

In vitro antischistosomal activity of *Artemisia annua* and *Artemisia afra* extracts

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ABSTRACT

Background: Schistosomiasis, a neglected tropical disease, imposes substantial health and economic burdens on impoverished groups living in predominantly rural areas. Praziquantel (PZQ) is the only drug available for treatment, and it is not completely efficacious. *Artemisia annua* and *Artemisia afra* infusions were proposed to possess antischistosomal activities in a recently retracted publication of a clinical trial, leading to our investigation *in vitro*.

Objective: The objective was to identify the main components of the infusions and evaluate the *in vitro* antischistosomal activities of traditionally prepared infusions as well as hexane and dichloromethane (DCM) extracts of the infusions of *A. afra* and *A. annua*.

Methods: Infusions of *A. afra* and *A. annua* were submitted to liquid-liquid partitioning with n-hexane and DCM to provide samples for *in vitro* bioassays using newly transformed schistosomulas (NTS) and adult *Schistosoma mansoni* worms obtained from infected mice. The viability of the NTS and adult *S. mansoni* was visually scored via microscopic readout.

Results: Nine phytochemicals comprising coumarins and organic acids were identified. *A. afra* and *A. annua* infusions and extracts possess potent *in vitro* antischistosomal activities against NTS, at 100 µg/ml. However, the *A. afra* infusions exhibited better activities against NTS than the *A. annua* infusion. The *A. afra* hexane- and DCM extracts presented IC₅₀ values that are similar to PZQ (1.5 µg/ml) and approximately five times lower than the comparison drug artesunate (11.6 µg/ml) against NTS. Low IC₅₀ values for both these extracts were also obtained in phenotypic assays with adult *S. mansoni*.

Conclusion: *A. afra* shows greater antischistosomal potential than *A. annua*. Thus, further studies are necessary to identify the active molecule(s) responsible for the notable antischistosomal activity of *A. afra*.

Introduction

The neglected tropical disease, schistosomiasis (bilharzia), is caused by the *Schistosoma* trematode fluke species of which *S. mansoni*, *S. japonicum*, and *S. haematobium* are the three main important parasites for human infection (Colley et al. 2014; CDC, 2018; McManus et al. 2018; WHO, 2021). Infectious cercariae penetrate the host's skin and transform into schistosomulas, which travel the vascular system and eventually reach the host's liver, where they mature into adult worms.

Fertilised eggs are produced by adult female worms inside the host's veins to either induce inflammation that leads to morbidity, or to be cast back into the environment (Colley et al. 2014).

Populations in rural tropical and sub-tropical regions are mainly affected (CDC, 2018; Colley et al. 2014; McManus et al. 2018; WHO, 2021). Malnutrition, anaemia, and underdeveloped cognitive function among infected children are common (CDC, 2018). Inadequate sanitation, unsafe water supply, food insecurity, and the widespread occurrence of parasites contribute to the vulnerability of endemic

Abbreviations: DCM, dichloromethane; IC₅₀, Half-maximal inhibitory concentration; NTS, newly transformed schistosomula; PZQ, Praziquantel.

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communities to infection (Colley et al. 2014; McManus et al. 2018; WHO, 2021). However, prevalence is increasing due to migration patterns and increased tourism (WHO, 2021). For example, schistosomiasis remains a frequent infection among Europeans who travel to and migrate from susceptible areas (Lingscheid et al. 2017). Overall, some 800 million people are at risk of acquiring infection, with transmission occurring in 78 countries (Boubacar et al. 2017; WHO, 2021).

In 2019, the World Health Organization (WHO) estimated that 236 million people, of which 90% resided in Africa, required treatment. Current treatment consists mainly of praziquantel (PZQ), which is an effective and safe treatment option and is included in schistosomiasis control programmes (CDC, 2018; McManus et al. 2018). This drug is currently on the WHO's list of essential medicines and although effective against all adult *Schistosoma* flatworm species, it is, however, ineffective against the immature- and egg phase of the flatworms (WHO, 2019; Xiao et al. 2018). Even though it is considered the safest among all anthelmintic drugs, the potential for emergence of drug resistance and its effectiveness against only the adult stage of the parasite indicate that new and safe medications, effective against all stages, are needed (Cioli et al. 2014).

