

Antifungal Activity of Natural Naphthoquinones and Anthraquinones against *Madurella mycetomatis*

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Eumycetoma, the fungal form of the neglected tropical disease mycetoma, is a crippling infectious disease with low response rates to currently available antifungal drugs. In this study, a series of natural naphthoquinones and anthraquinones was evaluated for their activity against *Madurella mycetomatis*, which is the most common causative agent of eumycetoma. The metabolic activity of *Madurella mycetomatis* as well as the viability of *Galleria mellonella* larvae upon treatment with

quinones was investigated. Several hydroxy-substituted naphthoquinones exhibited activity against *Madurella mycetomatis*. In particular, naphthazarin (5,8-dihydroxy-1,4-naphthoquinone) was identified as a considerably active antifungal compound against *Madurella mycetomatis* (IC₅₀ = 1.4 μM), while it showed reduced toxicity to *Galleria mellonella* larvae, which is a well-established *in vivo* invertebrate model for mycetoma drug studies.

Introduction

Mycetoma is classified as a neglected tropical disease (NTD) and characterized by grain formation in tumor-like lesions leading to severe malformation upon long-term infection with a heterogeneous group of filamentous bacteria (actinomycetoma) or fungi (eumycetoma).^[1] Most eumycetoma cases are caused by *Madurella mycetomatis* infection.^[2] Most actinomycetoma cases are caused by *Nocardia brasiliensis*. The therapy options and prognosis strongly depend on the causative agent(s). While actinomycetoma can be cured with common antibiotics in more than 90% of the cases, the treatment of eumycetoma with antifungals such as itraconazole and terbinafine is much less successful (with curing rates between 8 to 50%), and necessitates long-term treatment as well as amputation of affected limbs in advanced cases.^[3,4]

Since mycetoma is a poverty-associated disease, efforts to improve mycetoma treatments are scarce and alternative drug design approaches are warranted. One approach taken is the

screening and repurposing of drugs present in compound libraries. The screening of chemical boxes from the Medicines for Malaria Venture (MMV, Geneva, Switzerland) led to the identification of fenarimols as promising drug candidates for the treatment of *M. mycetomatis*.^[5] This development culminated in the establishment of an open science research network for the discovery of new eumycetoma drugs (MycetOS), which has identified further promising compound classes such as benzimidazoles, thiazoles, and melanin inhibitors, in addition to fenarimols.^[6,7]

Melanin biosynthesis is a vital factor for *M. mycetomatis*, which forms black grains during its development in the host, and melanin protects the fungus from stress factors and antifungal azole drugs.^[8] Combination of melanin inhibitors with itraconazole showed promising *in vivo* therapeutic effects in *M. mycetomatis*-infected *Galleria mellonella* larvae.^[9] Since naphthalene and naphthoquinone derivatives are components of the fungal melanin biosynthesis pathway, a closer look at the therapeutic potential of naphthoquinones appears to be reasonable.

There is a plethora of biologically active naphthoquinones. Natural naphthoquinones are wide-spread colorful plant metabolites. Its most prominent example is lawsone (2-hydroxy-1,4-naphthoquinone), also known as the coloring agent of the henna plant *Lawsonia inermis*, which has well-documented antioxidant and antimicrobial properties.^[10,11] Lawsone methyl ether (2-methoxy-1,4-naphthoquinone, MNQ) reportedly inhibited *Penicillium digitatum* and *Fusarium proliferatum* growth.^[12,13]

The natural naphthoquinone plumbagin was isolated from the roots of *Plumbago* sp., and is the main antifungal ingredient of Ayurvedic skin care products using "chitrak churna", the root powder made from South Asian *Plumbago* plants.^[14] Modern research confirmed the antifungal activity of plumbagin against *Candida albicans*.^[15,16] Juglone, the nor-analog of plumbagin, was isolated from common walnut *Juglans regia* and black walnut *Juglans nigra*.^[17,18] Juglone showed antifungal activity, which was enhanced by formulation with poly(*D,L*-lactic-co-glycolic acid) (PLGA) nanoparticles.^[19]

