

Effects of Lead Phytochemicals of *Radix Scutellariae* on *Acanthamoeba*

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PURPOSE. *Acanthamoeba* keratitis (AK), which is associated with noncompliant use of contact lenses, remains difficult to treat due to delayed diagnosis and paucity of therapeutic agents. Although improvements in activity against *Acanthamoeba* infection have been achieved in disinfecting solutions for soft contact lenses, such modifications have not been extended to those for special rigid gas permeable (RGP) contact lenses, which are increasingly used for myopia control in children. Phytochemicals present in herbs used for traditional Chinese medicine may be effective as therapeutic or preventive agents. The purpose of this study was to investigate amoebicidal properties of lead phytochemicals of *Radix scutellariae* alone and in combination with multipurpose (disinfecting) solutions (MPS) for RGP lenses.

METHODS. Viability of *Acanthamoeba castellanii* and *A. polyphaga* trophozoites was determined following exposure to four phytochemicals: baicalin, baicalein, wogonoside, and oroxylin A and both alone and in combination with four RGP MPS, using a modified stand-alone technique.

RESULTS. As individual agents, wogonoside and oroxylin A showed highest activity against *A. castellanii* and *A. polyphaga* trophozoites, respectively. For both organisms, the combination of baicalein and oroxylin A was superior. Effectiveness of MPS alone did not exceed 0.27 log reduction, but addition of combined baicalein and oroxylin A resulted in 0.92 and 0.64 log reductions of *A. castellanii* and *A. polyphaga*, respectively.

CONCLUSIONS. The combination of baicalein and oroxylin A enhanced the activity of MPS for RGP contact lenses against trophozoites of two pathogens, *A. castellanii*, and *A. polyphaga*, and offers a potential therapeutic and/or preventative agent for AK.

Keywords: acanthamoeba, contact lenses, keratitis, phytochemicals, TCM

Acanthamoeba infection is the cause of a rare but severe form of eye infection which is associated with the use of contact lenses.^{1,2} It is present in wide range of environments and has been isolated from tap water,³ swimming pools,⁴ and soil,⁵ as well as contact lens cases.⁶ Contamination of contact lens cases is associated with use of tap water,⁶ and this should be avoided in the care of contact lenses. Most contact lens wearers use multipurpose solutions (MPS) for cleaning, rinsing, and disinfecting. Unlike hydrogen peroxide systems, which have been reported to have good activity against *Acanthamoeba* if sufficient time is allowed before neutralization,^{7,8} MPS vary in their efficacy against this organism.⁹⁻¹³ Newer formulations containing dual antimicrobial agents have been introduced for soft lenses which may be more effective,¹⁴⁻¹⁶ but further evaluation is needed. With respect to solutions for rigid gas permeable (RGP) lenses, such combination disinfectants are not yet available. Although RGP lenses are less frequently prescribed for routine vision correction, they are becoming increasingly popular for orthokeratology use and are considered by most optometrists to be the most effective method for reducing progression of myopia.¹⁷ Nevertheless, regardless of the effectiveness of the solutions, a proportion of contact lens wearers will fail to comply with correct care

and routines, increasing their risk of ocular infections, including *Acanthamoeba* keratitis (AK).^{18,19}

AK is usually treated with nonspecific disinfecting agents, including chlorhexidine (0.02%) and polyhexamethylene biguanide (PHMB) (0.02%), but late diagnosis is associated with poor prognosis for a full recovery and may result in vision loss.²⁰ Although the current treatments can be effective, disinfecting agents do have cytotoxic effects on the cornea which may exacerbate symptoms and delay recovery.^{21,22} There are reports of use of propamidine, which, although it has toxic side effects, has shown good results when used early in the infection in combination with chlorhexidine or neomycin, but this agent is not available in all countries.^{23,24} For successful treatment, topical antimicrobials need to be applied to the eye hourly for several days and then for a further 2 to 3 weeks for most cases. Some patients require treatment for 12 months or even longer, and most patients need combination therapy. Therefore, there is a need for alternative treatments of AK.²⁰

