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Panax quinquefolium hairy root extracts and their effect in connection with antibiotics against pathogenic bacteria – a preliminary study

SUMMARY

The aim of the present study was to determine the level of ginsenosides in extracts from hairy root A, B, G clones of *Panax quinquefolium* and their action with antibiotics against clinical bacterial isolates. The content of ginsenosides (the key biologically active compounds) were determined in tested extracts using HPLC. The activity of extracts with antibiotics was established by microdilution broth method. Total triterpene saponin content was 14.68, 14.32 and 10.07 mgg^{-1} d.w. for root culture clones A, B and G, respectively. Our research indicates that the addition of extracts mainly from B and G clone hairy root cultures to antibiotics allow to reduce the ampicillin and tetracycline effective concentration respectively against *Enterococcus faecalis* and both *Escherichia coli* and *Acintobacter baumannii*.

Keywords: ginseng, ginsenosides, connection with antibiotic

INTRODUCTION

Ginsenosides are triterpene saponins isolated from various members of the genus *Panax* including *Panax quinquefolium*. Most of these compounds belong to the dammarane or the oleananetype. The dammarane-type ginsenosides can be further classified into protopanaxadiol-type ginsenosides (PPD) and protopanaxatriol-type ginsenosides (PPT) based on their glycone moieties. The 20S-protopanaxadiol derivatives (PPD) have hydroxyl groups at positions C3, C12, and C20, and sugar moieties attached to the β-OH at C-3 and/or C-20, while the 20S-protopanaxatriol derivatives (PPT) have hydroxyl groups at positions C3, C6, C12, and C20 and sugar moieties at the α-OH at C-6 and/or β-OH at C-20. The metabolites Rb1, Rb2, Rc and Rd are 20S-protopanaxadiol derivatives (Rb group), while the saponins Re and Rg1 are 20S-protopanaxatriol derivatives (Rg group) (9). Exemplary structures of ginsenosides are shown in Figure 1.

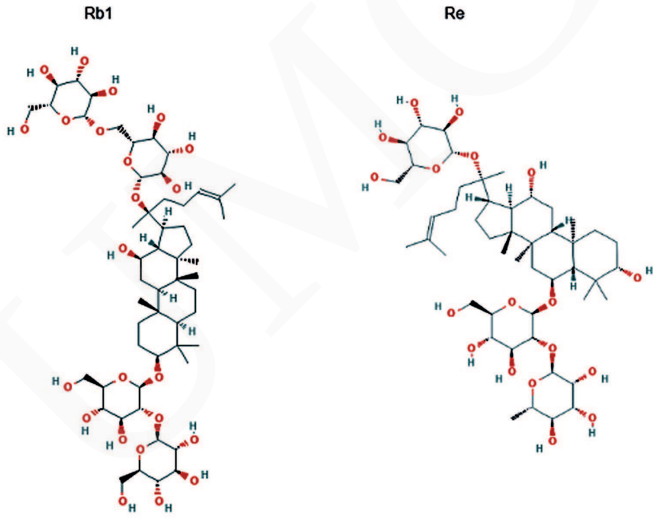

Fig. 1. Structure of ginsenoside Rb1, belonging to protopanaxadiol derivatives and Re belonging to protopanaxatriol derivatives.

Ginsenosides are considered as main pharmacologically active compounds of ginseng. They act on central nervous, immune system and cardiovascular systems. Ginseng has antioxidative, vasorelaxation, antiinflammatory, anticancer, antiallergy activities, as well as hypoglycemic and phytoestrogenic effects (3).

