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INHIBITORY PROPERTIES OF LITHIUM, SODIUM AND POTASSIUM *o*-, *m*- AND *p*-COUMARATES AGAINST *ESCHERICHIA COLI* O157:H7*

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ABSTRACT

Background. The aim of this paper was to assess the inhibitory properties of salts of phenolic acids against *Escherichia coli* O157:H7 ATCC 8739. *Escherichia coli* O157:H7 is a pathogen which is able to produce verotoxins provoking hemorrhagic diarrhea in humans. There is a strong need for the effective natural methods eliminating *E. coli* O157:H7 from food.
Methodology. The following salts were tested: sodium, potassium and lithium salts of *ortho*-coumaric, *meta*-coumaric and *para*-coumaric acids. The 1%, 2%, 3%, 4% and 5% water solutions of each substance were prepared. Agar-well diffusion method was applied. Petri dishes were incubated at 35°C for 24 h. At the end of the incubation period, inhibition zones which appeared on the medium Petri dishes were calculated in millimeters.
Results. It was found that lithium salt of *o*-coumaric acid, potassium salt of *o*-coumaric acid, lithium salt of *m*-coumaric acid and sodium salt of *m*-coumaric acid were most effective towards *E. coli* O157:H7, while potassium salt of *m*-coumaric acid, a sodium salt of *p*-coumaric acid were slightly less effective and lithium salt of *p*-coumaric acid did not possess any antimicrobial activity.
Conclusion. The salts of phenolic acids having various structural features showed different characteristics towards foodborne pathogens. Such findings indicate that phenolic acids and their salts may be a potential bio-alternative for chemical food preservation.

Key words: *Escherichia coli* O157:H7, antimicrobial activity, phenolic salts

INTRODUCTION

Escherichia coli O157:H7 is known to be a food-borne pathogen able to produce verotoxins called shiga toxins responsible for provoking the hemorrhagic colitis and hemolytic-uremic syndrome in humans. There is a demand for effective natural methods allowing to eliminate *E. coli* O157:H7 from food products. Such outbreaks are usually related to the consumption of cattle origin meat products. Many researchers have proved a gastrointestinal system of cattle to function like a reservoir of this pathogen. There have been many well-developed meat-processing techniques which reduce a risk of contaminated beef products transferred to customers. A big number of studies have indicated *E. coli* O157:H7 is able to survive in manure for three months after defecation. Such an ability of this foodborne pathogen to survive in manure constitutes an environmental, as well as a food safety concern.

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Food poisoning caused by a wide variety of different pathogens constitutes a great problem not only for customers but also food producers who try to apply different preservation methods. It is a huge risk of food contamination caused by some pathogenic and spoilage microflora which is simultaneously a reason for a relatively high number of illnesses. Another issue is that many pathogens are highly resistant to antibiotics (Meng et al., 1998; Perreten et al., 1998; Stermitz et al., 2000). A wide variety of food products are preserved with synthetic preservatives which is a reason of customers' worries as well. This is why a higher interest is observed in the implementation of completely new and effective nontoxic antimicrobial substances. It has been indicated that there are a high number of different phenolic compounds found in extracts of spices and herbs which might be added to food products as a natural and effective way of preservation (Smid and Gorris, 1999).

Spices and herbs are commonly known to have been added to food products even in the ancient times. They found their application as flavouring agents, in folk medicines and food preservatives (Beuchat, 1994; Nakatani, 1994; Cutler, 1995). Many spices and herbs, due to the fact that they contain some phenolic substances, are able to prolong the shelf-life of food products by possessing some antioxidant, bacteriostatic and bactericidal activity (Beuchat and Golden, 1989). Spices and herbs are considered to be safe products, not causing any risk for people's health (Smid and Gorris, 1999). Natural phenolic substances which are present in spices and herbs seem to be an appealing alternative for synthetic food additives. They enjoy a great popularity due to their antimicrobial activities and medicinal benefits, as well as their flavour and fragrance features. The extracts from different plants are very popular nowadays due to the fact that they contain some bioactive principles which might be used as natural food preservatives (Voravuthikunchai et al., 2004).

