Antimikrobni učinak etanolnog ekstrakta sjemenki i pulpe ploda grejpa

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Antimicrobial activity of grapefruit seed and pulp ethanolic extract

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Antibacterial and antifungal activity of ethanolic extract of grapefruit (*Citrus paradisi* Macf., *Rutaceae*) seed and pulp was examined against 20 bacterial and 10 yeast strains. The level of antimicrobial effects was established using an *in vitro* agar assay and standard broth dilution susceptibility test. The contents of 3.92% of total polyphenols and 0.11% of flavonoids were determined spectrometrically in crude ethanolic extract. The presence of flavanones naringin and hesperidin in the extract was confirmed by TLC analysis. Ethanolic extract exibited the strongest antimicrobial effect against *Salmonella enteritidis* (*MIC* 2.06%, *m/V*). Other tested bacteria and yeasts were sensitive to extract concentrations ranging from 4.13% to 16.50% (*m/V*).

Keywords: *Citrus paradisi* (*Rutaceae*), grapefruit seed and pulp extract, antimicrobial activity

»Grapefruit Seed Extract« (GSE) is a commercial product derived from the seeds and pulp of grapefruit (*Citrus paradisi* Macf., *Rutaceae*). Chemical research revealed the presence of flavonoids (1, 2), ascorbic acid, tocopherols, citric acid (3), limonoids (4–6), sterols and minerals (7) in grapefruit seeds and pulp. GSE is commonly reported to have a powerful antimicrobial activity. However, only a few scientific reports of antibacterial and antifungal *in vitro* effects could be found in the literature. These studies mostly dealt with the preservation of vegetables and fruits (8), peanuts (9, 10), beef (11) and chicken meat (12). An *in vivo* study is related to the GSE activity on the intestianl micro-flora of patients suffering from atopic eczema (13). It has been shown to help inhibit the proliferation of *Candida*, a yeast that can impinge upon probiotic bacteria and affect the gastrointestinal tract health. The antibacterial efficacy, mechanism of action and *in vitro* toxicity of a commercial GSE were investigated recently (14, 15). It has been found that the extract disrupts the bacterial membrane and liberates the cytoplasmic contents within 15 minutes. The latest *in vitro* investigation showed that the commercial 33% grapefruit-

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water glycerol solution exerted potent antifungal activity against the yeast-like fungi strains and lower activity against dermatophytes and molds (16).

GSE products, commonly 33% water-glycerol solutions, are widely used as naturopathic remedies, natural foodstuff supplements, disinfectant and sanitizing agents as well as preservatives in food and cosmetic industry. However, some of commercially available products are not fully natural. Scientific studies showed that the composition of self-made extracts of grapefruit seeds was quite different from that of some commercial extracts. Artificial agents, such as benzethonium chloride, triclosan and methyl parabene, were identified in commercially available products (17, 18). Preservatives were detected in all the antimicrobially active extracts. Researchers have found that products not containing any preservatives and several self-made preparations failed to show antimicrobial efficacy and concluded that antimicrobial activity being attributed to GSE is merely due to the synthetic preservative agents it contains (19). Therefore, GSE has become a subject of contraversy. The present study contributes to the identification of the antibacterial and antifungal effects of the self-made ethanolic extract of grapefruit seeds and pulp.

EXPERIMENTAL

Samples

Self-made ethanolic extract of *Citrus paradisi* Mecf. (*Rutaceae*) was prepared from commercially available grapefruits. Air dried powdered plant material (juiceless pulp and seeds, in quantitative ratio 4:1) was extracted with 70% ethanol in a Soxhlet apparatus for 6 h. After cooling, the solvent was removed using rotary evaporator and dry residue was chemically analysed. For microbiological test, 33% (*m/V*) extract was prepared using 70% ethanol.

Phytochemical analysis

TLC analysis was carried out on Kieselgel 60 F_{254} (precoated 0.2 mm thick plastic plates, Merck, Germany) using the mobile phase ethyl acetate/formic acid/acetic acid/water (100:11:11:27, *V/V*). Visualisation of flavonoids and phenolic acids was achieved by spraying the sheet with 1% methanolic solution of diphenylboric acid aminoethyl ester followed by 5% ethanolic solution of polyethylene glycol 4000. The chromatogram was evaluated under UV light at 365 nm (20). For reference purposes, naringin and hesperidin (Roth, Germany) were used.