Until recently, antibiotics have been considered the most effective method of combating bacterial infections. They have successfully prevented or treated local infections, as well as systemic disorders. In developing countries, where sanitation is still poor, antibiotics decrease the morbidity and mortality caused by food-borne or poverty-related infections (8, 24, 41). However, bacteria have been known to develop resistance to antibiotics since their discovery. Initially, as new compounds were being quickly developed and introduced onto the market, the scale of the problem was not alarming. Despite this, at the end of the 20th century, the problem of antibiotic resistance had risen to dangerously high levels in all parts of the world, making effective treatment of some infectious diseases impossible (7, 27, 30, 41). The rapid spread of multidrug resistant bacteria is a consequence of the abuse of antibiotics and chemotherapeutics and the diversity

of bacterial resistance mechanisms (5, 22, 26). Expensive treatment is often ineffective and leads to more general or fatal infections.

Therefore, new antibiotics or natural compounds, that could enhance the effect of exiting antibiotics, are being sought (21, 31–33). One potential source of such natural compounds is represented by various plant extracts that are considered to be effective in fight against certain bacteria. Phytochemicals affect bacteria through various mechanisms (35). For example, some inhibit RNA synthesis and efflux pumps interact with the cytoplasmic membrane and with DNA changing the permeability of cell membranes, disrupt energy production by inhibiting some enzymes at the plasma membrane, and interfere with catabolic enzymes and the electron transport chain (34). Literature data also indicate that such plant secondary metabolites as saponins, alkaloids, phenols and phenolic acid, and essential oils may be suitable for combating microorganisms, including those resistant to antibiotics (2, 31–33).

Our previous studies have described the generation of *P. quinquefolium* (American ginseng) hairy root culture and the optimization of its ginsenoside production (11, 12) and examined the antibacterial activity of American ginseng hairy root extracts against a range of standard bacterial and yeast strains (13). The present study is the continuation of this issue by making a novel determination of the ability of *P. quinquefolium* B and G clones of hairy root extracts after the conditions optimization of efficient ginsenoside production to enhance the action of antibiotics against clinical isolates of *Enteroccocus faecalis, Eschericha coli*, and *Acinetobacter baumannii*. These genera of bacteria were included into the study because of their increasing resistance to recommended antibiotics (10, 17, 19, 28).

MATERIALS AND METHODS

Extraction and isolation of saponins from hairy root cultures of *P. quinquefolium*

Hairy roots were obtained by *P. quinquefolium* transformation with *A. rhizogenes* A4 strains, as described previously (14). The cultures were grown in 300 mL Erlenmeyer shake flasks with 80 mL of modified, hormone-free B-5 medium (6, 11). The ginsenoside extraction procedure and the method of quantitative saponin content determination have been described by Kochan et al. (11) in the paper entitled "The study of the effect of *P. quinquefolium* hairy root extracts in combination with antibiotics against clinical isolates of *Enterocccus faecalis, Eschericha coli* and *Acinetobacter baumannii*".

The study included examples of multidrug resistant clinical isolates obtained from patients with chronic respiratory infections. *Enterocccus faecalis* was isolated from nasal discharge, *Eschericha coli* from throat swab and *Acinetobacter baumannii* from sputum. The bacteria were identified according to standard microbiological methods with the use of Columbia Agar (bioMérieux), Enterococcosel Agar (Emapol), Mac Conkey Agar (bioMérieux). They were identified to the species with the use of API 20 Strep, API 20 E and API 20 NE tests (bioMérieux). The susceptibility testing to recommended antibiotics was carried out by disc-diffusion method with the use of discs (Bio-Rad).Two-fold dilution series of the antibiotics ranging from 1024 to 0.5 µg mL^{-1} in Mueller-Hinton Broth medium (Emapol) were prepared using 96-well microtiter plates. Ampicillin was used for the clinical isolate of *Enterococcus faecalis*, and tetracycline for *Escherichia coli* and *Acinetobacter baumannii*. The MIC value, i.e. the lowest concentration of the assayed antibiotic that inhibits the visible growth of the tested bacteria, was detected after 24 h of incubation at 37°C under aerobic conditions. The MBC was determined by transferring all the cultures which were cultivated in higher than MIC concentrations on Columbia Agar medium (bioMérieux) and incubating them at 37°C for 18 h. After incubation time the growth levels of cultures were checked.

