

A STUDY OF ANTIMICROBIAL ACTIVITY OF POLYPHENOLS DERIVED FROM WOOD

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Due to the spreading and increasing drug resistance of pathogens, the search for novel antibiotics is becoming ever more important. Plant-derived polyphenols are a vast and promising class of compounds with a potential to fight infectious diseases. Still, they are not routinely used in clinical practice. No reports on the *in vivo* studies of these compounds have been presented. The aim of our work was to compare the antimicrobial activity of resveratrol (stilbene), dihydroquercetin and dihydromyricetin (flavonols) extracted from the bark and wood of conifers against the dermatophytes *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Candida albicans*. Using the radial diffusion assay, we established that dihydroquercetin, resveratrol and dihydromyricetin exhibit high activity against *S. aureus* even at the smallest possible concentrations of 0.22, 0.15, and 0.15 mM, respectively. In contrast, the highest achievable concentrations of these compounds in the solutions (21.5, 15.5 and 15.0 mM for dihydroquercetin, resveratrol and dihydromyricetin, respectively) have no effect on the growth of *P. aeruginosa* and *C. albicans*. These findings suggest that polyphenols derived from conifers could have a potential to be used as a medicine for topical application to treat bacterial infections of the skin caused by *S. aureus*.

Keywords: polyphenols, flavonoid, stilbene, antimicrobial agents, resveratrol, antimicrobial activity, antioxidants, wood

Funding: this work was supported by the Ministry of Education and Science of the Russian Federation (Subsidy Contract No 14.616.21.0083 since July 17, 2017; ID RFMEFI61617X0083).

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Received: 13.06.2018 **Accepted:** 20.07.2018

DOI: 10.24075/brsmu.2018.040

ИССЛЕДОВАНИЕ АНТИМИКРОБНОЙ АКТИВНОСТИ ПОЛИФЕНОЛОВ ИЗ ДРЕВЕСНОГО СЫРЬЯ

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В связи с выработкой патогенами лекарственной устойчивости к используемым антибиотикам поиск антимикробных агентов нового типа приобретает все большую актуальность. Растительные полифенолы — обширный и перспективный в борьбе с инфекционными заболеваниями класс соединений. Однако они почти не используются в медицинской практике, а результаты их биологических испытаний *in vivo* в литературе отсутствуют. Целью работы было провести сравнительное исследование антимикробной активности препаратов полифенолов ресвератрола (стильбен), дигидрокверцетина и дигидромирицетина (флавонолы), выделенных из коры и древесины хвойных пород, в отношении дерматофитов: *Staphylococcus aureus*, *Pseudomonas aeruginosa* и *Candida albicans*. В тесте на подавление роста газона индикаторных культур в условиях радиальной диффузии из лунки установлено, что все три соединения проявляют высокую активность в отношении *S. aureus*: концентрации 0,22 мМ для дигидрокверцетина, 0,15 мМ для ресвератрола и 0,15 мМ для дигидромирицетина превышают предел эффективности. Напротив, в отношении *P. aeruginosa* и *C. albicans* даже максимально возможные с учетом растворимости концентрации полифенольных соединений 21,5 мМ, 15,5 мМ и 15,0 мМ не оказывают какого-либо эффекта на рост культур. Полученные данные позволяют рассматривать полифенолы из хвойных растений в качестве перспективного наружного средства для лечения бактериальных инфекций кожи, вызываемых *S. aureus*.

Ключевые слова: полифенолы, флавоноиды, стильбены, антимикробные средства, ресвератрол, антимикробная активность, антиоксиданты, древесное сырье

Финансирование: исследование поддержано Министерством образования и науки Российской Федерации (Соглашения о предоставлении субсидии № 14.616.21.0083 от 17.07.2017, уникальный идентификатор RFMEFI61617X0083).

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Статья получена: 13.06.2018 **Статья принята к печати:** 20.07.2018

DOI: 10.24075/vrgmu.2018.043

Polyphenols are secondary metabolites massively produced by plants. They have found wide application in the pharmaceutical industry and are routinely used as basic ingredients in complementary medicine. So far, over 8,000 phenolic compounds of plant origin have been identified. They are classified into simple phenols, derivatives of hydroxy cinnamyl and hydroxy benzyl alcohols, flavonoids, stilbenes, and lignans [1]. The largest class of polyphenols is constituted by flavonoids. Their core is formed by a flavan nucleus: a structure consisting of two aromatic rings linked by a chain of three carbon atoms [2]. Flavonoids (chalcones, catechins, anthocyanins, leucoanthocyanins, flavones, flavanones, and flavonols) are found in abundance in bark, flowers, seeds and fruits. Stilbenes, another group of polyphenols, have two benzene rings and are related to flavonoids.

Stilbenes exert antioxidant and antimicrobial activity protecting plants from the harmful effects of phytopathogens, ozone and ultraviolet light. Stilbenes are found in various phyla and in conifers in particular [3]. The most famous representative of stilbenes is resveratrol. Other stilbenes, including pterostilbene, pinosylvin and rhaponticin, have also exhibited a similar type of activity [2].

