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In Vitro Examination of the Antimicrobial Activity of Phytosterol Ester Against Saprophytic and Pathogenic Microorganisms

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Running title: ***In Vitro*** **Examination of the Antimicrobial Activity of Phytosterol Ester Against Microorganisms**

Abstract
The intake of pytosterols, phytostanols and their esters has proven health beneficial effects on the human body – they lower the serum cholesterol levels and have potential in inhibiting cancers. The antimicrobial activity of the phytosterol ester against pathogenic and saprophytic microorganisms at different pH values was determined using the well-diffusion method. The phytosterol ester demonstrated antimicrobial activity against the pathogens *Staphylococcus aureus, Listeria monocytogenes* and *Escherichia coli* and against the saprophytes *Aspergillus niger*, *Aspergillus awamori*, *Fusarium moniliforme*, *Rhizopus* sp. and *Penicillium chrysogenum*, the highest antimicrobial activity against the saprophytes being in the pH range between pH=6,0 and pH=7,5. The obtained results opened up the possibilities for phytosterol ester application in the composition of different kinds of food with different pH values. Upon their intake the consumers would obtain the proven phytosterol ester health benefits and the addition of phytosterol ester would also serve as a biopreservative.

Practical applications
The intake of pytosterols, phytostanols and their esters has proven health beneficial effects on the human body – they lower the serum cholesterol levels and have potential in inhibiting cancers. The obtained results opened up the possibilities for phytosterol ester application in the composition of different kinds of food with different pH values. Upon their intake the consumers would obtain the proven phytosterol ester health benefits and the addition of phytosterol ester would also serve as a biopreservative.

Key words: phytosterol ester, antimicrobial activity, pathogenic, saprophytic

Introduction

Phytosterols are a group of naturally occurring sterol compounds found in plants with chemical structures close to those of cholesterol, the only difference being in their side-chain configuration or extra double bond (Baker et al, 1999). Phytostanol and phytosterol esters are obtained via esterification of plant stanols or sterols with fatty acids from common vegetable oils. Thus, the ester fatty acid composition is similar to the parent vegetable oil used as a source of the fatty acids. Phytosterol or phytostanol esterification modifies the physical properties from high-melting crystalline powders with low oil solubility into liquid or semi-liquid substances that can easily be included in the composition of a variety of (fat containing) foods (Phytosterols, phytostanols and their esters (CTA) 2008).

Although phytosterols have been used to treat hypercholesterolemia since the 1950s (Miettinen, 2001), it is only in the last few decades that they have found application as ingredients in the composition of functional foods. Phytosterols and phytostanols, in free or esterified form, are added to foods because of their properties to reduce cholesterol absorption in the gastro-intestinal tract and thereby lower blood cholesterol levels. Phytostanol- and phytosterol esters can be used as a fat replacer because the phytostanol/sterol moiety of the ester molecule does not provide any energy to the body (Phytosterols, phytostanols and their esters (CTA) 2008). Phytosterols have also shown potential in inhibiting cancers of the stomach, lung, ovaries and breasts (Woyengo et al. 2009). The European Food Safety Association (EFSA) and USA Food and Drug Administration (FDA) have accepted plant sterols as food ingredients (EFSA 2009, FDA 2009). Today, plant sterols and stanols have many applications as food additives and food ingredients (Ostlund et al. 2003). Due to the increased consumer awareness and the official recognition of their safety status, the number of dairy products containing plant sterols or stanols and their esters has increased considerably (Ozer and Kirmaci, 2010). Phytosterols are now being added to a number of commercially available foods, including fat-based spreads, salad dressing, yogurt, and cheese, to facilitate reduction of serum cholesterol levels, each type of food being characterized by its specific pH value (Kwak et al., 2005), turning these foods into functional foods. Both phytosterol and phytostanol esters give an enhanced creamy texture to low fat dairy products (yoghurt/drinking yoghurt). They might also improve food product flavor by masking bitterness and hence reduce the amount of sugar or other sweetener required to obtain a pleasant taste and mouthfeel (e.g. in soy drinks). Phytosterols, phytostanols and their esters are incorporated into a variety of foods and beverages and supplements, produced by a growing number of food- and beverage manufacturers (Phytosterols, phytostanols and their esters (CTA) 2008). Furthermore, the ester added to various food products show excellent stability at different pH values during long term storage (up to at least a year). Phytostanol and phytosterol esters are also stable in milk and fermented milk and products with viable bacteria like yoghurts and yoghurt drinks (Phytosterols, phytostanols and their esters (CTA) 2008). Phytosterols also have antimicrobial activity and provide an added benefit as a food preservative (Monu et al., 2008). For example, ß-sitosterol obtained from methanol extracts of a Brazilian plant, *Cissus sicyoides*, was shown to exhibit antimicrobial activity against *Bacillus subtilis* (Beltrame et al., 2002) in Mueller-Hinton broth. Moshi et al., 2004 also reported that purified dichloromethane extracts from the plant *Uvaria scheffleri* resulted in the inhibition of *Candida albicans* using an agar diffusion assay (Monu et al., 2008).