Certain antimalarial drugs, such as mefloquine and derivatives of artemisinin, extracted from *Artemisia annua* L. (Asteraceae), were shown to be effective against the immature schistosome stages rather than against the adult flatworms (Keiser et al. 2009; Bergquist et al. 2017). Ethanolic *A. annua* extracts have also been shown to possess lethal effects against adult *S. mansoni* worms *in vitro* (Abd Ellah et al. 2021). In experimental animals, the artemisinin derivative: artemether, was also shown to prevent the maturing of *S. mansoni* worms, if treatment is applied during the larval stages of the parasite (Xiao et al. 2000). Currently, artemisinin and its derivatives are used for treatment of malaria, and therefore the prospect that other *Artemisia* species might yield new antiparasitic compounds, including antischistosomal compounds, should be investigated (De Almeida et al. 2016; Gruessner et al. 2019). *Artemisia afra* Jacq. ex Willd. (Asteraceae) is closely related to *A. annua* but does not contain artemisinin (Van der Kooy et al. 2008; Du Toit and Van der Kooy, 2019). Reviews documenting the studies on *A. afra* indicated that *A. afra* has been tested for *in vitro* activity against *Plasmodium falciparum*, parasitic gastrointestinal nematodes, *Trypanosoma* species and *Leishmania donovani* with good to moderate activity (Liu et al. 2009; Du Toit and Van der Kooy, 2019). The fact that *A. afra* does not contain artemisinin but does show interesting activity against various parasites, therefore makes it a species of interest for further studies.

In a recent publication (Munyangi et al. 2018), it was reported that a clinical trial involving infusions prepared from leaves and twigs of both *A. annua* and *A. afra* might offer an alternative treatment for schistosomiasis, with *A. afra* being as efficacious as *A. annua* in eliminating *S. mansoni* parasite eggs, as indicated by the examination of patient faecal smears. Although the trial received a published critique highlighting serious shortcomings of the study and hence raising considerable doubts on the results observed (Argemi et al. 2019), followed by article retraction (Munyangi et al. 2020), our attention was drawn to the fact that despite no artemisinin being present in *A. afra* (van der Kooy et al. 2008), it could still display antischistosomal activity similar to that of *A. annua*, which does contain artemisinin.

We therefore decided to retrace the first steps of the pharmacological studies by assessing the *in vitro* antischistosomal activity of *A. annua* and *A. afra* infusions as well as hexane and DCM extracts, in order to investigate the role played by artemisinin in the antischistosomal activity, as was also suggested by Gruessner et al. (2019).

The aim of the current study was to comparatively evaluate traditionally prepared *A. afra* and *A. annua* infusions, n-hexane and DCM extracts prepared from the infusions, and the extracted infusions for their antischistosomal activities *in vitro*.

Material and methods

Chemicals and reagents

n-Hexane and DCM used for liquid-liquid partitioning of infusions were purchased from Merck (Darmstadt, Germany) and used without purification. Ultrapure water for the preparation of infusions was obtained from a Replib Ultrapure water system (Boston, MA, USA). Scopolin (CAS number: 531-44-2), scopolin (92-61-5), chlorogenic acid (327-97-9), neochlorogenic acid (906-33-2), 4-caffeoylquinic acid (905-99-7), 1,5-dicaffeoylquinic acid (DCQA) (19870-46-3), 3,4-DCQA (14534-61-3), 3,5-DCQA (2450-53-5), 4,5-DCQA (57378-72-0), luteolin (491-70-3), and quercetin (117-39-5) were purchased from Alfa Biotechnology (Chengdu, China) and all were of >98% purity.

Plant material

Commercial *A. annua* (cultivar A3) plant material was donated by ANAMED International e.V. (Winnenden, Germany). *A. annua* is commercially cultivated to contain high levels of artemisinin, which can act as a chemical marker for species identification. LCMS analysis was conducted to quantify artemisinin and thereby positively identify the plant material.

Leaves and twigs of *A. afra* were collected at Bronkhorstbaai (BB), South Africa and in the Botanical Garden (BG) of North-West University in April 2019, after which the plant samples were oven-dried for seven days at 40°C to remove moisture. All dried leaves were then separated from the twigs to obtain the material used for this study. Voucher herbarium specimen samples of both *A. afra* samples were deposited at the A.P. Goossens Herbarium at North-West University (PUC0015454 (*A. afra* BB) and PUC0015456 (*A. afra* BG)).

