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Antimicrobial activity of *Mahonia aquifolium* and two of its alkaloids against oral bacteria

Summary

Extracts or alkaloids isolated from Mahonia aquifolium *exhibit antimicrobial activity against Gram-positive and Gram-negative bacteria, fungi, and protozoa. In this study the bacteriostatic and bacteriocidal activities of a* M. aquifolium *extract and two of its major alkaloids, berberine chloride and oxyacanthine sulphate, were tested* in vitro *against nine different oral bacteria.*

Minimum inhibitory concentrations were in the range from ⩽0.0031% to 0.1993% for the M. aquifolium *extract, from 0.002% to >0.125% for berberine chloride, and from 0.0156% to >0.0625% for oxyacanthine sulphate. The values for the minimum bactericidal concentrations were in the same range, indicating that the test substances most probably acted in a bactericidal manner. The most susceptible bacterium against all three test substances was* Porphyromonas gingivalis.

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Key words: Mahonia aquifolium; *antimicrobial; oral bacteria; minimum inhibitory concentration*

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Introduction

Mahonia aquifolium (Pursh) Nutt., a member of the family *Berberidaceae,* is an evergreen bush originally found in the Pacific Northwest of the United States of America. For its decorative yellow flowers and blue fruits (Fig. 1), it was cultivated and spread to other parts of the world, including Europe (Frohne & Pfänder 2004). Parts of the plant have been used in medicine for centuries in North America and India, as well as in homeopathy (McCutcheon et al. 1994, Lampert et al. 1998). *M. aquifolium* and especially its root and bark contain many alkaloids like berberine,

Fig. 1 Mahonia aquifolium *(a) in spring and (b) in autumn with fruits.*

jatrorrhizine, palmatine, and oxyacanthine, which are thought to be responsible for the pharmacological properties of this plant. Berberine, which gives the root and the bark their yellow colour, is generally considered the physiologically dominant alkaloid (Iwasa et al. 1998).

Recently, interest increased as several clinical studies indicated that topical *M. aquifolium* ointments might be a safe and effective treatment for patients suffering from mild to moderate psoriasis (Augustin et al. 1999, Gulliver & Donsky 2005, Bernstein et al. 2006). Additionally, berberine exhibits cytotoxic effects against several carcinomas (Iwasa et al. 2001, Jantova et al. 2003), it has antimutagenic activities (Cernakova et al. 2002), and a hypoglycaemic effect (Zhou et al. 2007). Berberine has been identified as a novel cholesterol-lowering drug both, *in vitro* and *in vivo,* whose mode of action is distinct from that of statins (Kong et al. 2004). Furthermore, it is a candidate for the treatment of Alzheimer's disease (Zhu & Qian 2006, Asai et al. 2007).

Extracts or alkaloids isolated from *M. aquifolium* as well as from other plant species have been shown to be active against bacteria, fungi, and protozoa (Subbaiah & Amin 1967, Amin et al. 1969, Kuroda et al. 1976, McCutcheon et al. 1994, Iwasa et al. 1997, Park et al. 1999, Andenmatten 2000, Vollekova et al. 2001, Cernakova & Kostalova 2002, Kim et al. 2002, Nyasse et al. 2002, Vollekova et al. 2003, Slobodnikova et al. 2004, Han & Lee 2005, Quan et al. 2006). Microorganisms tested include various Gram-positive and Gram-negative bacteria like *Propionibacterium acnes,* coagulase-negative staphylococci and *S. aureus, Escherichia coli, Pseudomonas aeruginosa, Vibrio cholerae,* and *Mycobacterium tuberculosis.* Eukaryotic organisms include *Candida* spp., *Malassezia* spp., dermatophytes, filamentous fungi, and protozoa like *Entamoeba histolytica.*

Bacteria involved in oral diseases like caries and periodontitis have not been tested so far. Since *M. aquifolium* and its alkaloids have interesting antimicrobial properties, the aim of this study was to compare the antibacterial activity of a *M. aquifolium* extract and two of its major alkaloids, berberine and oxyacanthine, against nine representative oral bacteria.

