

EVALUATION OF ANTIMICROBIAL POTENTIAL OF DIFFERENT COMMERCIAL GRAPEFRUIT SEED EXTRACTS

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ABSTRACT

Extracts made from grapefruit (*Citrus x paradisi* Macfad.) seed are recognized for strong bactericidal and fungicidal properties. Bacterial resistance to antibiotics is an emerging issue, and therefore isolation and characterization of novel antimicrobials of natural origin represent the real challenge for investigators worldwide. The aim of this study was to evaluate the antimicrobial activity of three commercial GSE (Grapefruit Seed Extract) products against six bacterial and one fungal strain, through the agar well diffusion method, determination of minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC), and minimum fungicidal concentration (MFC). Obtained results indicate strong antimicrobial effects of investigated extracts, with different microbial susceptibility, including the multidrug-resistant (MDR) bacteria. The highest inhibitory potential is conducted for Citrofit®, while CitroBiotic® has shown the weakest effect. These results are in concordance with the obtained MIC and MBC values. It could be concluded that frequent use of commercial GSEs is justified due to their powerful antimicrobial activity. These findings are promising in terms of utilization of natural products as novel antimicrobial agents.

Keywords: grapefruit seed extract, agar well diffusion, MIC, MBC, antibacterial, antifungal, multidrug-resistant strains

INTRODUCTION

Antimicrobial resistance is recognized as one of the leading global health problems of 21st century by all major regulatory, economic and political bodies. Finding the new antimicrobials of natural origin, investigation of their *in vitro* and *in vivo* activity, as well as defining their precise chemical composition occupies investigators worldwide during the last decades. Grapefruit (*Citrus x paradisi*), well known plant from Rutaceae family, is used in traditional medicine as the treatment for different diseases and conditions such as: ulcers, urinary and gastrointestinal infections, cardiovascular diseases, diabetes, cancer, and regulation of body weight and lipid metabolism (Çiçek Polat *et al.*, 2018). Grapefruit seed extract (GSE) is commercial product derived from the seed and pulp of grapefruit (Han *et al.*, 2015), and mainly represent 33% water-glycerol solution widely used as naturopathic remedy, food supplement, disinfectant and preservative in food and cosmetic industry (Cvetnić & Vladimir-Knežević, 2004). GSE has powerful antimicrobial properties, including antibacterial, antifungal, antiviral, and antiparasitic activity, and for this reason has attracted much attention from the general public (Reagor *et al.*, 2002; Cvetnić & Vladimir-Knežević, 2004; Xu *et al.*, 2007; Lim *et al.*, 2010). Main chemical substances responsible for antimicrobial effects of GSEs are polyphenolic compounds such as catechin, epicatechin, epicatechin-3-O-gallate, dimeric, trimeric and tetrameric procyanidins (Aloui *et al.*, 2014). In addition, commercially available GSEs contain some artificial agents such as benzethonium chloride, triclosan and methyl paraben, but investigation of antimicrobial potential of fully natural grapefruit seed extracts has showed that antimicrobial activity could not be attributed only to the synthetic preservative agents (Cvetnić & Vladimir-Knežević, 2004). GSE as the commercial product is available on the market under various names, such as: Citricidal®, CitroBiotic®, Citrofit®, Citrosept®, NutriBiotic® and many others. The aim of this study was *in vitro* examination of antimicrobial activity of three different commercially produced GSEs, and determination of the sensitivity degree of Gram-positive and Gram-negative bacteria and fungi on tested extracts.

MATERIAL AND METHODS

Investigated microbial strains and extracts

Antibacterial and antifungal activity of three commercial GSEs: Citrofit® (Vedicon, Austria), CitroBiotic® (Sanitas GmbH & Co., Germany), and NutriBiotic GSE Liquid Concentrate® (NutriBiotic, California, USA) is evaluated against following microorganisms: *Bacillus subtilis* subsp. *spizizenii* ATCC 6633, *Staphylococcus aureus* subsp. *aureus* ATCC 33591 (MRSA strain), *Enterococcus faecalis* ATCC 29212, *Escherichia coli* ATCC 35218 (ESBL producing strain), *Salmonella enterica* subsp. *enterica* serovar Enteritidis ATCC 13076, *Pseudomonas aeruginosa* ATCC 9027, and *Candida albicans* ATCC 10231. According to the manufacturer, Citrofit® contains 33% of grapefruit seed extract, water and vegetable glycerine; CitroBiotic® is 83% glyceric extract of grapefruit seed and peel, combined with water and ascorbic acid. NutriBiotic GSE Liquid Concentrate® contains grapefruit seed extract (33%) and vegetable glycerine (67%).

