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Chemical Composition of East Asian Invasive Knotweeds, their Cytotoxicity and Antimicrobial Efficacy Against Cariogenic Pathogens: An *In-Vitro* Study

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Background: Giant knotweeds originating from East Asia, such as *Reynoutria japonica*, and *Reynoutria sachalinensis*, and their hybrid such as *Reynoutria* x *bohemica*, are invasive plants in Europe and North America. However, *R. japonica* is also a traditional East Asian drug (*Polygoni cuspidati rhizoma*) used in Korean folk medicine to improve oral hygiene. The aim of this study was to evaluate the antibacterial activity of acetone extracts of *Reynoutria species* against dominant caries pathogen such as *Streptococcus mutans* and alternative pathogens, as well as characterize the phytochemical composition of extracts and examine their cytotoxicity.

Material/Methods: Ultrasonic extraction was used to obtain polyphenol-rich extracts. The extracts were characterized by HPLC-DAD-ESI-MS. To test bacterial viability, the minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) against *S. mutans, S. salivarius, S. sanguinis,* and *S. pyogenes* were determined. The cytotoxicity of the extracts to human fibroblasts derived from gingiva was evaluated using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay.

Results: The *R. japonica* extract had the highest bacteriostatic and bactericidal activity against pathogens causing caries, mainly dominant caries pathogen *S. mutans* (mean MIC 1000 µg/mL and MBC 2000 µg/mL), which was most likely associated with a higher content of stilbene aglycons and anthraquinone aglycons in the extract. Moreover, the *R. japonica* extract demonstrated the lowest cytotoxic effect on human fibroblasts and exhibited cytotoxic activity only at the concentration causing the death of all *S. mutans*.

Conclusions: The results indicate that the *R. japonica* acetone extract can be considered as a natural, antimicrobial agent for caries control.

MeSH Keywords: Dental Caries • Dental Research • Medicine, East Asian Traditional • Plants, Medicinal

Abbreviation: **MIC** – minimal inhibitory concentration; **MBC** – minimal bactericidal concentration

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Background

Dental caries are induced by dental plaque, the community of microorganisms (biofilm), and it is the most common tooth disease affecting humans worldwide [1,2]. Among bacterial pathogens, *S. mutans* are considered crucial cariogenic pathogens [3] but other Gram-positive bacteria such as *S. sanguinis* and *S. salivarius*, are also commonly associated with dental caries [4,5]. The mechanical removal of dental plaque by tooth brushing and flossing is an effective method for caries prevention. However, most people brush their teeth improperly and, thus, remove plaque insufficiently [6,7]. Consequently, mouthwashes and dentifrices are recommended for dental biofilm control as adjuncts to mechanical removal [8,9]. The gold standard antimicrobial substance is chlorhexidine digluconate, which when used as an addition to a toothpaste or mouthwash, can have undesirable side effects including tooth staining, taste alteration, and the development of hypersensitivity reactions [10-12], which understandably encourages the search for new remedies. *Reynoutria japonica* Houtt. (syn. *Fallopia japonica* (Houtt.) Ronse Decr., *Polygonum cuspidatum* Sieb. & Zucc.) is a traditional East Asian drug also known as *Polygoni cuspidati rhizoma*. Among numerous traditional uses, it has been applied in Korean folk medicine to maintain oral hygiene [13]. *R. japonica* has been considered in Europe and North America as an invasive plant for a long time, however, it has recently been included in the European Pharmacopoeia (European Pharmacopoeia, 2017) [14] which creates the possibility of using it as a traditional botanical drug. In Europe, *R. sachalinensis* (F. Schmidt) Nakai, is commonly found, which is morphologically similar to *R. japonica* and its hybrids *R. x bohemica* Chrtek & Chrtková. However, *R. sachalinensis* and *R. x bohemica*, which are often confused with *R. japonica*, are not included in European Pharmacopoeia. Recent studies have shown that a methanolic extract of *R. japonica* rhizomes and its fractions demonstrated antibacterial activity against *Streptococcus mutans* [13,15,16]. A previous study revealed that these plants contained high levels of potentially antibacterial polyphenols including stilbenes, anthraquinones, and phenylpropanoid glycosides; however, the amount of these constituents differed significantly between plant species [17]. Previous research also determined that extraction of *Reynoutria sp.* rhizomes with 70% acetone instead of methanol made it possible to obtain a plant extract that was richer in polyphenols.

Considering that available in Europe, *Reynoutria* sp. might be a source of anticaries compounds, investigating its biological effects in the dental field is needed. Therefore, the aim of this study was to evaluate the antibacterial activity of acetone extracts of two *Reynoutria* sp. and one hybrid against dominant caries pathogen *S. mutans* and three other pathogens commonly associated with dental caries: *S. sanguinis, S. salivarius,* and *S. pyogenes*. Another aim of this study was to evaluate the cytotoxicity of these plant extracts to human fibroblasts. The final aim of this study was to characterize the phytochemical composition of the extracts. Our study hypothesis was that acetone extracts of *Reynoutria sp.* exhibit bactericidal effect against model cariogenic pathogens with low cytotoxicity.

Material and Methods

Solvents and reagents

Chromatography solvent LC-MS-grade acetonitrile, water, and formic acid was obtained from Merck. Other solvents of analytical grade were purchased from POCh.