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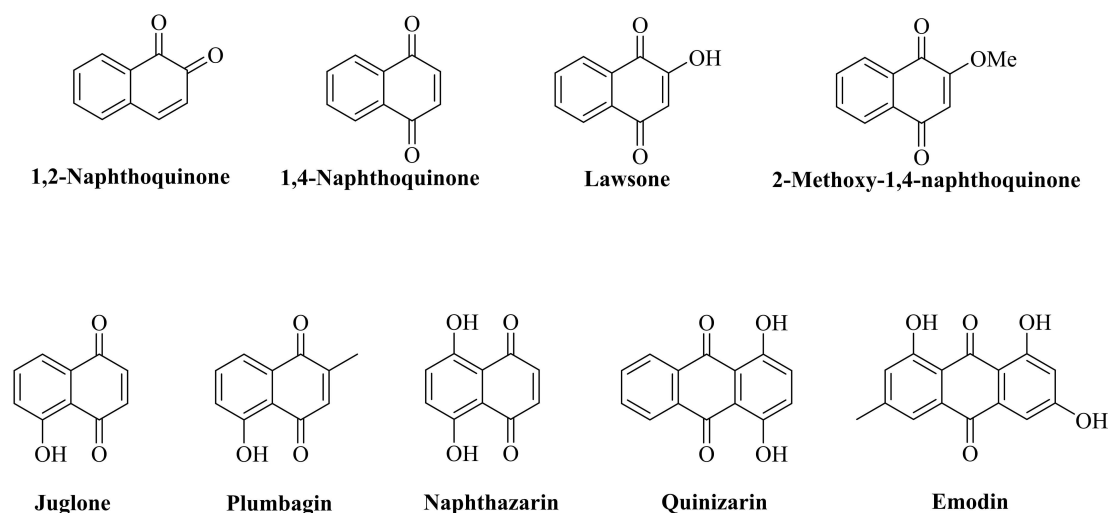


Figure 1. Structures of naphthoquinones and anthraquinones used in this study.

Naphthazarin was found in the Manchurian walnut *Juglans mandshurica* and the roots of Boraginaceae plants, and this compound exhibited protective effects on a Parkinson's disease model at sub-toxic concentrations.^[20,21] Investigation of the anticancer activity of naphthazarin unveiled noteworthy microtubule depolymerizing properties for this compound.^[22]

Marketed (semi-)synthetic antiparasitic naphthoquinone drugs such as buparvaquone also possess antifungal activities, and buparvaquone showed higher *in vivo* activity than itraconazole against *Sporothrix brasiliensis* in a *Galleria mellonella* model.^[23]

The class of natural anthraquinones is likewise a treasure trove of biologically active compounds. Quinizarin was found in dyer's madder (*Rubia tinctorum*) and showed selectivity for *Candida albicans* topoisomerase I when compared with human topoisomerase I.^[24] Emodin occurs in medicinal herbs such as *Rheum palmatum*, *Polygonum cuspidatum* and *Polygonum multiflorum*, which have been used in TCM (traditional Chinese medicine) for centuries, while emodin itself presented various biological properties such as antioxidant, anticancer, antimicrobial, anti-diabetic, immune-suppressive, hepatoprotective and neuroprotective effects.^[25,26] Its antifungal activity was documented upon treatment of *Candida albicans*, and suppression of (1,3)- β -D-glucan synthase as well as inhibition of fungal CK2 were identified as possible modes of action of emodin in *Candida albicans*.^[27–29]

The cytotoxic and antifungal activity of naphthoquinones can be primarily attributed to reactive oxygen species (ROS) formation as well as interference with redox cycles and associated proteins.^[30] While a panel of antiparasitic nitroimidazole and nitrofurans-based drugs failed to exert antifungal activity against *M. mycetomatis*, other redox-active compounds such as niclosamide and its analog MMV665807 revealed potent activity against various *M. mycetomatis* and *Actinomyces* strains.^[31]

To the best of our knowledge, there is no data available about the anti-mycetoma activity of natural naphthoquinones

and anthraquinones. Hence, in this study, the antifungal activity of a panel of naphthoquinone and anthraquinone derivatives against *M. mycetomatis* was investigated and discussed for the first time.

