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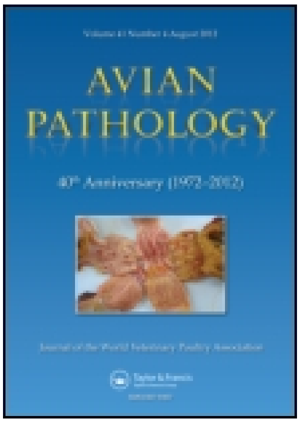

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Antihistomonal effects of artemisinin and *Artemisia annua* extracts *in vitro* could not be confirmed by *in vivo* experiments in turkeys and chickens

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Five different *Artemisia annua*-derived materials (i.e. dry leaves, pure artemisinin, and hexane, dichloromethane or methanol extracts of leaves) were screened for their *in vitro* activities against six clonal cultures of *Histomonas meleagridis.* Except for the methanol extract, all tested materials displayed *in vitro* activity against all tested protozoal clones. Neither the dry plant material, extracts nor artemisinin showed any antibacterial activity against the xenic bacteria accompanying the six *H. meleagridis* clones at concentration levels identical to the antihistomonal setting. The dichloromethane extract of dry leaves (Ext-DCM) (minimal lethal concentration = 1.0 mg/ml) and artemisinin (half-maximal inhibitory concentration = 1.295 mg/ml) had the most promising antihistomonal properties and were therefore subsequently tested in a standardized experimental infection model in both turkeys and chickens infected with clonal *H. meleagridis.* There were no differences between treatment groups, where all infected turkeys showed severe clinical histomonosis and demonstrated severe typhlohepatitis typical for histomonosis. Consistent with the infection model used, the infected chickens did not show any adverse clinical signs but contracted severe lesions in their caeca 7 and 10 days post infection (d.p.i.), liver lesions were absent to mild after 7 d.p.i. and progressed to severe lesions at 10 d.p.i.; thus no differences between treatment groups were observed. In conclusion, neither artemisinin nor Ext-DCM was able to prevent experimental histomonosis in turkeys and chickens at the given concentrations, which is contrary to the antihistomonal effect noticed *in vitro* even though the same clonal culture was used. The results of this study therefore clearly demonstrate the importance of defined *in vivo* experimentation in order to assess and verify *in vitro* results.

Introduction

Histomonosis is a parasitic disease in gallinaceous birds, primarily affecting turkeys and chickens. It causes severe lesions in the caecum and the liver and can lead to high mortality rates, especially in turkeys (McDougald, 2005). Infection with *Histomonas meleagridis* in poultry flocks has re-emerged since the ban of effective treatments (McDougald, 2005; Callait-Cardinal *et al.*, 2007; Stokholm *et al.*, 2010).

Previously used drugs have not yet been replaced resulting in an urgent need for new curative or prophylactic treatments. Several *in vitro* and *in vivo* experimental studies on chemotherapeutics have shown variable outcomes in finding a new and efficient therapy against *H. meleagridis* infections (Hu & McDougald, 2002; Hafez & Hauck, 2006; Bleyen *et al.*, 2009; Hafez *et al.*, 2010; Hauck *et al.*, 2010b). Within recent years a trend towards non-chemotherapeutic alternative means has been set in the combat of histomonosis. Despite this awareness there is still only a limited number of *in vitro* studies on the effects of natural compounds on *H. meleagridis* available (Zenner *et al.*, 2003; Grabensteiner *et al.*, 2007, 2008; Hauck & Hafez, 2007; Arshad *et al.*, 2008; van der Heijden & Landman, 2008a). The situation is similar when it comes to evaluating the impact of natural compounds on histomonosis *in vivo* (Duffy *et al.*, 2004, 2005; Hafez & Hauck, 2006; Grabensteiner *et al.*, 2008; van der Heijden & Landman, 2008b).

Artemisia annua has been used as an herbal infusion in traditional Chinese medicine for treatment of fevers, including malaria (Klayman, 1985). The sesquiterpene lactone artemisinin is one of the main active compounds of this medicinal plant and has been shown to be effective against various *Plasmodium* spp., including *Plasmodium falciparum* that causes the most severe

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form of malaria in humans (Qinghaosu Antimalaria Coordinating Research Group, 1979). Artemisinin-based combination therapies are presently recommended as first choice for uncomplicated antimalarial treatment by the World Health Organization (2010). Furthermore, several studies have shown promising effects of artemisinin against other pathogenic protozoa, including poultry coccidia (Allen *et al.*, 1997, 1998; Brisibe *et al.*, 2008; del Cacho *et al.*, 2010). It has been demonstrated that supplementation of different levels of dried *A. annua* leaves in feed reduced the oocyst excretion in chickens experimentally infected with *Eimeria* spp. (Allen *et al.*, 1997, 1998; Brisibe *et al.*, 2008). Supplementing dried leaves or leaf extracts directly into poultry diets is an easy way to administer feed supplements in poultry flocks.

Since the discovery of artemisinin as an antimalarial drug several hypotheses on its mode of action have been suggested. The most plausible mode of action may be attributed to the cleavage of its endoperoxide bridge (Klayman, 1985; Olliaro *et al.*, 2001). An iron-dependent mechanism leads to the cleavage of the endoperoxide bridge, producing free radicals that selectively target and inhibit the sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPases pump (Eckstein-Ludwig *et al.*, 2003). This mechanism has also been proposed in avian *Eimeria* spp., where inhibition of coccidian sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPases was demonstrated recently (del Cacho *et al.*, 2010). The molecular basis of metabolic processes in histomonads, such as if Ca^{2+}-dependent ATPases are present, has not yet been reported. In addition, it has been suggested that artemisinin disrupts the mitochondrial membrane in the malaria parasite (Li *et al.*, 2005). Recently, it was demonstrated that artemisinin and its derivatives are distributed to malarial mitochondria, where they induce production of reactive oxygen species resulting in depolarization of the mitochondrial wall (Wang *et al.*, 2010).

Therefore, one aim of the present study was to investigate whether a panel of extracts of *A. annua* leaves, as well as pure artemisinin, causes similar antiprotozoal effects on *H. meleagridis in vitro.* Significant inhibitory impact of the plant derivates on histomonads *in vitro* should be verified *in vivo* in a second step. Recently established *in vitro* propagated clonal cultures of *H. meleagridis* (Hess *et al.*, 2006b) were used for screening the compounds *in vitro* (Grabensteiner *et al.*, 2008) and to assess their effect in a standardized experimental infection model in both turkeys and chickens (Hess *et al.*, 2006a).