Materials and Methods

Microorganisms and Growth Conditions

The bacteria studied in this work were: *Streptococcus mutans* ATCC 25175, *Streptococcus sanguinis* ATCC 10556, *Streptococcus anginosus* ZIB 6006 (clinical isolate, School of Dental Medicine [UZM] Basel), *Lactobacillus salivarius* subsp. *salivarius* DSM 20555, *Actinomyces naeslundii* ATCC 12104, *Aggregatibacter (Actinobacillus) actinomycetemcomitans* ZIB 1001 (clinical isolate, UZM Basel), *Fusobacterium nucleatum* OMZ 274 (Werner-Felmayer et al. 1988), *Prevotella intermedia* ATCC 25611, and *Porphyromonas gingivalis* ZIB 3071 (clinical isolate, UZM Basel).

The bacteria were grown on Columbia blood agar plates (Columbia Agar Base [BBL Becton Dickinson, Allschwil, Switzerland] supplemented with 4 mg/l hemin, 1 mg/l menadione, and 50 ml/l human blood) at 36 °C. Streptococci were grown in air for 24 h, *A. actinomycetemcomitans* and *L. salivarius* subsp. *salivarius* in air +10% CO_2 for 24 h, and anaerobes in 10% CO_2, 10% H_2, 80% N_2 for 5–10 days. Liquid media used were: Todd Hewitt Broth (BBL, Becton Dickinson, Allschwil, Switzerland) for streptococci and *A. actinomycetemcomitans;* Thioglycolate Broth (Oxoid, Pratteln, Switzerland) supplemented with 4 mg/l hemin and 1 mg/l menadione for *F. nucleatum, A. naeslundii,* and *L. salivarius* subsp. *salivarius;* Cooked Meat Medium (Oxoid, Pratteln, Switzerland) for *P. intermedia;* and Todd Hewitt Broth (BBL, Becton Dickinson, Allschwil, Switzerland) supplemented with 4 mg/l hemin and 1 mg/l menadione for *P. gingivalis.* These conditions were used for the growth of the bacteria as well as for the determination of the minimal inhibitory concentration (MIC) and the minimal bactericidal concentration (MBC).

Test Substances

An extract (Batch AWT 1445) of *M. aquifolium* from powdered stem bark was obtained from Pentapharm (Aesch, Switzerland). The total alkaloid content was 0.848% (w/v), with 0.118% berberine and 0.125% oxyacanthine, as determined by HPLC analysis. The galenic formulation used for this study was an aqueous solution containing 19.6% glycerine and 10% ethanol. This solution was first filter-sterilized through both 0.45 µm and 0.22 µm pore-size filters (Millex-HV and Millex-GV resp., Millipore, Zug, Switzerland). Afterwards, 6% dimethyl sulfoxide (DMSO) was added to improve the solubility. Therefore, the resulting total alkaloid content was 0.7971%, with 0.1109% berberine and 0.1175% oxyacanthine. The inhibitory effect of the galenic formulation with 19.6% glycerine and 10% ethanol without the *M. aquifolium* extract was tested as well.

Berberine chloride was purchased from Fluka (No. B3251, Sigma-Aldrich, Buchs, Switzerland) and oxyacanthine sulfate from Roth (Lot No. 05729455, Karlsruhe, Germany). Both substances were dissolved in an aqueous solution containing 6% DMSO and subsequently filter-sterilized through 0.22 µm pore-size filter (Millex-GV, Millipore, Zug, Switzerland). The stock solutions used for the determination of the MIC and the MBC contained 0.25% berberine chloride and 0.125% oxyacanthine sulfate, respectively.