Agar well diffusion method

Agar well diffusion method is performed according to the Balouiri *et al.* (2016). Investigated bacterial strains were at first cultured overnight at 37 °C in Mueller Hinton (MH) medium (HiMedia Laboratories Pvt. Limited, India) and fungal strain in Sabouraud 4% Glucose Agar (SGA) (Sigma-Aldrich, USA). Following the recommendations of EUCAST (2017), the inoculum was prepared from overnight cultures of test microorganisms in sterile saline solution to obtain a uniform homogeneous turbidity corresponding to the 0.5 McFarland standard, and a microbial cell concentration of 1-2 x 10⁸ CFU/ml. Inoculation was performed using sterile swabs soaked in a suspension of test microorganisms. After inoculation, the seeded agar plates were left at room temperature for 15 minutes to allow the better absorption of the applied inoculum into the substrate. After the substrate absorbed the inoculum, plates were drilled in order to make wells with diameter of 8 mm. 50 µl of investigated grapefruit seed extracts, were transferred into the wells and incubated for 16 to 18 hours at 37 °C. All extracts

were triple tested. Antimicrobial activity of tested GSEs was evaluated based on diameter of inhibition zones, after the extract diffusion in the medium. Four different antibiotics: Colistin (10 µg), Streptomycin (10 µg), Ampicillin (10 µg) and Amoxicillin (25 µg), as well as standard antifungal drug Nystatin (100 IU), all made by Oxoid™, Great Britain, were used as positive control.

Broth microdilution method

Broth microdilution method (CLSI, 2018) was applied in order to determine the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of tested grapefruit seed extracts. Considering the antifungal activity, we used the term MFC (minimum fungicidal concentration). Serial dilutions were made in sterile 96 well microtiter plate. Amoxicillin (Sigma-Aldrich, USA) and Nystatin (Sigma-Aldrich, USA) were used as reference, prepared in initial concentration of 1024 µg/ml and 1000 µg/ml (5916 IU/ml), respectively. Dimethyl Sulfoxide, DMSO (Sigma-Aldrich, USA) was used as the solvent. At first, 100 µL of liquid MH medium was added to each well in a microtiter plate, except in the wells of the first column. Amount of 200 µl of tested GSEs at initial concentration of 330 µg/ml was added in the first microtiter column. Then, 100 µl of extract was transferred from the first column to the second, and after mixing 100 µl of content, transferred from the second to the third column. This procedure was repeated to the tenth column, from which 100 µl of content was finally removed. In this way, in each row of the microtiter plate, from position 2 to position 10, a serial two-fold dilution of the test substance in the range of 165 to 0.64 µg/ml was obtained. The last row in the microtiter plate contained double serial dilution of antibiotic, starting from 512 to 2 µg/ml. Wells from the second to the eleventh column were then inoculated with the 5 µl of test microorganisms' suspension, at the concentration of 1-2 x 10⁸ CFU/ml, and final concentration of 5 x 10⁵ CFU/ml was obtained. Column 11 containing 100 µl of standard inoculum without tested GSEs served as the positive control, while column 12 containing only the sterile liquid medium and DMSO was used as the negative control. Each test was performed twice. Microtiter plates were incubated for 16-18 hours at 37 °C.

Resazurin assay

Reading of MIC is done manually using an inverted mirror and after that 30 µl of 0.015% Resazurin sodium salt solution (Sigma-Aldrich, USA) was added to all wells. Resazurin dye allows easier interpretation of the results (Elshikh et al., 2016). The wells containing the living microorganisms were coloured pink, while those with death microorganisms, destroyed by the test substance, were stained blue. All columns with no visible colour change represent the concentration of the extract above the MIC value. The last position in the row where the blue colour remained, while there is a presence of pink colour in the next position, represents MIC.