Preparation of plant material infusions and extracts

Infusions were prepared by adding leaf material (2 g) to boiling deionised water (200 ml). The mixture was allowed to infuse for 10 min, after which it was filtered. The filtrate (2 ml) was then again filtered through a polytetrafluoroethylene (PTFE) syringe filter (0.45 µm, 25 mm) into a high-performance liquid chromatography (HPLC) vial for chemical analysis. The remaining infusion was frozen at -80°C, followed by freeze-drying to yield a primary dried infusion sample.

For liquid-liquid partitioning, infusions were prepared as described above. After the initial filtration, the infusion (200 ml) was cooled to room temperature (25°C), transferred into a separating funnel, and sequentially extracted with n-hexane (200 ml) and DCM (200 ml). The organic extracts were evaporated to dryness using a rotary evaporator (Buchi R200). The dried organic extracts were reconstituted in 5 ml of methanol, of which 1 ml of each was transferred into HPLC vials for analysis, whereas the remaining solutions were again dried to yield the extracts for bioactivity testing. One ml of the residual aqueous extract (extracted infusion) was transferred into a HPLC vial for analysis, whilst the remaining liquid was frozen at -80°C, followed by freeze-drying to yield a dried extracted infusion sample.

HPLC analysis

A Shimadzu iNexera LC-2040 HPLC equipped with a quaternary pump, autosampler, column oven, and photodiode array detector was used to analyse the samples. Separation and chemical fingerprinting were performed on an Agela C18 4.6 × 150 mm, 5 micron column (Agela Technologies, Tianjin, China). For the primary infusion samples, a stepwise gradient mobile system was used at a flow rate of 0.8 ml/min. The eluents consisted of water + 0.1% formic acid (A) and methanol (MeOH) + 0.1% formic acid (B). The gradient system started at 20% B at time 0-1, increased to 40% B after 6 min, where it was kept at 40% until 9 min, whereafter it increased to 60% after 11 min. It was kept at 60%

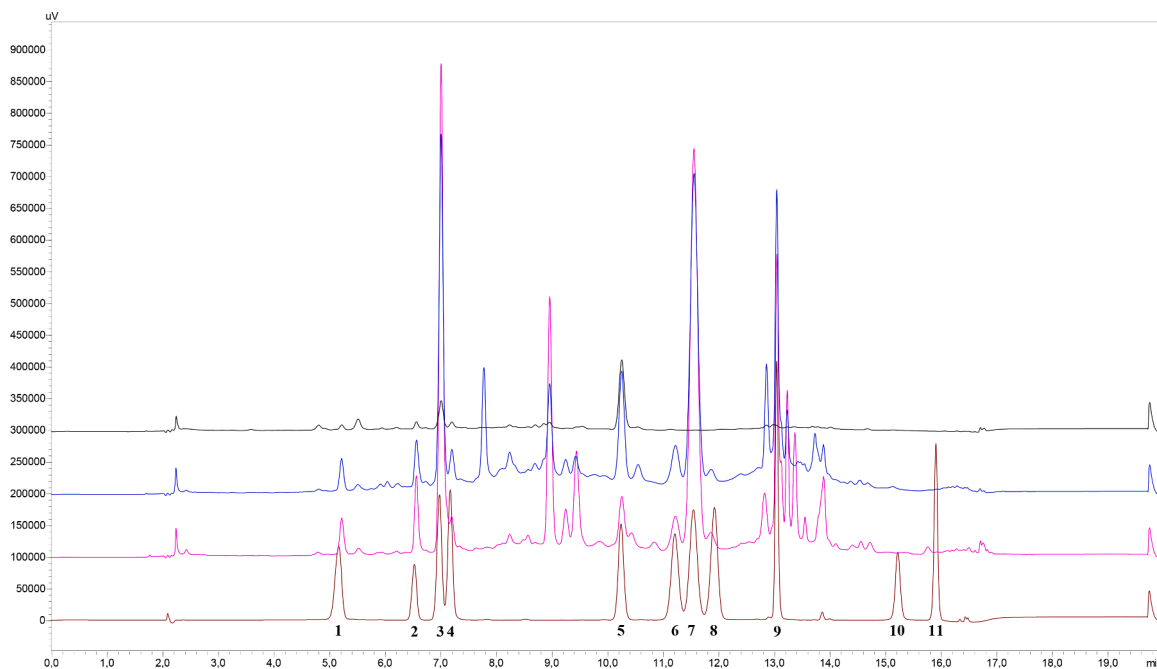


Fig. 1. Overlaid HPLC chromatograms of the infusions and the reference standard mixture (RSM). From top to bottom: *A. afra* (BG), *A. afra* (BB), *A. annua*, and RSM, as measured at 254 nm. The elution order for the reference standards is: [1] neochlorogenic acid, [2] scopoline, [3] chlorogenic acid, [4] 4-caffeoylquinic acid, [5] scopoletin, [6] 3,4-dicaffeoylquinic acid (DCQA), [7] 3,5-DCQA, [8] 1,5-DCQA, [9] 4,5-DCQA, [10] quercetin, and [11] luteolin.