In order to apply phytosterol esters in the composition of various food products as biopreservatives, it is mandatory to examine their antimicrobial activity against saprophytic and pathogenic microorganisms.

The purpose of the present study was to examine the antimicrobial properties of phytosterol ester against saprophytic and pathogenic microorganisms and to evaluate its antimicrobial activity against saprophytic microorganisms at different pH values.

Materials and methods

Test-microorganisms

Pathogenic microorganisms – *Proteus vulgaris* G, *Candida albicans* NBIMCC 74, *Listeria monocytogenes* 1, *Listeria monocytogenes* 2, *Staphylococcus aureus* Al, *Staphylococcus aureus* 25923, *Salmonella abony* NTCC 6017, *Salmonella enteritidis*, *Escherichia coli* ATCC 25922, *Escherichia coli* ATCC 8739, *Pseudomonas aeruginosa* NBIMCC 1390.

Saprophytic microorganisms – *Aspergillus niger*, *Aspergillus awamori*, *Fusarium moniliforme*, *Rhizopus* sp., *Bacillus subtilis*, *Bacillus cereus*.

All strains have been deposited in the culture collection of the Department of "Microbiology" at UFT, Plovdiv.

Media

LBG – agar. Composition (g/dm^3): tryptone - 10; yeast extract - 5; NaCl - 10; glucose – 10; agar - 15; pH = 7.5. Sterilization - 20 min at 121°C.

For the in vitro examination of the antimicrobial activity of the phytosterol ester against saprophytic microorganisms at different pH values of the medium (LBG-agar), the pH of the medium was adjusted using 8M HCl and 10M NaOH solutions.

Phytosterol ester. The used phytosterol ester was Phytosterol ester (Soybean) by HSFBiotech®.

Preparation of the suspensions of the test pathogenic microorganisms

The test pathogenic microorganisms were cultured on LBG-agar at 37±1°C for 24-48 hours. Using sterile loop biomass of the developed test pathogenic microorganisms was suspended in sterile saline solution in order to obtain suspensions of the test pathogenic microorganisms. The concentrations of the suspensions (cfu/cm^3) were determined by preparing appropriate tenfold dilutions and spread plating on LBG-agar. The inoculated Petri dishes were incubated at 37±1°C until the formation of countable single colonies.

Preparation of the suspensions of the test saprophytic microorganisms

The test saprophytic microorganisms were cultured on LBG-agar until spore formation. 5 cm^3 of saline solution were pipetted in the tubes with the sporulated saprophytic microorganisms in order to obtain suspensions of the saprophytes with concentration of 10^7cfu/cm^3. The concentration of the suspension of *Bacillus cereus* was determined by preparing appropriate tenfold dilutions and spread plating on LBG-agar and the inoculated Petri dishes were incubated at 37±1°C until the formation of countable single colonies. The concentrations of the other 4 test saprophytic microorganisms were determined using counting chamber.

Determination of the antimicrobial activity of phytosterol ester against saprophytic and pathogenic microorganisms – well-diffusion method

Melted and cooled to 40°C LBG-agar medium was inoculated with 1% spore suspensions of the test saprophytic microorganisms – *Aspergillus niger*, *Aspergillus awamori*, *Penicillium* sp. and *Rhizopus* sp. (concentration of the suspensions – 10^7cfu/cm^3). The final concentration of saprophytic microorganisms in the LBG-agar medium was 10^5cfu/cm^3. The inoculated medium was poured in Petri dishes (20cm^3 in each Petri dish) and allowed to solidify.