We previously conducted a small-scale study of the effects of three herbs used in treatment of infections in traditional Chinese medicine (TCM) protocols on viability of *Acanthamoeba* organism which revealed that *Radix scutellariae* had an antimicrobial effect on *A. royreba*.²⁵ However, the activity of this herb was found to vary considerably between sources and



TABLE. Major Antimicrobial Agents of Multipurpose Solutions for RGP Lenses

Name	MPS	MRDT, hours	Ingredients	Manufacturer
A	AMO TotalCare	4	EDTA/PHMB 0.0001%	Abbott Medical Optics, Santa Ana, CA, USA
B	Boston Advance	4	CHX 0.003%/EDTA 0.05%/PHMB 0.0005%	Bausch & Lomb, Rochester, NY, USA
C	Boston Simplus	4	CHX 0.003%/PHMB 0.0005%	Bausch & Lomb, Rochester, NY, USA
D	MeniCare Plus	4	EDTA/PHMB 0.0005%	Menicon Co., Nagoya, Japan

CHX, chlorhexidine; EDTA/PHMB, Ethylenediaminetetraacetic acid/Polyhexamethylene biguanide; MPS, multipurpose (disinfecting) solutions; MRDT, manufacturer's recommended disinfecting time.

batches of the dried product or its extracts, making it difficult to draw any conclusion about its effectiveness. The antimicrobial properties of the herb are due to the presence of phytochemicals, and the levels of these agents vary depending on climatological conditions, harvesting time and methods, storage conditions, and extraction procedures.^{26,27} Because *R. scutellariae* appeared to have potential for use in ocular solutions, the effects of its viability on exposure to human corneal epithelial cells (HCEC) was determined by trypan blue exclusion, methylthiazole tetrazolium assay production, and fluorescein isothiocyanate staining and flow cytometry. There was no significant increase in viability, apoptosis, or early necrosis in a comparison between HCEC and control cells exposed to the extract.²⁸

Determining the lead phytochemicals in *R. scutellariae* and testing those compounds at known concentrations against *Acanthamoeba* could demonstrate whether the phytochemicals had potential as therapeutic or preventive agents incorporated into multipurpose contact lens solutions. This study tested the effects of four main phytochemicals, determined by mass spectrometry/gas chromatography from an extract of *R. scutellariae* alone and in combination on two pathogenic *Acanthamoeba* species, and investigated the compatibility of these agents with existing MPS for RGP lenses.

METHODS

Preparation of *Acanthamoeba*

Acanthamoeba castellanii (product 30234; American Type Culture Collection, Manassas, VA, USA) and *A. polyphaga* (product 30461; American Type Culture Collection) were used in this study. For the production of trophozoites, *Acanthamoeba* organisms were grown axenically at 25°C without shaking in 10 mL of peptone-yeast extract-glucose (PYG) medium at pH 6.5. The PYG medium was changed every other day. When the cells reached approximately 90% confluency on the lower surface of the culture flasks, the flasks were vigorously agitated, and the contents transferred into 15-mL tubes and then centrifuged at 90g for 10 minutes at room temperature. The supernatant was discarded, and the pellet was washed three times with Page's amoebic saline (PAS) and then resuspended in fresh PAS. The final inoculum was adjusted to 10⁶ trophozoites/mL by dilution with PAS, and the number was determined manually, using a hemocytometer.

Preparation of Lead Phytochemicals of *R. scutellariae*

Investigation of a hot water extract of *R. scutellariae*, using spectrometry/gas chromatography revealed that this extract of the herb contained five lead compounds with possible antimicrobial effects. These phytochemicals, baicalin, baicalein, wogonin, wogonoside and oroxylin A (Zipharm International; Toronto, Ontario, Canada), were first tested against *A. royreba*. All these compounds, except for wogonin, caused 0.4

or more log reduction in viability in a pilot study (unpublished data). For this study, stock solutions of 100 mM of each of the remaining four phytochemicals were prepared in dimethyl sulfoxide (DMSO) for testing against the two pathogenic species of *Acanthamoeba*.