Plant materials and extract preparation

The plant raw material was collected from the same spot and extract preparation method was the same as described in the authors previous study [17].

Rhizomes of the studied plants were collected in the last week of September from synanthropic habitats in remote areas of the city of Wroclaw (Poland): *R. japonica* (51°07.404' N 17°04.146' E), *R. sachalinensis* (51°06.190' N 17°08.635' E), *R. x bohemica* (51°05.666' N 17°01.746' E). All raw materials were collected just before the onset of dormancy. Species were identified by Klemens Jakubowski (MSc Botany) from the Botanical Garden of Medicinal Plants herbarium based on the morphology of vegetative and generative organs according to available floras and the pharmacopoeia monograph. Air-dried and powdered rhizomes of *R. japonica*, *R. sachalinensis,* and *R. x bohemica* (400 g of each species) were extracted in 4 steps with 70% acetone (each extraction in an ultrasonic bath, 2 hours). The solvent was evaporated under reduced pressure and 50 mg of dried extracts were dissolved in 80% MeOH in volumetric flasks to get a 5 mg/mL concentration. Then, the solutions were filtered through a 0.22 µm Chromafil syringe membrane (Macherey-Nagel) to autosampler vials and injected into the HPLC system. For the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay and antibacterial activity, a stock solution was prepared using dried extracts by dissolving 100 mg of an extract in 1 mL of DMSO. By taking an appropriate amount of the stock solution, different concentrations of extracts (250–4000 µg mL^{-1} for the antimicrobial test and 5–2000 µg mL^{-1} for the cytotoxicity evaluation) were investigated.

HPLC apparatus

The Ultimate 3000RS system (Thermo Dionex) equipped with a low-pressure quaternary gradient pump was used, including a vacuum degasser, an autosampler, a column compartment,

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a diode array detector, and a high-resolution time-of-flight mass spectrometer (Bruker qTOF Compact, Bruker Daltonik) equipped with electrospray ionization (ESI). The system was controlled by Bruker Hystar.

UHPLC-DAD-MS conditions

The Kinetex C_{18} 2.6 µm analytical column (150×2.1 mm), (Phenomenex) was maintained at 30°C. The mobile phase consisted of solvent A (water: formic acid at 100: 0.1, v/v) and B (acetonitrile: formic acid at 100: 0.1, v/v). Gradient elution was programmed as follows: 0–22 minutes 15–22% B, 22–33 minutes 22–95% B, flow rate 0.3 mL/minute; column equilibration at 12 minutes between injections. UV-Vis spectra were recorded in the range of 200–450 nm. Chromatograms were acquired at 305 nm. The high-resolution time-of-flight mass spectrometer was equipped with electrospray ionization (HPLC/UV/ESI-HR-TOF-MS). ESI-MS conditions: splitless, nebulizer pressure 30 psi; dry gas flow 8 L/minute; dry temperature 250°C and capillary voltage 2.2 kV for negative ion mode. The analysis was carried out using a scan from 50 m/z to 2200.

Antimicrobial testing

For the purpose of this *in vitro* study, we selected 4 bacterial strains: S. *mutans* which is considered a crucial pathogen in dental caries, *S. sanguinis* and *S. salivarius,* which are commonly associated with dental caries, and *S. pyogenes*. Among the evaluated bacteria, *S. pyogenes*, also known as group A *Streptococcus* (GAS), is the only one not associated with dental caries directly; however, it is unique for its ability as a human pathogen to cause a wide variety of clinical infections and post-infectious sequelae, and it is a model for Gram-positive bacteria.

The following microorganisms were used in this study: *Streptococcus mutans* ATCC 25175, *Streptococcus sanguinis* ATCC 13419, *Streptococcus salivarius* ATCC 10556, and *Streptococcus pyogenes ATCC 12344*.

Preparation of bacterial suspension

Each bacterial species was inoculated on plates containing Columbia Agar with 5% sheep blood (BioMerieux) and incubated at 37°C for 24 hours. Then the colonies were transferred from the plates to normal saline to obtain a suspension absorption rate equal to a 0.5 McFarland standard solution; the density of the cell suspension was assessed spectrophotometrically (Densima, bioMerieux). The resultant suspension contained 1.5×10^8 CFU/mL.

Minimum inhibitory concentration (MIC)

The minimum inhibitory concentration (MIC) was determined using agar dilution. The Muller-Hinton agar (MHA, Oxoid) supplemented with 5% defibrinated sheep blood (MHA-B) was used. To prepare agar dilution plates, the stock solutions of extracts were incorporated at 7 volumes (from 1.25 mL to 20 mL) to obtain concentrations ranging from 250 to 4000 µg/mL. The stock solutions (100 mg/mL) were added to warm MHA-B (50°C) and poured into 9 cm Petri dishes. Suspensions of the test strains were diluted 1: 10 in normal saline, and 20 mL of each suspension was transferred onto the agar surface. Two plates without extracts and 1 plate with only 4% DMSO were inoculated to serve as growth controls and be used to check for contamination. MHA-B plates were incubated microaerophilically at 37°C for 24 hours. The lowest concentration of the extract that completely inhibited the growth of bacteria was considered the MIC.