until 13 min, increasing to 100% B at 14 min, where it was kept at 100% until 17 min. The system was re-equilibrated for 3 min at 20% B. For the hexane, DCM and extracted infusion samples the gradient started at 20% B, changing to 100% B after 20 min, and holding at 100% B until 25 min, after which the system was reconditioned at 20% B for 5 min. A mixture of the reference compounds was also analysed using these two chromatographic systems. The choice of reference compounds was based on literature regarding the major compounds found in *A. annua* infusions (Van der Kooy et al. 2008; Mouton and Van der Kooy, 2014; Du Toit and Van der Kooy, 2019).

Liquid chromatography mass spectrometry analysis

A validated method using an Agilent Ultivo electrospray ionisation triple quadrupole mass spectrometer was used to analyse all samples for artemisinin content (Lee et al., 2022). A Kinetix 2.6 μm C18 100 \times 2.1 mm column was used, and the gradient mobile system was as follows: 0.0 min 35% B, 1.0 min 35% B, 2.5 min 100% B, 3.9 min 100% B, 4.0 min. 35% B, and a post run time of 1.5 min to re-equilibrate the system. Total run time was 5.5 min. A multiple reaction monitoring (MRM) method with the following transitions was used for quantitation of artemisinin; 283.2 m/z to 265.1 m/z (collision energy 4 V) and 247.1 m/z. (collision energy 8 V). The optimized settings were as follow: gas temperature 300°C, N₂ gas flow 10 L/min, nebulizer pressure 35 psi, capillary voltage 4000V, fragmentor voltage 85V.

Drug-screening assay with *S. mansoni* newly transformed schistosomula (NTS) and adult worms

All samples were first diluted in 100% dimethyl sulfoxide (DMSO) (Sigma-Aldrich, Schaffhausen, Switzerland) to obtain stock solutions of 10 mg/ml. The dried *A. afra* (BB) infusion sample was equally divided (1:1) into two samples and the one dissolved in DMSO, as above, and the second in ultrapure water (Milli-Q® Advantage A10 purification system, Merck) to a concentration of 10 mg/ml. All stock solutions were vortexed and sonicated. For *in vitro* assays with NTS and adult *S. mansoni*, stock solutions were further diluted with supplemented M199 medium

(Gibco, Waltham, MA, USA) and supplemented RPMI 1640 medium (Gibco), respectively. Working solutions of 1 mg/ml and 100 $\mu\text{g/ml}$ were prepared.

NTS were obtained by mechanically transforming cercariae of *S. mansoni* (Liberian strain), which were obtained from infected intermediate host snails (*Biomphalaria glabrata*). Adult *S. mansoni* were collected by dissecting the mesenteric veins of infected mice at day 49 post-infection.

The phenotypic screening was conducted as described by Lombardo et al. (2019). In brief, approximately 30-40 NTS were incubated with the test samples in a final volume of 250 μl of M199 medium supplemented with 5% (v/v) fetal calf serum (FCS) (Bioconcept AG, Allschwil, Switzerland), 1% (v/v) penicillin/streptomycin (Sigma-Aldrich), and 1% (v/v) antibacterial/antifungal solution for up to 72 hrs at 37°C and 5% CO₂, using transparent flat-bottom 96 well plates (Sarstedt AG, Sevelen, Switzerland). The final drug concentration in the well was 100 $\mu\text{g/ml}$ or 10 $\mu\text{g/ml}$ depending on the working solution that was used. To determine the IC₅₀ of the samples, dose response assays in a concentration range of 6.25 $\mu\text{g/ml}$ to 100 $\mu\text{g/ml}$ and 0.621 $\mu\text{g/ml}$ to 10 $\mu\text{g/ml}$ were conducted, starting at a concentration of 100 $\mu\text{g/ml}$ and 10 $\mu\text{g/ml}$, respectively. Each sample was tested in triplicate, and the experiments were repeated once.