The content of total polyphenols in the crude ethanolic extract was determined by the method of Schneider (21) involving Folin-Chiocalteu reagent and tannic acid as standard. The analyses were carried out at 720 nm. Determination of the flavonoid fraction in the crude ethanolic extract was performed according to the *European Pharmacopoeia* (22). After acid hydrolysis, the formed flavonoid aglycones were spectrophotometrically determined at 425 nm by creating a complex with $AlCl_3$. The content of flavonoids in the extract was calculated as hyperoside. The measurements were carried out using a Helios Gamma & Delta UV-Visible spectrophotometer (Spectronic Unicam, UK)

Microbiological tests

Ten Gram-positive (*Bacillus cereus* ATCC 11778, *B. subtilis* NCTC 8236, *Sarcina flava* MFBF*, *S. lutea* ATCC 9341, *Staphylococcus aureus* ATCC 6538P, *S. aureus* ATCC 29213, *S. epidermidis* MFBF*, *Enterococcus faecalis* ATCC 20201, *Streptococcus* sp. MFBF*, *Listeria monocytogenes* MFBF*) and ten Gram-negative (*Escherichia coli* O:157 MFBF*, *E. coli* O:128 MFBF*, *Shigella sonnei* MFBF*, *Salmonella enteritidis* MFBF*, *Yersinia enterocolitica* O:9 MFBF*, *Citrobacter freundii* MFBF*, *Klebsiella oxytoca* MFBF*, *Proteus mirabilis* MFBF*, *P. vulgaris* MFBF*, *Pseudomonas aeruginosa* ATCC 27895) bacteria strains, as well as ten yeast strains (*Candida albicans* MFBF* 1, C. *albicans* MFBF* 2, C. *albicans* MFBF* 3, *C. krusei* MFBF*, C. *krusei* MFBF* K1, C. *tropicalis* MFBF, C. *tropicalis* MFBF* T1, *C. parapsilosis* MFBF*, *Saccharomyces cerevisiae* MFBF* V1, *Kluyveromyces maxianus* MFBF CC4) were tested.

Antimicrobial activity testing was based on the agar diffusion method and standard serial broth dilution assay. The agar diffusion method was performed according to the *European Pharmacopoeia* (22). Testing inoculum with 10^4–10^5 cells (0.5 mL portion) was swabbed on solidified Müeller Hinton agar (Merck) for bacteria and on Sabouraud dextrosa agar for yeasts. Metal cylinders 8 × 6 × 10 mm in diameter were then placed on the agar. Twenty-five and fifty microlitres of test solutions were applied. The same volume of 70% ethanol was also tested as control. After a 2 h period of diffusion at 4 °C, the agar plates were incubated for 18 h at either 37 °C for bacteria, or 25 °C for yeasts. The diameters of the clear growth inhibition zones around the cylinder were measured.

Minimal inhibitory concentration (*MIC*) values were evaluated by the dilution susceptibility test (23). Test strains were grown in a nutrition medium containing progressively lower dilutions of the test extract and incubated at 37 °C for bacteria or 25 °C for yeasts. Last two tubes were free of test extract and served as a growth control in broth and 70% ethanol. A sample was deemed free of viable germs if the nutrient solution appeared clear on visual inspection after 18 h. The lowest concentration of the test extract (*m/V*) preventing appearance of turbidity was considered to be *MIC*. All samples showing no turbidity were subcultured into Müeller Hinton or Sabouraud agar. The lowest extract concentration from which the microorganisms did not recover and grow when transferred to fresh medium was the minimal microbicidal concentration (*MMcC*).

RESULTS AND DISCUSSION

The results of spectrometric determination showed that the crude ethanolic extract of grapefruit seeds and pulp contained 3.92 ± 0.40% total polyphenols ($\bar{x}$ ± SD, n = 3). The content of flavonoids was 0.110.02% ($\bar{x}$ ± SD, n = 3). The presence of flavanones naringin and hesperidin in the ethanolic extract was confirmed by the TLC method.

Self-made 33% (*m/V*) ethanolic extract of grapefruit seeds and pulp (GSE) was screened for antimicrobial activity against 20 bacterial strains and 10 yeast strains by the

* Collection of microorganisms of the Institute of Microbiology, Faculty of Pharmacy and Biochemistry, University of Zagreb, Croatia

agar diffusion method (Table I). 70% ethanol as the control did not show any zones of inhibition. The investigated extract was active against all Gram positive bacteria, but exerted no inhibiting effect on the growth of the tested Gram negative bacteria. GSE exibited the largest zones of inhibition for *Listeria monocytogenes* (16 mm), *Streptococcus faecalis* (15 mm) and *Bacillus subtilis* (14 mm). Ethanolic extract showed lower activity