Sterile melted LBG-agar medium was poured in Petri dishes and after the solidification of the agar, the dishes were spread plated with suspensions of the pathogenic microorganisms (*Proteus vulgaris* G, *Candida albicans* NBIMCC 74, *Listeria monocytogenes* 1, *Listeria monocytogenes* 2, *Staphylococcus aureus* Al, *Staphylococcus aureus* 25923, *Salmonella abony* NTCC 6017, *Salmonella enteritidis*, *Escherichia coli* ATCC 25922, *Escherichia coli* ATCC 8739, *Pseudomonas aeruginosa* NBIMCC 1390.) and of *Bacillus cereus* and *Bacillus subtilis*.

Three solutions of the phytosterol ester in distilled water were prepared with concentrations 0,26%; 0,36% and 0,72%, respectively. The solutions were prepared using maltodextrin and carrageenan because the phytosterol ester is not soluble in water.

In the inoculated with the test microorganisms Petri dishes wells (d=6mm) were prepared and 60µl of the corresponding phytosterol ester solution with the corresponding concentration were pipetted in each well. The results were recorded as diameters of the inhibition zones, in millimeters, after 24 – 48 hours of incubation of the Petri dishes at optimal temperature for the growth of the corresponding test-microorganism (at 30°C for the saprophytic microorganisms and at 37°C for the pathogenic microorganisms).

The experiments were performed in quadruplicate. Using MS Office Excel 2010 the mean values and the standard deviations were calculated.

Results and discussion

Antimicrobial activity of phytosterol ester against pathogenic microorganisms

The growing interest towards inclusion of phytosterol esters in the composition of various foods is due to its inherent health beneficial effects. In a series of experiment was determined the antimicrobial activity of the phytosterol ester against pathogens causing foodborne diseases as well as saprophytes causing food spoilage. The results of the study on the inhibitory effect of the phytosterol ester against some of the most common

gastro-intestinal pathogenic microorganisms are summarized in Table 1.

No inhibition of the growth of the pathogens *Staphylococcus aureus* Al, *Salmonella abony* NTCC 6017, *Salmonella enteritidis*, *Escherichia coli* ATCC 8739, *Pseudomonas aeruginosa* NBIMCC 1390, *Candida albicans* 74 by any of the phytosterol ester concentrations included in the study was observed. Experimental data indicate that the phytosterol ester influenced the growth of *Staphylococcus aureus* ATCC 25093, *Escherichia coli* ATCC 25922 and the two strains of *Listeria monocytogenes* included in the present study (Table 1). The growth inhibition was strain-specific, not species-specific. This demonstrated the mandatory inclusion of more than one strain of a given species in every study on antimicrobial activity in order to obtain quality conclusions.

The phytosterol ester did not suppress the growth of the representatives of the genera *Salmonella* sp., *Pseudomonas* sp., *Proteus* sp. and *Candida albicans*. To summarize, the phytosterol ester had very weak influence on the growth of pathogenic bacteria and yeasts (Table 1).

Antimicrobial activity of phytosterol ester against saprophytic microorganisms

The inhibitory activity of the phytosterol ester against saprophytic microorganisms increased with increasing the concentration of the phytosterol ester up to 0.36%. Higher concentrations did not influence significantly the growth of fungi of the genera *Aspergillus*, *Penicillium* and *Fusarium*. The phytosterol ester had virtually no effect on the growth of the spore-forming bacteria of the genus *Bacillus*. No inhibition of the growth of the saprophytic microorganisms *Bacillus subtilis* and *Bacillus cereus* by any of the phytosterol ester concentrations included in the study was observed (Table 2).

Each type of food has a certain pH value and shelf life. Thus, the antimicrobial activity of the phytosterol ester against saprophytes at different pH values was examined. The antimicrobial activity was determined in the pH range from 5.0 to 8,0 at different concentrations of the phytosterol ester (0.18%; 0.26%; 0.36% and 0.72%). The results of these experiments are shown in Table 3 to Table 6.