Materials and Methods

***In vitro* experiments.** H. meleagridis *cultures.* In the first experiment, six different clonal cultures of *H. meleagridis* (Table 1) were used to evaluate the effect of artemisinin and *A. annua* extracts. Clonal cultures were established through micromanipulation (Hess *et al.*, 2006b). The screening was carried out on available low passage numbers of the clonal cultures, since it is well known that pathogenicity declines with increasing passages (Tyzzer, 1936; Hess *et al.*, 2008). All experiments were performed in 2 ml Eppendorf tubes using protozoal cultures propagated for 48 h in Medium 199 supplemented with Earle's salts, L-glutamine, 25 mM HEPES, L-amino acids (M199; Gibco®, Invitrogen™, Lofer, Austria), 2 mg/ml rice starch (Sigma-Aldrich, Vienna, Austria) and 15% foetal calf serum (Gibco®, Invitrogen™) at 40°C prior to testing. Cell suspensions of 10^6 protozoa/ml were prepared by counting the number of viable cells in a Neubauer counting chamber (Bright-Line® haemocytometer; Hausser Scientific, Horsham, Pennsylvania, USA, supplied by Sigma-Aldrich, Vienna, Austria) using Trypan blue (0.4%) (Gibco®, Invitrogen™, Lofer, Austria) to exclude non-viable cells. After centrifugation at 500 × g for 5 min, the protozoa were resuspended in M199 with 15% foetal calf serum without rice starch and the cell concentration was adjusted to 10^6 protozoa/ml.

Table 1. *Clonal* H. meleagridis *cultures used for screening of antiprotozoal properties of artemisinin and* A. annua *extracts.*

Clonal culture	Abbreviation	Passage numbers
Histomonas meleagridis/Chicken/Hungary/5009-C2/05	Hm2	45 to 47
Histomonas meleagridis/Turkey/Austria/2877-C3/05	Hm3	21 to 23
Histomonas meleagridis/Turkey/Austria/5642-C4/05	Hm4	38, 40 to 41
Histomonas meleagridis/Turkey/Austria/2922-C6/04	Hm6	24 to 26
Histomonas meleagridis/Chicken/Austria/8175-C7/06	Hm7	19 to 21
Histomonas meleagridis/Turkey/Germany/4114-C18/05	Hm18	110 to 112

A. annua *materials.* Dry leaves from seed propagated *Artemisia annua* (cv. Artemis, F2 seeds, Mediplant, Conthey, Switzerland) cultivated at the Department of Food Science, Aarhus University, Årslev, Denmark were finely ground before use. Artemisinin (purity > 99%) was obtained from Xiang Xi Holley Pharmaceutical Co., Ltd (Shanghai, China). Crude extracts from fresh thawed or dried *A. annua* leaves were made using hexane, dichloromethane or methanol. The crude extracts were filtered using a glass funnel and filter paper (AGF675, 400 mm, white ribbon filter, ashless; Frisenette, Knebel, Denmark) and evaporated at 30°C *in vacuo* using a rotary evaporator before the oily precipitate (extract) was used in further experiments.

Artemisinin and extracts were suspended (artemisinin, 1000 mg/ml) or dissolved (leaf extracts, 75 mg/ml) in dimethyl sulphoxide (DMSO) (purity 99.9%; Merck, Darmstadt, Germany). These were kept as stock solutions throughout the experiment. Dry leaf powder was put directly into 2 ml Eppendorf tubes and suspended in 100 μl M199 with 15% foetal calf serum prior to efficacy screening.

Experimental set-up. A test system previously described by Grabensteiner *et al.* (2007) was used. For each tested clonal culture, negative and positive controls were included, consisting of 10^5 protozoa/ml in fresh culture medium without the addition of *A. annua* materials (negative control) or with the addition of 0.4 mg/ml dimetridazole (positive control). The concentration levels of the materials in the test cultures were: dry leaf powder, 5, 10, 20, and 40 mg/ml; artemisinin, 5, 10, and 20 mg/ml; hexane extract (Ext-HEX), 500, 1000, and 1500 μg/ml; dichloromethane extract (Ext-DCM), 500, 1000, and 1500 μg/ml; and methanol extract (Ext-MeOH), 500, 1000, and 1500 μg/ml. Furthermore, hexane, dichloromethane and methanol were tested at 0.15% and DMSO at 2% added to aliquots consisting of 10^5 protozoa/ml in fresh culture medium to assess the maximal effect of solvents in the test cultures.

Test cultures were put in 2 ml Eppendorf tubes and consisted of 100 μl compound solution (= stock solution diluted 1:10 with M199 with 15% foetal calf serum prior to inoculation), 800 μl culture medium consisting of M199, 2 mg/ml rice starch, and 15% foetal calf serum and 100 μl cell suspension with 10^6 protozoa/ml, thus starting with 10^5 protozoa/ml in all test cultures. Eppendorf tubes were incubated at 40°C for 48 h. Protozoan multiplication in all samples was evaluated 24 and 48 h after inoculation by counting the number of viable cells as described above. The mean of two counts was recorded for each replicate. Complete inhibition was confirmed by inoculation of 100 μl from the bottom of the respective cell suspension into 900 μl fresh medium without addition of any test material, where they were evaluated after 48 h of incubation at 40°C. The lowest concentration for a given test material that led to complete inhibition in all clonal cultures with no live or motile protozoa after 24 h of incubation was determined as the minimal lethal concentration (MLC) (Grabensteiner *et al.*, 2007).

In a second experiment, the effects of Ext-DCM from thawed fresh *A. annua* and dried leaves, respectively, against *H. meleagridis*/Turkey/Austria/2922-C6/04 were compared. The tested concentrations were 500, 1000, and 1500 µg/ml for both extracts. The experimental set-up and controls were as described above.

A third experiment was set up to determine the half-maximal inhibitory concentration (IC_{50}) of artemisinin, which is the concentration inhibiting 50% of the protozoal growth compared with the untreated control. In a similar set-up to the first experiment, protozoa from *H. meleagridis*/Turkey/Austria/2922-C6/04 were used to evaluate the inhibitory properties of artemisinin. Concentrations of artemisinin in the test solutions for determining the IC_{50} were 10^1, 2×10^1, 4×10^1, 10^2, 2×10^2, 4×10^2, 10^3, 2×10^3, 4×10^3, 10^4, 2×10^4 and 4×10^4 µM. The IC_{50} was enumerated by graphical extrapolation using GraphPad Prism® 5 for Windows (GraphPad Software, San Diego, California, USA; www.graphpad.com).