Minimum bactericidal concentration (MBC)

Extract dilutions were made in a concentration range from 250 µg/mL to 4000 µg/mL in 500 mL sterile test tubes containing Brain Heart Infusion broth (BHI, bioMerieux). The contents of each tube were inoculated with 0.1 mL of the bacterial suspension and then were incubated at 37°C for 24 hours. After incubation, the microbial growth was determined by plating 20 µL samples from the tubes on a nutrient agar medium: Columbia Agar with 5% sheep blood (bioMerieux) and Agar (BHIA, bioMerieux). The MBC was defined as the lowest concentration of the extract that did not permit any visible growth on the appropriate agar plate after the incubation period.

Cell culture

This *in vitro* study was performed on human gingival fibroblasts obtained from a primary culture. The study protocol was accepted by the Bioethics Commission of Wrocław Medical University, No. KB-434/2017. The tissue cultures of human gingival fibroblasts were derived from healthy adult volunteers undergoing minor surgical procedures of epithelial-connective tissue grafting. The gingival biopsy was provided by the Department of Dental Surgery. The epithelial-connective tissue fragment was removed from the hard palate in a small portion about 1–2 mm^2 using the "punch" method. This method allows you to obtain the connective tissue layer located closest to the epithelium, which is characterized by significantly better keratosis [18]. The obtained material was transported in a nutritional medium (Dulbecco Modified Eagle Medium, DMEM) to the cell culture laboratory. The DMEM contained 10% fetal calf serum (FCS) and antibiotics (penicillin 100 IU/mL, streptomycin 100 µg/mL, and amphotericin B 100 µg/mL). To obtain a stable cell culture, the cells were

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Table 1. Antibacterial activity (MIC and MBC) of extracts of *Reynoutria* species.

Strains	*R. japonica*		*R. sachalinensis*		*R. bohemica*	
	MIC	MBC	MIC	MBC	MIC	MBC
Streptococcus mutans ATCC 25175	1000	2000	1000	4000	1000	4000
Streptococcus sanguinis ATCC® 10556	750	2500	1000	4000	1000	3000
Streptococcus pyogenes ATCC 12344	1500	4000	1500	4000	3500	4000
Streptococcus salivarius ATCC® 13419	3500	>4000	4000	>4000	>4000	>4000

>4000: not detected by the highest concentration of tested samples; controls (4% DMSO), v/v, final concentration showed no MIC against all the strains tested. MIC – minimal inhibitory concentration (µg/ml); MBC – minimal bactericidal concentration (µg/ml).

isolated according to the procedure described and patented by Saczko et al. [19] (Patent No.: P 3812045). Our study involved selecting the best passages of the most efficient fibroblast derived from one patient. The cells were grown in polystyrene flasks with a 25 cm^2 cell culture surface (Falcon) as a monolayer in a DMEM (Sigma-Aldrich, St Louis, MO, USA), which contained 2 mM L-glutamine, 10% fetal bovine serum (FBS, Sigma-Aldrich, St Louis, USA) and 50 µg/mL streptomycin (Sigma-Aldrich, St Louis, USA) at 37°C in 5% CO_2. For the experiments, the cells were removed by trypsinization (trypsin 0.25% and EDTA 0.02%; Sigma-Aldrich, St Louis, MO, USA) and washed with phosphate-buffered saline (PBS).

Cytotoxicity evaluation using MTT assay

The viability of cells was determined by the MTT assay (Sigma-Aldrich, St Louis, MO, USA) after incubation with different concentrations of the tested extracts. The MTT assay was used to estimate the mitochondrial metabolic function through a spectroscopic measurement of mitochondrial dehydrogenase. To conduct the experiment, the cells were seeded into 96-well microculture plates at 1×10^4 cells/well and grown for 24 hours. Then, after a 24 hours or 72 hours incubation with investigated concentrations of the tested extracts (from 5 to 2000 µg/mL), the study was performed according to the manufacturer's protocol. An MTT solution was prepared in PBS (5 mg/mL). The culture medium was carefully removed from the wells of the plate and 100 µL of the MTT solution was added to each well. The plate was incubated at 37°C for 2 hours. After incubation, 100 µL of the MTT solvent (4 mM HCl in absolute isopropanol) was added to each well. The liquid was pipetted in each well until the formazan crystals dissolved entirely. The absorbance was determined using a multi-well scanning spectrophotometer at 570 nm (Enspire Perkin Elmer, Multiplate Reader, USA). The mitochondrial metabolic function was expressed as a percentage of viable treated cells in relation to untreated control cells.

Statistical analysis

Each assay was performed in quadruplicate. The outcome, data were summarized as mean ± standard deviation and analyzed. One-way analysis of variance (ANOVA) was performed, followed by the Tukey test, for comparison of multiple means. The level of significance was $P<0.05$. The results were analyzed statistically with Statistica 13 (StatSoft Poland).

Results

Antibacterial effect of the extracts

The values of MIC and MBC of the *R. japonica, R. sachalinensis*, and *R. x bohemica* extracts tested against *S. mutans*, *S. sanguinis*, *S. pyogenes*, and *S. salivarius* are presented in Table 1. Among the evaluated plant extracts, the 70% acetone extract of *R. japonica* revealed the highest activity against *Streptococcus sp.*, while the MIC and MBC values against *S. mutans* were 1000 units and 2000 units respectively. The remaining plant extracts have also exhibited a moderate antimicrobial activity.