Experimental data in Table 3 and Table 4 for fungi of the genus *Aspergillus* (*Aspergillus niger* and *Aspergillus awamori*) indicate that high inhibitory effect was achieved at a concentration of the phytosterol ester of 0.26%. Higher phytosterol ester concentrations did not significantly affect the growth of *Aspergillus* sp. The optimum pH values of the medium (food) for the manifestation of the antimicrobial activity against *Aspergillus awamori* was pH = 6,5, and against *Aspergillus niger* it was pH = 7,0 - 7,5.

Similar results on the influence of the pH of the medium (food) at various concentrations of the phytosterol ester were obtained in the examination of the antimicrobial activity of the different concentrations of the phytosterol ester against *Penicillium chrysogenum* (Table 5), the highest activity being determined at pH = 6,5.

All three fungal species used as saprophytic test-microorganisms so far reproduce by conidia spores. Although each has its own sporulation apparatus, the spores of all three species are exospores. Probably this is the reason for the high antimicrobial activity of the phytosterol ester at a concentration of 0.72% in the medium.

A different trend was observed in the examination of the influence of different concentrations of the phytosterol ester at different pH values on the growth of *Fusarium moniliforme* (Table 6). The highest antimicrobial activity was determined at pH = 6,0 and pH = 7,5. It is noteworthy that high inhibitory activity was defined at the lower concentrations of the phytosterol ester (0,18% and 0,26%) and it was significantly influenced by the pH of the medium (Table 6). The antimicrobial activity of the higher concentrations of the phytosterol ester (0.36% and 0.72%) was low, regardless of the pH of the medium. This effect is probably related to the way of multicellular spore forming in the genus *Fusarium* and therefore at this fungal representative was determined the highest antimicrobial activity ($d_{\text{inhibition zone}} = 21,83 \pm 0,29$ mm).

In the research of Kavita et al., 2014 sterol-containing plant extracts have demonstrated antimicrobial activity against *Escherichia coli* ATCC 8739, *Staphylococcus aureus* ATCC 25923, *Bacillus subtilis* ATCC 6051 and

Pseudomonas aeruginosa ATCC 9027. The researchers concluded that Gram-positive bacteria were more susceptible than Gram-negative bacteria which complies with the results in the present paper.

Conclusion

In addition to its health beneficial properties the phytosterol ester exhibited pronounced antimicrobial activity against saprophytic fungi. The antimicrobial activity depended on the pH of the medium. The phytosterol ester exercised the highest inhibitory effect on fungal growth at pH = 6,0 - 7,5. The obtained results are of particular importance for the application of the phytosterol ester in the composition of different kinds of food aiming at both obtaining its proven health benefits and suppressing saprophytic fungal growth.

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Table 1. Antimicrobial activity of the phytosterol ester against pathogenic microorganisms

	Inibition zone, d_{mm} Pathogenic test-microrganism, (10^5 cfu/cm^3)			
	***Staphylococcus aureus* ATCC 25923**	***Escherichia coli* ATCC 25922**	***Listeria monocytogenes* 1**	***Listeria monocytogenes* 2**
0,18% PSE	9,33±0,58	10,33±0,58	9,67±0,58	not found
0,26% PSE	9,33±0,58	10,33±0,58	12,00±0,00	not found
0,36% PSE	9,33±0,58	10,33±0,58	12,33±0,58	9,67±0,58
0,72% PSE	9,67±0,58	10,33±0,58	12,33±0,58	11,33±0,58

**data are given as mean value ± standard deviation (n=4)*

Table 2. Antimicrobial activity of the phytosterol ester against saprophytic microorganisms

	Inibition zone, d_{mm} Saprophytic test-microorganism				
	***Aspergillus niger*, 3x10^5cfu/cm^3**	***Aspergillus awamori*, 1,2x10^5cfu/cm^3**	***Fusarium moniliforme*, 1,12x10^5cfu/cm^3**	***Rhizopus* sp., 2,3x10^5cfu/cm^3**	***Penicillium chrysogenum* 4x10^5 cfu/cm^3**
0,18% PSE	9,33±0,58	9,67±0,58	not found	10,33±0,58	12,33±0,58
0,26% PSE	11,33±0,58	14,00±1,00	19,67±0,58	11,00±1,00	13,00±0,50
0,36% PSE	11,63±0,58	14,33±0,58	15,33±0,58	11,67±0,58	15,33±0,58
0,72% PSE	11,63±0,58	14,00±0,00	15,67±0,58	12,33±0,58	16,67±0,58