Antibacterial effect. Bacteria present in the same monoeucaryotic *Histomonas* cultures as in the antiprotozoal setting were isolated using selective media—Columbia 5% sheep blood agar (aerobe, 37°C for 24 h; Biomerieux, Vienna, Austria), MacConkey agar (aerobe, 37°C for 24 h; LAB M, Heywood, Lancashire, UK), Chromocult® Coliform Agar (aerobe, 37°C for 24 h; Merck), Schaedler 5% sheep blood agar (anaerobe, 37°C for 24 h; Biomerieux), and Sabouraud Gentamycin Chloramphenicol agar (aerobe, 42°C for 48 h; Biomerieux)—and biochemical characterization methods—that is, catalase test (Bactident® Catalase; Merck) and *Escherichia coli* typing sera F1, F21, F103 for avian pathogenic *E. coli* (O1, O2, O78; Veterinary Laboratories Agency, New Haw, Addlestone, Surrey, UK).

The antibacterial activity was assessed using the disc diffusion method (Bauer *et al.*, 1966; Clinical and Laboratory Standards Institute, 2008). Preparation of inoculum followed the Clinical and Laboratory Standards Institute Direct Colony Suspension Method (Clinical and Laboratory Standards Institute, 2008): therefore, colonies from agar plates grown for 24 h were suspended in PBS (Gibco®, Invitrogen™) and bacteria were evenly spread over the surface of the agar plates with sterile cotton swabs. Mueller Hinton plates (Biomerieux) were used for *E. coli* and *Proteus* spp., whereas Columbia 5% sheep blood agar plates were used for *Streptococcus* spp. and *Staphylococcus* spp. Volumes of 20 µl of the test solutions in concentrations identical to those in the first experiment were loaded onto empty Sensi-discs (Oxoid Ltd, Cambridge, UK). Discs were loaded with 20 µl PBS as negative controls or with 10 µg meropenem (Oxoid Ltd) as positive controls.

Statistical analysis. All assays were performed in duplicate and repeated independently three times. The data analysis and statistical calculations were made using one-way analysis of variance followed by Tukey's multiple comparison test (GraphPad Prism® 5 for Windows; GraphPad Software, San Diego, California, USA; www.graphpad.com). $P \leq 0.05$ was considered significant.

***In vivo* experiments.** Based on the results from the *in vitro* experiments a bird experiment was set up in order to investigate the effect of artemisinin and Ext-DCM on a virulent clonal culture of *H. meleagridis* (*H. meleagridis*/Turkey/Austria/2922-C6/04) in turkeys and chickens (Table 2). Administration of artemisinin was done via feed and Ext-DCM via drinking water.

Experimental birds and housing. Sixty-five 1-day-old turkey poults (Big 6; Aviagen Turkeys Ltd, Tattenhall, UK) were randomly split into five groups with equal gender ratio (Groups I, II, III, IV and V) (Table 2). One hundred 1-day-old specific pathogen free chicks (VALO; Lohmann Tierzucht GmbH, Cuxhaven, Germany) were randomly split into four groups (Groups VI, VII, VIII and IX) (Table 2). On the first day of life all birds were individually marked using the Swiftack™ system (Heartland Animal Health Inc., Fair Play, Missouri, USA) before they were housed in pens on deep litter (wood shavings) in rooms under negative pressure. Birds without challenge infection were kept apart from the challenged birds in order to prevent contamination. Medicated or unmedicated water and feed were provided *ad libitum*.

All procedures performed on the birds were approved by the institutional ethics committee and licensed by the Austrian Government (licence number 68.205/0103-II/3b/2011).

Feed and A. annua *extracts.* Birds were supplemented with artemisinin in the feed or *A. annua* extract in the drinking water according to their groups (Table 2). Supplementation started at day 1 of life and was continued throughout the experimental period. The dosage was determined due to the obtained *in vitro* results and followed the protocol of Grabensteiner *et al.* (2008), in which *in vivo* dosages were calculated as two-fold of the MLC or IC_{50}.

All feed, medicated and unmedicated, used in the investigation were produced at the Department of Animal Science, Aarhus University, Viborg, Denmark. The nutritional composition of the base diets was adjusted according to the age of birds. Artemisinin (purity ≥ 99%; Trademax Pharmaceuticals & Chemicals Co., Ltd, Shanghai, China) was added to the diets in concentrations of 100 and 2600 mg/kg feed. Thus, both turkey and chicken feed were split into an unmedicated base diet, a diet supplemented with 100 mg/kg artemisinin and with 2600 mg/kg artemisinin.

Ext-DCM from dried *A. annua* leaves was dissolved in DMSO and administered as follows: from day 1 of life until day 15, the turkeys of Group I got drinking water supplied with 0.2% pure extract; from day 16 onwards the concentration was decreased to 0.1% due to reduced water intake. The chickens of Group VI were supplied with 0.1% extract in the drinking water from day 1 of life and onwards.

Infection of the birds with a clonal culture of H. meleagridis. At 14 days old, the birds were infected with *H. meleagridis*/Turkey/Austria/2922-C6/04 (Hess *et al.*, 2006b) (*in vitro* passage 22). All turkey poults in Groups I, II, III and IV received 3×10^5 *H. meleagridis* by the cloacal route using a conventional Eppendorf pipette. Birds of Group III died or had to be euthanized before inoculation was undertaken (see below). The chickens of Groups VI, VII and VIII were inoculated both orally and cloacally with 3×10^5 *H. meleagridis*. For the oral inoculation, a crop tube placed on a syringe (1 ml Omnifix F solo; B. Braun

Table 2. *Overview of bird species, treatments and challenge infection with* H. meleagridis/*Turkey/Austria/2922-C6/04.*

Group	Treatment	Number of birds	Challenge inoculum 300,000 *H. meleagridis*
I	Ext-DCM 0.2% in drinking water (days 1 to 15); 0.1% (day 16 onwards)	15 turkeys	Cloacally
II	Artemisinin 100 mg/kg feed	15 turkeys	Cloacally
III	Artemisinin 2600 mg/kg feed	15 turkeys	Cloacally
IV, infection control	None	15 turkeys	Cloacally
V, negative control	None	5 turkeys	None
VI	Ext-DCM 0.1% in drinking water	30 chickens	Orally and cloacally
VII	Artemisinin 100 parts/10^6 in feed	30 chickens	Orally and cloacally
VIII, infection control	None	30 chickens	Orally and cloacally
IX, negative control	None	10 chickens	None

Melsungen AG, Melsungen, Germany) was used. The required numbers of *H. meleagridis* were adjusted into a volume of 300 µl Medium 199 + 15% foetal calf serum. Following inoculation, all birds were deprived from feed and water for 5 h.