Identification of major constituents in *Reynoutria species*

All identified compounds are presented in Table 2, while the chromatogram of the studied extracts is presented in Figure 1. The HPLC-DAD-HR-MS analysis of extracts revealed differences in the composition of compounds. The chromatogram comparing peak amounts in different extracts demonstrated that the *R. japonica* extract contained the highest amounts of compounds 2, 3, and 5 identified as stilbenes: resveratroloside, piceid, and resveratrol respectively; and compound 16 identified as emodin. On the other hand, the *R. sachalinensis* extract contained the highest amounts of compounds 9, 13, and 15 identified as phenylpropanoids: hydropiperoside, vanicoside B, and vanicoside A respectively, and contained a small amount of emodin with no stilbenes. The phytochemical profile of *R. x bohemica* was intermediate between the 2-parent species.

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Table 2. Retention times, UV $\lambda_{max,}$ MS data and ion formula suggestion of the constituents present in the acetone extracts of rhizomes of *R. japonica*, *R. sachalinensis* and *R. x bohemica*.

	Compound	tR [min]	UV [nm]	qTOF m/z [M-H]-	Error (ppm)	Ion formula**	Meas. m/z Other ions
1	Piceatannol glucoside	3.2	220, 305, 319	405.1191	0.2	C20H21O9	243.0661 [piceatannol]
2	Resveratrolside	3.6	218, 304, 315	389.1242	1.5	C20H21O8	227.0713 [resveratrol]
3	Piceid	5.9	218, 308, 318	389.1242	–0.5	C20H21O8	227.0713 [resveratrol]
4	Epicatechin-3-O-gallate	6.4	220, 279	441.0827	0.3	C22H17O10	–
5	Resveratrol	10.6	218, 306, 319	227.0714	0.7	C14H11O3	–
6	N-trans-feruloyltyramine	11.0	220, 282, 325	312.1241	0.4	C18H18NO4	–
7	Emodin-glucoside	12.8	221, 247, 269, 281, 423	431.0984	–1.5	C21H19O10	–
8	Torachrysone	15.4	220, 312	245.0819	2.9	C14H13O4	–
9	Hydropiperoside	15.4	222, 298, 313	779.2193	–1.9	C39H39O17	–
10	(3,6-*O*-di-p-coumaroyl)-β-fructofuranosyl-(2→1)-(2'-*O*-acetyl-6'-*O*-feruloyl)-**b**-glucopyranoside	15.9	220, 298, 315	851.2404	2.5	C42H43O19	–
11	Vanicoside C	17.3	220, 298, 313	821.2298	1.6	C41H41O18	–
12	Tatariside B	18.1	220, 298, 313	893.2510	0.3	C44H45O20	–
13	Vanicoside B	19.0	222, 298, 315	955.2666	–0.5	C49H47O20	477.1284 $[M-H]^{2-}$
14	Questin	20.2	222, 286, 430	283.0612	0.5	C16H11O5	–
15	Vanicoside A	21.0	222, 298, 315	997.2772	–0.2	C51H49O21	498.1335 $[M-H]^{2-}$
16	Emodin	25.2	221, 248, 267, 288, 430	269.0455	0.6	C15H9O5	–
17	Physcion	29.5	222, 266, 288, 430	–	–	–	–

Cytotoxicity of the extracts to human fibroblasts derived from gingiva

The results obtained using the MTT assay are presented in Figures 2 and 3. The study showed that the *R. japonica* extract exhibited the lowest cytotoxic effect among all tested extracts. It decreased cell viability to 50% only after reaching the highest concentration of 2000 µg/mL, after both 24 hours and 72 hours of incubation. What is more, in the concentration of 1000 µg/mL, in which it revealed an inhibitory effect on the *S. mutans* growth (Table 1), it caused a slight decrease in cell viability (about 6%) after a 24-hour incubation. However, longer incubation (72 hours) decreased cell viability to about 48%.

Discussion

S. mutans and *S. sanguinis* showed much greater sensitivity to the tested extracts than *S. pyogenes* and *S. salivarius*. Still, it is important to note that *S. mutans* is a dominant caries pathogen [20]. When considering the obtained results, it appears that the *R. japonica* extract, if used at concentrations between >1000 and <2000 µg/mL, may be a useful antimicrobial agent for caries control. In the indicated range of concentrations, it caused the inhibition of growth of *S. mutans* and *S. sanguinis* and death of the most these bacteria; at the same time, it did not cause any cytotoxicity to normal human fibroblasts. Moreover, the *R. japonica* extract at concentrations from 5 to 500 µg/mL, following a 24-hour incubation, caused a significant increase in cell viability (up to 38% compared to the control) as shown in Figure 2. This result suggests that the *R. japonica* extract could have a stimulatory effect on normal human fibroblasts and might be used for healing gingiva wounds, where gingival fibroblasts play an active role and are committed to repopulating damaged tissues [21]. It seems most likely that differences in the activity of the studied extracts observed in the antimicrobial assay and the MTT test resulted from disparities in chemical compositions of these extracts. The *R. japonica*