Table I. Inhibition of bacterial and yeast growth by GSP

Microorganisms	Inhibition zone (mm)	*MIC* (%, *m/V*)
Gram positive bacteria		
Bacillus cereus ATCC 11778	12	8.25
Bacillus subtilis NCTC 8236	14	8.25
Sarcina flava MFBF	12	8.25
Sarcina lutea ATCC 9341	11	8.25
Staphylococcus aureus ATCC 6538P	10	8.25
Staphylococcus aureus ATCC 29213	12	8.25
Staphylococcus epidermidis MFBF	10	8.25
Streptococcus faecalis ATCC 20201	15	4.13
Streptococcus sp. MFBF	12	4.13
Listeria monocytogenes MFBF	16	4.13
Gram negative bacteria		
Escherichia coli O:157 MFBF	–	4.13
Escherichia coli O:128 MFBF	–	4.13
Shigella sonnei MFBF	–	8.25
Salmonella enteritidis MFBF	–	2.06
Yersinia enterocolitica O:9 MFBF	–	8.25
Citrobacter freundi MFBF	–	16.50
Klebsiella oxytoca MFBF	–	8.25
Proteus mirabilis MFBF	–	16.50
Proteus vulgaris MFBF	–	16.50
Pseudomonas aeruginosa ATCC 27895	–	8.25
Yeasts		
Candida albicans MFBF 1	9	16.50
Candida albicans MFBF 2	10	8.25
Candida albicans MFBF 3	11	8.25
Candida krusei MFBF	12	8.25
Candida krusei MFBFK1	12	8.25
Candida tropicalis MFBF	13	16.50
Candida tropicalis MFBF T1	12	16.50
Candida parapsilosis MFBF	10	16.50
Saccharomyces cerevisiae MFBF V1	13	8.25
Kluyveromyces maxianus MFBF CC4	13	16.50

ATCC – American Type Culture Collection
NCTC – National Collection of Type Cultures
MFBF – Collection of microorganisms of the Institute of Microbiology, Faculty of Pharmacy and Biochemistry, University of Zagreb, Croatia

Table II. Antimicrobial efficacy of GSE

Microorganisms	GSE concentration (%, *m/V*)				
Bacteria	16.50	8.25	4.13	2.06	1.03
Bacillus cereus ATCC 11778	–	±	+	+	+
Bacillus subtilis NCTC 8236	–	±	+	+	+
Sarcina flava MFBF	–	±	+	+	+
Sarcina lutea ATCC 9341	–	–	+	+	+
Staphylococcus aureus ATCC 6538P	–	–	+	+	+
Staphylococcus aureus ATCC 29213	–	–	+	+	+
Staphylococcus epidermidis MFBF	–	–	+	+	+
Streptococcus faecalis ATCC 20201	–	–	–	+	+
Streptococcus sp. MFBF	–	–	–	+	+
Listeria monocytogenes MFBF	–	–	–	+	+
Escherichia coli O:157 MFBF	–	±	±	+	+
Escherichia coli O:128 MFBF	–	–	±	+	+
Shigella sonnei MFBF	–	–	+	+	+
Salmonella enteritidis MFBF	–	–	–	–	+
Yersinia enterocolitica O:9 MFBF	–	–	+	+	+
Citrobacter freundi MFBF	–	+	+	+	+
Klebsiella oxytoca MFBF	–	–	+	+	+
Proteus mirabilis MFBF	–	+	+	+	+
Proteus vulgaris MFBF	–	+	+	+	+
Pseudomonas aeruginosa ATCC 27895	–	–	+	+	+
Yeasts					
Candida albicans MFBF 1	–	+	+	+	+
Candida albicans MFBF 2	–	–	+	+	+
Candida albicans MFBF 3	–	–	+	+	+
Candida. krusei MFBF	–	–	+	+	+
Candida krusei MFBF K1	–	–	+	+	+
Candida tropicalis MFBF	–	+	+	+	+
Candida tropicalis MFBF T1	–	+	+	+	+
Candida parapsilosis MFBF	–	+	+	+	+
Saccharomyces cerevisiae MFBF V1	–	–	+	+	+
Kluyveromyces maxianus MFBF CC4	–	+	+	+	+

– no growth of microorganism (bactericidal/fungicidal activity of GSE)
± slight growth of microorganism (bacteristatic activity of GSE)
+ normal growth of microorganism (no activity of GSE)
For other symbols see Table I.

