THE ACTIVITY OF DIFFERENT EXTRACTS FROM PANAX QUINQUEFOLIUM L. CULTURES AGAINST PATHOGENIC STAPHYLOCOCCUS AUREUS WITH RESPECT TO GINSENOSIDE CONTENT

Monika Sienkiewicz¹, Anna Głowacka¹, Edward Kowalczyk² and Ewa Kochan³

¹Environmental Biology Department, Medical University of Lodz, Poland
²Pharmacology and Toxicology Department, Medical University of Lodz, Poland
³Pharmaceutical Biotechnology Department, Medical University of Lodz, Poland

*Corresponding author: monika.sienkiewicz@umed.lodz.pl

Abstract: Ginsenosides can be isolated from various cultures of Panax quinquefolium L., American ginseng. The aim of the study was to determine the antibacterial activity of extracts from leaves, stalks, hairy root cultures and field roots of P. quinquefolium L. containing ginsenosides against Staphylococcus aureus isolates obtained from various clinical materials. The agar well diffusion assay was used to evaluate microbial growth inhibition at various concentrations of extracts. The susceptibility of the clinical isolates to recommended antibiotics was determined with the disk-diffusion method. The results showed that the tested extracts inhibited the growth of all S. aureus clinical isolates, including MRSA (methicillin-resistant S. aureus) with MIC values ranging from 0.5 mg/mL to 1.7 mg/mL. The level of antimicrobial activity of extracts depends on the ginsenoside content. Both field roots and hairy root cultures represent excellent sources of these metabolites. Extracts with ginsenosides were found to inhibit multidrug-resistant staphylococci and can be a valuable complement to antistaphylococcal therapy.

Key words: antibacterial effect; minimal inhibitory concentration; plant secondary metabolites

Received: March 30, 2015; Revised: April 28, 2015; Accepted: April 28, 2015

INTRODUCTION

American ginseng (Panax quinquefolium L.) is a native North American member of the Araliaceae family, and is a perennial understory herb associated with deciduous forests. Ginsenosides (triterpene saponins) present in ginseng extract are considered to be its main biologically active components. Ginsenosides are divided into three groups, depending on their type of aglycone and sugar moieties: the Rb group (including Ra1, Ra2, Rb1, Rb2, Rb3, Rc, Rd, Rg3, Rh2), the Rg group (including Rg1, Rg2, Re, Rh1), and the Ro group (oleanolic acid) (Wang et al., 2012). The most common ginsenosides are Rb1, Rb2, Rc, Rd, Rg1 and Re, and these are responsible for the majority of pharmacological effects of ginseng on the nervous, cardiovascular, reproductive and metabolic systems (Wang et al., 2009; Ernst 2010; Cho 2012; Kim 2012). Ginsenosides also have anti-fatigue, anti-hyperglycemic, anti-obesity, anticancer, antioxidant and anti-aging properties (Wang et al., 2009; Yuan et al., 2010). The growing phenomenon of bacterial resistance to antibiotics provides a strong incentive to study the antimicrobial activity of extracts from various medicinal species, such as ginseng. However, studies of antimicrobial activity have so far been confined to the reports of field-grown plants and concern standard strains of bacteria (Yan-Qing, 2013, Lee et al. 2013).

Our previous studies demonstrated that the hairy root extracts from three clones containing ginsenosides at concentrations between 0.8 mg/mL and 1.4 mg/mL inhibited the growth of standard
strains of bacteria (S. aureus ATCC 4330, S. aureus ATCC MR3, E. faecalis ATCC 29212 Van, E. faecalis ATCC 51299 VanB, E. faecium ATCC 35667 Van, E. coli ATCC 35218, E. coli ATCC 25922, P. aeruginosa ATCC 27853 and yeast (C. albicans) ATCC 10231) (Kochan et al., 2013). The aim of this study was to compare the antibacterial activity of extracts from ginseng leaves, stalks, hairy root cultures and field roots against clinical strains of S. aureus, MRSA and MSSA (methicillin-sensitive S. aureus) with different patterns of resistance to recommended antibiotics.

