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In vitro Effect of *Arnebia densiflora* and *Artemisia annua* Plant Extracts against *Theileria annulata* Schizonts

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ABSTRACT

Background: This study investigated the *in vitro* activities of naphthoquinones and artemisinin contained in the extracts obtained from *Arnebia densiflora* and *Artemisia annua* plants against peripheral blood mononuclear cells infected with *Theileria annulata* schizonts. For this purpose, the n-hexane extract of *A. densiflora* and the petroleum ether and methanol extracts of *A. annua* were obtained.

Methods: Non-infected and infected cells were cultivated and 2 $\times 10^6$ cells were seeded in each well in 0.5 ml of RPMI 1640 medium. The n-hexane extract of *A. densiflora* was tested six times at concentrations of 125, 62.5, 15.625, 7.8125 and 3.9062 mg/ml. *A. annua* petroleum ether and methanol extracts were also tested six times at 125, 62.5, 31.25, 15.625, 7.8125, 3.9062 and 1.9531 mg/ml concentrations on PBMCs. A Mann-Whitney U test was used to determine differences between control and experimental groups.

Result: The *A. densiflora* extract killed both non-infected and infected cells at significant levels ($P<0.05$) compared to the control group at all tested concentrations. *A. annua* methanol extract had no adverse effect on non-infected cells and the effect on infected cells was insufficient, with the only observed significant effect at a concentration of 125 µg/ml. The *A. annua* petroleum ether extract had no adverse effect on non-infected cells and killed infected cells at significant levels ($P<0.05$) at concentrations of 15.625 µg/ml and above at 24 hours. At 48 hours, all infected cells were killed at a concentration of 62.5 µg/ml. In conclusion, *A. annua* petroleum ether extract can be used in future *in vivo* studies for Theileriosis at these effective concentrations.

Key words: *Arnebia densiflora*, *Artemisia annua*, Artemisinin, Naphthoquinones, *Theileria annulata*.

INTRODUCTION

Tropical theileriosis is a protozoan infection that causes high mortality and low productivity in imported high-grade cattle and crossbreeds, thereby negatively affecting economies (Agina *et al*., 2020).

Control of tropical theileriosis is achieved by i) vector control by application of acaricides, ii) drug administration against the disease agent and iii) use of attenuated macro schizont-infected cell culture vaccines for protection against the disease. Acaricide application has many disadvantages, such as high prices, residual drug waste in the meat and milk of cattle, contamination of the environment and resistance in ticks. Vaccines used for protection against the disease may be insufficient in controlling the disease due to inadequate amounts produced and difficulties in transportation and administration (Karagenc and Eren, 2002; Ghosh *et al.,* 2006). Although antiprotozoal drugs such as halofuginone, parvaquone and buparvaquone are used in the treatment of cattle infected with *Theileria annulata*, treatment is very expensive and unsuccessful if not started early (Maharana *et al*., 2016; Nampoothiri, 2021).

Despite the availability of synthetic drugs whose dosage and stability can be easily adjusted, the use of herbal products as an alternative in the treatment of many diseases is becoming widespread in both developed and developing societies (Sofowora, 2013).

Various studies have been performed to determine the effects and possible toxic characteristics of herbal medicines which have been used traditionally for centuries (Efferth, 2017; Shahrajabian *et al.,*2020). Considering that there are more than nine thousand natural plant species in Turkey, there is great potential in terms of active herbal ingredients.

Naphthoquinones and artemisinin are molecules used in veterinary and human medicine against blood parasites (Sanford *et al*., 2016; Bhat *et al.,* 2018; Wang *et al*., 2019). Naphthoquinones are used in parasitology for the treatment of some protozoan infections. It has been observed that naphthoquinone derivatives inhibit the mitochondrial electron transport in *Theileria parva*, causing the death of the parasite (Dobbeleare *et al*., 2000). In addition to these,

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naphthoquinones are used for the treatment of malaria, Toxoplasmosis and *Pneumocystis pneumonia* (Srivastava *et al*., 1999; Mone *et al*., 2021).