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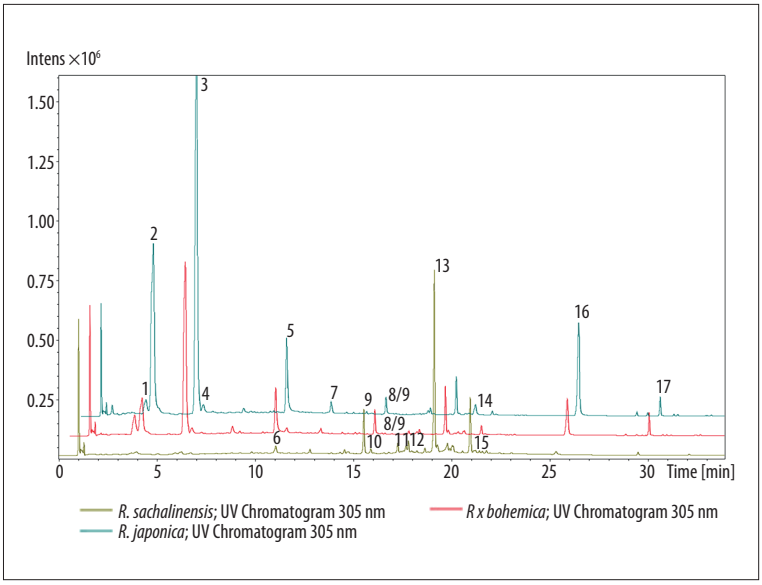

Figure 1. HPLC chromatograms of the acetone extract of rhizomes of *Reynoutria japonica*, *R. x bohemica* and *R. sachalinensis* with detection at 305 nm.

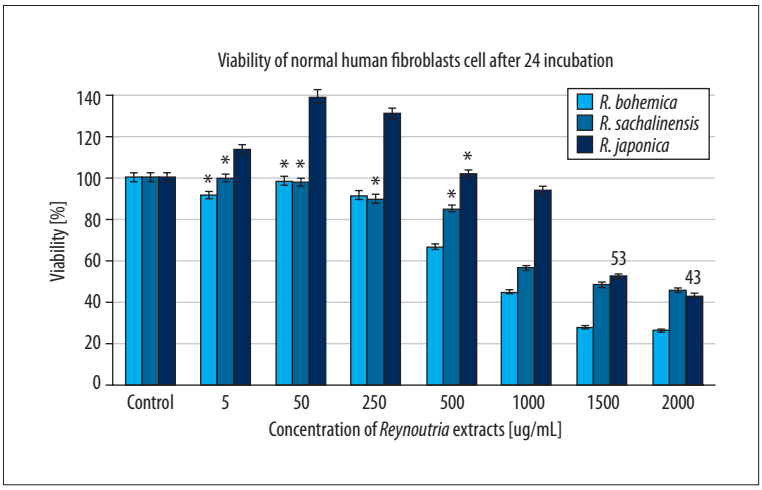

Figure 2. Viability of normal human fibroblasts cell line after 24-hour incubation following increasing concentrations of extracts. Viability is expressed as the percentage of the control cells (cells without extracts). Error bars shown are means ± standard deviation for n=4. * statistically non significant for $P \leq 0.05$.

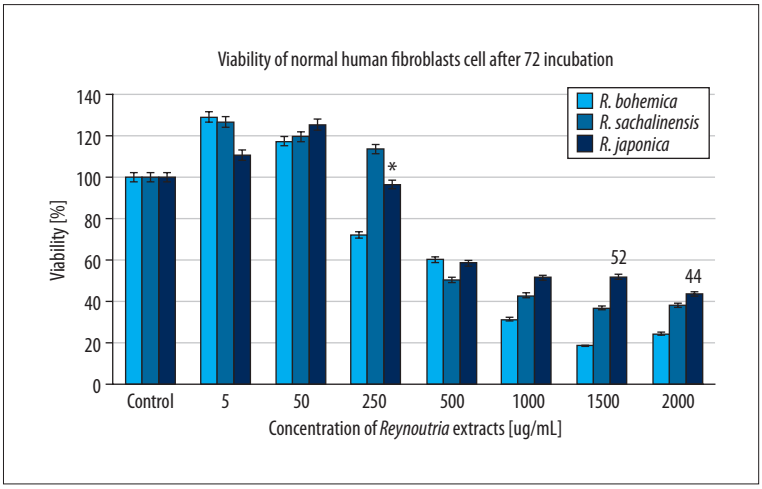

Figure 3. Viability of normal human fibroblasts cell line after 72-hour incubation following increasing concentrations of extracts. Viability is expressed as the percentage of the control cells (cells without extracts). Error bars shown are means ± standard deviation for n=4. * statistically non significant for $P \leq 0.05$.

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extract contained the highest amounts of stilbenes (resveratroloside, piceid, and resveratrol) and anthraquinones (emodin and physcion) among the tested extracts.

Previous studies on acetone extracts of the same species, which determined the amounts of certain compounds in these extracts, revealed that *R. japonica* contained 40.43 mg of piceid in 1.0 g of a raw extract and 3.54 mg/g of resveratrol, whereas *R. bohemica* contained 20.18 mg/g and 2.24 mg/g of piceid and resveratrol respectively [17]. The *R.* x *bohemica* extract also contained a much smaller amount of emodin (8.33 mg/g in the *R.* x *bohemica* extract versus 21.73 mg/g of a raw extract of *R. japonica*). *R. sachalinensis* contained even less anthraquinones and no detectable stilbenes; instead, it contained a considerable amount of phenylpropanoid esters (mainly vanicoside B).