Numerous research have been carried out to investigate the antimicrobial activities of different plant extracts towards particular types of foodborne pathogens (Beuchat, 1994; Lis-Balchin and Deans, 1997; Smith-Palmer et al., 1998; Hara-Kudo et al., 2004). Moreover, it is quite hard to clearly assess the antimicrobial activities of plant extracts due to the fact that a low number of plant samples was tested, different test methods were used and diverse bacterial strains were applied (Shan et al., 2007). It is unambiguously confirmed that phenolic compounds present in spices and herbs highly attributed towards their antioxidant and pharmaceutical properties (Cai et al., 2004; Shan et al., 2005; Wu et al., 2006). There is a number of scientists who believe that the phenolic compounds coming from spices and herbs possess a beneficial role in the preservation of unwanted microflora growth (Hara-Kudo et al., 2004). The interest in the discovery of natural antibacterial substances has encouraged many researchers to carry out investigations on the relationship between bacterial inhibition and total phenolic content of spices and herbs. Some earlier examinations indicated a high correlation between antioxidant activity and total phenolic content in some kinds of spices and herbs (Cai et al., 2004; Shan et al., 2005).

However, as no sufficient reliable data on the correlation between antibacterial activity and antioxidant capacity of spices and herbs exist, numerous investigations should be carried out to confirm their effectiveness. The aims of this examination were assessing and comparing *in vitro* antibacterial activity of nine salts of phenolic substances and assessing their lowest inhibitory concentrations towards *E. coli* O157:H7.

MATERIAL AND METHODS

Sample preparation

The salts were prepared by dissolving appropriately weighed amount of *o*-, *m*- and *p*-coumaric acids (hydroxycinnamic acids) in aqueous solutions of lithium, sodium and potassium hydroxides in a stoichiometric ratio 1:1. The anhydrous compound was obtained after the evaporation of the solvent in the water bath, and drying at 120°C in a dryer. Acids such as *o*-, *m*- and *p*--coumaric ones were bought in Linegal Chemicals Sp. z o.o. and lithium, sodium and potassium hydroxides were bought in POCH S.A. The 1%, 2%, 3%, 4% and 5% water solutions of each substance were prepared to check their antimicrobial activity towards *E. coli* O157:H7.

Microbial strain

The strain *E. coli* O157:H7 ATCC 8739 used for microbiological analysis was obtained from ATCC

collections. Culture of *E. coli* O157:H7 was maintained in 5% glycerol in temperature –20°C.

Preparation of liquid bacterial culture in tryptic soy broth

A sixteen-h-old culture inoculated in tryptone soy broth (bioMérieux, Warsaw, Poland) at temperature 35°C was taken for further experiment. The optical density of this culture after inoculation was determined at 625 nm (Ultraspec III, Pharmacia, Sweden). The incubation was stopped when the optical density achieved a value in the range of 0.8–1.0. The culture suspensions were diluted to an absorbance of 0.1 and used as such for the antimicrobial tests.

Placing the culture dilution on a plate with medium

The medium used for further experiment was Mueller Hinton agar with sheep blood on sterilised Petri dish (Ø 10 cm; bioMérieux, Warsaw, Poland). A 1 ml of a sixteen-h-old culture diluted to achieve an absorbance of 0.1 was placed onto the surface of pre-dried Mueller Hinton agar with sheep blood Petri dishes (Ø 10 cm), then they were allowed to remain in contact with bacteria for 20 min at room temperature.

Agar-well diffusion method

The antimicrobial activity of the phenolic substances was assayed by the Agar-well diffusion method (Perez et al., 1990). Five equidistant holes were made in each Petri dish using sterile cork borers (Ø 7 mm). 0.05 ml of each salt at five different concentrations was added to each hole using a pipettor (Eppendorf). Negative controls were tryptone soy broth samples. The inoculated plates were incubated at 35°C for 24 h.

Measuring the inhibition zone diameter

At the end of the incubation period, inhibition zones which appeared on the medium Petri dishes were calculated in millimeters. Antibacterial activity was evaluated by measuring the diameter of inhibition zone of the tested bacteria expressed in millimeters. All tests were performed in triplicate.