Likewise, artemisinin is a molecule used in human medicine against *Plasmodium* infections (Sanford *et al*., 2016; Wang *et al*., 2019), which has also been stated to be effective against some other protozoans and helminths (Kim *et al*., 2002; Mishina *et al*., 2007; Lam *et al*., 2018). The cytotoxic effects of artemisinin and its semi-synthetic derivatives have been investigated and it has been reported that these substances can be effective on cancer cells (Woerdenbag *et al*., 1993; Zhang *et al*., 2018) and also on Sars-CoV-2 virus, recently (Farmanpour-Kalalagh *et al*, 2022).

So far, *in vitro* and *in vivo* trials have limitedly been carried out with various extracts of different plants against *T. annulata*, *T. parva* and *T. lestoquardi* species. In *in vitro* experiments, *Curcuma longa* and *Pavetta schumanniana* plant extracts were tested against *T. annulata*, while different plants have been tested against *T. parva* and *T. lestoquardi* species and it has been determined that they have antitheilerial effects (Farah *et al*., 2012, 2013, 2014, 2015a).

This study aimed to determine the levels of artemisinin contained in the n-hexane extract of *Arnebia densiflora* and the petroleum ether and methanol extracts of *Artemisia annua* and their in-vitro activity against *T. annulata* schizonts to explore whether these extracts are promising for future *in vivo* trials.

MATERIALS AND METHODS

Collection, drying and extraction of *Arnebia densiflora* with n-hexane

The *A. densiflora* (Ledeb. ex Nordm.) Ledeb. plant was collected from calcareous hills 10 km from the Eskişehir Alpu district of Turkey in June 2020. The roots of the collected plants were removed, dried in the open-air shade, mechanically powdered (drug) and extracted with n-hexane at room temperature in a Soxhlet extractor (Bozan, 1994).

Analysis of *Arnebia densiflora* extracts

Analysis of the extracts obtained from *A. densiflora* roots was performed by thin-layer chromatography (TLC)-densitometry and isolated naphthoquinones determined (Bozan *et al.,* 1997).

Collection, drying and extraction of *Artemisia annua* with petroleum ethers and methanol

In order to evaluate the solubility of artemisinin, solvents with different polarities were applied to the plant samples. The above-ground parts of *A. annua* collected from open land in Bursa, Turkey were obtained in September 2020. Flowers and leaves were dried in the open-air shade and mechanically powdered. Extracts were obtained with petroleum ether and methanol maceration in an ultrasonic water bath (Christen and Veuthey, 2001).

Extracts obtained from *A. densiflora* and *A. annua* were stored in dark-coloured glass bottles at -20°C.

Gas chromatography analysis of extracts from *Artemisia annua*

Artemisinin in petroleum ether and methanol extracts of *A. annua* was separated according to retention time in a Chrompack capillary column and evaluated according to the relative percentage (Peng *et al.,* 2006).

Assessment of anti-theilerial activity of plant extracts

Isolation of non-infected peripheral blood mononuclear cells (PBMCs) and those infected with *Theileria annulata* schizont

Non-infected and *T. annulata* schizont-infected PBMCs were used in the study. Non-infected PBMCs were isolated from the peripheral blood of a calf with Ficoll-Paque (Biochrom AG) and cultivated (Brown, 1987). The medium used in cultivation (RPMI 1640) contained 10% heat-inactivated neonatal calf serum (NCS), 2 mM L-glutamine, 100 µg/ml streptomycin and 100 U/ml of penicillin. Passages two and three of *T. annulata* from previously cultivated Osmanbükü-İbrahim Özkan isolate were used in the experiments.

Determination of *in vitro* efficacy of plant extracts

T. annulata schizont-infected and non-infected PBMCs were placed on separate plates and fresh medium was added to these cells as the first control group. As the second control group, 0.5% DMSO, used in solubilizing the extracts, was added to both cell groups at the highest concentration of 8 ml. This process was repeated six times for both control groups.

The effects of extracts on non-infected and *T. annulata* schizont-infected PBMCs were determined in 24-well plates. In one plate, 2×10^6 non-infected PBMCs were seeded to each well in 0.5 ml of medium (Wilkie *et al*., 1998). On the second plate, 1×10^5 *T. annulata* schizont-infected PBMCs were seeded to each well in 0.5 ml of medium.

The n-hexane extract of *A. densiflora* was tested six times at concentrations of 125, 62.5, 15.625, 7.8125 and 3.9062 µg/ml. *A. annua* petroleum ether and methanol extracts were tested six times at 125, 62.5, 31.25, 15.625, 7.8125, 3.9062 and 1.9531 mg/ml concentrations. The concentrations were determined by our preliminary trials and similar studies mentioned herein.