Examination of the birds and sampling procedures. All birds were examined daily to detect any adverse clinical signs (e.g. diarrhoea, anorexia, behavioural changes) and mortality. Feed and water consumption were recorded daily. Body weight was measured weekly. Cloacal swabs were taken three times a week starting prior to infection in order to re-isolate and monitor the *H. meleagridis* excretion according to the protocol described recently (Hess *et al.*, 2006a). All birds were sampled for blood once a week.

Euthanasia and post-mortem sampling. At 7 and 10 days post infection (d.p.i.), 15 chickens from each infected group (Groups VI, VII and VIII) and five chickens from the negative control (Group IX) were killed. Turkeys that survived the challenge were killed at termination of the experiment at 5 weeks of age (Hess *et al.*, 2006a). Euthanasia due to severe histomonosis or killing of chickens at specific time points was performed by intravenous anaesthesia with thiopental followed by bleeding.

Pathological examination was performed on all birds. Lesions indicative for histomonosis in the caeca and the livers were noted with scores ranging from 0 for no lesions to 4 describing the most severe lesions, according to recently described protocols (Windisch & Hess, 2010; Zahoor *et al.*, 2011).

Statistical analysis. Mortality data were analysed using the Gehan–Breslow–Wilcoxon test followed by the Bonferroni multiple comparison test, with a sigificance level $P \leq 0.05$.

Results

***In vitro* experiments.** *Antiprotozoal effect.* Antiprotozoal properties after 48 h of incubation are presented in detail in Table 3. For all treatments of the six clonal cultures, the number of viable protozoa is presented as the mean with standard deviations of the six replicates.

Addition of 5 mg dry leaf powder/ml did not result in significant reduction in any of the six clonal cultures (Hm2, Hm3, Hm4, Hm6, Hm7 and Hm18). In Hm3, Hm6, Hm7 and Hm18, protozoal growth was significantly lower at dry leaf powder levels of 10, 20 and 40 mg/ml. Hm2 showed a significant increase in protozoal growth when treated with 5 or 10 mg/ml dry leaf powder. Complete growth inhibition was seen at 20 and 40 mg/ml in Hm2, Hm3, Hm6, Hm7 and Hm18, whereas complete inhibition of cell proliferation was only seen at 40 mg/ml dry leaf powder in Hm4. The MLC for dry leaf powder was determined to 40 mg/ml after 24 h.

For artemisinin, complete inhibition of protozoa proliferation was not observed in any of the six clonal cultures at 24 or 48 h following incubation with different concentrations (5, 10 and 20 mg/ml). However, significant reductions of histomonads were noticed after incubation for 48 h with artemisinin in the six cultures, ranging from 56.5 to 95.3% for 5 mg/ml, 70.3 to 96.9% in 10 mg/ml and 83.7 to 96.8% in 20 mg/ml compared with the untreated controls.

Growth of clonal cultures receiving 0.5 mg/ml Ext-HEX was significantly reduced in Hm2, Hm3 and Hm7, and the multiplication declined in all six clones at concentrations of 1.0 and 1.5 mg/ml Ext-HEX after 48 h. However, only 1.5 mg/ml resulted in complete inhibition in the six clonal cultures at 24 h of incubation. The MLC was determined to 1.5 mg/ml for Ext-HEX.

When adding Ext-DCM to the protozoa cultures, the three tested concentrations (0.5, 1.0 and 1.5 mg/ml) showed a significant inhibitory effect against histomonads of all clones. Complete inhibition of the cultures after 24 h of incubation was observed at Ext-DCM levels of 1.0 mg/ml and 1.5 mg/ml (MLC = 1.0 mg/ml).

In contrast to the other two extracts (Ext-HEX and Ext-DCM), Ext-MeOH was not able to induce complete inhibition in any of the tested clonal cultures of *H. meleagridis.* For Hm2, Hm3, Hm6 and Hm7 the growth after 48 h at the three concentration levels was not significantly different from the non-treated controls. Only Hm4 was significantly inhibited after 48 h at the three concentrations of 0.5, 1.0 and 1.5 mg/ml. A significant increase in number of viable histomonads was observed after addition of 0.5 mg/ml Ext-MeOH in cultures of Hm18.

Comparison of two sources of Ext-DCM. The tested concentrations of Ext-DCM extracted from dried *A. annua* leaves showed the same pattern in inhibition of *H. meleagridis*/Turkey/Austria/2922-C6/04 as Ext-DCM extracted from fresh leaves. Similar MLCs were confirmed in both extracts (1 mg/ml), thus justifying the use of dry leaf extracts in the *in vivo* study.

Determination of IC_{50} for artemisinin. The dose–response curve for artemisinin is shown in Figure 1, from which the IC_{50} for artemisinin after 48 h was determined by graphical interpolation to 4586 µM, which equals 1.295 mg/ml in test solution.

Antibacterial effect. In total, 19 bacterial strains were isolated. *E. coli* strains (8/19) were isolated at least once from all six *H. meleagridis* clonal cultures. *E. coli* serotypes O1, O2, or O78 were isolated from Hm3, Hm4, Hm6, and Hm7, *Streptococcus* spp. (5/19) were isolated from Hm3, Hm4, Hm6, and Hm7, *Proteus* spp. (5/19) were isolated from Hm2, Hm3, Hm4, and Hm18, and one *Staphylococcus* sp. was isolated from Hm18.

No inhibitory effect of dry leaf powder, artemisinin, Ext-HEX, Ext-DCM or Ext-MeOH was observed in any of the 19 isolated bacterial strains from the six investigated *H. meleagridis* clones.