Results and Discussion

The natural naphthoquinones lawsone, 2-methoxy-1,4-naphthoquinone, juglone, plumbagin, and naphthazarin, and the natural anthraquinones quinizarin and emodin were selected for antifungal testing based in their previously documented function as antibiotic and/or antimycotic agents (Figure 1). 1,2-Naphthoquinone and 1,4-naphthoquinone were added to this study for comparison purposes. Initially, the *in vitro* antifungal activity of the test compounds at concentrations of 100 μ M and 25 μ M was investigated against the most common *M. mycetomatis* MM55 strain (Table 1).

Except for the two lawsone derivatives (i.e., lawsone and 2-methoxy-1,4-naphthoquinone) and quinizarin, all tested quinone derivatives showed promising antifungal activities

Table 1. Metabolic activity (in%)^a upon treatment of *M. mycetomatis* isolate MM55 with test compounds at doses of 100 μ M and 25 μ M. Itraconazole served as positive control.

Compound	100 μ M	25 μ M
1,2-Naphthoquinone	19.2 \pm 3.0	11.5 \pm 2.4
1,4-Naphthoquinone	11.7 \pm 0.8	7.0 \pm 0.6
Lawsone	42.7 \pm 5.1	84.7 \pm 16.7
2-Methoxy-1,4-naphthoquinone	42.5 \pm 8.2	57.5 \pm 16.6
Juglone	13.0 \pm 2.0	7.1 \pm 3.6
Plumbagin	7.2 \pm 0.8	5.6 \pm 3.5
Naphthazarin	13.2 \pm 2.9	7.4 \pm 1.9
Quinizarin	79.5 \pm 11.5	47.5 \pm 4.9
Emodin	13.6 \pm 3.1	8.2 \pm 2.8
Itraconazole ^b	2.34 \pm 4.49	-1.04 \pm 2.37

^aExperiments were performed in triplicate \pm SD (calculated using GraphPad Prism 7). ^bValues taken from ref. [7].

against *M. mycetomatis* MM55 fungi at a concentration as low as 25 μM . Plumbagin, juglone, naphthazarin, 1,4-naphthoquinone, emodin and 1,2-naphthoquinone were all inhibiting *M. mycetomatis* growth at a concentration of 25 μM . For these compounds accurate IC_{50} values were determined. Here, naphthazarin was identified as the most active compound with an IC_{50} value of 1.4 μM (Figure 2), which is in the reported activity range of the positive control itraconazole with an IC_{50} value of 1.1 μM .^[7] The other quinones exhibited distinctly lower activities with IC_{50} values between 10–12 μM (Table S1, Supporting Information). In addition, a MIC_{50} value of 8.0 μM was obtained for naphthazarin upon testing against ten *M. mycetomatis* strains (Table 2).

Because of its promising *in vitro* activity against *M. mycetomatis*, naphthazarin was selected for *in vivo* experiments using *Galleria mellonella* wax moth larvae. Initially, the toxicity of naphthazarin to the larvae was evaluated. Treatment of larvae with a single dose of naphthazarin (0.152 $\mu\text{g}/\text{kg}$) showed no toxic effects, and all treated larvae survived. Thus, a dose of 0.152 $\mu\text{g}/\text{kg}$ naphthazarin appeared to be safe, while its close analog juglone was reported to be toxic to *G. mellonella* larvae at doses of 0.4, 4.0, and 40 $\mu\text{g}/\text{larvae}$.^[9] Next, larvae infected