The micro-dilution broth method was used to determine the effect of different concentrations of extracts from the A, B, and G hairy root clones on the activity of antibiotics against the

tested bacteria. The extracts were mixed with Mueller-Hinton Broth to obtain concentrations of 0.5 mg mL^{-1}, 1 mg mL^{-1}, 1.5 mg mL^{-1} and 2.0 mg mL^{-1} (concentrations close to MIC and higher were selected according to the preliminary study), and were added to the number of double dilutions of antibiotic in Mueller-Hinton Broth medium on the 96-well microtiter plates. An inoculum containing $1.5 \cdot 10^8$ CFU of each tested bacteria was prepared. 10 µl from the suspension per well was added to a mixture of broth with various concentrations of antibiotics and extracts (CLSI, 2015). The MIC was determined as the lowest concentration of antibiotic in the extract which inhibits the visible growth of bacteria after 24 h of incubation at 37°C under aerobic conditions. The investigations were repeated twice.

STATISTICAL ANALYSIS

All treatments were performed in triplicate. Data was analysed using the Kruskal-Wallis test. Any relationship at $p \leq 0.05$ was considered as significant. Statistica Version 13.1 software was used for all statistical analyses (STATSoft).

RESULTS

Ginsenosides content in three clones of *P. quinquefolium* hairy roots

Three lines of *P. quinquefolium* hairy roots were carried on modified B-5 medium. Total ginsenoside content was 14.76, 14.32 and 10.08 mgg^{-1} d.w. respectively for clones B, A, and G (Table 1). The studied cultures synthesized both protopanaxadiol and protopanaxatriol derivatives; however, the percentage of saponins belonging to the Rb group (Rb1+Rb2+Rc+Rd) or the Rg group (Rg1+Re) in relation to all studied metabolites varied between the tested lines of hairy roots (Fig. 1); for example, clone A accumulated more Rb group metabolites (73.47%) than other hairy roots. Different results were observed for protopanaxatriol derivatives: the highest share of Rg group saponins (57.69%) was found in clone G.

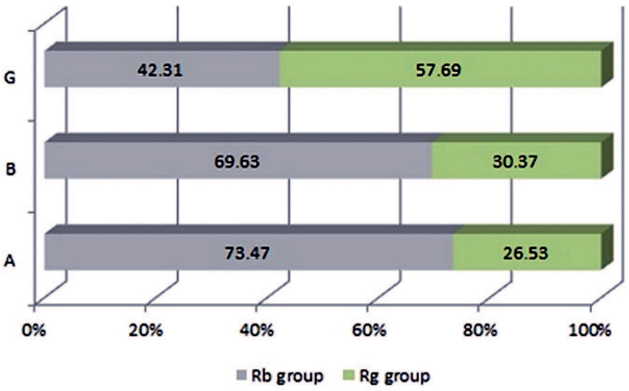

Fig. 1. The percentage content of protopanaxadiol and protopanaxatriol derivatives in relation to all studied ginsenosides in three lines of *P. quinquefolium* hairy roots.

The content of individual ginsenosides also varied between the studied hairy root cultures (Table 1), with the pattern Rc>Rb1>Re>Rb2>Rg1> Rd observed for clone A with Rc and Rb1 as quantitatively dominant saponins and the least amount of Rd, while the pattern Rb2>Re>Rb1>Rc>Rg1>Rd was observed for clone B with metabolite Rb2, being significantly predominant; as in clone A, the lowest content was determined for saponin Rd.

A different pattern was observed for clone G: Re>Rb1 >Rg1>Rc>Rd>Rb2. The main ginsenoside, i.e. metabolite Re, constituted 41.51% of total examined saponin content (Table 1), being twice that calculated for Re in the A (16.77%) and B (20.78%) clones. In contrast to clone B, the lowest level was found for saponin Rb2.