Statistical analysis

All data were subjected to statistical analysis. The presented results are arithmetic means from three replicates. They were processed statistically with the Microsoft Excel spreadsheet, on the basis of tests (t-Student's test, ANOVA) that examine differences between two independent or dependent sample means. Results were presented as means ±standard deviations. All tests were carried out at the significance level of $\alpha = 0.05$.

RESULTS AND DISCUSSION

In the present study, the antimicrobial effects of nine salts of phenolic acids possessing different chemical structures were tested against a gram-negative bacteria *E. coli* O157:H7. Coumarates are present in spices and herbs in form of free acids, not as their salts. The reason why salts of coumaric acids were used for examination instead of pure coumaric acids was the fact that acids were unable to be solved in water and for examination in this research water solutions containing different contents of active substance were needed to check their antimicrobial acitivity. It is commonly known that *p*-coumaric acid predominantly appears in natural spices and herbs. On the other hand, salts of coumaric acids were soluble in water and they were used for the examination. The salts proved to possess a significantly different inhibitory activity towards the investigated strain. There was a significant variation in the antibacterial activities of nine phenolic compounds. The antimicrobial properties of the phenolic salts and their potential application in pathogen elimination were quantitatively estimated by the measurement of inhibition zone and zone diameter. The antimicrobial activities of salts of phenolic acids against *E. coli* O157:H7 are presented in Figures 1–3.

Figure 1 shows the antimicrobial activities of lithium, sodium and potassium *o*-coumarates on *E. coli* O157:H7 growth.

It can be said that the salts of *o*-coumaric acid show a relatively wide spectrum against the gram-negative bacteria. It was observed that lithium salt of *o*-coumaric acid was indicated to have the most inhibitory activity towards *E. coli* O157:H7 compared with sodium and potassium salts of *o*-coumaric acid (taking into account corresponding concentrations). The inhibition zone diameter for lithium salt of *o*-coumaric acid was measured as 23 mm at 5% concentration of an active substance, 21 mm at 4% concentration, 12 mm at 3%

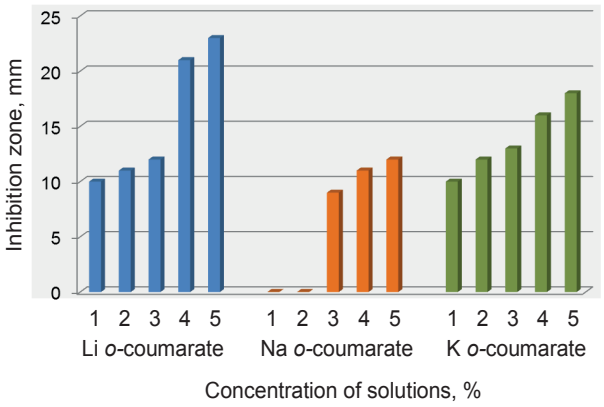

Fig. 1. Relationship between diameter of inhibition zone (mm) and active substance concentration (%) of lithium, sodium and potassium *o*-coumarates towards *E. coli* O157:H7

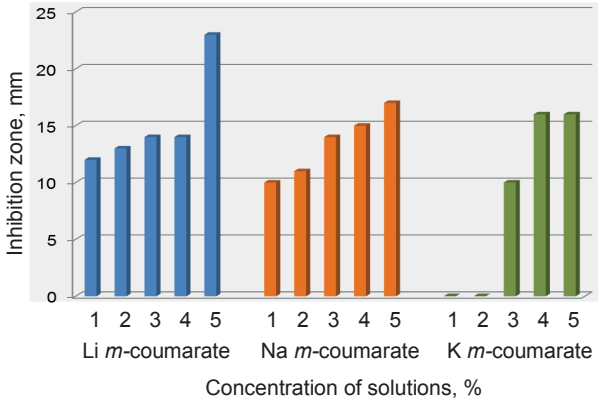

Fig. 2. Relationship between diameter of inhibition zone (mm) and the active substance concentration (%) of lithium, sodium and potassium *m*-coumarates towards *E. coli* O157:H7

concentration, 11 mm at 2% concentration and 10 mm at 5% concentration.