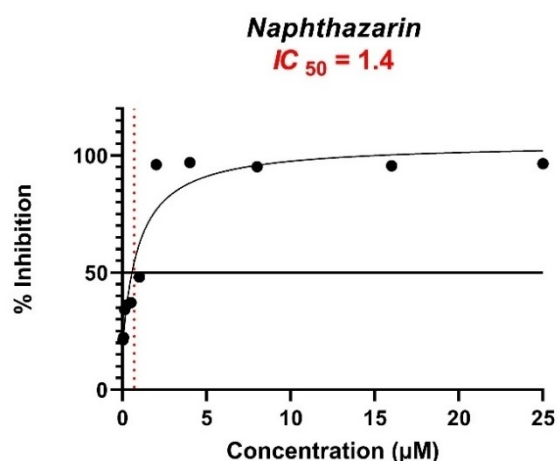


Figure 2. Antifungal activity (dose-response curve and IC_{50}) of naphthazarin when tested against *M. mycetomatis* MM55 ($n = 1$).

Table 2. Antifungal activity (MIC) of naphthazarin against ten genetically and geographically diverse <i>M. mycetomatis</i> isolates.		
Strain	Naphthazarin MIC (μM)	Itraconazole MIC (μM)
MM55	2.0	0.09
MM49	4.0	0.09
I1	8.0	0.04
I3	16.0	0.35
I11	8.0	0.35
P1	4.0	0.04
AI1	8.0	0.04
SO1	8.0	0.09
Peru72012	4.0	0.04
CBS247.48	8.0	0.09
MIC_{50}^a	8.0	0.09

^a MIC_{50} values were obtained from the individual MIC values of ten different *M. mycetomatis* isolates ($n = 1$).

with *M. mycetomatis* were used for *in vivo* activity tests with naphthazarin (0.152 $\mu\text{g}/\text{kg}$, administered thrice 4 h, 28 h, and 52 h after infection), and the observed results were compared with the survival of untreated larvae and of larvae treated with the positive control amphotericin B (Figure 3). Amphotericin B was chosen as positive control because it prolonged survival in the *G. mellonella* model in contrast to azoles, which were inactive.^[32] As expected, amphotericin B showed improved survival rates when compared with untreated infected larvae, however, naphthazarin showed no improvement in larvae survival. Although after one day the survival rate was slightly better than in the untreated group, many larvae died shortly after the second dose of naphthazarin on day 2, and after four days, all larvae of the naphthazarin group were dead. Based on this finding, the administration of reduced doses of naphthazarin (less than 0.152 $\mu\text{g}/\text{kg}$) and/or longer recovery times between the first, the second and the third dose (more than 24 h) appear to be feasible in future *in vivo* experiments with *M. mycetomatis*-infected *G. mellonella* larvae in order to achieve more promising results for naphthazarin. Reduced doses appear to be reasonable given the high *in vitro* activity of naphthazarin with its MIC_{50} value of 8 μM .

Reduced or non-toxic doses of naphthazarin might also be applied in combination with other non-toxic antifungals in order to reach an optimum of antifungal activity with a minimum of side-effects. The application of sophisticated formulation systems such as PLGA, which was successfully applied for juglone before, can be another way to improve the *in vivo* activity and to reduce toxic effects of naphthazarin.^[19] Toxic *in vivo* effects of naphthoquinone drugs are mainly based on an imbalance of the host antioxidant system upon treatment.^[33] Juglone-resistant moths were able to reduce juglone to harmless 1,4,5-dihydroxynaphthalene in their gut.^[34,35] In cell-based systems, addition of *N*-acetylcysteine (NAC) detoxified plumbagin and juglone based on their reactive C3 atom (in contrast to lawsone, which was inactive in this study), and, thus, might also be applied in combination with naphthazarin to reduce its toxicity.^[36] However, it should be

