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Antimicrobial properties of natural coniferous rosin in the European Pharmacopoeia challenge test

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Rosins (resins) are natural products of the coniferous trees. Purified rosin from the trunk of Norway spruce (*Picea abies*) is antibacterial against the gram-positive bacteria, but not against the gram-negative bacteria in agar plate diffusion test. In this study, we examined the antimicrobial properties of the coniferous rosin against bacteria and yeasts using the European Pharmacopoeia (EP) challenge test. The microbes tested were *Staphylococcus aureus,* methicillin-resistant *Staphylococcus aureus* (MRSA)*, Escherichia coli, Pseudomonas aeruginosa, Bacillus subtilis*, and *Candida albicans*. To prepare challenge media, purified rosin was mixed with a biologically inert salve in varying concentrations. The microbes were inoculated (5×10^5 microbes (bacteria) or 5×10^4 microbes (yeast, *C.albicans*)) into 10 g of the rosin-containing challenge medium for 14 days at maximum. Samples were taken from the media for re-cultivation of the microbes at time intervals of 1 h, 24 h, 4, 7, and 14 days. The microbicidal efficacy of the challenge media was estimated by reduction of the number of the colony forming units (CFU) of microbes in the test samples. A reduction of more than 10^3 CFU for bacteria and 10^2 CFU for fungi in 7 days was considered to indicate a significant microbicidal action. Pure rosin was antimicrobial within 24 h against all microbes tested. The 0.5% rosin-salve medium (w/w) did not differ in microbicidal effects from the rosin-free salve medium (control). A raise of the rosin concentration resulted in increase of the microbicidal effect of the rosin-salve medium against all micro-organisms tested. Rosin concentration of 10% (w/w) in the medium significantly reduced the colonization of *S. aureus* (including MRSA) within 24 h and significantly reduced the colonization of all other micro-organisms within 4 days. Rosin is strongly microbicidal against a wide range of microbes, against both gram-positive and gram-negative bacteria, and against *C. albicans*, in the EP challenge test. The minimum concentration of rosin is 10% (w/w) to prevent the preservation of the microbes in the rosin-salve media.

Key words: Rosin; European Pharmacopoeia (EP) challenge test; fungus, yeast; dermatophytes; Candida albicans; gram- negative bacteria.

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Rosins (resin; pitch) are natural products of the coniferous trees. For centuries, the rosin from Norway spruce (*Picea abies*) has been a part of the traditional medicine in Finland and Sweden in self-treatment of various diseases, for example, skin sores and infections (1, 2). In this practice, the rosin is mixed with animal fat or butter by boiling to prepare a therapeutic salve.

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In microbiological analyses, the coniferous rosin is shown to be antimicrobial against a surprisingly large range of microbes. In agar diffusion tests, the rosin exhibits antimicrobial effects against both bacteria and fungi but, among the bacteria, the antibacterial effect seems to be limited to gram-positive bacteria (3). Correspondingly, in agar diffusion tests, rosin is antifungal against dermatophytes, but not against yeasts, such as *Candida albicans* (4). These tests may, however, be handicapped and confused by the fact that the coniferous rosin is not, or at least poorly water soluble. This poor water solubility may violate the agar diffusion tests.

In this study, we examined the antimicrobial and antifungal properties of the coniferous rosin in the European Pharmacopoeia (EP) challenge test. In this test, the rosin is mixed with a biologically inert salve medium, and this salve is used as a challenge medium into which the micro-organisms are inoculated. The reduction in re-growth of the inoculated microbes (preservation of the microbes in the challenge medium as a function of time) is applied as an indicator of the bacteriostatic or fungistatic effect of the challenge medium. In particular, we wanted to estimate the minimum inhibitory concentration of the rosin in salve medium that will be bacteriostatic and fungistatic in the EP challenge test.