It was observed that potassium salt of *o*-coumaric acid had a lower influential effect on the growth inhibition of *E. coli* O157:H7 than lithium salt. The inhibition zone diameter for potassium salt of *o*-coumaric acid was measured as 18 mm at 5% concentration of an active substance, 16 mm at 4% concentration, 13 mm at 3% concentration, 12 mm at 2% concentration and 10 mm at 5% concentration.

Among the studied *o*-coumarates, sodium salt of *o*-coumaric acid has the lowest inhibitory activity towards *E. coli* O157:H7. The inhibition zone diameter was measured as 12 mm at 5% concentration of an active substance, 11 mm at 4% concentration and 9 mm at 3% concentration. 1 and 2% concentration of sodium salt of *o*-coumaric acid did not possess any inhibitory activity towards *E. coli* O157:H7. It can be concluded that 3% concentration of this salt is the lowest minimum inhibitory concentration.

Figure 2 shows the antimicrobial activities of lithium, sodium and potassium *m*-coumarates on *E. coli* O157:H7 growth.

It was indicated that the salts of *m*-coumaric acid showed also a relatively wide spectrum against the gram-negative bacteria. It was observed that lithium salt of *m*-coumaric at 5% concentration possessed the biggest inhibitory activity towards *E. coli* O157:H7 compared with sodium and potassium salts of *m*-coumaric acid. The inhibition zone diameter for this concentration amounted to 23 mm which is the same as for a 5% solution of lithium *o*-coumarate. The inhibition zone diameter of lithium salt of *m*-coumaric was measured as 14 mm at 4% concentration, 14 mm at 3% concentration, 13 mm at 2% concentration and 12 mm at 1% concentration.

It was observed that sodium salt of *m*-coumaric acid had a lower inhibitory effect on the growth inhibition of *E. coli* O157:H7 than lithium salt. The inhibition zone diameter for sodium salt of *m*-coumaric acid was measured as 17 mm at 5% concentration of an active substance, 15 mm at 4% concentration, 14 mm at 3% concentration, 11 mm at 2% concentration and 10 mm at 5% concentration.

Potassium salt of *m*-coumaric acid was indicated to have the lowest inhibitory activity towards *E. coli* O157:H7 compared with the other studied *m*-coumarates. The inhibition zone diameter was 16 mm at 5% concentration of an active substance, 15 mm at 4% concentration and 10 mm at 3% concentration. 1 and 2% concentration of potassium salt of *m*-coumaric acid did not possess any inhibitory activity towards *E. coli* O157:H7. It can be concluded that 3% concentration of this salt is the lowest minimum inhibitory concentration.

Figure 3 shows the antimicrobial activities of lithium, sodium and potassium *p*-coumarates on *E. coli* O157:H7 growth.

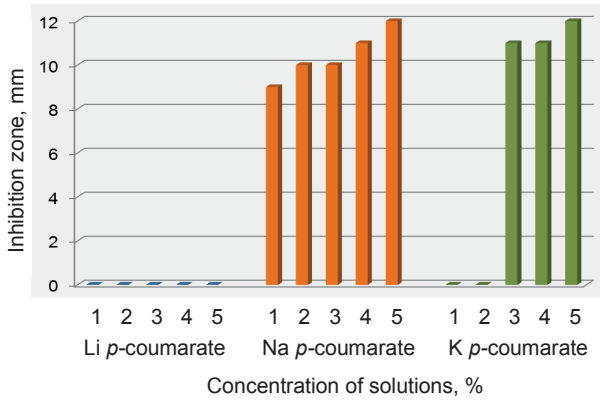

Fig. 3. Relationship between diameter of inhibition zone (mm) and the active substance concentration (%) of a lithium, sodium and potassium *p*-coumarates towards *E. coli* O157:H7

It was observed that sodium and potassium salts of *p*-coumaric at 5% concentration possessed the highest inhibitory effect towards *E. coli* O157:H7 compared with lithium compound. The inhibition zone diameter for this concentration raised to 12 mm. The inhibition zone diameter of sodium salt of *p*-coumaric was 11 mm at 4% concentration, 10 mm at 3% concentration, 10 mm at 2% concentration and 9 mm at 1% concentration.

