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Investigation of the antimicrobial effect of black cumin seed oil using Staphylococcus aureus strains

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1. Summary

As customer awareness grows, there is an increasing consumer demand for minimally processed food products containing reduced amounts of additives. In order to preserve the safety and stability of foodstuffs, the use of preservatives is necessary in most cases, but their amounts can be reduced or they can be replaced by using alternative preservatives. There are numerous research findings in the scientific literature that support the antimicrobial effect, oxidative stability and scavenger abilities of black cumin seed oil. These properties, combined with its beneficial physiological effects, make it particularly suitable for use as a natural preservative.

The goal of this work was to investigate the antimicrobial effect of black cumin seed oil in the case of *Staphylococcus aureus*, a pathogen relevant from a food safety point of view. In order to be able to map the mode of action more accurately, we worked with erythromycin, and to test the suitability of the oil in a combined preservation process, it was combined with organic acids.

2. Introduction

Food-borne illnesses affect about 3 million people annually in Hungary **[10]**, and so efforts to minimize food safety risks are of utmost importance **[15], [25]**.

The use of preservatives can offer a partial solution, however, there has been an increasing demand on the parts of consumers to reduce the amounts of additives in foodstuffs, because harmful health effects, sometimes legitimately, are attributed to them **[9]**. Using alternative substances with antimicrobial effects the amounts of preservatives added to foodstuffs can be reduced, without any additional adverse health effects.

The extract of the seeds of black cumin (*Nigella sativa*), a plant native to Southern Europe, North Africa and Southwest Asia, inhibits several isolates of bacterial origin, especially Gram-positive cocci, with a wide working spectrum **[1], [4]**. Its positive effect on the human body – mainly in the case of digestive, gynecological and respiratory diseases – has been proven by phytochemical studies **[3], [14], [31]**. Its blood glucose stabilizing, cholesterol-lowering, gastric mucosal regenerative and parasiticidal effects are also supported by numerous studies **[1], [2], [7], [8], [21], [28]**.

Figure 1 illustrates the main active components of black cumin seed oil, as determined by HPLC analysis: thymoquinone (TQ), dithymoquinone (DTQ), thymol (THY) and thymohydroquinone (THQ) **[23]**, of which TQ exhibits the highest biological activity **[14]**. Based on preliminary research, TQ could be one of the new antibacterial and multiresistance-modifying agents **[16]**. If its Efflux pump inhibiting effects can be confirmed by further tests, it can be helpful in the fight against the rapidly spreading multidrug resistance (MDR), posing an international problem **[22], [24], [26], [30]**.

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3. Materials and methods

3.1. Materials used

In our experiments, 100% pure black cumin seed oil was used, containing 9.6 mg TQ per ml, according to information from the manufacturer (Amazing Herbs Rt.). Extraction of the oil was carried out by cold pressing, therefore, it contained significant amounts of lipophilic components (e.g., antioxidants, TQ derivatives) **[19]**.

We worked with 10 *Staphylococcus aureus* strains, obtained from the National Collection of Agricultural and Industrial Microorganisms of Szent István University. Our choice was based on literature data, according to which *Staphylococcus aureus* appeared to be the most sensitive the effects of black cumin seed oil **[16], [27]**. Code numbers of the strains and information on their sources are summarized in ***Table 1.***

Experiments were carried out using nutrient agar: 28 g of powdered medium (composition: 1 g of beef extract, 5 g of peptone, 5 g of NaCl, 2 g of yeast extract, 15 g agar-agar) was dissolved in 1000 ml of distilled water, and then sterilized at 121 °C for 15 minutes.

3.2. Applied method

For the qualitative analysis of natural antimicrobial substances, the agar diffusion method is considered to be the most efficient procedure **[32]**, therefore, in the case of black cumin seed oil, the agar well diffusion method was used. A microbe suspension of 10^7 TKE/ml concentration was used, 1 cm^3 of which was pipetted onto a sterilized Petri dish. Plate pouring was performed with 15 cm^3 of 45 °C temperature medium. Into the wells of 10 mm diameter, drilled into the solidified agar plates, 100 µl of the component to be analyzed was pipetted. Samples were incubated for 24 hours at 37 °C, and at the end of the incubation period, diameters of the inhibition zones were determined.