Minimal Inhibitory Concentration (MIC) and Minimal Bactericidal Concentration (MBC)

Broth macrodilution testing was performed according to the Clinical and Laboratory Standards Institute CLSI (CLSI, formerly NCCLS) guidelines (NCCLS 1997a, NCCLS 1997b). First, 1 ml of each of the stock solutions was diluted into 1 ml double-concentrated liquid media, resulting in 0.125% berberine chloride, 0.0625% oxyacanthine sulfate, and 0.3986% *M. aquifolium* extract, respectively. Then, two-fold serial dilutions of the test substances were made with 1x liquid media. Eight dilution steps were tested for each of the test substances.

Suspensions of the test strains were prepared in the corresponding liquid media to a density of approximately 5×10^6 cfu/ml. The correct bacterial counts were determined by plating dilutions of

the suspensions onto appropriate agar plates. A 100 µl volume of bacterial suspension was added to each of the 2 ml serial dilutions of the test substances. Aerobes and microaerophilic bacteria were incubated at 36 °C for 24 h and anaerobes for 5–10 days at the conditions described above.

The MIC corresponded to the lowest concentration of an antimicrobial substance that completely inhibited visible bacterial growth. Minimal bactericidal concentration values were determined by plating 100 µl from all tubes without visible growth onto Columbia blood agar plates. The lowest concentration of test substance that killed ≥ 99.9% of the initial test inoculum was defined as the minimum bactericidal concentration (MBC). The susceptibility tests were performed three times for each bacterial strain.

To estimate whether the addition of DMSO had an influence on the growth of the bacteria, we inoculated *S. mutans* with various concentrations of DMSO in Todd Hewitt Broth (0.5%, 1%, 2%, 4%, 6%, 8%, 10%, and 20%). With the exception of 20% DMSO, there was no visible effect on growth. However, DMSO concentrations of 4% and higher resulted in a slight decrease of cell counts. To compare the three test substance, DMSO was added to all three of them as well as to the growth controls.

Results

Tables I and II summarize the MIC and MBC values found for berberine chloride, oxyacanthine sulfate, and the *M. aquifolium* extract. The MIC values for berberine chloride were between 0.0020% and >0.1250%, and between 0.0156% and >0.0625% for oxyacanthine sulfate. The *M. aquifolium* extract was the most active substance tested, with MIC values between ≤ 0.0031% and 0.0996%. *Porphyromonas gingigvalis,* a potentially periodontopathogenic black-pigmented bacteria, was by far the most sensitive bacterial species. The galenic formulation containing 19.6% glycerol and 10% ethanol showed some antibacterial activity as well, especially against *P. intermedia* and *A. actinomycetemcomitans.* The MBC values were in the same range as the MIC values.

Discussion

The *M. aquifolium* extract inhibited all nine oral bacterial species with MIC values between ≤ 0.0031% and 0.1993%, corresponding to ≤ 0.031 mg/ml to 1.993 mg/ml. The difference between the MIC and the MBC values was very narrow, indicating that the test substances most probably acted in a bactericidal manner. In a similar study the antimicrobial activity of *Melaleuca alternifolia* was tested against the same nine oral bacteria also used in this study (Kulik et al. 2000). MIC and MBC values for a tea tree oil solution and a tea tree gel were a factor of 10 higher than those found in this study with *M. aquifolium.* In contrast, the MIC and MBC values for chlorhexidine digluconate were approximately 100 times lower.

The antimicrobial activity of *M. aquifolium* crude extract has already been tested against some bacteria with MIC values in a similar range as for the oral bacteria (0.025 mg/ml to 0.5 mg/ml, Slobodnikova et al. 2004). There are diverging results about the antifungal activity of *M. aquifolium* (McCutcheon et al. 1994, Andenmatten 2000, Vollekova et al. 2001, Vollekova et al. 2003). There are only a few studies about the antimicrobial activity of the bisbenzylisochinoline alkaloid oxyacanthine, which was the least active of the substances tested in this study. In the study of

Tab. I Minimal inhibitory concentrations (MIC, in %).