***In vivo* experiments.** *Observations prior to challenge infection.* The birds in Groups I, II, IV and V showed no decrease in activity, clinical signs or depression, whereas turkeys administered artemisinin 2600 mg/kg feed (Group III) started to show lower feed consumption. At days 5 to 7 following feeding, seven out of 15 birds from Group III died unexpectedly. At the same time, the remaining birds of Group III displayed increasing depression and anorexia and were therefore killed humanely on day 7. Post-mortem findings in Group III were: distended gallbladder (approximately $0.5 \times 0.5 \times 2$ cm^3; 15/15 birds), fatty-appearing pale liver (only present in killed birds; 8/15 birds), enlarged kidneys with increased tubular appearance (15/15 birds), urate deposits in ureters (15/15 birds), empty intestines (9/15 birds), soft long bones (9/15 birds) and beaks (6/15 birds).

Furthermore, it was observed that the water containing 0.2% Ext-DCM had a very pronounced strong herbal odour, which possibly decreased the intake of water of those turkeys (Group I) at 2 weeks of age to 60 to 70%

Table 3. *Results of the* in vitro *activities of artemisinin,* A. annua *dry leaves and extracts against six clonal cultures of* H. meleagridis *after a 48-h incubation period expressed in number of protozoal cells*

	Hm2		Hm3		Hm4		Hm6		Hm7		Hm18	
Treatment	Mean and SD[a] (10^4 protozoa)	Reduction[b] (%)	Mean and SD (10^4 protozoa)	Reduction (%)	Mean and SD (10^4 protozoa)	Reduction (%)	Mean and SD (10^4 protozoa)	Reduction (%)	Mean and SD (10^4 protozoa)	Reduction (%)	Mean and SD (10^4 protozoa)	Reduction (%)
Control	70 ± 12.1		45 ± 14.4		52 ± 8.5		39 ± 14.2		53 ± 8.0		80 ± 21.5	
DMSO 2%	64 ± 21.1	8.7	25 ± 3.5^{A}	43.7	45 ± 4.9	13.4	29 ± 12.2	24.8	50 ± 9.3	5.0	100 ± 26.0	−25.9
Dimetridazole 0.4 mg/ml	0 ± 0.0^{A}	100.0	0 ± 0.0^{A}	100.0	0 ± 0.0^{A}	100.0	0 ± 0.0^{A}	100.0	0 ± 0.0^{A}	100.0	0 ± 0.0^{A}	100.0
Hexane 0.15%	73 ± 23.2	−4.5	43 ± 14.8	3.6	39 ± 13.6	23.7	46 ± 13.3	−18.5	50 ± 4.8	6.2	73 ± 10.9	8.7
Dichloromethane 0.15%	70 ± 19.7	−0.2	40 ± 12.1	10.2	42 ± 18.8	17.7	40 ± 10.1	−2.7	49 ± 2.7	8.2	66 ± 18.9	17.0
Methanol 0.15%	73 ± 19.9	−5.4	34 ± 12.0	23.7	43 ± 11.3	16.3	41 ± 9.4	−6.1	50 ± 9.6	4.9	86 ± 16.3	−7.9
Dry plant 5 mg/ml	126 ± 27.4^{A}	−80.9	55 ± 18.6	−21.8	63 ± 35.6	−21.8	43 ± 45.5	−10.4	63 ± 4.3	−19.9	89 ± 23.9	−11.5
Dry plant 10 mg/ml	100 ± 21.9^{A}	−43.5	7 ± 5.1^{A}	85.5	36 ± 25.5	29.9	9 ± 11.5^{A}	75.9	27 ± 11.7^{A}	48.4	21 ± 16.7^{A}	74.0
Dry plant 20 mg/ml	0 ± 0.0^{A}	100.0	0 ± 0.0^{A}	100.0	0 ± 0.2^{A}	99.8	0 ± 0.0^{A}	100.0	0 ± 0.0^{A}	100.0	0 ± 0.0^{A}	100.0
Dry plant 40 mg/ml	0 ± 0.0^{A}	100.0	0 ± 0.0^{A}	100.0	0 ± 0.0^{A}	100.0	0 ± 0.0^{A}	100.0	0 ± 0.0^{A}	100.0	0 ± 0.0^{A}	100.0
Artemisinin 5 mg/ml	11 ± 5.4^{A}	83.7	5 ± 1.9^{A}	89.4	2 ± 1.5^{A}	95.3	5 ± 3.0^{A}	87.9	23 ± 9.7^{A}	56.5	5 ± 4.1^{A}	94.2
Artemisinin 10 mg/ml	8 ± 4.4^{A}	88.1	3 ± 1.8^{A}	92.9	2 ± 1.0^{A}	95.6	3 ± 3.1^{A}	92.1	16 ± 5.0^{A}	70.3	2 ± 1.3^{A}	96.9
Artemisinin 20 mg/ml	4 ± 1.6^{A}	94.7	2 ± 1.1^{A}	95.1	3 ± 1.2^{A}	93.9	3 ± 2.3^{A}	93.4	9 ± 6.5^{A}	83.7	3 ± 1.8^{A}	96.8
Ext-HEX 0.5 mg/ml	28 ± 7.8^{A}	59.9	21 ± 6.1^{A}	53.4	50 ± 29.6	2.8	38 ± 8.4	2.5	27 ± 9.7^{A}	48.5	63 ± 25.9	21.5
Ext-HEX 1.0 mg/ml	0 ± 0.0^{A}	100.0	0 ± 0.3^{A}	99.6	0 ± 0.1^{A}	99.9	2 ± 4.2^{A}	95.6	0 ± 0.0^{A}	100.0	0 ± 0.0^{A}	100.0
Ext-HEX 1.5 mg/ml	0 ± 0.0^{A}	100.0	0 ± 0.0^{A}	100.0	0 ± 0.0^{A}	100.0	0 ± 0.0^{A}	100.0	0 ± 0.0^{A}	100.0	0 ± 0.0^{A}	100.0
Ext-DCM 0.5 mg/ml	6 ± 6.3^{A}	91.9	5 ± 4.6^{A}	89.4	27 ± 13.8	47.2	5 ± 7.6^{A}	86.8	25 ± 8.0^{A}	53.2	42 ± 20.7^{A}	47.8
Ext-DCM 1.0 mg/ml	0 ± 0.0^{A}	100.0	0 ± 0.0^{A}	100.0	0 ± 0.0^{A}	100.0	0 ± 0.0^{A}	100.0	0 ± 0.0^{A}	100.0	0 ± 0.0^{A}	100.0
Ext-DCM 1.5 mg/ml	0 ± 0.0^{A}	100.0	0 ± 0.0^{A}	100.0	0 ± 0.0^{A}	100.0	0 ± 0.0^{A}	100.0	0 ± 0.0^{A}	100.0	0 ± 0.0^{A}	100.0
Ext-MeOH 0.5 mg/ml	88 ± 9.9	−26.6	53 ± 7.9	−17.0	19 ± 4.5^{A}	62.6	43 ± 6.3	−11.4	54 ± 6.0	−2.1	134 ± 22.9^{A}	−68.3
Ext-MeOH 1.0 mg/ml	80 ± 10.0	−14.6	38 ± 7.0	16.5	20 ± 6.0^{A}	61.2	30 ± 3.0	23.2	50 ± 10.1	4.6	107 ± 30.9	−35.0
Ext-MeOH 1.5 mg/ml	71 ± 13.7	−1.6	22 ± 3.8^{A}	51.6	15 ± 3.0^{A}	70.5	23 ± 9.3	41.7	48 ± 8.3	9.1	73 ± 18.5	8.2