The inhibition zone diameter for potassium salt of *p*-coumaric acid was 12 mm at 5% concentration of an active substance, 11 mm at 4% concentration and 11 mm at 3% concentration. The lowest concentrations (1 and 2%) of potassium salt of *p*-coumaric acid did not possess any inhibitory activity towards *E. coli* O157:H7. It can be concluded that 3% concentration of this salt is the lowest minimum inhibitory concentration. Lithium salt of *p*-coumaric acid did not exhibit any inhibitory activity against *E. coli* O157:H7.

The salts of *p*-coumaric acid showed relatively low antimicrobial activities against the gram-negative bacteria in comparison with the salts of *o*-coumaric acid and the salts of *m*-coumaric acid. There were many previous studies which have reported the antibacterial activity of phenolic compounds against foodborne pathogens (Cai et al., 2004; Shahidi and Chandrasekara, 2010). However, it is not easy to compare directly the results of various types of research to present reasonable relationships between antibacterial activity, the concentration of phenolic active compounds due to the fact that there is a significantly low number of phenolic salts tested, different determination methods and different bacterial strains applied (Ceylan and Fung, 2004; Terpine and Abramovic, 2010). The results of the carried out experiment showed the importance of phenolic compounds in the antibacterial activity of spice and herb extracts against foodborne pathogens (Hara-Kudo et al., 2004; Kim et al., 2007).

The highly positive correlations between the inhibitory activity and a total concentration of active phenolic substance content was observed. The present study indicates that the inhibitory growth activity stays in a close correlation with the concentration of phenolic compounds (Russell, 1991).

The inhibitory growth activities of various phenolic compounds can provoke different modes of action. It is known that some essential oils are able to degrade the cell wall, come into interaction with the composition and destroy cytoplasmic membrane (Sikkema et al., 1994; Helander et al., 1998; Ultee et al., 1999; Lambert et al., 2001). They can also ruin membrane protein, interfere with membrane integrated enzymes (Raccach, 1984), provoke leakage of cellular components, make cytoplasm coagulation, deplete the proton motive force, alter fatty acid and phospholipid constituents, weaken enzymatic mechanisms for energy production and metabolism, change nutrient absorption and electron transport (Taniguchi et al., 1988), have an effect on the synthesis of DNA and RNA and damage protein translocation, as well as the function of the mitochondrion in eukaryotes (Raccach, 1984, Nychas, 1995, Qingming et al., 2010).

All such mechanisms do not constitute separate aims. The mode of action of antimicrobial phenolic compounds is highly dependent on the kind of microorganisms and stays in a huge correlation with their cell wall structure and the outer membrane arrangement. It is commonly known that plants such as spices and herbs are rich in complex phenolics including phenolic acids, flavonoids, tannins, lignans, coumarins, quinines (Zaika, 1988; Nakatani, 1994). Due to their desirable antimicrobial activities they should constitute a subject of further investigation. The mechanisms

of action of each phenolic compound towards different microorganisms are very difficult to explain (Kalemba and Kunicka, 2003; Burt, 2004). Furthermore, there is a necessity to carry out further investigations in order to explain the mechanisms of inhibitory activity and chemical structure of each phenolic compound (Ceylan and Fung, 2004; Cushnie and Lamb, 2005; Madhujith and Shahidi, 2007).

Many studies were previously carried out on the antibacterial activity of phenolic acids present in plant extracts (Ultee et al., 1999; Lamber et al., 2001). However, it is relatively difficult to compare the results of various investigations to assess the relations between antibacterial activity of phenolic acids. The reason is that there was a relatively low number of phenolic acids tested against different pathogens and different determination methods were applied. However, the results of previous investigations indicated that phenolic acids and their salts are biologically active against foodborne pathogens (Nychas, 1995; Ceylan and Fung, 2004).