Phenotypic assays with adult worms were performed in a similar manner. At least three worms (both sexes) were incubated with RPMI 1640 supplemented with 5% FCS and 1% penicillin/streptomycin at 37°C and 5% CO₂ for 72 hrs at concentrations of 100 $\mu\text{g/ml}$ and 10 $\mu\text{g/ml}$, respectively. Transparent flat-bottom 24-well plates were used, and the total volume was 2 ml. The experiments were conducted in duplicate and repeated once.

For all assays, negative controls (using the highest concentration of DMSO without exceeding 1.5% (v/v)) were included. The viability of the NTS and adult *S. mansoni* was visually scored via microscopic readout at selected time points (24 h, 48 h, and 72 h), using reference points such as motility, morphology, and granularity. Drug effects were calculated (Microsoft Excel) from mean viability values (\pm SD) of treated parasites in relation to the control, and IC₅₀ values were calculated using GraphPad Prism (Version 8.2.1).

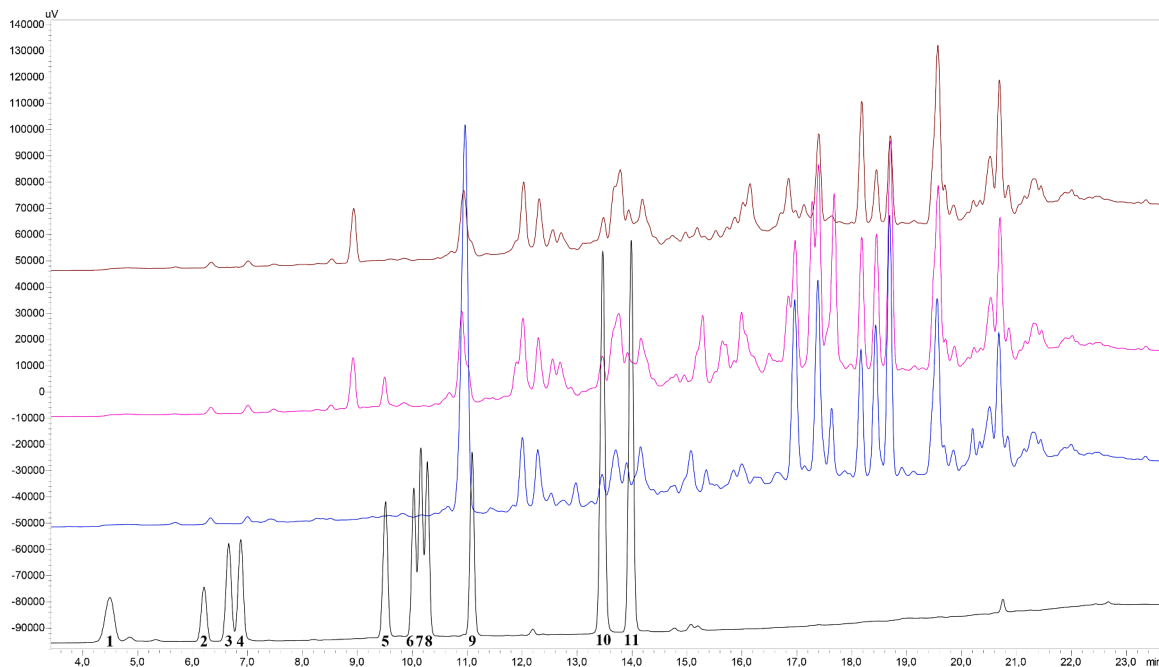


Fig. 2. Overlaid HPLC chromatograms of the hexane extracts of the infusions and the reference standard mixture (RSM). From top to bottom: *A. afra* (BG), *A. afra* (BB), *A. annua*, and RSM, as measured at 254 nm. The elution order for the reference standards is: [1] neochlorogenic acid, [2] scopoline, [3] chlorogenic acid, [4] 4-caffeoylquinic acid, [5] scopoletin, [6] 3,4-dicaffeoylquinic acid (DCQA), [7] 3,5-DCQA, [8] 1,5-DCQA, [9] 4,5-DCQA, [10] quercetin, and [11] luteolin.