(inhibition zone of 10–12 mm) against *Bacillus cereus*, *Sarcina flava*, *S. lutea*, *Staphylococcus aureus*, *S. epidermidis* and *Streptococcus* sp. MFBF. The data also showed that GSE inhibited the growth of all the tested yeasts. An inhibition zone of 13 mm was observed for *Saccharomyces cerevisiae*, *Kluyveromyces maxianus* and *Candida tropicalis* MFBF. The other tested strain of *Candida tropicalis* and two strains of *C. krusei* showed a zone of 12 mm.

The lowest antifungal activity of GSE (inhibition zones ranging from 9 mm to 11 mm) was observed against the tested strains of *Candida albicans*.

Table I also presents the results of the broth dilution susceptibility test, in which the GSE to be tested (33%, *m/V*) was serially diluted up to 0.06% (*m/V*). In nutrient broth, GSE was effective against all the tested microorganisms, even Gram negative bacteria. Tested bacteria were sensitive to extract concentrations ranging from 2.06% to 16.50% (*m/V*). The strongest effect of the extract (*MIC* 2.06%, *m/V*) was observed against *Salmonella enteritidis*. The growth of *Listeria monocytogenes*, *Streptococcus* strains and *Escherichia coli* was inhibited by 4.13% (*m/V*) GSE. *Bacillus cereus*, *B. subtilis*, *Sarcina flava*, *S. lutea*, *Staphylococcus aureus*, *S. epidermidis*, *Shigella sonnei*, *Yersinia enterocolitica*, *Klebsiella oxytoca* and *Pseudomonas aeruginosa* were sensitive to a higher extract concentration (8.25%, *m/V*). The highest *MIC* value 16.50% (*m/V*) was estimated for *Citrobacter freundi*, Proteus *mirabilis* and *P. vulgaris*. GSE exibited antifungal activity against all tested yeasts in concentrations from 8.25% to 16.50% (*m/V*). *Saccharomyces serevisiae*, *Candida krusei* and two strains of *C. albicans* showed higher sensitivity than the other tested yeasts.

Table II also represents the results of the broth dilution susceptibility test. As it can be seen, the extract concentration of 16.50% (*m/V*) was bactericidal/fungicidal for all the tested bacteria and yeasts. Previously determined minimal inhibitory concentrations of GSE (Table I) were found to be microbicidal for a large part of tested microorganisms, with the exception of *Bacillus cereus*, *B. subtilis*, *Sarcina flava* and *Escherichia coli*. In those cases, ethanolic extract exerted bacteristatic activity.

Our results showed clear differences between the antimicrobial effects of self-made GSE and some commercially available preparations reported previously (14–16). Despite the fact that some commercial extracts were found to be superior to the tested self-made ethanolic extract, the latter still showed slight, but constant activity against all the tested bacteria and yeasts.

CONCLUSIONS

Results reported here contribute to the knowledge of the antimicrobial efficacy of GSE. It has been established that the fully natural ethanolic extract of grapefruit seeds and pulp affects the tested bacteria and yeasts remarkably, but exerts less antimicrobial efficacy compared to some commercial preparations reported in the literature. These differences may be partly caused by the differences in the contents of polyphenols, especially flavonoids. This allows the conclusion that antibacterial and antifungal properties of commercially available products should not necessarily be the consequence only of the presence of synthetic preservative agents, as some authors claim. Since there is not enough scientific evidence to support the medical use of GSE, further phytochemical and biological investigations are needed.

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S A Ž E T A K

Antimikrobni učinak etanolnog ekstrakta sjemenki i pulpe ploda grejpa

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Ispitano je djelovanje etanolnog ekstrakta sjemenki i pulpe ploda grejpa (*Citrus paradisi* Macf., *Rutaceae*) na 20 sojeva bakterija i 10 sojeva kvasnica. Antibakterijski i antifungalni učinak ekstrakata testiran je postupkom difuzije na hranjivom agaru i standardnom metodom dilucije. Spektrofotometrijski je određeno da suhi ekstrakt sadrži 3,92% ukupnih polifenola, dok je udio flavonoida iznosio 0,11%. Prisutnost flavanona naringina i hesperidina u etanolnom ekstraktu potvrđena je tankoslojnom kromatografijom. Ekstrakt je pokazao najsnažniji učinak na vrstu *Salmonella enteritidis* (*MIC* 2,06%, *m/V*), dok je na ostale ispitane bakterije i kvasnice djelovao u koncentracijama od 4,13% do 16,50% (*m/V*).

Ključne riječi: Citrus paradisi (*Rutaceae*), plod grejpa, ekstrakt sjemenki i pulpe, antimikrobni učinak

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