Minimum bactericidal concentration (MBC) and minimum antifungal concentration (MFC) assays

After the determining of minimum inhibitory concentration (MIC), the determination of the minimum bactericidal concentration (MBC) and minimum fungicidal concentration (MFC) was proceeded (CLSI, 2018). The volume of 10 µl of content from the well whose position was marked as MIC, as well as from the previous three wells in the same row with no observed microbial growth, was transferred on agar plates with MH/SGA medium. Inoculated plates were incubated at 37 °C for 18-24 hours. The MBC/MFC was recorded as the value

closest to the determined MIC value, at which there was no growth of microorganisms after incubation. All experiments were performed in triplicate.

Statistical analysis

Performed tests were done in three replications, therefore descriptive statistical parameters: mean values ± standard deviation (SD) were calculated, using the Microsoft Office 2019 Excel (Microsoft Corporation, USA). In order to determine the presence of statistically significant differences between collected data, One-way ANOVA and *post hoc* Fisher's Least Significant Difference (LSD) test (STATISTICA 10; StatSoft.Inc.) were applied, at the significance level of p<0.05.

RESULTS AND DISCUSSION

Antibacterial and antifungal assays

All three investigated GSEs showed inhibitory effects against reference microorganisms, but with different intensity. Overall results suggest Citrofit® as the strongest GSE, with widest inhibition zones observed in the case of *B. subtilis* subsp. *spizizenii* (31.00±0.00) and MRSA (30.00±0.00). The most sensitive Gram-negative strain to this extract was *S. enterica* subsp. *enterica* serovar Enteritidis (24.30±1.15). Citrofit® caused the greatest inhibition of *C. albicans* (26.30±0.57) as well, which is significantly different in comparison to the standard antimycotic (Tab 1). These results are in accordance with the investigation of Krajewska-Kulak et al. (2003), where 33% grapefruit-water glycerol solution also exhibited strong activity against the yeast-like fungi. NutriBiotic® performed balanced antibacterial activity, with the greatest zones on *P. aeruginosa* (25.30±1.52), followed by slightly different inhibition of multidrug-resistant ESBP producing *E. coli* (25.00±1.00), MRSA, *E. faecalis*, *S. enterica* subsp. *enterica* serovar Enteritidis (24.60±0.57), and *B. subtilis* subsp. *spizizenii* (22.60±1.52). Both Citrofit® and NutriBiotic® have shown significantly higher antibacterial and antifungal activity compared to the CitroBiotic® (Tab 1). A possible explanation for such results could be the fact that CitroBiotic® preparation is completely free of benzethonium chloride, benzalkonium chloride and triclosan, as well as of organochlorine- and organophosphorus-pesticides (Manufacturer Report No. K 2465 FN - III and K 2465 FN - I). There is a chance that some of the listed substances may act as antimicrobial agents. Statistical analysis has shown that antimicrobial activity of tested GSEs was significantly higher from antibacterial and antifungal effects of tested antimicrobials (Tab 1). None of four used antibiotics didn't cause inhibition of MRSA, in contrast to the effects of GSEs. The obtained results coincide with the data that GSE has a pronounced effect on methicillin-resistant *Staphylococcus aureus* (Su et al., 2012), which indicates that investigated extract could be used in the treatment of infections caused by this multiresistant bacterial strain. In addition, ESBP producing *E. coli* was inhibited by Colistin and Streptomycin, but with lower rate in comparison to the GSEs (Tab 1). In most cases, Gram-positive bacteria have shown greater sensitivity to the investigated extracts compared with Gram-negative bacteria, which is in line with previous findings (Reagor et al., 2002; Hegggers et al., 2004; Xu et al., 2007; Bangwo et al., 2015). This could be explained by the fact that Gram-positive bacteria have only a thick layer of peptidoglycan in their cell wall, which makes them susceptible to inhibitory substances, while Gram-negative bacteria have an outer lipid membrane in their cell wall, as a kind of barrier, making them less sensitive. GSE probably acts by destroying the cell membrane and ultimately leading to lysis and death of the bacterium (Hegggers et al., 2004; Bhatia et al., 2015).