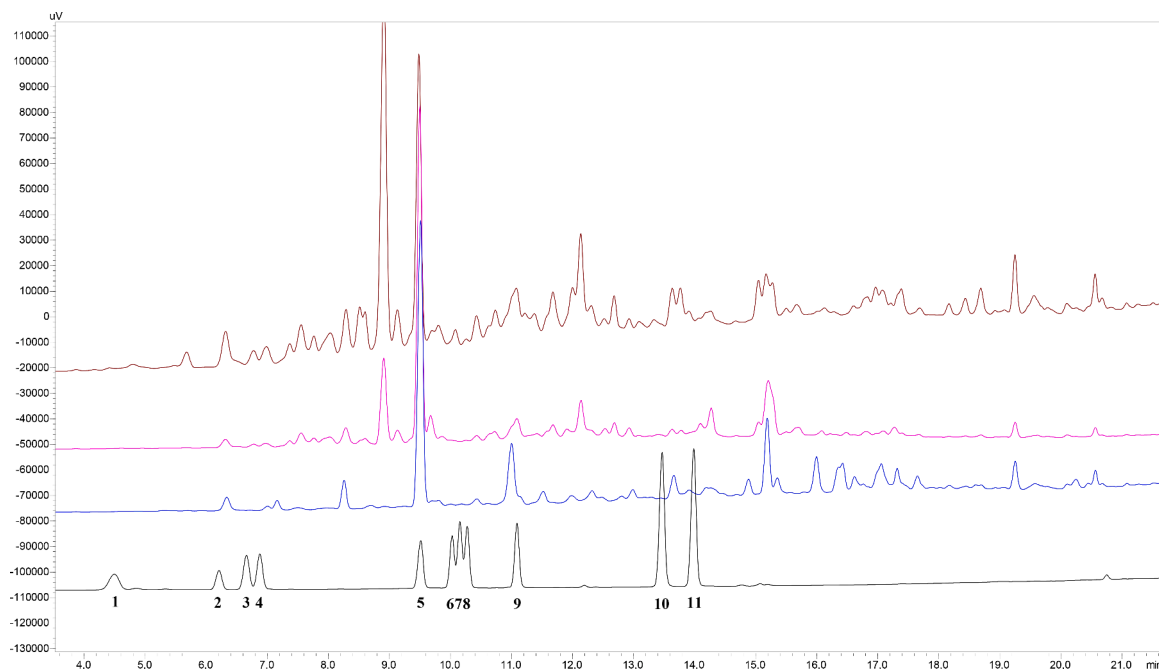


Fig. 3. Overlaid HPLC chromatograms of the DCM extracts of the infusions and the reference standard mixture (RSM). From top to bottom: *A. afra* (BG), *A. afra* (BB), *A. annua*, and RSM, as measured at 254 nm. The elution order for the reference standards is: [1] neochlorogenic acid, [2] scopoline, [3] chlorogenic acid, [4] 4-caffeoylquinic acid, [5] scopoletin, [6] 3,4-dicaffeoylquinic acid (DCQA), [7] 3,5-DCQA, [8] 1,5-DCQA, [9] 4,5-DCQA, [10] quercetin, and [11] luteolin.

Results and discussion

No trace of artemisinin could be found in both the *A. afra* samples, whereas the *A. annua* sample contained 0.36% artemisinin after extraction of all plant material samples with organic solvents and LCMS analysis as described by Lee et al. (2022). Analysis of the infusion samples confirmed that both the *A. afra* samples were devoid of artemisinin and the *A. annua* infusion contained 0.25% artemisinin, which

equates to an extraction efficiency of roughly 70%. Liquid partitioning with hexane and DCM extracted 91% and 9% respectively of the available artemisinin, with the extracted infusion sample of *A. annua* containing undetectable levels of artemisinin.

Fig. 1-4 illustrates the HPLC chromatograms of all samples as compared to 11 reference standards. The infusions contained neochlorogenic acid, scopoline, chlorogenic acid (major component), scopoletin, and four dicaffeoylquinic acids with 3,5- and 4,5-