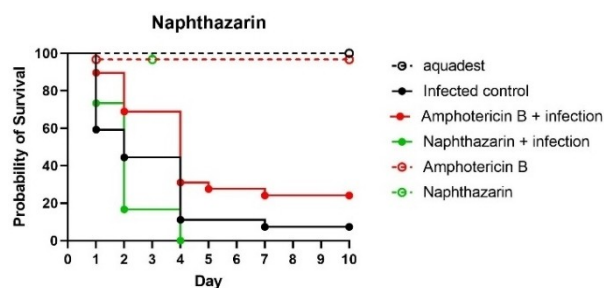


Figure 3. Survival curve of *G. mellonella* larvae infected with *M. mycetomatis* and treated with naphthazarin. The dashed line represents non-infected larvae treated with aquadest. The black line represents *M. mycetomatis* infected *G. mellonella* larvae receiving no treatment. The red line represents *M. mycetomatis* infected larvae treated with 1 mg/kg amphotericin B 4 h, 28 h and 52 h after infection. The green line represents *M. mycetomatis* infected larvae receiving 0.152 $\mu\text{g}/\text{kg}$ naphthazarin 4 h, 28 h and 52 h after infection. The red dashed line shows healthy larvae treated with 1 mg/kg amphotericin B on three consecutive days, while the green dashed line indicates treatment of healthy larvae with 0.152 mg/kg naphthazarin.

taken into account that naphthazarin reacts more slowly with NAC than juglone and plumbagin.^[37] In addition to NAC, vitamin C was able to reduce toxic effects of juglone on porcine oocytic cells and mouse embryos.^[38,39] It is noteworthy that vitamin C potentiated juglone *in vivo* antitumor activity in mice bearing Ehrlich ascites tumors, and, thus, it might be a suitable additive for *in vivo* applications of naphthazarin, too.^[40,41]

Conclusions

The screening of a small panel of naphtho- and anthraquinone derivatives led to interesting results and the identification of a new lead compound. The naphthoquinone naphthazarin showed promising *in vitro* activity against the fungus *M. mycetomatis* fungi and might be considered for future *in vivo* experiments with adjusted dose and treatment schemes considering its toxic effects at repeatedly administered high doses. Synthetic fine-tuning of the naphthazarin molecule as well as combination of antifungal naphthazarin with other fungicides (azoles) and/or additives (vitamin C) might also be a useful strategy to optimize activity and to reduce systemic toxicity. Community contributions to this research are welcome via the open science MycetOS infrastructure.^[42]

Experimental Section

General

Naphthoquinones (1,2-naphthoquinone, 1,4-naphthoquinone, lawsone, 2-methoxy-1,4-naphthoquinone, juglone, plumbagin, and naphthazarin) and anthraquinones (quinizarin and emodin) were obtained from Sigma-Aldrich, Alfa-Aesar, and TCI, and these compounds were used for the biological experiments without further purification.

Compound screening

The antifungal activity of the compounds was tested against a geographically and genetically diverse set of *M. mycetomatis* isolates. These included strains MM49 and MM55 from Sudan, strains I1, I3 and I11 from India, strain P1 from Mali, strain AI1 from Algeria, strain SO1 from Somalia, strain Peru72012 from Peru and strain CBS247.48 whose country of origin was unknown. All isolates were originally obtained from patients and identified and maintained in the Erasmus Medical Centre, Rotterdam, The Netherlands. *In vitro* susceptibility testing was performed as reported previously.^[7] Mycelia were cultivated in RPMI 1640 medium supplemented with 0.35 g/L L-glutamine (Capricorn-Scientific, Germany) and 1.98 mM 4-morpholinepropanesulfonic acid (MOPS, Sigma, USA) at 37 °C. *M. mycetomatis* mycelia in RPMI 1640 medium were sonicated for 10 s at 20 μm (Soniprep, Beun de Ronde, The Netherlands) and centrifuged at 2600×g for 5 min. The mycelia were washed and resuspended in fresh RPMI 1640 medium to obtain a fungal suspension of 68% to 72% transmission at 660 nm (Novaspec II spectrophotometer). Per compound 1 μL at a given concentration was added to a round-bottom 96-well microtiter plate (Corning, USA) along with 100 μL of fungal suspension. Plates were sealed and incubated at 37 °C for 7 days. After incubation, 100 μL of 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-