MATERIALS AND METHODS

Ground plants and hairy root cultures

The plant material used for the study was obtained thus: (i) From experimental fields at the Agricultural University of Lublin as roots, leaves and stalks of soil-grown 3-year-old P. quinquefolium L. (Fig. 1). Organs from field cultivation were harvested in the middle of September from 3-year-old P. quinquefolium L. plants grown on dusty, light, loamy sand characterized by neutral pH, very high phosphorus content, and average potassium and magnesium content; (ii) from in vitro culture as hairy root cultures. The hairy roots of P. quinquefolium L. were obtained after infection with an agropine-type strain of Agrobacterium rhizogenes ATCC 15834. The transformation was verified by PCR analysis as described earlier (Kochan et al., 2012). Hairy root cultures were grown in 300-mL shaken Erlenmeyer flasks with 80 mL of hormone-free, liquid, B-5 medium (Gamborg et al., 1968). The average inoculum size was about 300 mg fresh weight (f.w.) and 28.9 mg dry weight (d.w.). The cultures were maintained in the dark at 26°C on a rotary shaker (100 rpm) and subcultured every 28 days. Fresh roots, after drying on absorbent paper, were dried at room temperature and processed for ginsenoside extraction and HPLC analysis.

Sample preparation

Samples of 1 g of dry raw material, weighed to 0.1 g tolerance, were placed in 250-mL flasks. They were extracted 3 times with 50 mL of 80% methanol for 30 min at boiling temperature under a reflux condenser. The combined methanol extracts were evaporated until dryness in a vacuum evaporator under lowered pressure at 60°C. The flask with dried residues was placed in a desiccator filled with a drying agent. The dried methanolic extract was weighed.

The dried methanolic extracts were dissolved in 5 mL of 50% HPLC methanol. Samples of 2 mL were placed on a solid phase extraction (SPE) column with octadecyl (C18) as reverse phase. The column was first rinsed with 10 mL of 100% HPLC methanol and 10 mL of distilled H2O to prepare the column to receive the sample. A 2-mL sample was passed through the column, which was then washed with 10 mL of distilled H2O and 10 mL of 30% HPLC methanol to selectively remove impurities. Ginsenosides were selectively eluted using 10 mL of 100% HPLC methanol and were evaporated until dryness in a vacuum evaporator under lowered pressure at 60°C. Dry extracts were used for HPLC analysis.

Ginsenosides Rb1, Rb2, Rc, Rd, Re and Rg1 were purchased from C. Roth GmbH+Co, Karlsruhe, Germany. A standard stock solution consisting of a mixture of the Rb1, Rb2, Rc, Rd, Re and Rg1 ginsenosides (10 mg/mL of each ginsenoside) was prepared in methanol of HPLC grade (J.T. Baker, Netherlands). A series of standard operating solutions of different concentration were obtained by diluting the mixed standard stock solution.
HPLC analysis of ginsenosides

Dried extracts obtained using the SPE procedure were dissolved in 2 mL of methanol (HPLC grade) and filtered through a 0.2-μm pore diameter Millex®-FG Hydrophobic Fluoropore filter (PTFE). Aliquots of 20 μL were then introduced to a liquid chromatography system consisting of LiChroART® 250-4, Waters 600 Controller pump and UV-VIS Waters 996 detector combined with a Pentium 60 PC running Millennium software. A reverse C₁₈ column was employed to separate ginsenosides. Two different mixtures of acetonitrile with water were used as eluent. A 30:70 acetonitrile to water ratio was used for determination of the ginsenosides Rb₁, Rb₂, Rc, Rd (flow rate 2 mL/min, analysis time 45 min), and an 18:82 ratio was used for determination of the ginsenosides Rg₁ and Re (flow rate 3 mL/min, analysis time 40 min.). Ginsenoside detection was performed at a wavelength of 203 nm. Quantification of ginsenosides (mg/g d.w.) was carried out by comparing retention times and peak areas between standards and samples.

Staphylococcus aureus clinical isolates

The clinical isolates of S. aureus were collected in 2011 and 2012 from a range of clinical materials recovered from patients of the Internal Medicine, Surgery and Dermatology departments of two of the Medical University hospitals in Lodz. The thirty bacterial strains used in our investigations were isolated from the respiratory tract (n=6), abdominal cavity (n=8), postoperative wounds (n=10) and skin lesions (n=6).

S. aureus clinical isolates were cultured on Columbia agar (bioMérieux), on mannitol salt agar (bioMérieux), and the ability of bacteria to produce catalase and coagulase was determined (bioMérieux). Bacteria were identified to species level using API Staph tests (bioMérieux). The tested strains were cultivated on Columbia agar medium with 5% blood and incubated at 37°C for 24 h. Bacterial suspensions with an optical density of 0.5MF were prepared with the bioMérieux densitometer.