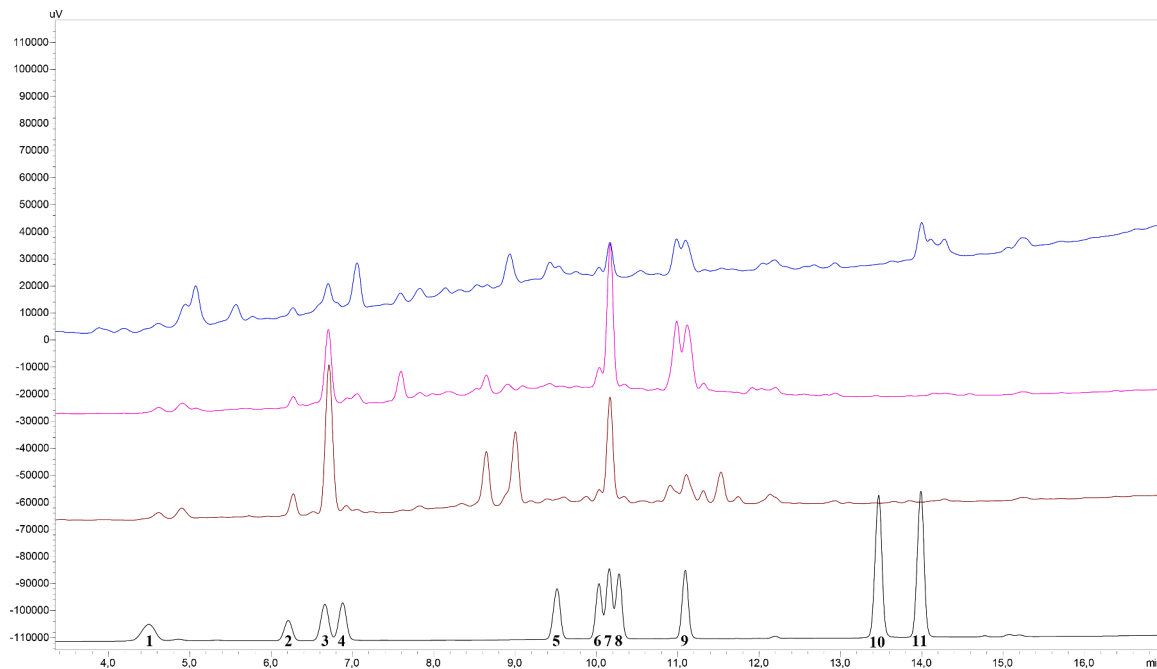


Fig. 4. Overlaid HPLC chromatograms of the remaining extracted infusions and the reference standard mixture (RSM). From top to bottom: *A. afra* (BG), *A. afra* (BB), *A. annua*, and RSM, as measured at 254 nm. The elution order for the reference standards is: [1] neochlorogenic acid, [2] scopoline, [3] chlorogenic acid, [4] 4-caffeoylquinic acid, [5] scopoletin, [6] 3,4-dicaffeoylquinic acid (DCQA), [7] 3,5-DCQA, [8] 1,5-DCQA, [9] 4,5-DCQA, [10] quercetin, and [11] luteolin.

Table 1

In vitro antischistosomal activities of *A. afra* and *A. annua* infusions and extracts.

Samples	NTS, % inhibition (SD)					<i>S. mansoni</i> adult worms, % inhibition (SD)				
	10 µg/ml 24 h	10 µg/ml 72 h	100 µg/ml 24 h	100 µg/ml 72 h	IC ₅₀ (µg/ ml) 72 h	10 µg/ml 72 h	100 µg/ml 24 h	100 µg/ml 48 h	100 µg/ml 72 h	IC ₅₀ (µg/ml) 72 h
<i>A. afra</i> (BG) -infusion	14.3*	15.4*		90.0 (10.0)			15.6*	24.1*	9.2 (5.3)	>100
<i>A. afra</i> (BB) -infusion	0.0*	1.4 (1.9)	80.7 (33.5)	100.0 (0.0)	17.8	1.2*	66.7 (15.3)	75.9 (4.8)	74.0 (2.2)	
<i>A. afra</i> (BB) -infusion [§]	10.3 (5.7)	12.0 (7.5)	100.0 (0.0)	100.0 (0.0)	29.1	11.7 (6.7)	19.6 (4.6)	35.2 (3.5)	42.7 (10.7)	>100
<i>A. afra</i> (BB) -extracted infusion		14.5 (4.1)		95.8 (36.7)	>50	4.9*				
<i>A. afra</i> (BB) -hexane extract	97.1 (4.0)	100.0 (0.0)	100.0*	100.0 (0.0)	1.8	41.25 (8.05)	100.0 (0.0)	100.0 (0.0)	100.0 (0.0)	10.4
<i>A. afra</i> (BB) -DCM extract	100.0 (0.0)	100.0 (0.0)	88.2*	100.0 (0.0)	1.7	28.85 (7.75)	100.0 (0.0)	100.0 (0.0)	100.0 (0.0)	8.9
<i>A. annua</i> -infusion		53.8 (34.5)		48.0 (25.7)	>50					
<i>A. annua</i> -extracted infusion		51.3 (43.7)		67.2 (26.5)						
<i>A. annua</i> -hexane extract		11.2 (7.0)		100.0 (0.0)	77.6					
<i>A. annua</i> -DCM extract		4.9 (6.8)		100.0 (0.0)	45.8					
Artesunate				100.0*	11.6*					
Praziquantel [#]					1.5					0.05