Song et al. [13] demonstrated that a methanolic extract of *R. japonica* rhizomes significantly inhibited the growth of *S. mutans* with the MIC range of 1.0–2.0 mg/mL, depending on the strain, whereas the MBC was 4.0 mg/mL. The results presented in the Table 1 indicate that the acetone extract of *R. japonica*, which was the subject of our study, revealed a higher bactericidal activity against *S. mutans* as well as a higher bacteriostatic activity against *S. sanguinis* and *S. pyogenes*. Active fractions separated from the *R. japonica* methanolic extract by Song et al. [15] demonstrated a high bacteriostatic and bactericidal activity against *S. mutans* with the minimum inhibitory concentration (MIC) range of 31.3–125 µg/mL and the minimum bactericidal concentration (MBC) range of 0.5–1 mg/mL. The HPLC analysis of the most active fraction revealed the presence of resveratrol, emodin, and physcion, which comprised of approximately 16.2%, 18.9%, and 2.07% of the fraction mass respectively [22]. Similarly, the ethyl acetate fraction obtained from the methanolic extract revealed a significant antibacterial activity, exhibited the MIC range of 0.125–1.0 mg/mL, depending on bacterial strains, and the MBC range of 0.5–2.0 mg/mL. The HPLC-UV analysis demonstrated that the ethyl acetate fraction consisted of polydatin, resveratrol, anthraglycoside B, and emodin, while anthraglycoside B and emodin were the main components [16]. When considering the aforementioned results, it may be suggested that the antibacterial activity depends on the presence and amount of anthraquinones (mainly emodin) and stilbenes (mainly resveratrol) in the fraction.

Xu et al. [20] noticed that emodin significantly inhibited the *S. mutans* (ATCC 25175) growth in a dose-dependent manner (0.5–2 mg/mL). Moreover, emodin inhibited the production of acid and insoluble glucans by *S. mutans*, thereby reducing caries induction in rats. In the study by Coenye et al. [23], anthraquinones, especially emodin at concentrations below the MIC, reduced the *S. mutans* biofilm formation on hydroxyapatite. The MIC for emodin was higher than 250 µg/mL, but the growth of biofilms in the presence 5 µg/mL of emodin was reduced to 10.9%. The authors suggested that the antibiofilm effect of emodin was caused by the insertion of a planar molecule into the cell membrane and/or by binding the same molecule to membrane-embedded molecules, including proteins.

Yim et al. [24] analyzed stilbenes and oligostilbenes isolated from leaves and stems of *Vitis amurensis* Rupr. (Vitaceae) and evaluated their antimicrobial activity against 2 oral pathogens: *S. mutans* and *S. sanguinis*. Four stilbenes, trans-ε-viniferin (the most active), piceatannol, trans-resveratrol, and amuresin G, exhibited the highest antibacterial activity. Piceatannol and trans-resveratrol (also present in our acetone extracts) demonstrated a considerable activity against *S. mutans* with the MIC of 50 µg/mL. These compounds also displayed a certain activity against *S. sanguinis* with the MIC values of 50 and 25 µg/mL respectively. Conversely, the glycosides of piceatannol and resveratrol did not inhibit any microbial growth leading to a suggestion that the glycosylation of piceatannol and resveratrol caused a virtually complete loss of antibacterial activity. The results of this study make is possible to conclude that a high antimicrobial activity of the *R. japonica* rhizome was influenced by the content of stilbene aglycones, such as resveratrol and piceatannol, but not stilbene glycosides such as piceid. However, our study revealed that even the *R. sachalinensis* extract, which did not contain stilbenes, and which possess a very small amount of anthraquinones, showed a significant antimicrobial activity with the MIC at 1 mg/mL for *S. mutans* and *S. sanguinis*. This could mean that other substances contained in the *R. sachalinensis* extract also have antibacterial properties. The extract of *R. sachalinensis* contains the highest amount of phenylopropanoid glycosides, mainly vanicoside B. Saito et al. [25] showed that there was antibacterial activity of extracts and fractions from rhizomes and leaves of *R. sachalinensis* against several Gram-positive and Gram-negative bacteria, but streptococci were not among the tested strains. This is the first paper on the antibacterial activity of the *R. sachalinensis* extract against caries pathogens. In future research, the antimicrobial activity of dominant compounds (vanicoside B, vanicoside A, hydropiperoside) of the extract should be investigated to explain its antimicrobial effect.

The MTT assay is used to assess cell metabolic activity. Viable cells with active metabolism convert MTT into a purple-colored formazan product. Dead cells are not able to convert MTT into formazan, thus color formation, measured spectrometrically near 570 nm, is a marker that reflects viable cell metabolism [26,27].

Antioxidants like ascorbic acid are known to interfere with tetrazolium reduction assays through reducing tetrazolium salts non-enzymatically and lead to increased absorbance values in assay wells [28–30]. Antioxidants present in plant extracts might also alter the reliability and sensitivity of the MTT assay [31].