MATERIALS AND METHODS

Antimicrobial preservation challenge test according to the European Pharmacopeia (Chapter 5.1.3)

The European Pharmacopoeia (EP) challenge test is widely recognized as a standard by cosmetic and pharmaceutical industries in the EU countries (5, 6). The EP test consists of a specific set of common micro-organisms, against which the antimicrobial action of the challenge medium (preservation of the micro-organisms in the challenge medium) is tested. The test is performed by adding a specific number of bacteria or fungi as challenge micro-organisms. The microbes will remain viable and re-cultivable from the challenge medium if the medium does not exert any bacteriostatic or fungistatic influences.

The density of the inoculation is in the range of 10^4–10^6 CFU/mL or CFU/g according to the EP (5). The re-growth of bacteria from the challenge medium is usually assayed at 1 h, 48 h, 7 days, 14 days, and 28 days. To focus on early inhibition rate, modified time intervals were used. In this study, time intervals were 1 h, 24 h, 4 days, 7 days, and 14 days. Samples taken at different time intervals are cultured in specific microbiological media, and a reduction in number of the microbial colony forming units (CFU) in test samples as function of time is used as a measure of the antimicrobial influence.

According to the EP Challenge test criteria, a reduction of bacteria by at least 10^2 CFU within 48 h and at least 10^3 within 7 days is considered to indicate a significant antibacterial activity of the medium (5). Correspondingly, a reduction in molds/yeasts of at least 10^2 CFU within 14 days is considered fungistatic.

Coniferous rosin and preparation of rosin-salve media

Coniferous rosin was mechanically ripped and collected from trunks of the Norway spruce in Finnish Lapland, and purified by dissolving with alcohol, filtered and dried (patent pending FI123787). The purified dry rosin is a soft-solid and sticky material in room or body temperatures (20–37 °C). Rosin-salve media for the EP challenge tests were prepared by mixing the ethanol solution of purified rosin (50% rosin in concentrated ethanol) with a biologically inert salve, so that the rosin content in the final salve medium was 0.5%, 2%, or 10% after evaporation of the alcohol. Pure rosin-alcohol mixture was also tested, and the salve medium without rosin was used as a control.

The salve base in challenge media consisted (INCI) of a mixture of petrolatum, paraffinum liquidum, hydrogenated castor oil, cera microcrystallina, sorbitan oleate, cera alba and stearic acid. These rosin media are soft creams that are stable at least for 3 years at room temperature regarding their antimicrobial activity, and the medium does not change in composition in at least 3 years as analysed using the gas-liquid chromatography (LGC; acetone extract). In LGC, acetone extracts of both purified rosin and the rosin-salve media are similar in chemical composition containing p-coumaric acid, resin acids and lignans. On rough average, 10%, 60% and 30% of the purified trunk rosins from Norway spruce are p-coumaric acid, resins acids or lignans, respectively.

Bacteria and fungi tested

The standard set of micro-organism strains used in EP challenge tests by Laboratory of Microbiology, Department of Public Health, Hjelt Institute, Helsinki University, Helsinki, Finland, was applied. The tested micro-organisms were: *Staphylococcus aureus* ATCC 6538, methicillin-resistant *Staphylococcus aureus* (MRSA) ATCC 43300, *Escherichia coli* ATCC 10538, *Pseudomonas aeruginosa* ATCC 15442, *Klebsiella pneumonia* ATCC 27736, *Bacillus subtilis* ATCC 6633, and *C. albicans* ATCC 10231.

Inoculation of microbes into the challenge medium

Each microbe tested was added as 1 mL aliquots [5×10^5 cfu/mL (bacteria) and 5×10^4 CFU/mL (yeast, *C.albicans*)] into 10 g of the rosin-salve challenge medium. The medium was kept (incubated) in room temperature and samples, as duplicates, were taken 1 h, 24 h, 4, 7, and 14 days after the inoculation for the re-cultivation of the micro-organisms in specific microbiological culture media.