**data are given as mean value ± standard deviation (n=4)*

Table 3. Antimicrobial activity of the phytosterol ester against *Aspergillus awamori* at different pH values

Aspergillus awamori, N = $5x10^5$cfu/cm^3 **Inibition zone, d_{mm}**				
48 h	0,18% PSE	0,26% PSE	0,36% PSE	0,72% PSE
pH = 5,0	13,67±0,58	16,33±0,58	17,67±0,58	18,00±0,00
pH = 5,5	12,33±0,58	14,33±0,58	18,00±0,00	19,67±0,58
pH = 6,0	13,33±0,58	15,00±0,00	15,00±0,00	17,67±0,58
pH = 6,5	15,33±0,58	17,67±0,58	18,00±0,00	18,00±0,00
pH = 7,0	12,67±0,58	15,33±0,58	17,00±0,00	18,67±0,58
pH = 7,5	12,67±0,58	15,00±0,00	15,33±0,58	15,33±0,58
pH = 8,0	15,00±0,00	15,33±0,58	15,33±0,58	17,67±0,58

**data are given as mean value ± standard deviation (n=4)*

Table 4. Antimicrobial activity of the phytosterol ester against *Aspergillus niger* at different pH values

Aspergillus niger, N = $5x10^5$cfu/cm^3 Inhibition zone, d_{mm}				
	0,18% PSE	0,26% PSE	0,36% PSE	0,72% PSE
pH = 5,0	10,00±0,50	10,33±0,58	10,33±0,58	11,00±0,00
pH = 5,5	10,33±0,58	14,83±0,29	11,67±0,58	11,00±0,50
pH = 6,0	13,00±0,50	14,83±0,76	11,67±0,58	11,33±0,58
pH = 6,5	14,50±0,50	16,67±0,58	11,00±0,00	11,00±0,50
pH = 7,0	15,33±0,58	17,50±0,50	14,67±0,58	14,33±0,58
pH = 7,5	15,67±0,58	17,67±0,58	14,50±0,50	14,67±0,58
pH = 8,0	15,00±0,50	17,00±1,00	13,00±0,50	14,67±0,58

**data are given as mean value ± standard deviation (n=4)*

Table 5. Antimicrobial activity of the phytosterol ester against *Penicillium chrysogenum* at different pH values

Penicillium chrysogenum, N = $5x10^5$cfu/cm^3 Inhibition zone, d_{mm}				
48 h	0,18% PSE	0,26% PSE	0,36% PSE	0,72% PSE
pH = 5,0	10,33±0,58	12,67±0,58	12,67±0,58	13,33±0,58
pH = 5,5	11,33±0,58	13,33±0,58	14,50±0,50	14,83±0,29
pH = 6,0	12,33±0,58	14,83±0,29	16,67±0,58	17,33±0,58
pH = 6,5	12,67±0,58	16,67±0,58	16,83±0,29	17,33±0,58
pH = 7,0	10,33±0,58	12,00±0,50	12,67±0,58	13,33±0,58
pH = 7,5	12,33±0,58	13,00±0,50	15,33±0,58	16,67±0,58
pH = 8,0	10,33±0,58	12,50±0,50	13,67±0,58	14,83±0,29

**data are given as mean value ± standard deviation (n=4)*

Table 6. Antimicrobial activity of the phytosterol ester against *Fusarium moniliforme* at different pH values

Fusarium moniliforme, N = $8x10^5$cfu/cm^3; Inhibition zone, d_{mm}				
	0,18% PSE	0,26% PSE	0,36% PSE	0,72% PSE
pH = 5,0	17,33±0,58	18,50±0,50	16,67±0,58	10,17±0,29
pH = 5,5	13,17±0,29	16,67±0,58	15,67±1,15	14,33±0,58
pH = 6,0	20,00±1,00	19,67±0,58	17,17±0,29	12,33±0,58
pH = 6,5	14,50±0,50	14,17±0,76	11,00±1,00	10,17±0,29
pH = 7,0	17,17±0,29	19,50±0,50	14,67±1,15	11,50±0,50
pH = 7,5	20,00±0,00	21,83±0,29	15,17±0,29	12,67±0,58
pH = 8,0	11,00±1,00	17,67±0,58	17,17±0,29	12,67±0,58

**data are given as mean value ± standard deviation (n=4)*