The plates were incubated at 37°C and 5% CO_2. Cell growth was checked under an inverted microscope at 24 and 48 hours. A cell suspension of 150 µl was taken from the wells for each dilution from which total cell numbers and live and dead cell proportions were determined by hemocytometry (Barker, 2004).

Cytotoxicity (LC_{50}) tests were conducted on the data of DMSO and all experimental groups.

Statistical analysis

Statistical analysis was performed using SPSS 11.5 software. The arithmetic means and standard errors of the per cent proportions of dead PBMCs of the control and experimental groups were calculated at 24 and 48 hours. For comparison, a Mann-Whitney U test was used. Statistical significance was accepted as $P<0.05$.

RESULTS AND DISCUSSION

As a result of the extraction of *A. densiflora* with n-hexane, 4.12 g of dark purple-coloured extract from 90.5 g of the drug was obtained. *A. annua* extraction from 100 g of drug with petroleum ether yielded 2.28 green-coloured extract, and with methanol 5.56 g of dark green-coloured extract.

Naphthoquinones isolated from the extract obtained from *A. densiflora* roots by TLC-densitometry were teracrylalkannin, β,β-dimethylacrylalkannin, α-methyl-n-butylalkannin, and isovalerylalkannin. Results of the gas chromatographic analysis of the amount of artemisinin in the petroleum ether and methanol extracts of *A. annua* are shown in Table 1.

The *A. densiflora* extract had a negative effect on non-infected PBMCs at all concentrations tested (Table 2). At 24 and 48 hours, all tested concentrations of *A. densiflora* extract on infected PBMCs were found to be statistically different compared to the control group (Table 2). These results show that *A. densiflora* extract was effective against infected PBMCs even at the smallest concentration tested. At concentrations of 62.5 µg/ml and above, all infected cells died at 24 hours, whereas the mortality rate in the control group was very low. Cytotoxicity (LC_{50}) values are about 6.63 µg/ml for infected cells and about 66.95 µg/ml for non-infected cells at 24 hours.

In the efficacy trials of *A. annua* petroleum ether extract on non-infected PBMCs, no statistically significant results were found between the control group and the experimental group in terms of dead cell ratios observed at 24 hours. At the 48th hour of the experiment, only the results obtained from the concentration of 125 µg/ml were found to be statistically significant (Table 3). This finding revealed that *A. annua* petroleum ether extract should be tested at concentrations lower than 125 µg/ml, as higher concentrations may damage non-infected cells. Cytotoxicity (LC50) values are about 23.43 µg/ml for infected cells and about 125 µg/ml for non-infected cells at 24 hours.

In the efficacy trials of *A. annua* petroleum ether extract against infected PBMCs, the results obtained with 125, 62.5, 31.25 and 15.625 µg/ml concentrations at 24 hours and 62.5 and 31.25 µg/ml at 48 hours were found to be significantly different compared to the control group. There was no statistically significant difference between the other concentrations and the control group at 24 or 48 hours (Table 3). These results showed that *A. annua* petroleum ether extract killed all infected PBMCs at concentrations of 62.5 µg/ml and above at 48 hours, whereas the mortality rate in the control group was low.

Table 1: Gas chromatography analysis of artemisinin extracted from *A. annua*.

Extract	Artemisinin (mg/100 g)
Petroleum ether	4.54±0.10
Methanol	1.14±0.07

Table 2: Dead cell ratios from efficacy trials of *A. densiflora* n-hexane extract against non-infected and infected PBMCs (n = 6).

Concentration of extract (µg/ml)	Non-infected PBMCs		Infected PBMCs	
	24th hour	48th hour	24th hour	48th hour
	Mean (%) ±SE	Mean (%) ±SE	Mean (%) ±SE	Mean (%) ±SE
125	50.17±3.094**	57.85±2.121**	100±0.0**	-
62.5	46.85±2.211**	56.07±1.860**	100±0.0**	-
15.625	45.85±2.118**	58.30±3.244**	95.13±3.125**	100±0.0**
7.8125	45.60±3.875**	56.07±2.774**	70.60±3.464*	91.48±4.615**
3.9062	31.25±1.474*	48.87±1.618**	29.62±3.312*	74.57±3.565*
Control	25.93±1.602	39.90±1.188	6.85±1.422	12.92±2.145

SE: Standard error, *: P<0.05, **: P<0.01.