Interpretation of the results

All micro-organisms inoculated were re-cultivable 1 h after inoculation from all rosin-salve media in same extent as from the control challenge media (salve medium without rosin) (Table 1). This level of re-growth was considered strong (+ + +; more than 10^3 CFU/mL; >log3) and considered as a reference in calculations of the bacteriostatic and fungistatic influences. The reduction of the regrowth from the reference (+ + +; more than 10^3 CFU/mL; >log3) to strong (+; 1–10 CFU/mL; log1) or absent (–; no regrowth of microbes) was considered to indicate a significant bacteriostatic or fungistatic effect, corresponding the growth reduction of bacteria by 10^3 or of fungi by 10^2 CFU/mL or more.

RESULTS

The test results are presented in Table 1. Rosin dissolved in ethanol immediately killed all inoculations. None of the microbes were re-cultivable in the 1-h test sample from the rosin-alcohol medium. All microbes were re-cultivable from the rosin-salve media within 1 h when the rosin

Table 1. Intensity of the re-growth of the micro-organisms in samples from rosin-salve challenge media in different time intervals. Abbreviation + + + indicates the growth of 10^3 CFU/mL of micro-organisms in the sample under testing. Correspondingly, the abbreviations + + and + indicate the growth intensities of 10^2 CFU/mL and 1–10 CFU/mL, respectively. The abbreviation (–) indicates that no micro-organisms could be re-cultivated from the sample

	Exposure time	*S. aureus*	*MRSA*	*E. coli*	*Ps. aeroginosa*	*B. subtilis*	*C. albicans*
Rosin, purified, dissolved in alcohol (ethanol)	1 h	–	–	–	–	–	–
	24 h	–	–	–	–	–	–
	4 days	–	–	–	–	–	–
	7 days	–	–	–	–	–	–
Rosin, purified, alcohol evaporated	1 h	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +
	24 h	–	–	–	–	+	–
	4 days	–	–	–	–	–	–
	7 days	–	–	–	–	–	–
Rosin 0.5% in salve base	1 h	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +
	24 h	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +
	4 days	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +
	7 days	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +
	14 days	–	–	–	–	+	+
Rosin 2% in salve base	1 h	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +
	24 h	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +
	4 days	+ +	+ +	+ + +	+ + +	+ + +	+ + +
	7 days	+	+	+ +	+ +	+ + +	+
	14 days	–	–	–	–	+	+
Rosin 10% in salve base	1 h	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +
	24 h	+	–	+ + +	+ + +	+ + +	+
	4 days	–	–	+	+	+	–
	7 days	–	–	–	–	–	–
	14 days	–	–	–	–	–	–
Salve base	1 h	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +
	24 h	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +
	4 days	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +
	7 days	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +
	14 days	+	+	–	+	+ +	+ +

Bacillus subtilis, *Candida albicans, Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus,* MRSA, methicillin-resistant *Staphylococcus aureus*.

was alcohol-free (alcohol evaporated). However, the challenge of the microbes to pure purified rosin significantly reduced the number of all inoculated bacteria within 24 h. The challenge to 10% rosin-salve medium reduced the number of staphylococci within 24 h and, correspondingly, also significantly reduced the colonization of all other microbes within 4 days. The pure salve medium (control) or the 0.5% rosin-salve medium did not show any reduction in re-growth of the microbes within 7 days. As there occurred a significant reduction in colonization of microbes in pure salve media (controls) after 14 day, all data from the 14 day challenges were considered unreliable, and were omitted. The 2% rosin-salve medium showed significant antimicrobial effect against staphylococci and *C. albicans* within 7 days, but it did not influence *B. subtilis* and showed mild effects against *E. coli* and *P. aeruginosa*.

DISCUSSION

This study shows that the coniferous rosin is not only strongly microbicidal against a wide range of microbes in the EP challenge test, including both gram-positive (*S. aureus*, MRSA, *B. subtilis*) and gram-negative bacteria (*E. coli, P. aeruginosa*) but also against yeasts, like *C. albicans*. According to this test, not only the rosin alone but also rosin-salves are widely and significantly bacteriostatic and fungistatic when the rosin concentration (minimal concentration to prevent the preservation of the micro-organisms) in the salve mixture is 10% or more.