Functionally, stilbenes and flavonoids belong to a class of phytoalexins, natural antibiotics of plants that protect the latter against infectious diseases and are synthesized in response to bacterial or fungal infections. It is reasonable to assume that phytoalexins will also demonstrate their bactericidal activity *in vitro*. As a rule, the extracts of herbs rich in flavonoids, such as chamomile, St. John's wort, plantain, and marigold, have a marked antibacterial effect [4]. In recent years, there has been growing interest in phytoalexins stimulated by the challenge of drug resistance and the ability of bacteria to form biofilms normally studied in such important model pathogens as *Mycobacterium tuberculosis*, *Streptococcus pneumoniae*, *Neisseria gonorrhoeae*, and *Streptococcus mutans* [5]. The most promising polyphenols capable of neutralizing these bacteria are a group of red wine polyphenols responsible for the "French paradox", such as quercetin, kaempferol, resveratrol, and some others [6]. However, despite a surge of interest in this problem, the mechanisms of antibacterial activity of stilbenes and flavonoids remain obscure, although it is known that all subclasses of flavonoids have bactericidal and fungicidal effects [7]. For example, resveratrol inhibits growth of *Helicobacter pylori*, a pathogen that causes peptic ulcer, *in vitro* [8], enhances phagocytosis of *C. albicans* [9] and suppresses phagocytosis of *S. aureus* and *E. coli*; this effect is mediated by the TLR-2 receptors of the innate immunity.

Although there is a huge body of evidence about the antimicrobial activity of plant-derived polyphenols, their use in clinical practice is limited to herbal extracts with unknown or only roughly estimated concentrations of active ingredients. As of today, a few companies and organizations, including TransMIT Gesellschaft für Technologietransfer mbH (Germany) and Favorsky Irkutsk Institute of Chemistry, have launched production of purified polyphenols derived from the bark and wood of conifers. These formulations could be a good option in the treatment of pyogenic and inflammatory conditions caused by infection, considering a rapid spread of multidrug-resistant bacteria.

In this study, we aimed to measure the antimicrobial activity of commercial polyphenols produced from the available raw materials against dermatophytes *S. aureus*, *P. aeruginosa* and *C. albicans* and to compare it between the studied compounds and with the activity of the antimicrobial ointments for topical application routinely used to treat similar infections.

METHODS

Resveratrol and dihydromyricetin extracted from conifer wood were courtesy of TransMIT Gesellschaft für Technologietransfer mbH (Germany). Dihydroquercetin was provided by the laboratory of Favorsky Irkutsk Institute of Chemistry. Quality control by ¹H-nuclear magnetic resonance spectrometry was performed on the Bruker AM-300 spectrometer (Bruker Daltonics GmbH; Germany). Resveratrol used in the study was a homogenous 100% trans-isomer.

The studied compounds were dissolved in nonpyrogenic injection-grade normal saline (Escom; Moscow) and passed through Sterile Minisart® syringe filters with polyether sulfone membranes (Sartorius; Germany). Solutions of polyphenolic compounds were stored in 50-ml polypropylene screw-cap tubes at +4 °C for 1 month away from direct sunlight.

Levomecol (Nizhfarm; Nizhny Novgorod) is an ointment for topical application used to treat purulent wounds, venous ulcers, inflammation of the skin, and burns. Its active substances are dioxymethyltetrahydropyrimidine (4.0%) and chloramphenicol (0.75%). For our experiments, the ointment was weighted in sterile Eppendorf tubes and diluted tenfold in 96% ethanol.

Clotrimazole (Glaxo-Wellcome Poznan; Poland) formulated as an ointment containing 1% of the active substance was diluted in 96% ethanol in the same way as levomecol was.

Strains of human pathogens

Pathogenic *S. aureus* (ATCC 25923), *P. aeruginosa* (ATCC 27853), and *C. albicans* (NCTC 2625) were provided by Tarasevich State Institute of Standardization and Control (Moscow). The strains were cultured for 18–20 hours in an agarized meat-peptone broth supplemented with 0.1% glucose (for bacteria) and 1% glucose (for *C. albicans*).

Twenty milliliters of the meat-peptone agar were applied onto 9-cm sterile plastic Petri dishes. The plates were then slightly air-dried at room temperature and heated in an air incubator to +37 °C.

The prepared 1% agarose was poured into 2-ml glass tubes and cooled down to 40 °C in the water bath. The melted agarose from each tube was combined with 5×10⁵ CFU of each pathogenic culture, mixed, transferred to a culture plate containing the corresponding growth medium, and the upper layer of the mix was evenly distributed on the plate surface.

One hour after the first layer of the culture was applied, 4-mm wells were made in the agar using a sterile stainless-steel puncher.

Solutions of polyphenolic compounds with concentrations close to the solubility limit were prepared using 96% ethanol as a solvent: 0.43 M for resveratrol, 0.31 M for dihydromyricetin, and 0.3 M for dihydroquercetin. These solutions, as well as stock solutions of levomecol and clotrimazole, were then diluted 10-, 100- and 1000-fold in 96% ethanol. Aliquots of each dilution (5 µl) were mixed with 15 µl of sterile deionized water. Twenty microliters of the obtained mix were introduced into the wells. Each dilution was tested in three replicates in different culture plates. After the dilutions in the wells were air-dried, the plates were placed in the incubator preheated to +37 °C and incubated for 40 hours. Clear areas (zones of inhibition) formed in the bacterial and fungal lawns were measured using a pair of calipers.