Table 1 Diameter of inhibition zones* (mm) obtained through the agar well diffusion method

| Microbes | Investigated GSEs | | | | Standard antimicrobials | | | |
|--|-------------------------|-------------------------|-------------------------|----------------------|-------------------------|------------------------|------------------------|-------------------------|
| | Citrofit® | CitroBiotic® | NutriBiotic® | Colistin | Streptomycin | Ampicillin | Amoxicillin | Nystatin |
| <i>Bacillus subtilis</i> subsp. <i>spizizenii</i> ATCC 6633 | 31.00±0.00 ^a | 18.50±0.57 ^c | 22.60±1.52 ^a | 10±0.00 ^b | 20±0.00 ^d | 22±0.00 ^{b,e} | 25±0.00 ^{b,d} | - |
| <i>Staphylococcus aureus</i> subsp. <i>aureus</i> ATCC 33591 (MRSA strain) | 30.00±0.00 ^a | 12.00±0.00 ^c | 24.60±0.57 ^a | NI | NI | NI | NI | - |
| <i>Enterococcus faecalis</i> ATCC 29212 | 28.00±0.00 ^a | 15.60±2.08 ^c | 24.30±0.57 ^a | NI | 15±0.00 ^d | 9±0.00 ^{b,e} | NI | - |
| <i>Escherichia coli</i> ATCC 35218 (ESBL producing strain) | 21.60±0.57 ^a | 14.00±0.00 ^c | 25.00±1.00 ^a | 10±0.00 ^b | 12±0.00 ^d | NI | NI | - |
| <i>Salmonella enterica</i> subsp. <i>enterica</i> serovar Enteritidis ATCC 13076 | 24.30±1.15 ^a | 16.30±1.15 ^c | 24.60±0.57 ^a | 11±0.00 ^b | 15±0.00 ^d | NI | 18±0.00 ^{b,d} | - |
| <i>Pseudomonas aeruginosa</i> ATCC 9027 | 18.30±0.57 ^a | 12.50±0.50 ^c | 25.30±1.52 ^a | 17±0.00 ^b | 14±0.00 ^d | NI | NI | - |
| <i>Candida albicans</i> ATCC 10231 | 26.30±0.57 ^a | 13.30±0.57 ^c | 15.60±0.57 ^a | - | - | - | - | 20.60±1.15 ^f |

*Results are Mean ± SD

NI=No inhibition

Columns that don't share the same letters, differ significantly at p<0.05 after *post hoc* LSD test.

Determination of minimum inhibitory concentration (MIC)

In terms of antibacterial and antifungal activity, tested GSEs have showed MIC values of <0.64-82.50 µg/ml (Tab 2). Comparing the three GSEs used in this investigation, Citrofit® has showed a higher antimicrobial activity than CitroBiotic® and NutriBiotic®. Furthermore, antimicrobial activity of this

extract almost completely surpassed the activity of Amoxicillin and Nystatin. Tested antibiotic didn't cause the growth inhibition of MRSA, *E. faecalis* and ESBL producing *E. coli*, while all tested extracts were successful in this sense (Tab 2).

Table 2 Minimum inhibitory concentration (MIC) of tested extracts and controls

| Microbes | MIC* (µg/ml) of GSEs | | | Control (µg/ml) |
|--|----------------------|--------------|--------------|---------------------|
| | Citrofit® | CitroBiotic® | NutriBiotic® | |
| <i>Bacillus subtilis</i> subsp. <i>spizizenii</i> ATCC 6633 | <0.64 | 82.50 | 41.25 | 8.00 ¹ |
| <i>Staphylococcus aureus</i> subsp. <i>aureus</i> ATCC 33591 (MRSA strain) | <0.64 | 10.32 | 5.16 | NI ¹ |
| <i>Enterococcus faecalis</i> ATCC 29212 | 20.63 | 20.63 | 20.63 | NI ¹ |
| <i>Escherichia coli</i> ATCC 35218 (ESBL producing strain) | 20.63 | 20.63 | 20.63 | NI ¹ |
| <i>Salmonella enterica</i> subsp. <i>enterica</i> serovar Enteritidis ATCC 13076 | 10.32 | 20.63 | 41.25 | 256.00 ¹ |
| <i>Pseudomonas aeruginosa</i> ATCC 9027 | <0.64 | 10.32 | 2.58 | NI ¹ |
| <i>Candida albicans</i> ATCC 10231 | <0.64 | 82.50 | 41.25 | 7.81 ² |