Table 3: Dead cell ratios from efficacy trials of *A. annua* petroleum ether extract on non-infected and infected PBMCs (n = 6).

Extract concentration (µg/ml)	Non-infected PBMCs		Infected PBMCs	
	24th hour	48th hour	24th hour	48th hour
	Average (%)±SE	Average (%)±SE	Average (%)±SE	Average (%)±SE
125	33.08±2.324	53.33±4.950*	100±0.0***	-
62.5	29.89±0.956	39.46±2.196	91.59±4.581***	100±0.0***
31.25	30.22±1.770	41.94±1.782	68.80±5.097***	74.25±4.991***
15.625	25.53±1.695	38.25±1.810	30.31±4.703**	14.91±3.373
7.8125	25.37±1.863	38.01±1.609	14.68±2.010	10.06±2.047
3.9062	27.15±1.497	40.26±1.980	9.33±3.391	5.74±0.655
1.9531	25.47±1.661	34.88±2.526	9.92±1.643	8.17±1.586
Control	28.08±2.460	36.84±1.501	9.16±2.702	9.04±1.969

SE: Standard error, *: P<0.05, **: P<0.01, ***: P<0.001.

In the efficacy trials of *A. annua* methanol extract against non-infected PBMCs, there were no statistically significant differences between the control group and experimental group in terms of death rates observed at 24 and 48 hours (Table 4).

The results obtained from the efficacy trials of *A. annua* methanol extract against infected PBMCs at concentrations of 125 µg/ml and 62.5 µg/ml at 24 hours and 125 µg/ml at 48 hours were statistically different from the control group. At 24 and 48 hours, no statistically significant results were found between the other experimental groups and the control group (Table 4). This finding revealed that *A. annua* methanol extract was not effective against infected PBMCs at concentrations < 62.5 µg/ml. Cytotoxicity (LC_{50}) values are about 206.61 µg/ml for infected cells and about 226.44 µg/ml for non-infected cells at 24 hours.

0.5% DMSO used for dissolving the extracts, at the highest concentration of 8 µl, did not show any statistically negative effects (Table 5). A cytotoxicity test (LC_{50}) was conducted on the DMSO used in the lab and values were about 30 µg/ml for infected cells and about 17.22 µg/ml for non-infected cells at 24 hours.

In today's world, there is growing interest in treatment trials using plant extracts. Herbal materials, which can be used as models for many synthetic drugs, can enlighten us as to the structures of the active ingredients they contain, and as such are indispensable elements of modern medicine. In addition, when used well, they create great economic potential for countries (Salmerón-Manzano, 2020).

Bovine Theileriosis can be diagnosed in a variety of ways, both early and late phases (Al-Hosary *et al*., 2020). So the treatment in time is important to protect against more economical losses.

The plant extract *A. densiflora* is rich in naphthoquinones, but there is no study on its effectiveness on Theileriosis. Despite *A. annua* having a rich artemisinin content, there are few studies on the effectiveness of this plant extract on Theileriosis. For example, a semi-synthetic artemisinin derivative, arteether, has been applied to the Theileria-infected cattle *in vivo*. Intramuscular injection of arteether at 5 mg/kg body weight for three consecutive days revealed a per cent efficacy of 66.66% (Khawale *et al*., 2019). Besides that, another synthetic artemisinin-based drug derived from *A. annua*, Arthemeter 80, has effectiveness against *T. lestoquardi* 48 h after exposure in-vitro was 0%, 14%, 30% and 45% at concentrations of 0.01, 0.1, 1.0 and 10 mg/L, respectively (Farah *et al*., 2015b). Ethanolic extract of *A. absinthium* leaves has also been tested against *Theileria equi in vitro*. The extract exhibited an EC_{50} value of 1371.5±17.3 µg/mL against the human foreskin fibroblast (HFF) cell line (Batiha *et al*., 2019). Although our study was not in-vivo, the findings of this research are important as it is one of the first studies in this field and conducting *in vitro* studies is essential in terms of helping to guide and plan future *in vivo* studies. Furthermore, *Arnebia* or *Artemisia* spp. plant trials above were conducted on the species *Theileria lestoquardi* or *T. equi*. However, *T. annulata* schizonts were used in our study for the first time in terms of those two plants.