Susceptibility testing

Susceptibility testing was carried out with the use of the disk-diffusion method. (Jorgensen and Turnidge, 2007). The following antibiotics (Becton Dickinson) were used for testing the susceptibility of S. aureus: FOX – cefoxitin (30 μg), P – penicillin (10IU), E – erythromycin (15 μg), DA – clindamycin (2μg), TE – tetracycline (30μg), TGC – tigecycline (15 μg), C – chloramphenicol (30 μg), CIP – ciprofloxacin (5 μg), RA – rifampin (5 μg), GM – gentamicin (10 μg), SXT – trimethoprim/sulfamethoxazole (1.25 μg /23.75 μg), LZD – linezolid (30 μg), FD – fusidic acid (10 μg), QDA – quinupristin/dalfopristin (15 μg), and also for VA –vancomycin (30 μg), DPC – daptomycin (15μg). Cultures were incubated at 37°C for 16-18 h under aerobic conditions on Mueller-Hinton II agar. The results were interpreted according to Clinical and Laboratory Standard Institute guidelines (EUCAST, 2012).

Determination of minimal inhibitory concentration (MIC)

The extracts from leaves, stalks, hairy root cultures and roots of P. quinquefolium L. growing in the ground were weighed and diluted in ethanol at concentrations from 95% to 97% w/v, which was used as a stock solution. An inoculum containing 1.5 x10⁸ CFU (0.1 mL) per spot was applied to the surface of the Columbia agar medium at concentrations from 0.5 mg/mL to 1.6 mg/mL of tested extracts. The minimal inhibitory concentration (MIC) was determined by the agar well diffusion assay after 24 h of incubation at 37°C under aerobic conditions on Columbia agar. Three independent analyses of the antibacterial activity of the extracts were performed. Control media containing only ethanol at concentrations used in diluting the extracts did not inhibit the growth of the bacteria.

Statistical analysis

All treatments were replicated in triplicate. The results were subjected to a Kruskal-Wallis test. The level of significance was set at P≤0.05. Spearman’s
rank correlation coefficient between MIC value and ginsenoside content was calculated. STATISTICA version 10 (STAT Soft, Poland) software was used for all calculations.

RESULTS

Characteristics of extracts from field plants and hairy root cultures

Six ginsenosides were quantified in extracts from organs of P. quinquefolium L. taken from field-cultivated plants and hairy root cultures: Rb1, Rb2, Rc, Rd (protopanaxadiol derivatives), Rg1 and Re (protopanaxatriol derivatives). HPLC analysis of ginseng saponins confirmed that plant material from both sources contained all examined ginsenosides. The highest amount of saponins, about 35 mg/g d.w., was found in roots from field cultivation. Leaves and hairy roots contained lower amounts, and stalks the least (Table 1). Our findings also reveal that the levels of individual ginsenosides differed between the studied extracts (Table 1). An analysis of the amount of protopanaxatriol derivatives indicated that Re predominates quantitatively: its level was about 4-times higher than Rg1 content in extracts from organs cultivated in the field and 2.5 times greater in hairy root cultures. Other observations were recorded for derivatives of protopanaxadiol. The Rb1 saponin was seen to dominate quantitatively in both types of roots, Rb2 in leaves and Rd in stalks.

Susceptibility testing of S. aureus isolates to recommended antibiotics

The results of susceptibility testing showed that the S. aureus isolates were highly resistant to β-lactam (penicillin for 93.3% of isolates), aminoglycoside (gentamicin for 80.0%), macrolide (erythromycin for 66.6%), lincosamide (clindamycin for 60.0%) and tetracycline (for 53.3% of clinical isolates). Among the 30 tested clinical strains, 23 (76.6%) were resistant to methicillin, based on tests with cefoxitin. The resistance of Staphylococcus aureus to recommended antibiotics is presented in Fig. 2.