Also some past research investigated antimicrobial activities of different spice and herb extracts (Burt, 2004; Cushnie and Lamb, 2005; Mussatto et al., 2007). Its results proved the significance of phenolic compounds present in a variety of spice and herb extracts. The highly positive relationships can be observed between the antibacterial activity and antioxidant capacity of the extracts. It can be said that antibacterial activity stays in a close correlation with the concentration of phenolic compounds and the antioxidant capacity of the extracts (Cutler, 1995; Wood, 2007).

Other scientists have also proved that phenolic compounds coming from different plant sources might indicate different antimicrobial activity against various foodborne pathogens (Perreten et al., 1998). It can be said that polyphenols including tannins and flavonoids, constitute also very significant antibacterial substances. Others have checked that many plant flavonoids such as epigallocatechin, catechin, myricetin and quercetin possess antimicrobial activity. Very high biological activity is a characteristic feature of many spices containing aromatic phenolic compounds such as thymol and carvacrol present particularly in oregano and thyme, eugenol and cinnamon (Fahosh et al., 2007; Shan et al., 2005, 2007).

CONCLUSION

It can be concluded that salts of phenolic acids express different antimicrobial activity against the tested foodborne pathogens. Many further experiments should be carried out in order to develop the most successful methods of elimination of pathogens from food products. There is a significant correlation between antibacterial activity and the concentrations of active phenolic substance content. Such phenolic compounds possess an influential effect towards the foodborne pathogen elimination from food. They constitute a natural alternative for chemical preservatives in the process of guaranteeing health safety and prolonging shelf-life of food products. However, if they are applied in a relatively high proportion in food, they might deteriorate its organoleptic and sensory features. Thus, their dose should be adjusted in a way to successfully eliminate unwanted strains, as well as not to cause unwanted flavour of food products. Phenolic compounds seem to be potential alternatives for synthetic bactericides and natural antioxidants. They may be applied not only in food industry but also in the pharmaceutical industry in order to prevent or treat pathogenesis caused by microorganisms and free radicals.

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AKTYWNOŚĆ PRZECIWDROBNOUSTROJOWA SOLI LITU, SODU I POTASU KWASU ORTO-, META- I PARA-KUMAROWEGO WOBEC *ESCHERICHIA COLI* O157:H7

STRESZCZENIE

Wstęp. Celem pracy była ocena właściwości hamujących soli kwasów fenolowych wobec *Escherichia coli* O157: H7 ATCC 8739. *Escherichia coli* O157: H7 jest drobnoustrojem chorobotwórczym zdolnym do produkcji werotoksyn wywołujących biegunkę krwotoczną u ludzi. Dlatego zachodzi potrzeba opracowania naturalnych metod skutecznie eliminujących występowanie *E. coli* O157: H7 w żywności.

Materiał i metody. Przedmiotem badań były sole sodu, potasu i litu kwasu orto-, meta- i parakumarowego. Przygotowane zostały roztwory: 1-, 2-, 3-, 4- i 5-procentowy każdej z soli. Zastosowano metodę dyfuzyjno--studzienkową w podłożu agarowym. Płytki Petriego były inkubowane w temperaturze 35°C przez 24 h. Pod koniec okresu inkubacji strefy zahamowania wzrostu zostały wyrażone w milimetrach.

Wyniki. Najlepszymi właściwościami przeciwdrobnoustrojowymi wobec *E. coli* O157: H7 charakteryzowała się sól litu kwasu o-kumarowego, sól potasu kwasu o-kumarowego, sól litu kwasu m-kumarowego i sól sodu kwasu m-kumarowego, natomiast sól potasu kwasu m-kumarowego, sól sodu kwasu p-kumarowego wykazały nieco mniejszą skuteczność, a sól litu kwasu p-kumarowego nie miała właściwości przeciwdrobnoustrojowych.

Wnioski. Sole kwasów fenolowych o zróżnicowanej budowie strukturalnej wykazały istotnie różne właściwości przeciwdrobnoustrojowe wobec bakterii chorobotwórczej występującej w żywności. Fakt ten dowodzi, że kwasy fenolowe i ich sole mogą być potencjalną alternatywą chemicznego konserwowania żywności.

Słowa kluczowe: *Escherichia coli* O157: H7, aktywność przeciwdrobnoustrojowa, sole fenolowe

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