends in the traditional procedure to enhance the quality and taste of food, these compounds could be employed as preservatives of food to inhibit corruption of food by *S. aureus*.

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