Antimicrobial activity of P. quinquefolium L. extracts

The tested extracts from leaves, stalks, hairy root cultures and field roots of P. quinquefolium L. demonstrated antistaphylococcal activity at concentrations from 0.5 mg/mL to 1.6 mg/mL. The highest activity against all tested S. aureus clinical isolates was demonstrated by field root extracts, with MIC values ranging from 0.5 to 0.7 mg/mL. Higher MIC values (0.9-1.0 mg/mL) were obtained for leaf extracts, while the activity of hairy root extracts ranged from 1.1 mg/mL to 1.4 mg/mL. The lowest activity was demonstrated by the extract obtained from stalks, with MIC values ranging from 1.4 mg/mL to 1.7 mg. The 0.5 mg/mL and 0.55 mg/mL extracts from field roots inhibited the growth of 30.5% MRSA cultures, while 42.9% of MSSA were susceptible to field root extract

Table 1. Ginsenoside contents in organs from field cultivation and in hairy roots cultures of P. quinquefolium. Each value is the mean of six replicates ± SE. Data analyzed using ANOVA Kruskal-Wallis test 0.05. The means with the same letter within the column do not differ significantly according to Kruskal Wallis test (p≤ 0.05)

<table>
<thead>
<tr>
<th>Plant material</th>
<th>Ginsenoside [mg/g d.w.]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Rb1</td>
</tr>
<tr>
<td>Hairy roots cultures</td>
<td></td>
</tr>
<tr>
<td>Field roots</td>
<td>10.767 ±1.216</td>
</tr>
<tr>
<td>Stalks</td>
<td>0.721 ±0.099</td>
</tr>
<tr>
<td>Leaves</td>
<td>1.999 ±0.299</td>
</tr>
</tbody>
</table>

Sienkiewicz et al.
at a concentration of 0.6 mg/mL. The 0.7 mg/mL leaf extract inhibited the growth of 48.9% of MSSA isolates, while 0.95 mg/mL inhibited 42.95%. The largest number of MRSA isolates (39.1%) was susceptible to this extract, with an MIC value of 0.95 mg/mL. The 1.0 mg/mL hairy root extract was clearly the most effective extract, both against MRSA (47.8%) and MSSA (57.1%) isolates. A large number of MRSA (30.4%) and MSSA (23.5% and 42.9%) clinical isolates were susceptible to the extract from stalks at concentrations of 1.6 mg/mL and 1.65 mg/mL, respectively. The antistaphylococcal activity of *P. quinquefolium* L. extracts is presented in Fig. 3.

**Relationship between antistaphylococcal activity and ginsenosides content of the tested extracts**

The statistical dependence between antistaphylococcal activity and saponin content of studied extracts was assessed. Table 2 shows the correlation between MIC value and ginsenoside level. A high negative correlation was observed between MIC value (both for MRSA and MSSA) and total saponins containing Rb and Rg groups, and with Rc and Re metabolites. A significant relationship was also noted between antistaphylococcal activity and the Rb1, Rd, and Rg1
contents. These results may confirm that ginsenosides are responsible for the antistaphylococcal activity of ginseng extracts.

DISCUSSION

Ginsenosides are distributed throughout the ginseng plant and are also detected in hairy root cultures (Li et al., 1996; Kochan et al., 2008; 2012, Qu et al., 2009; Li et al., 2012). The highest levels of six examined saponins were found in samples taken from field-cultivated roots. These observations confirm earlier studies (Kochan et al., 2008). Mizuno et al. (1994) demonstrated that cultivated and wild P. ginseng roots had similar contents of total examined saponins, and the ginsenosides Re and Rb1 were the dominant metabolites. It was found that field roots contained about twice as much saponin as the leaves. A similar relationship was observed by Proctor et al. (2011); however, the differences in the total amount of ginsenosides in both organs were significantly lower. In another study (Li and Wardle, 2002), the ginsenoside content of the roots and leaves of a 3-year-old American ginseng plant were found to be almost identical. However, these results contradict those of Li et al. (2012) and Kim et al. (2012), where higher ginsenoside contents were noted in the leaves than in the roots of Korean ginseng cultivated in the field (Kim et al., 2012) or cultured in an aeroponic system (Kim et al., 2012). These differences between the results presented in our paper and those of previous studies might be affected by seasonal fluctuation (Li and Wardle, 2002), geographical differences (Chung et al., 2012), age variations (Wang et al., 2007; Qu et al., 2009; Chung et al., 2012), cultivation methods (Lim et al., 2005; Chung et al., 2012; Kim et al., 2012), time of harvest (Kochan et al., 2008; Chung et al., 2012) and other factors (Yamaguchi et al., 1988; Li et al., 1996; Wang et al., 1999; Schlag et al., 2006; Shi et al., 2007; Qu et al., 2009; Proctor et al., 2011; Li and Wardle, 2012; Li et al., 2012). Although lower amounts of ginsenosides were found in hairy root cultures than in roots and leaves from field cultivation, it is worth noting that the ginsenosides from these cultures were extracted after 28 days of hairy root growth. The roots and leaves taken from ground cultivation, on the other hand, had been growing for 3 years. The saponin content in hairy root cultures described in the present study was comparable to or higher than the ginsenoside level seen in other hairy root cultures of ginseng (Yoshikawa 1997; Palazon et al., 2003; Yu et al., 2003; Kochan et al., 2013).