Statistical processing

The diameter of a halo in the bacterial/fungal lawn was measured with calipers with an accuracy of 0.5 mm. Every

Table. Evaluation of *in vitro* antimicrobial activity of resveratrol, dihydromyricetin and dihydroquercetin against *S. aureus*, *P. aeruginosa*, and *C. albicans* by radial diffusion

Compound	Dilution	The diameter of the inhibition zone (mm)		
		<i>S. aureus</i>	<i>P. aeruginosa</i>	<i>C. albicans</i>
Dihydroquercetin	1/10	9.7 ± 0.4	0	0
	1/100	8.3 ± 0.4	0	0
	1/1000	7.7 ± 0.4	0	0
Resveratrol	1/10	10.0 ± 0.0	0	0
	1/100	8.3 ± 0.4	0	0
	1/1000	7.3 ± 0.4	0	0
Dihydromyricetin	1/10	11.7 ± 0.4	0	0
	1/100	10.0 ± 0.0	0	0
	1/1000	8.7 ± 0.4	0	0
Levomecol	1/10	9.3 ± 0.4	6.3 ± 0.4	0
	1/100	6.3 ± 0.4	0	0
	1/1000	0	0	0
Clotrimazole	1/10	5.7 ± 0.4	0	12.3 ± 0.4
	1/100	0	0	8.3 ± 0.4
	1/1000	0	0	6.0 ± 0.0

measurement was done in three replicates in different culture dishes. For a series of 3 measurements, an arithmetic mean and a mean square error were calculated.

RESULTS

Sizes of the zones of inhibition in the bacterial and fungal lawns are shown in the Table.

Even the highest possible concentrations of the polyphenols tested in our experiment (21.5, 15.5 and 15.0 mM, respectively) exhibited zero antimicrobial activity against the gram-negative bacterium *P. aeruginosa* and fungus *C. albicans*. In contrast, the lowest possible concentrations of these polyphenolic compounds (0.22, 0.15, and 0.15 mM, respectively) were effective against the gram-positive *S. aureus*. *In vitro* activity of dihydromyricetin against *S. aureus* was slightly higher than that of resveratrol and dihydroquercetin.

Levomecol was active against *S. aureus* at the minimal concentration of 24.2 mM and against *P. aeruginosa* at 242.3 mM. The lowest active concentration of clotrimazole against *C. albicans* was below 3.4 mM.

DISCUSSION

The *in vitro* antimicrobial and antifungal activity of resveratrol and other polyphenols has been reported in several studies [10–12]. Some authors have tested the activity of phenolic compounds against human dermatophytes [13]. The study [14] demonstrates that resveratrol inhibits bacterial phagocytosis by macrophages through the interaction with the TLR2 receptor and the nuclear factor NF- κ B. There is evidence suggesting that resveratrol is capable of inhibiting retinal inflammation experimentally induced by *S. aureus* [15]. However, so far polyphenols have not been used as an alternative to antibiotics, which raises the question whether research should

be continued to obtain a comprehensive description of their specific biological activity.

Our experiments demonstrate that the molar antimicrobial activity of flavonol (dihydromyricetin and dihydroquercetin) and stilbene (resveratrol) against the gram-positive human pathogen *S. aureus* is high: it is even higher than the molar activity of some antibiotics traditionally used for topical application.

In contrast, the activity of those polyphenols against gram-negative *P. aeruginosa* and the pathogenic yeast *C. albicans* is so low that it could not be detected by the assay used in the study. This observation is, however, not consistent with the literature [13] reporting marked resveratrol activity against *S. aureus* and *P. aeruginosa* (in the range between 171 and 342 μ g/ml, i.e. 39–78 mM) and the microscopic fungi *Trichophyton mentagrophytes*, *Trichophyton tonsurans*, *Trichophyton rubrum*, *Epidermophyton floccosum*, and *Microsporum gypseum* (25–50 μ g/ml, i.e. 5.7–11.4 mM).

The obtained data suggest that plant-derived polyphenols, such as dihydromyricetin, have a potential to be used as a medicine for topical application to treat skin infections caused by staphylococci, including their drug-resistant strains. Therefore, further research is needed to investigate the antimicrobial activity of polyphenols using animal models and to assess the toxicity of these compounds towards animal cells, as well as their immunostimulatory and immunosuppressive effects [14, 15].

CONCLUSIONS

Our study has confirmed the marked antimicrobial activity of resveratrol, dihydromyricetin and dihydroquercetin exerted *in vitro* at concentrations of 0.15 mM and above. The most pronounced effect was observed for dihydromyricetin. The study suggests a significantly higher bactericidal activity of polyphenols in comparison with the traditionally used levomecol ointment active against *S. aureus* at 24.2 mM.

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