[a] Statistical differences from clonal cultures without treatment are indicated with uppercase superscript letters ($P \leq 0.05$). Data were analysed using one-way analysis of variance followed by Tukey's multiple comparison test. SD, standard deviation.

[b] Relative reduction of protozoan cells in comparison with the untreated clonal culture. " − " indicates an increase in growth compared with the untreated culture.

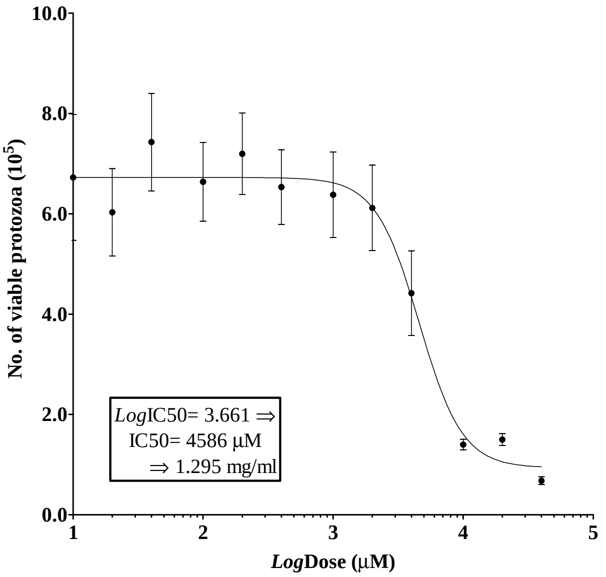

Figure 1. *Dose–response curve of* H. meleagridis/*Turkey/Austria/2922-C6/04 after 48 h of incubation with artemisinin (mean ± standard deviation,* n = *6).*

compared with turkeys of the remaining groups. For welfare reasons, based on the lower water consumption the concentration of Ext-DCM was thereafter reduced to 0.1% in Group I. No obvious differences in feed and water consumption were observed between the groups of chickens (Groups VI, VII, VIII and IX).

One turkey of Group IV (infected control group) died at day 1 of age with no apparent post-mortem findings or signs of infection. Two turkeys from Group I (application of 0.2% Ext-DCM) died within 2 days after challenge for reasons unrelated to histomonosis. One chicken in the negative control group (Group IX) died at 3 days old showing neither significant pathological lesions nor signs of bacterial infection.

Re-isolation of the parasite. No live histomonads were recovered from turkeys or chickens prior to challenge infection. From infected turkeys the protozoa were re-isolated from cloacal swabs at 2 d.p.i. and onwards in all infected groups (Groups I, II and IV). In Groups I and IV, 100% of the birds excreted the parasite at least once during the experiment. Similarly, 14 out of 15 turkeys of Group II (application of 100 parts/10^6 artemisinin) had positive re-isolations. No histomonads could be recovered from any of the five turkeys in the negative control group (Group V) throughout the experiment.

The excretion of histomonads from infected chickens was observed starting at 2 d.p.i. The number of chickens that were found positive by re-isolation at least once were: Group VI (0.1% Ext-DCM), 14/30 birds; Group VII (artemisinin 100 mg/kg feed), 12/30 birds; Group VIII (infected control group), 16/30 birds; and the negative control group (Group IX), 0/9 birds.

Morbidity, mortality and pathological findings in infected turkeys. All infected turkeys (Groups I, II and IV) showed various clinical signs of histomonosis, starting with general depression and ruffled feathers. Later on, sulphurous-coloured diarrhoea and sudden death became obvious in the afore-mentioned groups. Birds suffering from severe clinical signs were killed humanely. The cumulative mortality of turkeys that died or were killed due to histomonosis is shown in Figure 2. Two birds from Group II (artemisinin 100 mg/kg feed) and one from the infection control (Group IV) overcame the clinical signs at 18 d.p.i. and were regarded as having survived the challenge. Consequently, the experiment was terminated at 20 d.p.i. by killing those three birds and all turkeys of Group V.

Turkeys that died due to histomonosis displayed severe disease-specific lesions in the caeca and livers. Furthermore, necropsy of the three surviving turkeys revealed severe lesions in the caeca and livers similar to pathological changes of turkeys that died from the disease. The lesion scores observed in the caeca and livers are shown in Table 4.

None of the chickens in any of the infected groups (Groups VI, VII and VIII) displayed clinical signs or died due to histomonosis. Nevertheless, necropsy of the chickens showed that the birds from the infected groups (Groups VI, VII and VIII) had severe lesions (lesion score = 3 to 4) in their caeca at 7 d.p.i. (see Table 4). On the same day, the majority of the infected chickens had no or mild gross lesions in the livers. Birds from the same groups displayed severe caecal lesions on day 10 after infection, which were accompanied by inflammation and necrosis of the livers. Organs of non-infected chickens of Group V were found normal during post-mortem examination.

Discussion

The present *in vitro* experiment revealed significant dose-dependent reductions in protozoal counts of all six tested clones of *H. meleagridis* for the tested concentrations of artemisinin and Ext-DCM.