Our results show that ginsenosides from P. quinquefolium L. possess antistaphylococcal activity that does not depend on the level of bacterial resistance to antibiotics. This is of great importance for the application of ginsenosides as active antimicrobial agents against multiresistant strains, including MRSA. The investigation described in this paper found that the MIC of roots and leaves extracts ranged from 0.5 mg/mL to 1.0 mg/mL. In contrast, Kołodziej et al. (2013) demonstrated significantly lower antistaphylococcal activity against S. aureus ATCC 6538P with respective MIC values of 0.92 mg/mL and >21.4 mg/mL for roots and leaves extracts. In addition, the extracts from hairy roots cultures of P. quinquefolium L. demonstrate high antimicrobial activity against both S. aureus ATCC 4330 and S. aureus ATCC MR3 standard strain, as has been described in earlier studies (Kochan at al. 2013), and the clinical strains illustrated in this paper.

Hairy root cultures produced on a large scale can be a valuable source of these active compounds, whose synergistic action can be used to inhibit the emergence of resistant pathogens. Ginsenosides from Korean red ginseng have been shown to have antibacterial and synergistic activity (Sang Sung and Gun Lee, 2008). The authors demonstrated that the com-

<table>
<thead>
<tr>
<th>Ginsenosides</th>
<th>MIC MRSA</th>
<th>MIC MSSA</th>
</tr>
</thead>
<tbody>
<tr>
<td>total</td>
<td>-0.970</td>
<td>-0.973</td>
</tr>
<tr>
<td>Rg group</td>
<td>-0.963</td>
<td>-0.973</td>
</tr>
<tr>
<td>Rb group</td>
<td>-0.981</td>
<td>-0.973</td>
</tr>
<tr>
<td>Rb1</td>
<td>-0.776</td>
<td>-0.778</td>
</tr>
<tr>
<td>Rb2</td>
<td>-0.019</td>
<td>0.000</td>
</tr>
<tr>
<td>Rc</td>
<td>-0.980</td>
<td>-0.973</td>
</tr>
<tr>
<td>Rd</td>
<td>-0.759</td>
<td>-0.778</td>
</tr>
<tr>
<td>Rg1</td>
<td>-0.759</td>
<td>-0.778</td>
</tr>
<tr>
<td>Re</td>
<td>-0.989</td>
<td>-0.973</td>
</tr>
</tbody>
</table>

Table 2. Spearman’s rank correlation coefficient between MIC value and examined ginsenosides.
bination of ginsenosides and kanamycin possesses a synergistic or additive effect against MRSA: the ginsenosides were shown to interact with the membrane, thus increasing the permeability of the plasma membrane to kanamycin.

CONCLUSIONS

The antistaphylococcal activity of extracts from leaves, stalks, hairy root cultures and field roots of *P. quinquefolium* L. depend on the ginsenoside content. The extracts containing ginsenosides have antistaphylococcal activity, irrespective of the level of bacterial antibiotic resistance. Excellent sources of active ginsenosides are not only extracts from leaves and field roots, but also those of hairy root cultures.

Acknowledgments: The research was supported by Grant No. 502 13 771 from the Medical University of Lodz and has not been submitted elsewhere.

Authors’ contributions: MS designed and performed the experiments, analyzed the data and prepared the manuscript. AG, EK assisted in data analysis. EK performed experiments, analyzed the data and assisted in manuscript preparation. All authors read and approved the final manuscript.

Conflict of interest disclosure: The authors report no conflict of interest.

REFERENCES


European Committee on Antimicrobial Susceptibility Testing (EUCAST). Breakpoint Tables for Interpretation of MICs and Zone Diameters, version 2.0; valid from 1 January 2012; Available online: www.eucast.org (accessed on 16 December 2012).