4. Results

4.1. Minimum inhibitory concentration (MIC)

The minimum inhibitory concentration (MIC) value of black cumin seed oil for the *Staphylococcus aureus* strains was 50 mg/ml, equivalent to a TQ content of 0.48 µg/ml. Diameters of the inhibition zones are shown in ***Table 2***. Most resistant was the strain isolated from mammary gland inflammation (2033), while the most sensitive proved to be the strain used in the FDA's antibiotic sensitivity test (1755). It is important to note that the required microbe inhibition concentration in foods might be different from the 5% determined by the laboratory experiment, because the food matrix can influence the bacteriostatic or bactericidal effects through several interactions **[13].**

4.2. Combination with erythromycin

The combination with erythromycin, aimed at a more accurate understanding of the antimicrobial mode of action of black cumin seed oil, was preceded by the determination of the MIC value of the antibiotic. In the case of 8 of the 10 strains, values ranged between 3.5 and 7.0 µg/ml. The strain from human clinical samples, with a code number of 2174, and the strain isolated from pus, with a code number of 1186, proved to be resistant, therefore, in the case of these strains, the concentration of 7.0 µg/ml was used. The MIC values of erythromycin for the *Staphylococcus aureus* strains and the diameters of the inhibition zones that developed are summarized in ***Table 3.***

Results of the combination confirmed the additive effect found in earlier studies **[16]**, in which tetracycline was used, but the mode of action of the two antibiotics are similar: peptide chain elongation is inhibited by tetracycline in the 30S, and by erythromycin in the 50S ribosomal subunit **[5], [20]**. ***Table 4.*** illustrates that black cumin seed oil and erythromycin amplified each other's effects for most of the strains. In the case of strain no. 2174, resistant to erythromycin, there was a 100% increase in the inhibition zone, while in the case of the also resistant strain no. 1186, the increase was 50%. The efficacy of the combination is also supported by the nearly 50% increase in the inhibition zone of strain no. 2033, least sensitive to the effect of black cumin seed oil.

4.3. Combination with acetic acid and citric acid

Application of combinations with food acids was justified by the fact that the minimum inhibitory concentration of the oil is relatively high: 5%. At this concentration, it would probably reduce the enjoyment value of the foods (it has a characteristic peppery, slightly hot flavor) **[29]**, however, in a combined preservation process, together with another inhibitory factor, it could be effective at lower concentrations **[17]**. Results in ***Table 5*** clearly show that, in the 5 to 15 mg/ml concentration range, growth of the ten strains was inhibited to nearly the same extent by the food acids. As a result of the combinations, there was no significant increase in the inhibition zone in the case of most strains. For the acetic acid combinations, the diameters of the inhibition zones increased by 5 and 6 mm in the case of two strains (2399, 1314), while in the case of the citric acid combinations, a 5 mm increase in the inhibition zone was only observed for strain no. 2033, isolated from sheep mammary gland inflammation. The results obtained do not let us assume that the combinations could be effective.

5. Conclusions

Based on our experiments, combined preservation procedures are recommended as the main application possibility for black cumin seed oil, for example, together with substances of biological origin that are effective in low concentrations, such as nisin or pimaricin. In case of effective combinations, it could even be used to reduce the energy demand of gentle preservation procedures, such as high hydrostatic pressure treatment or modern irradiation methods.

Based on the results of studies aimed at understanding the mode of action, erythromycin resistant strains can be sensitized in the presence of black cumin seed oil. It is likely that, in the case of strains no. 2174 and 1186, due to the presence of TQ, the Efflux pump was unable to pump out erythromycin from the cell plasma, and so their resistance to erythromycin was eliminated. The increase in the sensitivity of the other strains can also be explained by the Efflux pump inhibiting effect of TQ: thanks to the lipophilic properties of the oil, it can penetrate the cell membrane itself, and can inhibit cell functions (e.g., ATP synthesis) **[11], [12], [18]**. In the combinations, erythromycin also entered the cells, blocking the growth of the *Staphylococcus aureus* strains by inhibiting protein synthesis. In this case, functioning of the cells was inhibited by the oil not only because of its hydrophobic properties, but also by helping erythromycin's entry into the cytoplasm, and so cells were subjected to a double stress effect. If further experiments succeed in supporting the Efflux pump inhibiting effect of black cumin seed oil, or the TQ extracted from it, it could provide real help in the fight against rapidly spreading multidrug resistant strains.

Combinations with food acids were not sufficiently effective, which could be explained by the fact that the antimicrobial effect of the acids is based mainly on the inhibition of membrane transport processes through decreasing the pH and eliminating the proton gradient **[6]**, and the Efflux pump inhibiting effect of TQ could not be exerted.

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