Table 1. Ginsenoside content in the studied clones of *P. quinquefolium* hairy root cultures

Clone	Ginsenosides [mg g^{-1}d.w.]±SE						
	Rb1	Rc	Rb2	Rd	Rg1	Re	Total
A	$3.30^{a} \pm 0.33$	$3.88^{a} \pm 0.79$	$2.12^{a} \pm 0.05$	$1.22^{a} \pm 0.18$	$1.40^{a} \pm 0.03$	$2.40^{a} \pm 0.02$	$14.32^{a} \pm 1.31$
B	$2.73^{b} \pm 0.09$	$1.84^{b} \pm 0.61$	$5.00^{b} \pm 0.65$	$0.65^{b} \pm 0.09$	$1.40^{a} \pm 0.08$	$3.05^{b} \pm 0.08$	$14.76^{a} \pm 0.46$
G	$2.44^{b} \pm 0.33$	$1.23^{b} \pm 0.18$	$0.08^{c} \pm 0.02$	$0.52^{b} \pm 0.03$	$1.63^{b} \pm 0.10$	$4.18^{c} \pm 0.18$	$10.08^{b} \pm 0.6$

Values in each column marked with the same letter mean they do not differ significantly according to the Kruskall-Wallis test ($p>0.05$)

The enhancement of the inhibitory effect of antibiotics against pathogenic bacteria by *Panax quinquefolium* hairy root extracts

In general, supplementation with extracts increased the activity of the tested antibiotics (Table 2). Supplementation of ampicillin with extracts obtained from B and G hairy root clones caused a significant reduction of MIC values of antibiotic against *E. faecalis* isolate from 64 µg mL^{-1} to <0.5 µgmL^{-1}. Similarly, the extracts from the B and G clones enhanced the action of tetracycline against *E. coli* and *A. baumannii* isolates, and the clone A extract slightly enhanced that of tetracycline to a lesser degree, mainly against the *E. coli* isolate.

Table 2. The effect of chosen concentrations of hairy root extracts and antibiotics on pathogenic bacteria

MIC of the antibiotics	Concentration of tested extracts from hairy root clones (mg mL^{-1})	MIC for combination of antibiotics and extracts from hairy root clones (µg mL^{-1})		
		A	B	G
Enterococcus faecalis 64 µg mL^{-1} (ampicillin)*	0.5	64	64	64
	1	1.0	<0.5	<0.5
	1.5	1.0	<0.5	<0.5
	2.0	<0.5	<0.5	<0.5
Escherichia coli 32 µg mL^{-1} (tetracycline)*	0.5	32	32	32
	1	8.0	1.0	1.0
	1.5	8.0	<0.5	<0.5
	2.0	4.0	<0.5	<0.5
Acinetobacter baumannii 128 µg mL^{-1} (tetracycline)*	0.5	128	128	64
	1	2.0	<0.5	<0.5
	1.5	1.0	<0.5	<0.5
	2.0	1.0	<0.5	<0.5

* ≥16 µg mL^{-1} resistance according to CLSI recommendation

DISCUSSION

In contrast to the earlier study on *P. quinquefolium* hairy roots cultivated in unmodified B-5 medium (13), our present findings indicate that the age of the culture, expressed as the number of passages and composition of the medium, may influence the total ginsenoside yield and the profile of individual metabolites. The total ginsenoside content of lines A, B, and G investigated in the present study was found to be approximately 14.68, 14.32, and 10.07 mg g^{-1} d.w. respectively. A similar investigation found that hairy root cultures of *P. ginseng* accumulated a lower level of total ginsenosides (2.58–5.44 mgg^{-1} d.w.) (18) than that of *P. quinquefolium* described in the present study. Among protopanaxadiol derivatives, ginsenoside Rb1 prevailed quantitatively in clones A and G, similar to other hairy root lines of ginseng (20, 38). In clone B, the main saponin, belonging to Rb group, was metabolite Rb2. This compound quantitatively dominated in leaves

of blossoming soil-grown 4-year-old *P. quinquefolium* plants (15). In addition, our findings confirm the previous ones indicating that the level of saponin Rd was present in significantly lower amounts than those of the other ginsenosides (38–40).