	Substance			
bacteria	berberine chloride[a]	oxyacanthine sulfate[a]	*M. aquifolium* extract[a]	galenic formulation
S. mutans	>0.1250	0.0625	0.0249	>5
S. sanguinis	0.0625	0.0625	0.0996	>5
S. anginosus	0.0625	>0.0625	0.0996	>5
L. salivarius	0.0625	>0.0625	0.0996	>5
A. naeslundii	0.0313	>0.0625	0.0415	5
A. actinomycetemcomitans	0.0625	>0.0625	0.0125	1.25
F. nucleatum	0.0156	>0.0625	0.0498	2.5
P. intermedia	0.0729	0.0156	0.1993	1.25
P. gingivalis	0.0020	0.0261	≤0.0031	2.5

[a] Mean of three experiments.

Tab. II Minimal bactericidal concentrations (MBC, in %).

	Substance			
bacteria	berberine chloride[a]	oxyacanthine sulfate[a]	*M. aquifolium* extract[a]	galenic formulation
S. mutans	>0.1250	0.0625	0.0498	>5
S. sanguinis	0.0625	>0.0625	0.0996	>5
S. anginosus	0.0625	>0.0625	0.0996	>5
L. salivarius	0.0625	>0.0625	0.0996	>5
A. naeslundii	0.0417	>0.0625	0.0498	5
A. actinomycetemcomitans	0.0625	>0.0625	0.0498	2.5
F. nucleatum	0.0156	>0.0625	0.0498	5
P. intermedia	0.0729	0.0208	0.1661	1.25
P. gingivalis	0.0020	0.0261	≤0.0031	2.5

[a] Mean of three experiments.

Kuroda et al. (1976) the MIC values of oxyacanthine against eight Gram-positive and Gram-negative bacteria ranged from 0.0625 mg/ml to 1 mg/ml, which is in the same concentration range as in our study.
Berberine chloride, on the other hand, was the more active alkaloid tested with MIC values between 0.02 mg/ml and >1.25 mg/ml. The chinolizine alkaloid berberine can be isolated from various plant species (Grycova et al. 2007). The mechanism by which it inhibits microbial growth is attributed to its ability to intercalate and form stable complex with DNA (Kumar et al. 1993). Already 40 years ago Subbaiah & Amin (1967) demonstrated its antimicrobial activity against *Entamoeba histolytica* both, *in vitro* and *in vivo.* Berberine has a moderate activity against fungi and yeasts (Tab. III). Interestingly, berberine shows a synergistic interaction with amphotericin B and fluconazole *in vitro.* In the case of amphotericin B, this synergic effect could be verified in a mouse model with disseminated candidiasis (Han & Lee 2005, Quan et al. 2006).
Different Gram-positive and Gram-negative bacteria have been tested as well (Tab. III). The MIC values of the oral bacteria tested in this study are in the same range. Berberine as an isolated compound is pumped out of the bacterial cell by multidrug resistance pumps (MDRs). However, the antimicrobial activity of berberine could be restored either by the synergistic action of a natural inhibitor of MDRs identified in several berberine producing *Berberis* plant species or by conjugating *in vitro* an MDR inhibitor to berberine (Stermitz et al. 2000, Ball et al. 2006).
The only clinical studies with *M. aquifolium* extracts so far were performed to assess the efficacy for patients with psoriasis. Although the first studies sometimes lacked appropriate controls, newer studies indicate that creams or ointments containing *M. aquifolium* extracts might be an effective treatment for patients with mild to moderate psoriasis (Gulliver & Donsky 2005, Bernstein et al. 2006). Side effects reported during these clinical trials were infrequent (Gulliver & Donsky 2005, Bernstein et al. 2006). The preparation used in one trial was a *M. aquifolium* extract in a patented liposome preparation (Bernstein et al. 2006).
In our study, DMSO had to be added to the substances to improve the poor water solubility of the compounds. Since this is not suitable for oral applications, other solubilisators and galenic formulations have to be tested.
Microorganisms in the dental plaque are organized as a biofilm, and have a drastically increased resistance to antimicrobial agents. Moreover, oral antimicrobial substances usually are applied for only 0.5 to one minute. Whether *M. aquifolium* and its alkaloids have a bactericidal activity also on bacteria in a biofilm and over a shorter exposure time can be answered by time-kill studies and the use of an *in vitro* biofilm model (Guggenheim et al. 2004).
The alkaloids berberine and oxyacanthine induce acute gastric lesions in mice at doses above 100 mg/kg and subacute lesions with 25 mg/kg doses (Küpeli et al. 2002). However, further studies are needed to determine the safety of *M. aquifolium,* especially the absorption through the oral mucosa and the gastrointestinal tract, since parts of the substance may be swallowed.