The challenge of bacteria to rosin dissolved in ethanol (rosin-alcohol mixture) immediately killed the inoculums, and the microbes were not re-cultivable from the medium after 1 h. This was not the case after the alcohol was evaporated and the rosin was alcohol-free. In the alcohol-free rosin media, the inoculated microbes remained alive and were re-cultivable as abundant colonies 1 h after the inoculation. However, a significant microbicidal effect of the rosin against all micro-organisms tested became apparent during the challenge of some hours and days. All these observations were concluded to indicate that the microbicidal influences observed in the present EP challenge tests are due to the actions of rosin.

The results are consistent with the earlier reports, but there also are some contradictions (3, 7). In the earlier agar diffusion tests, and in tests with broth media, coniferous rosin was antibacterial against the gram-positive bacteria only, like *S. aureus* and MRSA, but not, or in lesser extent, against the gram-negative bacteria (3, 8). In the present challenge study, however, the rosin was strongly and clearly bactericidal against the gram-negative bacteria as well. In addition, in agar plate diffusion tests, the rosin in over 10% concentrations has been shown to be antifungal against dermatophytic fungi (*Trichophyton* species), but not against *C. albicans* (4). However, the rosin was antifungal also against *C. albicans* in the present challenge test.

The poor water solubility of natural rosin may be an explanation for the differences and contradictions of why the agar diffusion test and the EP challenge test give dissimilar results. The bacteriostatic actions of rosin against the microbes may become more easily apparent in the challenge test than in the agar diffusion test. In our earlier agar diffusion test, the gram-negative bacteria were more resistant to rosin than the gram-positive bacteria (3). This became evident also in the present EP challenge test, where the gram-positive bacteria, like *S. aureus*, tended to be killed in the rosin media quicker and in lower rosin concentration than the gram-negative bacteria, like *E. coli*. However, the most rosin resistant bacterium in this study was the gram- positive spore forming bacterium, *B. subtilis*.

In estimation of the microbicidal efficacy by the EP challenge test, the minimum concentration of rosin in a rosin-salve media is around 10% (w/w) to create a significant microbicidal effect, and by contrariwise, the rosin concentrations below 10% allow the preservation of the micro-organisms in the medium. In concentration of 10% or more, the rosin is both bacteriostatic and fungistatic within 7 days against all microbes tested in this study, fulfilling, thereby, the criteria of good microbicidal efficacy of the coniferous rosin according to the EP challenge test criteria.

The exact mechanisms in antimicrobial actions of the coniferous rosin against microbes are not known. In LGC analysis, the resin acids are the major component of the coniferous rosins (9). These resin acids have been shown to be

antimicrobial and are, therefore, certainly mediators of the microbicidal effects of the natural coniferous rosins as well (3, 8, 10). Coniferous rosin (and resin acids) is basically insoluble in water. Resin acids (e.g. abietic acid or hydroxyabietic acid) are phenolic compounds with almost neutral acid dissociation constant (e.g. for abietic acid 7.28) and are present as both protonated and non-protonated forms at physiologic pH (12). One may assume that the non-protonated form of resin acid acts as proton acceptor outside the bacterial cell wall and membrane. Pronation may raise the lipid solubility of rosin and resin acids, increasing, thereby, the solubility of rosins into the biological tissues, e.g. bacterial cell wall, as well (11).

In culture experiments and electron microscopical studies, the coniferous rosin seems to destroy the bacterial cell wall and cell membrane (11). In electrophysiologic experiments, the rosin exposure decreased the cell membrane proton gradient in bacterial cells and this phenomenon was considered to be associated with a disruption of the proton transport in the membrane-bound ATPase, and resulting in uncoupling of the oxidative phosphorylation (11). Subsequently, the cell metabolism would cease and the supply of energy is lost. In electron microscopy, the cell walls are thickened; the cells are aggregated and finally degraded when the bacteria are exposed to rosin (11). When a plate (e.g. steel plate) is covered with a thin rosin film and microbe inoculates are set onto this film, the microbes are attached to and 'trapped' by the film. The bacteria are not re-cultivable from the film and seem to be destroyed if examined using the scanning and transmission electron microscopy (unpublished observations).

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