The n-hexane extract prepared from the roots of the *A. densiflora* plant collected from the Eskişehir region of Turkey was preferred because it is rich in naphthoquinones (Bozan, 1994). As a result of the study, it was determined that the extract had a negative effect on both non-infected and infected cells. This finding indicates that the extract is not suitable for *in vivo* trials at the concentrations tested. An argument for not experimenting with lower concentrations can be that although the effect level at the lowest concentration was statistically different, it was close to the

Table 4: Dead cell ratios from efficacy trials of *A. annua* methanol extract against non-infected and infected PBMCs (n=6).

Concentration of extract (µg/ml)	Non-infected PBMCs		Infected PBMCs	
	24th hour	48th hour	24th hour	48th hour
	Average (%)±SE	Average (%)±SE	Average (%)±SE	Average (%)±SE
125	26.85±1.722	39.920±3.2270	30.73±3.375**	23.171±5.1385*
62.5	26.53±1.929	35.940±1.6812	20.79±1.596*	13.143±2.5523
31.25	27.37±1.500	36.040±1.7583	17.26±1.596	8.286±1.4410
15.625	22.42±2.366	34.420±1.9369	12.50±1.177	5.843±2.3380
7.8125	24.13±0.701	35.800±2.3380	15.53±0.992	5.100±1.3645
3.9062	23.55±2.162	34.640±1.7195	12.26±0.817	7.014±1.1831
1,9531	22.27±0.992	33.900±2.5063	15.59±1.607	6.943±1.3282
Control	26.43±0.599	35.560±1.8405	12.26±2.558	6.371±2.6892

SE: Standard error, *: P<0.05, **: P<0.001.

Table 5: Dead cell ratios from efficacy trials of 0.5% DMSO against non-infected and infected PBMCs (n=6).

Amount of DMSO	Non-infected PBMCs		Infected PBMCs	
	24th hour	48th hour	24th hour	48th hour
	Average (%) ±SE	Average (%) ±SE	Average (%) ±SE	Average (%) ±SE
8 µl	26.578±1.3365	32.633±1.2204	14.544±2.2044	6.911±2.2378
Control	26.933±0.6726	31.683±1.4425	11.889±1.7220	5.189±1.3958

average mortality rate in the control groups. It is thought that even if the possible efficacy of lower concentrations on infected cells lasts, the adverse effect on non-infected cells may continue (Table 2).

The efficiency of *A. annua* methanol extract on infected cells-even at low levels- could be observed at a concentration of 125 µg/ml. Higher concentrations must be tried to get strong efficacy with non-infected PBMCs. These findings are consistent with the proportion of artemisinin contained in the extract. As a result, since the amount of artemisinin in the concentrations tested remained at very low levels, it could not show a sufficient effect. Thus, it is thought that this extract should be tried in higher concentrations.

More effective results were obtained from the petroleum ether extract of *A. annua*. At the tested concentrations of this extract, no adverse effects were observed in non-infected cells at 24 hours. However, it was determined that infected cells died at a concentration of 15.625 µg/ml compared to controls ($P<0.01$). At a concentration of 125 µg/ml, there was no adverse effect on non-infected cells, while all infected cells died, which is the optimal desired result. At 48 hours, the infected cells exposed to a concentration of 62.5 µg/ml died and the non-infected cells were not damaged ($P<0.001$). At a concentration of 125 µg/ml, there was a partial loss of non-infected cells at 48 hours.

Some data appear contradictory in tables 2, 3, 4 and 5 which show the dead cell ratios are more in some non-infected cells after a particular dose, in control groups and in the DMSO group as compared to infected cells. However, a high mitotic activity that occurs in parasite-infected cells possibly causes this conclusion. This situation is also reported by some researchers working on the cell cultivation of *Theileria* spp. (von Schubert *et al*., 2010).

CONCLUSION

Based on these findings, *in vivo* trials should be conducted at concentrations of 15.625-62.5 µg/ml which is important because it indicates the starting point for future trials. According to the results of this experiment, the petroleum ether extract of *A. annua* could have promising effects against *T. annulata* infection *in vivo*.

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