In the first step, the direct effect of dried *A. annua* leaves against mono-eukaryotic *H. meleagridis* was investigated in an *in vitro* setting. Furthermore, it was

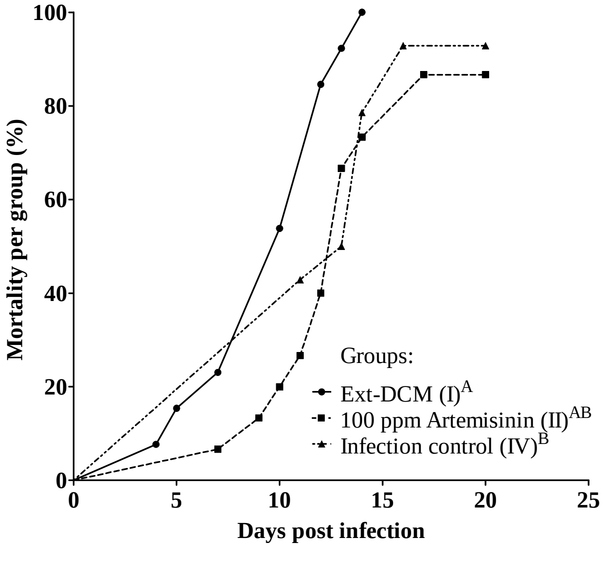

Figure 2. *Cumulative mortality due to histomonosis of turkeys challenged with* H. meleagridis/*Turkey/Austria/2922-C6/04. Groups I and II were treated with* A. annua *extract of leaves or artemisinin from the first day of life. Birds of Group IV were kept untreated before all turkeys were challenged at week 2 of life. Statistical difference between groups is indicated with different uppercase letters (*P *≤ 0.05).*

Table 4. *Median hepatic and caecal lesion scores (LS) of turkeys suffering from histomonosis and of chickens at 7 and 10 d.p.i.*

	Turkeys				Chickens					
	Mortality due to histomonosis (number of birds)	Lesions specific to histomonosis (number of birds)	Median LS		Mortality due to histomonosis (number of birds)	Lesions specific to histomonosis (number of birds)	Median LS (7 d.p.i.)		Median LS (10 d.p.i.)	
Treatment			Liver	Caecum			Liver	Caecum	Liver	Caecum
0.2% (0.1%) Ext-DCM	13/15	13/13	4	4	–[a]	–	–	–	–	–
0.1% Ext-DCM	–	–	–	–	0/30	30/30	0	4	3	4
Artemisinin 100 mg/kg feed	13/15	15/15	4	3	0/30	30/30	2	4	3	4
Infection control	13/14	14/14	4	3	0/30	30/30	0	4	3	4
Negative control	0/5	0/5	0	0	0/9	0/9	0	0	0	0

All birds were treated with artemisinin or *A. annua* leaf extract from first day of life and challenged at 2 weeks of age.
[a]Not applicable.

aimed that *in vitro* investigations in this study could deliver necessary data for a pre-selection of the tested materials and concentrations that were most promising for further *in vivo* testing.

As shown in Table 3 there are remarkably varying properties, although dose dependent, both within a clonal culture and between clones. In all six clonal cultures a trend towards growth enhancement was seen when adding 5 mg/ml dry leaf powder. This was only significant in Hm2, however, where 5 mg/ml resulted in 80.9% increase in protozoa counts when compared with the control within this clone. The increase in the number of protozoa may partly be explained by the ability of the *in vitro* cultivated *H. meleagridis* to use different starch sources as demonstrated recently by Hauck *et al.* (2010a), thus possibly also the starch fraction of *A. annua* (Brisibe *et al.*, 2009). A dose of 40 mg/ml dry leaf powder was the only dosage that inhibited protozoal multiplication in all six clonal cultures, thus resulting in absolute death of the parasites after 24 and 48 h, respectively.

With regard to artemisinin, the parent compound isolated from *A. annua*, significant dose-dependent reductions in protozoal counts were observed for all six clones. Nevertheless, none of the concentrations was able to induce a total inhibition of histomonad proliferation. Hence, no MLC could be determined although reduction rates ranged from about 85 to 95% for Hm2, Hm3, Hm4, Hm6 and Hm18. Only a few *in vitro* studies have tested artemisinin and not its derivatives against protozoa, which were mainly intracellular parasites assessed in cell cultures—for example, *Toxoplasma gondii* (Nagamune *et al.*, 2007; Hencken *et al.*, 2010) or *Neospora caninum* (Kim *et al.*, 2002). For evaluation of artemisinin IC_{50} values *in vitro*, two studies used procedures comparable with the present set-up against either *Trichomonas vaginalis* (Camuzat-Dedenis *et al.*, 2001) or *Leishmania* spp. (Sen *et al.*, 2010). Sen *et al.* (2010) obtained IC_{50} values on *Leishmania* spp. at artemisinin levels of 100 to 120 µM. This supports, as reviewed by White (2008) and Golenser *et al.* (2006), the *in vitro* activity of artemisinin on other protozoa being in the micromolar range. This concentration is considerably higher than the effective dose against the malaria parasite, which have IC_{50} values within the nanomolar range. In our first experiment, no MLC could be determined for artemisinin, and therefore the IC_{50} was determined based on the *in vitro* results to 4586 µM. This concentration is considerably higher than for malaria parasites. *H. meleagridis* is relatively different from obligate intracellular protozoa (e.g. *Plasmodium* spp.), for example by having resistant or cyst-like stages (Tyzzer, 1920; Zaragatzki *et al.*, 2010) that may explain the higher IC_{50}.

Ext-DCM was the most effective leaf extract, displaying complete inhibition of protozoal multiplication at 1.0 mg/ml in all clonal *H. meleagridis* cultures. This was superior to the Ext-MeOH, where no consistent inhibitory patterns were noticed between the six clonal cultures, and to some extent also the Ext-HEX, in which the MLC was determined to 1.5 mg/ml for the six tested *H. meleagridis* clones. This is in agreement with a recent study reporting that dichloromethane extracts from four different *Artemisia* spp. showed higher *in vitro* activity against bloodstream forms of *Trypanosoma brucei brucei* than methanol extracts from the same plant species (Nibret & Wink, 2010). In addition to artemisinin and its derivatives, *A. annua* extracts contain a range of essential oil components (Nibret & Wink, 2010) and phenolic compounds (Ferreira *et al.*, 2010). Camphor and 1,8-cineole were found to be the major components of *A. annua* L essential oil (Charles *et al.*, 1991), which are capable of protecting chickens from pathological lesions after experimental infection with *Eimeria acervulina* or *Eimeria tenella* (Allen *et al.*, 1997).

The comparison of Ext-DCM from dried *A. annua* leaves against Ext-DCM from fresh thawed *A. annua* leaves revealed identical MCLs, indicating similar *in vitro* antihistomonal properties. Therefore, further experiments were performed with the less laborious procedure using extracts of dry leaves.