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Our results suggest that *R. japonica* seems to have low cytotoxicity to normal human fibroblasts, but it should be considered whether the compounds present in the extracts interfere with the MTT test. Wang et al. [32] showed that emodin, which was found in *R. japonica* in a much larger amounts than in the remaining extracts, could precipitate from a culture medium given its poor solubility in water. This phenomenon induced a red shift of the emodin absorption curve and increased the overlap of the emodin and formazan absorption curves. At a high concentration of emodin, it might affect a high optical density of studied cells and suggest that emodin might promote the proliferation of cells. However, no precipitation was observed when the emodin concentration was ≤25 µg/mL. If you know the amount of emodin in the *R. japonica* extract, it is easy to calculate that this number has been exceeded the amounted 43.46 µg/mL only at the highest concentration of the extract (2,000 µg/mL). The next observed phenomenon included an observation that emodin could directly reduce the MTT tetrazolium salt to formazan. Formazan formation was correlated with the concentration of emodin, whether the serum was present or not, but it was significantly lower when the serum was present. However, only the highest emodin concentration (100 µg/mL) had a remarkable effect on MTT reduction. In addition, for another strong antioxidant, resveratrol, the high amount present in the *R. japonica* extract might reduce the MTT salt to its blue formazan by a cell-independent chemical reaction [31]. However, it should be noted that in our study the procedure applied in the MTT assay included the removal of the culture medium together with the extracts prior to adding the MTT solution. This ensured the removal of potentially interfering agents [31] and excluded the possibility that the phenomena described would occur. However, we recommend testing, in the future, the cytotoxicity of studied extracts also by using different methods, like sulforhodamine B assay (SRB), which is also considered a reliable test and even preferred by some researchers [33].

The investigation presented in this manuscript was focused on isolated *Streptococci* (*S. mutans*, *S. sanguinis*, *S. salivarius*, and *S. pyogenes)* as an *in vitro* model organisms for dental caries. *In vivo*, oral bacteria are in biofilms and the magnitude of other microorganisms in addition to streptococci are related to caries. Before clinical use, a further evaluation of selected extracts should be carried out for microorganisms obtained from the human host biofilm.

Conclusions

In our experimental study, we found that among the obtained extracts, the 70% acetone extract from the rhizome of *Reynoutria japonica* demonstrated the highest bacteriostatic and bactericidal activity against studied pathogens causing caries, in particular, the dominant caries pathogen *Streptococci mutans*, which was associated with a higher content of stilbene aglycons and anthraquinone aglycons. Furthermore, the *R. japonica* extract displayed low cytotoxicity in bacteriostatic concentrations towards *S. mutans*. and in concentrations below MIC it appears to have stimulatory effect on normal human fibroblasts, which might accelerate healing gingiva wounds.

The results obtained in this study were quite interesting, thus, we propose that acetone extract of rhizome of *R. japonica* can be considered in the future as an antimicrobial agent for caries control. However, it is important to be aware the limitations of the model system, which was focused only on *Streptococci species,* thus, the results are not sufficient to conclude utility of studied extract right now. Therefore, we recommend further detailed *in vitro* and *in vivo* studies related to other oral bacteria to evaluate and ensure effective therapeutic dosage of the studied extract.

Conflict of interest

None.

References:

1. Sampaio-Maia B, Caldas IM, Pereira ML et al: The oral microbiome in health and its implication in oral and systemic diseases. Adv Appl Microbiol, 2016; 97: 171–210
2. Kobierska-Brzoza J, Kaczmarek U: Genetic aspects of dental caries. Dent Med Probl, 2016; 53: 413–18
3. Zhang S: Dental caries and vaccination strategy against the major cariogenic pathogen, *Streptococcus mutans*. Curr Pharm Biotechnol, 2013; 14(11): 960–66
4. Chava VR, Manjunath SM, Rajanikanth AV, Sridevi N: The efficacy of neem extract on four microorganisms responsible for causing dental caries viz *Streptococcus mutans*, *Streptococcus salivarius*, *Streptococcus mitis* and *Streptococcus sanguis*: An *in vitro* study. J Contemp Dent Pract, 2012; 13(6): 769–72
5. Loesche WJ, Walenga A, Loos P: Recovery of *Streptococcus mutans* and *Streptococcus sanguis* from a dental explorer after clinical examination of single human teeth. Arch Oral Biol, 1973; 18(4): 571–75
6. Pesevska S, Ivanovski K, Mindova S et al: Bacterial contamination of the toothbrushes. J Int Dent Med Res, 2016; 9(1): 6–12
7. Badruddin IA, Kiptiyah NM, Prihartono N et al: The association between sweet food consumption, time of tooth brushing and dental caries experience in 12- to 15-year-old children in Indonesia (analysis of Indonesian health basic research data, 2013). J Int Dent Med Res, 2017; 10(Special Issue): 583–89
8. Baehni PC, Takeuchi Y: Anti-plaque agents in the prevention of biofilm-associated oral diseases. Oral Dis, 2003; 9: 23–29
9. Sreenivasan P, Gaffar A: Antiplaque biocides and bacterial resistance: A review. J Clin Periodontol, 2002; 29(11): 965–74
10. Parwani SR, Parwani RN, Chitnis PJ et al: Comparative evaluation of antiplaque efficacy of herbal and 0.2% chlorhexidine gluconate mouthwash in a 4-day plaque re-growth study. J Indian Soc Periodontol, 2013; 17(1): 72–77

Indexed in: [Current Contents/Clinical Medicine] [SCI Expanded] [ISI Alerting System] [ISI Journals Master List] [Index Medicus/MEDLINE] [EMBASE/Excerpta Medica] [Chemical Abstracts/CAS]