*Same values obtained through the duplicate.

¹Amoxicillin, initial concentration 1024 µg/ml

²Nystatin, initial concentration 1000 µg/ml (5916 IU/ml)

NI=No inhibition

Determination of MBC and MFC

The microdilution method of testing the minimum inhibitory concentration of Citrofit® has showed the most effective action on four microorganisms (*B. subtilis*, MRSA, *P. aeruginosa*, and *C. albicans*) with the MIC less than 0.64

µg/ml, whereby Gram-positive bacteria were more susceptible than Gram-negative. The same results were observed in the study of the minimum bactericidal and minimum fungicidal concentration following the results of the MIC, where Citrofit® had the strongest effect (Tab 2, Tab 3).

Table 3 Minimum bactericidal concentration (MBC) and minimum fungicidal concentration (MFC)

| Microbes | MBC and MFC* (µg/ml) of GSEs | | | Control (µg/ml) |
|--|------------------------------|--------------|--------------|--------------------|
| | Citrofit® | CitroBiotic® | NutriBiotic® | |
| <i>Bacillus subtilis</i> subsp. <i>spizizenii</i> ATCC 6633 | <0.64 | 165.00 | 82.50 | 16.00 ¹ |
| <i>Staphylococcus aureus</i> subsp. <i>aureus</i> ATCC 33591 (MRSA strain) | <0.64 | 20.64 | 10.32 | NI ¹ |
| <i>Enterococcus faecalis</i> ATCC 29212 | 41.26 | 41.26 | 41.26 | NI ¹ |
| <i>Escherichia coli</i> ATCC 35218 (ESBL producing strain) | 41.26 | 41.26 | 41.26 | NI ¹ |
| <i>Salmonella enterica</i> subsp. <i>enterica</i> serovar Enteritidis ATCC 13076 | 20.64 | 41.26 | 82.50 | 512 ¹ |
| <i>Pseudomonas aeruginosa</i> ATCC 9027 | <0.64 | 20.64 | 5.16 | NI ¹ |
| <i>Candida albicans</i> ATCC 10231 | <0.64 | 165.00 | 82.50 | 15.62 ² |

*Same values obtained through the triplicate.

¹Amoxicillin, initial concentration 1024 µg/ml

²Nystatin, initial concentration 1000 µg/ml (5916 IU/ml)

NI=No inhibition

CONCLUSION

Commercial GSEs investigated in this study have shown antimicrobial activity against all tested Gram-positive and Gram-negative bacteria, as well as the strain of pathogenic fungus *C. albicans*. The strongest inhibitory effects were observed for Citrofit®, especially against multidrug-resistant pathogens (MRSA and ESBL *E. coli*). Tested antimicrobials proved to be less effective against bacteria and *C. albicans* strain in comparison to the GSEs. There are differences between the antimicrobial activity of each extract, which could be attributed to the chemical content variation. It is known that phytochemical profile, as well as biological activity of plant products, could be related to the specific abiotic factors in the plant habitat, but also the conditions of mass production process. Also, there is a possibility that artificial additives, frequently present in such extracts, could be related to overall antimicrobial potential. It is promising that commercially available products of natural origin, possess such remarkable antimicrobial activity, especially in times where most synthetic antibiotics are less and less effective. However, it must be noted that investigated extracts could represent a dietary supplement and not an official treatment against infectious diseases. Wider medicinal use of GSEs should be supported with exact evidence regarding its phytochemicals, pharmacological and biological properties.

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