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Zusammenfassung

Extrakte oder isolierte Inhaltsstoffe von *Mahonia aquifolium* zeigen eine antimikrobielle Wirkung auf grampositive und gramnegative Bakterien, Pilze und Protozoen. In der vorliegenden Studie wurde die bakteriostatische bzw. bakterizide Wirkung eines *M. aquifolium*-Extraktes und von zwei der Hauptinhaltsstoffe, Berberinchlorid und Oxyacanthinsulfat, *in vitro* gegenüber neun verschiedenen oralen Bakterien untersucht.
Die Werte für die minimale Hemmkonzentration lagen für den *M. aquifolium*-Extrakt im Bereich von ⩽0,0031% bis 0,1993%, für Berberinchlorid von 0,002% bis >0,125% und für Oxyacanthinsulfat von 0,0156% bis >0,0625%. Die Werte für die minimale bakterizide Konzentration bewegten sich im gleichen Bereich, was auf eine bakterizide Wirkung der Testsubstanzen hindeutet. Der empfindlichste Keim gegen alle drei Testsubstanzen war *Porphyromonas gingivalis.*

Résumé

Des extraits ou des alcaloïdes isolés de *Mahonia aquifolium* exercent une activité antimicrobienne contre des bactéries Grampositives et Gram-négatives, des champignons et des protozoaires. Dans cette étude les activités bactériostatiques et bactéricide d'un extrait de *M. aquifolium,* ainsi que de deux de ses alcaloïdes majeurs, la chlorure de berbérine et le sulfate d'oxyacanthine ont été testées *in vitro* sur neuf bactéries buccales différentes.
Les concentrations inhibitrices minimales se situaient entre ⩽0,0031% et 0,1993% pour l'extrait de *M. aquifolium,* entre 0,002% et >0,125% pour la chlorure de berbérine, ainsi que

Tab. III MIC values of berberine.

Microorganisms	MIC range (in mg/ml)			References
Candida albicans	0.0125	–	>1 / 1.576[a]	Amin et al. 1969, Andenmatten 2000, Cernakova & Kostalova 2002, Isawa et al. 1997, Kim et al. 2002, Park et al. 1999, Slobodnikova et al. 2004, Vollekova et al. 2003
other *Candida* spp.	0.0031 / <0.004[b]	–	>0.5	Amin et al. 1969, Kim et al. 2002, Park et al. 1999, Slobodnikova et al. 2004, Vollekova et al. 2003
Malassezia spp.	0.05	–	>1	Andenmatten 2000, Vollekova et al. 2001
bacteria	0.0031	–	>2	Amin et al. 1969, Cernakova & Kostalova 2002, Isawa et al. 1997, Kim et al. 2002, Slobodnikova et al. 2004
oral bacteria	0.02	–	1.25	this study

[a] Cernakova & Kostalova 2002: 1.567 mg/ml; Vollekova et al. 2003: >1 mg/ml
[b] Amin et al. 1969: 0.0031 mg/ml; Park et al. 1999: <0.004 mg/ml

entre 0,0156% et >0,0625% pour le sulfate d'oxyacanthine. Les valeurs minimales de concentrations bactéricides, elles se situaient dans le même ordre de grandeur, indiquant que l'ensemble des substances testées avaient une action bactéricide. La bactérie la plus susceptible aux trois substances testées était *Porphyromonas gingivalis.*

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