At present histomonads need accompanying bacteria when cultured *in vitro*, but the role of the bacteria is not clear (McDougald, 2005). In order to assess whether the observed effects on *H. meleagridis* multiplication could be accounted as a direct or indirect effect, an antibacterial assay was performed on the accompanying xenic bacterial culture from all six clonal *H. meleagridis* cultures. No inhibitory effect on bacterial growth was noticed when treated with dried *A. annua* leaves, artemisinin or any of the three extract methods using compound concentrations as in the screenings for antihistomonal properties. It is known that artemether, a derivative of artemisinin, has no antibacterial effect on human hospital strains of *E. coli* and *Staphylococcus aureus* (Esimone *et al.*, 2002). Similar investigations found that artemisinin had no antibacterial effect on *S. aureus* (Dhingra *et al.*, 2000; Slade *et al.*, 2009). However, artemisinin showed antibacterial properties at 1 mg/ml against *E. coli*, *E. coli* NCTC 9002 and *Proteus vulgaris* (Dhingra *et al.*, 2000). In our study, the amount of artemisinin loaded onto the discs ranged between 100 and 300 μg/disc (20 μl each test solution per disc), which had no antibacterial effect on the bacterial strains isolated from the clonal histomonal cultures. This is in agreement with a study where no antibacterial effect of 100 μg/disc artemisinin was found on *E. coli* or *S. aureus* (Shoeb *et al.*, 1990). To the best of our knowledge, only a single study has addressed the antibacterial effect of essential oil components extracted from *A. annua* (Juteau *et al.*, 2002). These authors demonstrated that the oily extract showed no inhibitory effect on *E. coli* and *S. aureus*, whereas complete inhibition was obtained for *Enterococcus hirae* at 0.1 mg/ml. A few other studies have been carried out on extracts of leaves from other related *Artemisia* species describing large variations on the inhibitory effect on *E. coli*, *S. aureus* and *Proteus* spp. (Rabe & van Staden, 1997; Ahameethunisa & Hopper, 2010; Seddik *et al.*, 2010). The discrepancy between the efficacies of extracts may be explained by the different extraction methods, composition and purity of the tested extracts. Furthermore, extracts from different *Artemisia* species showing antibacterial effect were tested in concentrations several times higher than tested in the present work, in which a maximum of 10 to 30 μg extract per disc was used.

Combining the results of the antiprotozoal screening with the antibacterial tests, it is reasonable to assume that the observed inhibitory effect of dried *A. annua* leaves, artemisinin, Ext-HEX and Ext-DCM, is attributed to a direct effect on histomonads and could be regarded as antihistomonal. Ext-DCM and artemisinin were found to have the strongest antihistomonal effect in the *in vitro* studies and were therefore selected for further *in vivo* testing.

Turkeys received the challenge dose only cloacally as this is a proven route to establish infection in these birds (Liebhart *et al.*, 2008). Data about the comparative oral or cloacal infection of chickens are not available, but it was shown that a combination of both routes of application with virulent histomonads caused severe lesions in the caecum and/or the liver (Zahoor *et al.*, 2011). Therefore, chickens were infected via the crop and cloaca in order to ensure a successful infection.

Despite treatment with the test substances, the clinical outcome in turkeys was almost similar and of the same severity as noticed for the untreated but infected turkeys. Except three birds, all infected turkeys died or had to be killed due to severe clinical conditions.

Severe lesions in the caeca were present in all infected turkeys (median lesion score = 3 to 4) and chickens (median lesion score = 4), except for two turkeys (Group I) that were killed or died before the infection was established. Severe liver lesions were dominant in all infected turkeys regardless of treatment. In chickens, the liver affection progressed from very mild at 7 d.p.i. to severe at 10 d.p.i. independent of treatment. This indicates that neither artemisinin nor Ext-DCM had any protective effect on experimental histomonosis at the administered dose levels.

A possible explanation for the discrepancy between *in vitro* and *in vivo* efficacy of the present investigation is not obvious. It can be speculated that the low bioavailability (Titulaer *et al.*, 1990) and the considerable self-induced hepatic first-pass metabolism of artemisinins seen in mammals (Gordi *et al.*, 2005) may contribute insufficient concentrations of artemisinins in the birds. However, no information on the bioavailability and metabolism is yet available in poultry or avian species. An explanation for the difference in efficacy between *P. falciparum* and *H. meleagridis* could be that artemisinin and derivatives have a special affinity for malarial mitochondria (Wang *et al.*, 2010) and *H. meleagridis* does not possess mitochondria (Lindmark & Müller, 1973). In the *in vitro* experiments, the effective doses of artemisinin were in the micromolar range; therefore it can be suggested that the amount of artemisinin or the effective leaf extract fractions may have reached a level in which sufficient and lethal ratios of free radicals were obtained. Furthermore, no host interaction or metabolism was disturbing the direct effect on *H. meleagridis* when treated *in vitro*.

The post-mortem findings from the turkey group administered artemisinin 2600 mg/kg feed (Group III) may be indicative of intoxication, and further investigations are ongoing and will be discussed elsewhere. Although very little information on the toxicological profile of *A. annua* plant material and extracts, including artemisinin, in poultry is available, it has recently been investigated in broiler chickens (Arab *et al.*, 2009; Shahbazfar *et al.*, 2011). Hepatic and renal degeneration was seen histopathologically regardless of dose (17 to 136 mg/kg feed) after long-term oral administration of artemisinin, whereas neuronal degeneration seemed to be dose dependent, even though no clinical signs were present (Shahbazfar *et al.*, 2011). Furthermore, single doses of 1250 mg/kg and 2500 mg/kg showed similar patterns in clinical and histopathological findings, as well as bile retention in the liver (Arab *et al.*, 2009).

In conclusion, dry leaves and three extracts from *A. annua* as well as the main antimalarial constituent of this plant, artemisinin, were evaluated for the first time for their antihistomonal activities *in vitro* against six different clonal cultures of *H. meleagridis.* Four of the tested materials displayed *in vitro* activity against all protozoal clones. However, neither artemisinin nor Ext-DCM that were tested *in vivo* was able to prevent experimental histomonosis in turkeys or chickens at the given concentrations, although the clonal culture used for this investigation was one of the *in vitro* tested clones. Thus, the results of this study clearly demonstrate the importance of defined *in vivo* experiments in order to assess and verify *in vitro* results.

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