Determination of Amoebicidal Activities

The activity of the four phytochemicals against the two *Acanthamoeba* species was first determined. A fresh 0.2-mL aliquot of inoculum, prepared as described above, was added to 1.8 mL of PAS and each of the phytochemicals to give a final concentration of 400 μM and a final concentration of 10⁵ viable trophozoites/mL. Page's amoebic saline with an equal volume of DMSO was used as a negative control. The mixtures were left at 25°C for 4 hours and 6 hours, at which times samples were taken after mixing and were counted using the hemocytometer. Following examination of the effects of individual phytochemicals, those showing amoebicidal activity were combined to determine whether there were additive or synergistic effects.

To determine whether the phytochemicals could enhance the amoebicidal activity of MPS, the compounds were added to the solutions. Four commonly used MPS for RGP lenses were tested, and their major antimicrobial agent(s) are shown in the Table. A modified version of the stand-alone test was performed using the MPS alone and with the addition of the phytochemicals at 400 μM. DMSO was added to MPS without phytochemicals as control and to a PAS control as described above. The procedures were performed as above, replacing PAS with MPS in preconditioned plastic tubes. The resulting mixtures were vortexed and incubated at 25°C for the manufacturers' recommended disinfection times. Sampling and enumeration were performed as described above.

The phytochemicals were also added in combination with the MPS, and antimicrobial efficacy of the MPS with combined phytochemicals was determined as described for phytochemical alone. All estimations were performed in triplicate and standard deviations were determined.

RESULTS

When each phytochemical was used alone against *A. castellanii*, all four phytochemicals showed increased activity compared with the control. Wogoniside achieved a 0.5 log reduction in viability and baicalin 0.4 log reduction after 4 hours' exposure (Fig. 1a). In contrast, the most active phytochemical against *A. polyphaga* was oroxylin A, which produced a 0.4 log reduction (Fig. 1b). Increasing exposure time to 6 hours slightly increased the effects of all four phytochemicals against both organisms (Figs. 1c, 1d). Combinations of the more active agents were not always as active as the compounds alone, but baicalein and oroxylin A achieved a 0.65 and a 0.50 log reduction in viability of *A. castellanii* and *A. polyphaga*, respectively, after 4 hour's exposure (Figs. 1a, 1b).

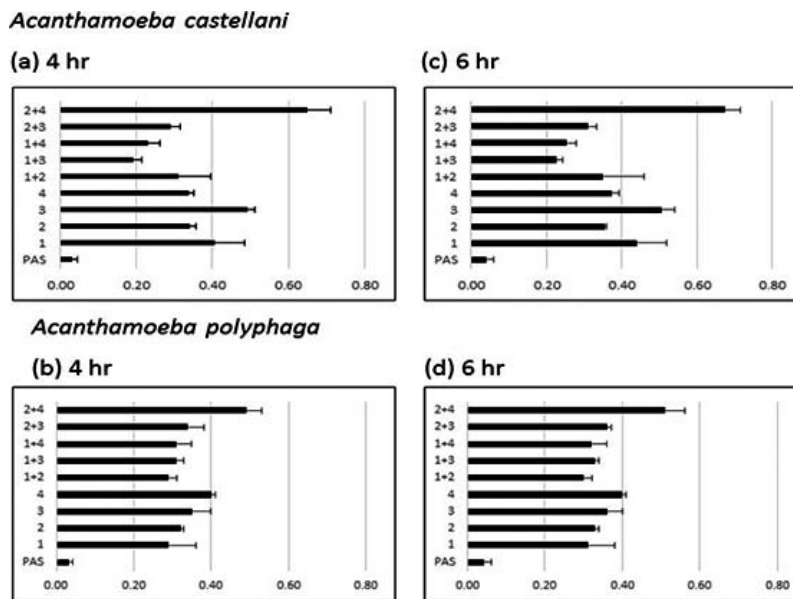


FIGURE 1. Log reduction in viability of *A. castellanii* and *A. polyphaga* exposed to lead phytochemicals of *R. scutellariae* for 4 (a, b) and 6 (c, d) hours. Phytochemical 1, baicalin; 2, baicalein; 3, wogonoside; 4, oroxylin A.