All hairy root cultures described in this paper contained more ginsenoside Re than Rg1. Similar results with reference to protopanaxatriol derivative content were also noticed for *P. ginseng* and *Panax* hybrid (*P. ginseng* x *P. quinquefolium*) hairy roots (18, 39).

The present report is the first to describe the ability of *Panax quinquefolium* hairy root extracts to enhance the action of recommended antibiotics against pathogenic bacteria. So far, only few studies have focused on the antimicrobial effect of ginseng extracts and they were related to the roots from field-grown plants. The antibacterial activity of a single ginsenosides and the total extract was described by Battinelli et al. (1). The authors found that total extract from *Panax ginseng* root did not show antimicrobial activity and they explained this fact as low ginsenosides concentration in the extract. It was inactive against all Gram-positive and Gram-negative microorganisms, but ginsenosides Re and Rg1 used alone possessed strong antibacterial activity against bacteria and yeast. Probably, the ability to interact with antibiotics of extracts noticed in our study could be related to the level of ginsenoside Re. The highest concentration of these ginsenosides were detected in B and G clones. Other authors reported that saponins isolated from the root of *P. quinquefolium* exhibited antimicrobial properties against three species of halitosis: *Fusobacterium nucleatum*, *Clostridium perfringens*, and *Porphyromonas gingivalis* (42). Furthermore, these saponins also inhibit the growth of bacteria that cause acne: *Propionibacterium acnes* (ATCC 11827 and ATCC 6919), *Staphylococcus epidermidis* (ATCC 12228), and *Staphylococcus aureus* (ATCC 25923). *P. acnes* ATCC6919 was mostly susceptible to the studied ginsenoside fraction with values of MIC and MBC both 64 μgmL^{-1}. For the remaining bacteria, the values of these parameters were higher (37).

An interesting study by Schmidt et al. (25) indicates that saponins have the potential to increase the action of antibiotics. To be more precise, that vancomycin-resistant *Enterococcus faecium* demonstrates increased susceptibility to the antibiotics gentamicin, teicoplanin or daptomycin when applied in combination with glycyrrhizin acid. Very good results were achieved, with the MIC values being reduced from 2 mg L^{-1} to $\leq$ 0.125 mg L^{-1} and from >8 mg L^{-1} to 1 mg L^{-1}, especially with the use of gentamicin. Similarly, Sung et al. (36) report that saponins from Korean red ginseng demonstrate synergistic or additive interaction with kanamycin or cefotaxime against methicillin-resistant *Staphylococcus aureus*.

Based on the levels of protopanaxadiol and protopanaxatriol derivatives in the studied hairy roots, it is possible that the antimicrobial activity of A, B, and

G clones is associated with their Rg group content. Clones B and G demonstrated very similar properties, both were more potent than clone A. In addition, both contained more Rg1 and Re metabolites than clone A, while clone G possessed 14% more Rg1 and 27% more Re than clone B.

The results obtained in this study have shown that *P. quinquefolium* extracts can probably enhance the inhibitory effects of antibiotics and could be potentially used to reduce the doses of antibiotics when treating patients. Further studies of extracts from several hairy root clones with different ginsenosides proportions were performed by using a checkerboard method which allows to define the type of interaction (additive or synergistic) for connections of extracts and antibiotics that are recommended.

CONCLUSION

The studied lines of *P. quinquefolium* hairy roots differ with regard to the quantitative content of individual ginsenosides. Our results indicate that the content of protopanaxatriol derivatives (Re and Rg1) probably favours antibacterial activity.

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