11. Chang YC, Huang FM, Tai KW, Chou MY: The effect of sodium hypochlorite and chlorhexidine on cultured human periodontal ligament cells. Oral Surg Oral Med Oral Pathol Oral Radiol Endod, 2001; 92(4): 446–50
12. Beaudouin E, Kanny G, Morisset M et al: Immediate hypersensitivity to chlorhexidine: Literature review. Eur Ann Allergy Clin Immunol, 2004; 36(4): 123–26
13. Song JH, Kim SK, Chang KW et al: *In vitro* inhibitory effects of Polygonum cuspidatum on bacterial viability and virulence factors of *Streptococcus mutans* and *Streptococcus sobrinus*. Arch Oral Biol, 2006; 51(12): 1131–40
14. European Pharmacopoeia. In European Directorate for the Quality of Medicines. 9th ed. Strasburg, France, 2017; 1481–83
15. Song JH, Yang TC, Chang KW et al: *In vitro* effects of a fraction separated from Polygonum cuspidatum root on the viability, in suspension and biofilms, and biofilm formation of mutans streptococci. J Ethnopharmacol, 2007; 112(3): 419–25
16. Ban SH, Kwon YR, Pandit S et al: Effects of a bio-assay guided fraction from Polygonum cuspidatum root on the viability, acid production and glucosyltranferase of mutans streptococci. Fitoterapia, 2010; 81(1): 30–34
17. Nawrot-Hadzik I, Granica S, Domaradzki K et al: Isolation and determination of phenolic glycosides and anthraquinones from rhizomes of various *Reynoutria species*. Planta Med, 2018; 84(15): 1118–26
18. Zucchelli G, Mele M, Stefanini M et al: Patient morbidity and root coverage outcome after subepithelial connective tissue and de-epithelialized grafts: A comparative randomized-controlled clinical trial. J Clin Periodontol, 2010; 37(8): 728–38
19. Saczko J, Dominiak M, Kulbacka J et al: A simple and established method of tissue culture of human gingival fibroblasts for gingival augmentation. Folia Histochem Cytobiol, 2008; 46(1): 117–19
20. Xu JS, Cui Y, Liao XM et al: Effect of emodin on the cariogenic properties of *Streptococcus mutans* and the development of caries in rats. Exp Ther Med, 2014; 8(4): 1308–12
21. Huynh NC, Everts V, Leethanakul C et al: Rinsing with saline promotes human gingival fibroblast wound healing *in vitro*. PLoS One, 2016; 11(7): 1–13
22. Pandit S, Kim HJ, Park SH, Jeon JG: Enhancement of fluoride activity against *Streptococcus mutans* biofilms by a substance separated from Polygonum cuspidatum. Biofouling, 2012; 28(3): 279–87
23. Coenye T, Honraet K, Rigole P et al: *In vitro* inhibition of *Streptococcus mutans* biofilm formation on hydroxyapatite by subinhibitory concentrations of anthraquinones. Antimicrob Agents Chemother, 2007; 51(4): 1541–44
24. Yim N, Ha do T, Trung TN et al: The antimicrobial activity of compounds from the leaf and stem of *Vitis amurensis* against two oral pathogens. Bioorg Med Chem Lett, 2010; 20(3): 1165–68
25. Saito M, Kawai Y, Yamazaki K et al: Antimicrobial activities of extracts from Sachaline Giant Knotweed Polygonum sachalinense. Food Sci Technol Int Tokyo, 1997; 3(3): 290–93
26. Huyck L, Ampe C, Troys M: The XTT cell proliferation assay applied to cell layers embedded in three-dimensional matrix. Assay Drug Dev Technol, 2012; 10(4): 382–92
27. Riss TL, Moravec RA, Niles AL et al: Cell viability assays. In: Sittampalam GS, Coussens NP, Brimacombe K et al. (eds.), Assay guidance manual [Internet]. Bethesda (MD): Eli Lilly & Company and the National Center for Advancing Translational Sciences; 2013; 785–96. *https://www.ncbi.nlm.nih.gov/books/NBK53196/*
28. Ulukaya E, Colakogullari M, Wood EJ: Interference by anti-cancer chemotherapeutic agents in the MTT-tumor chemosensitivity assay. Chemotherapy, 2004; 50(1): 43–50
29. Chakrabarti R, Kundu S, Kumar S, Chakrabarti R: Vitamin A as an enzyme that catalyzes the reduction of MTT to formazan by vitamin C. J Cell Biochem, 2000; 80(1): 133–38
30. Bernas T, Dobrucki J: Mitochondrial and nonmitochondrial reduction of MTT: interaction of MTT with TMRE, JC-1, and NAO mitochondrial fluorescent probes. Cytometry, 2002; 47(4): 236–42
31. Bruggisser R, von Daeniken K, Jundt G et al: Interference of plant extracts, phytoestrogens and antioxidants with the MTT tetrazolium assay. Planta Med, 2002; 68(5): 445–48
32. Wang X, Ge J, Wang K et al: Evaluation of MTT assay for measurement of emodin-induced cytotoxicity. Assay Drug Dev Technol, 2006; 4(2): 203–7
33. van Tonder A, Joubert AM, Cromarty AD: Limitations of the 3-(4,5 dimethylthiazol-2-yl)- 2,5-diphenyl-2H-tetrazolium bromide (MTT) assay when compared to three commonly used cell enumeration assays. BMC Res Notes, 2015; 8: 47

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