Of the four MPS tested for rigid lenses, MPS-D was the most active against *A. castellanii* achieving a 0.3 log reduction in viability (Table). A similar result was observed for *A. polyphaga* (0.27 log reduction). As the effects of the phytochemicals were less pronounced for *A. polyphaga* than for *A. castellanii*, they were tried in combination with MPS. Addition of each of the four phytochemicals individually to all four MPS resulted in increased activity against *A. polyphaga*. The increased effects were most pronounced for wogonoside and oroxylin A with MPS D. (Fig. 2) Combinations of the phytochemicals together with MPS enhanced the overall activity (Fig. 3) against both of the *Acanthamoeba* species. Combining baicalin and baicalein with MPS further enhanced the activity of all solutions against *A. castellanii*, increasing the log reduction up to 0.77 log for MPS-D, but did not enhance activity against *A. polyphaga* compared with the phytochemicals alone. The combination of baicalein and oroxylin A with MPS was most effective, increasing the activity of all four MPS above a 0.72 log kill for *A. castellanii*, with an optimal effect of 0.92 log reduction for these two compounds added to MPS-D (Fig. 3). For *A. polyphaga*, the combination of baicalein with either wogono-

side or oroxylin A and MPS D was most effective, giving a log reduction of 0.66 and 0.64, respectively.

DISCUSSION

All four phytochemicals, present in *R. scutellariae* affected the viability of pathogenic *Acanthamoeba*, with baicalin and wogonoside showing the greatest effects. Prolonging exposure from 4 to 6 hours did not increase the amoebicidal effects of these agents. When the phytochemicals were used in combination, the strongest effect was observed for the combination of baicalein and oroxylin. In contrast, most other combinations reduced the effectiveness compared to the compound alone. It is possible that clinical isolates may be more virulent and require higher concentrations of the agents, and further testing of such isolates is needed.

All four MPS used alone had little effect on the viability of *Acanthamoeba*, with only MPS-D achieving a log kill equivalent to the least active phytochemical. Combination of individual phytochemicals with MPS improved their activity, but not significantly above that of the phytochemicals alone. However, combined phytochemicals and MPS led to improved performance and almost 1-log reduction in numbers of viable *Acanthamoeba* organism in the case of MPS-D plus baicalein and oroxylin A for *A. castellanii*. This combination was the optimal for all MPS tested. It was interesting to note that the most active agent when used alone against *A. castellanii*, wogonoside, was less active in combination with other phytochemicals against both species, suggesting some antagonism. The activity of the most active individual agent against *A. polyphaga*, oroxylin A, was adversely affected only by the presence of baicalin. This antagonism was observed for both species. Combinations of active agents also had different effects which were somewhat associated with the MPS used. Although the active agents of the MPS may be similar, other additives present in the solutions may alter the effect of adding the phytochemicals. The activity of three of the combinations together with MPS-D exceeded that of any of the other solutions with any phytochemical combination against *A. castellanii*.

Acanthamoeba polyphaga

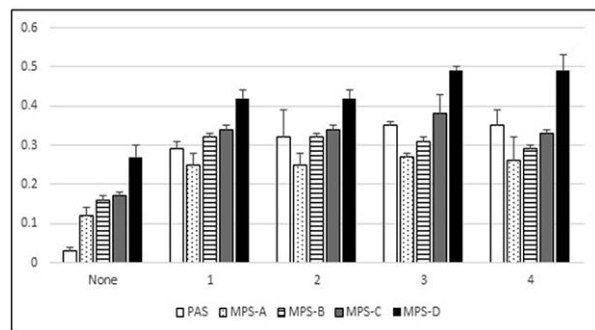


FIGURE 2. Log reduction in viability of *A. polyphaga* exposed to MPS alone and in combination with lead phytochemicals of *R. scutellariae* for 4 hours. Phytochemical 1, baicalin; 2, baicalein; 3, wogonoside; 4, Oroxylin A.