[(phenylamino)carbonyl]-2H-tetrazolium hydroxide (XTT) work solution (5 mL XTT (Sigma), 1 mg/mL in NaCl; 0.6 mL menadione (Sigma), 1 mM and 4.4 mL in NaCl) was added to each well, followed by incubation at 37 °C for 2 h and at room temperature for 3 h. The extinction of the supernatant was measured at 450 nm using a microplate reader (Epoch2, Biotek, USA). All assays were performed in triplicate.

Test compounds were investigated at concentrations of 100 μM and 25 μM against strain MM55. When compounds inhibited growth at 25 μM, the IC₅₀ values of these compounds were determined. For this reason, growth inhibition at concentrations ranging from 0.03 μM to 25 μM was determined and plotted. If an IC₅₀ < 8 μM was obtained, then the minimal inhibitory concentrations against the other isolates were determined as well.

Toxicity and *in vivo* activity in *Galleria mellonella*

In vivo efficacy was determined in larvae of the greater wax moth *Galleria mellonella*. Since *G. mellonella* is an invertebrate it is not subject to directive 2010/63/EU of the European law on animal testing. Larvae were housed in the dark in Petri dishes with Whatman paper. To determine the toxicity in *G. mellonella* larvae, a single dose of 0.152 μg/kg naphthazarin was injected in the last pro-leg of healthy larvae followed by monitoring of the survival for ten days. If no significant difference between the control and treated larvae was observed the compound was considered as non-toxic. Larvae were further used in infection studies to determine the *in vivo* activity of naphthazarin against *M. mycetomatis* according to our previously published protocol.^[5,32] Briefly, *M. mycetomatis* isolate MM55 mycelia were cultured in colorless RPMI 1640 medium supplemented with L-glutamine (0.3 g/L), 20 mM 4-morpholinepropanesulfonic acid (MOPS) and chloramphenicol (100 mg/L; Oxoid, Basingstoke, United Kingdom) for 2 weeks at 37 °C and sonicated for 2 min at 20 μm (Soniprep, Beun de Ronde, The Netherlands). The resulting homogenous suspension was washed with PBS and diluted to an inoculum size of 4 mg wet weight per larvae. 40 μL of the fungal suspension were injected in the last left pro-leg with an insulin 29 G U-100 needle (BD diagnostics, Sparks, Nevada, USA). Then, infected larvae were treated with 0.152 μg/kg naphthazarin at 4, 28, and 52 h after infection. Analogously, infected larvae were treated with 1 mg/kg amphotericin B as positive control based on its clinical application in humans. Treatment was given during the first three days after infection, followed by a seven-day observation period. Larvae were monitored for ten days in total. Larvae forming a cocoon were not considered for evaluation.

Statistical analysis

GraphPad Prism 7 (GraphPad Inc.) was applied for the Log-rank test and IC₅₀ calculations. A *p*-value smaller than 0.05 was considered to be significant.

Supporting Information

Additional supporting information (Table S1 with further IC₅₀ values, original NMR and MS spectra) may be found in the online version of the article at the publisher's website.

Author Contributions

MT, WWJS and BB designed the project. BB wrote the draft manuscript. JM performed the biological assay. MT and BB supervised the chemistry aspect, while WWJS supervised the biology aspect of the work. All authors reviewed, edited, and approved the manuscript for submission.

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Conflict of Interests

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Keywords: antifungal · eumycetoma · naphthazarin · naphthoquinone · neglected tropical disease

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