[§] dissolved in water;

* single experiment, no repetition;

[#] from Meister et al. (2014); NTS = newly transformed schistosomula; SD = standard deviation; IC₅₀ = Half-maximal inhibitory concentration; BG = botanical garden; BB = bronkhostbaai; DCM = dichloromethane; blank cells = data not available

dicaffeoylquinic acids as major components. The two flavonoids: luteolin and quercetin could not be detected in the infusion samples. The hexane extracts indicate that very small amounts, if any, of these marker compounds were extracted, and it mainly consists of as yet identified non-polar compounds. The DCM extracts transferred some of the marker molecules, most notably chlorogenic acid, into the organic phase. The extracted infusion samples contained most of the marker compounds, which indicates that these components might play a limited role in the observed bioactivity.

The results (Table 1) indicate that infusions of both *A. afra* and *A. annua* showed good activity, at 100 µg/ml, in the NTS bioassay. After

a treatment period of 72 hrs, *A. afra* (BG) afforded 90% inhibition, *A. afra* (BB) 100%, for both the DMSO- and water dissolved sample, and *A. annua* 48% inhibition. Further testing was conducted on *A. afra* (BB) and *A. annua* infusion samples, which yielded IC₅₀ values of 17.8 and 29.1 µg/ml for the two *A. afra* (BB) samples, respectively, and >50 µg/ml for the *A. annua* sample.

A. afra infusion samples were further submitted to bioassays conducted with adult *S. mansoni* worms, which overall delivered low to moderate inhibition, compared to the NTS bioassay results. Irrespective of the developmental stage of the parasite, it was interesting to note that by dissolving the *A. afra* (BB) infusion sample in DMSO, higher activities

resulted, with the highest being 75.9% inhibition at 100 µg/ml after 48 h, whilst the same infusion sample dissolved in water only delivered, at the highest, 42.7% inhibition at 100 µg/ml, together with an IC₅₀ value of > 100 µg/ml after 72 hrs of treatment.

No adult *S. mansoni* worm bioassays were conducted with the *A. annua* infusion, due to lower activity, at 100 µg/ml, and a higher IC₅₀ value, as compared to *A. afra* infusions, in the NTS bioassay.

The various extracts (hexane, DCM, and extracted infusions) of *A. afra* (BB) and *A. annua* were also tested in NTS bioassays. Both the hexane- and DCM extract of *A. afra* (BB) as well as of *A. annua*, afforded 100% inhibition, at 100 µg/ml after 72 h. The IC₅₀ values for the *A. afra* (BB) hexane- and DCM extract were found to be 1.8 and 1.7 µg/ml, respectively, and for the *A. annua* hexane- and DCM extract 77.6 and 45.8 µg/ml, respectively. Compared to the gold standard praziquantel (IC₅₀ = 1.5 µg/ml) (Meister et al. 2014) and to a semisynthetic comparator, artesunate (IC₅₀ = 11.6 µg/ml), both the hexane- and DCM extract of *A. afra* (BB) were therefore remarkably active.

Furthermore, the extracts of *A. afra* (BB) were also submitted to adult *S. mansoni* worm bioassays, which delivered 100% inhibition for both the hexane- and DCM extract at 100 µg/ml, after 24 hrs, 48 hrs, and 72 hrs of treatment. The IC₅₀ values were found to be 10.4 and 8.9 µg/ml after a 72 h incubation period for the *A. afra* (BB) hexane- and DCM extract, respectively.

Conclusion

The results indicate that *A. afra* is more active than *A. annua* against *S. mansoni*, and it seems that artemisinin plays a limited role in the observed bioactivity. The results also indicate that *A. afra* may contain highly active novel molecules with more in-depth research required to identify the molecule(s) responsible for these observed activities.

Declaration of competing interest

The authors declare no conflict of interest.

Ethical approval

The *S. mansoni* lifecycle is maintained at Swiss TPH and the animal work was carried out in accordance with Swiss national and cantonal regulations on animal welfare under the permission number 2070.

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