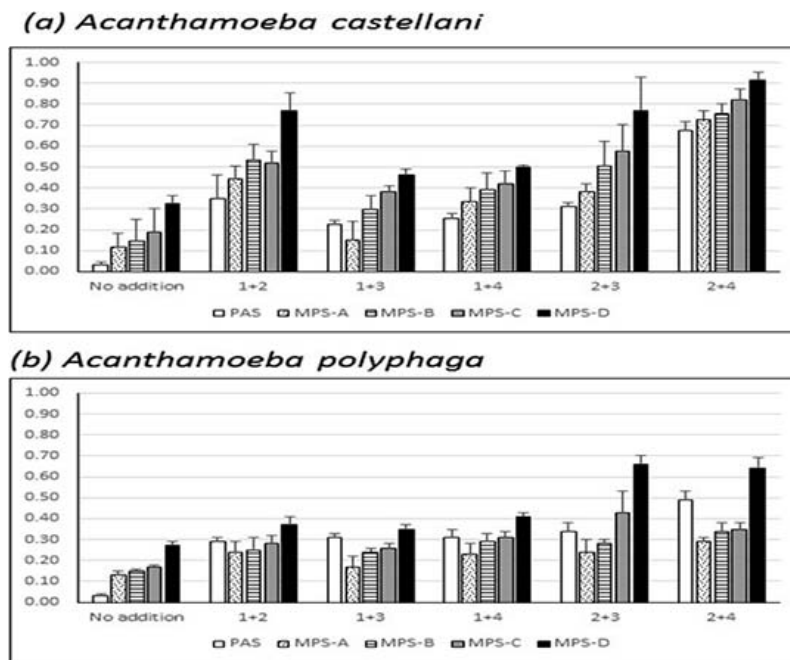


FIGURE 3. Log reduction in viability of (a) *A. castellani* and (b) *A. polyphaga* exposed to MPS alone and with combinations of lead phytochemicals of *R. scutellariae* for 4 hours. Phytochemical 1, baicalin; 2, baicalein; 3, wogonin; 4, oroxylin A.

Our results showed that the four MPS for RGP lenses tested lacked activity against *Acanthamoeba*. Noncompliant behavior combined with poor disinfecting capability of RGP solutions may increase risk of infection in subjects receiving orthokeratology treatment.²⁹ This study suggested that although further work is needed, a combination of baicalein and oroxylin A may be useful in the prevention or treatment of *Acanthamoeba* infection and, as synergistic effects were observed, that these agents could be also be added to current MPS.

R. scutellariae and its constituents have previously³⁰ been shown to have antibacterial properties, but we believe this is the first report of their activity against *Acanthamoeba*. We had previously noted effects of the parent herb against *Acanthamoeba* infection,²⁵ but results varied between batches of herb and herb extracts from different sources. Known chemical compounds can be synthesized and are therefore more predictable and reliable in their effects. Because constituents of *R. scutellariae* have antibacterial properties, their presence in multipurpose solutions may act against *Acanthamoeba* organisms not only by reducing its viability but also by reducing bacteria as its food source.

The phytochemicals identified in our *R. scutellariae* samples were similar to those identified in other studies.^{31,32} Some compounds have been shown to have useful properties and have been used in experimental treatments for cancer.³³ *R. scutellariae* has previously been shown to have minimum cytotoxic effects on HCEC,²⁸ and ingestion was not found to be harmful to rats or dogs.³¹ The herb has been used in TCM for many years seemingly without toxicity problems, but lack of toxicity of the individual agents requires confirmation.

This study has shown that phytochemicals present in *R. scutellariae* can affect the viability of *Acanthamoeba* organisms and thus have potential as therapeutic or prophylactic agents for AK. *Acanthamoeba* species are an important cause of keratitis, and the apparent compatibility of the phytochemicals with MPS for rigid lenses would make it possible to use them in MPS solutions and possibly reduce the incidence of AK. Such modification of RGP solutions has become imperative with the increasing popularity of overnight orthokeratology.

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