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In vitro determination of the anthelmintic potential of *Arum rupicola* Boiss var. *rupicola*

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Abstract: *Arum rupicola* Boiss var. *rupicola* is a preferred ingredient in traditional medicine in various countries, including Türkiye. The current study aims to establish the antiparasitic effect of *A. rupicola* Boiss var. *rupicola* on the eggs and larvae of *T. canis* in vitro. An ovicidal effect was not detected in the plant extracts, and embryogenesis in the *T. canis* eggs was not affected by the leaves' ethanolic extract and the distilled water extract of the rhizome during the incubation periods (6, 12, and 24 h). The larvicidal activity was observed in both extracts on the *T. canis* larvae after hatching. All *T. canis* larvae were motile after being incubated for 6 h with the leaf extract at 10 mg/mL. However, after 12 and 24 h of incubation at the same dose, the paralysed larvae rate was 9% and 15%, respectively. After 6, 12, and 24-h incubation with the leaf extract at 40 mg/mL, the paralysed larvae rates were 30%, 37%, and 42%, respectively. Dose and time factor analysis results were significant statistically in the leaf extracts ($p < 0.001$). The paralysed larvae rate was 10%, 12%, and 17% after 6, 12, and 24-h incubation, respectively, with the undiluted rhizome extract. Dose and time factor analysis results were statistically insignificant in the rhizome extracts ($p > 0.05$). Cytotoxic and haemolytic activities were observed only in the leaf extract. The paralysed larvae rate was 1.45 times higher in the leaf extracts than in the rhizome extracts ($p < 0.001$). Future studies may elucidate the effect of *A. rupicola* Boiss var. *rupicola* extracts on visceral larva migrans during in vivo experiments and determine the effectiveness of these plant extracts on other parasites.

Key words: Arum rupicola Boiss var. rupicola, extract, Toxocara canis, in vitro, ovicidal, larvicidal

1. Introduction

Today, chemical anthelmintics are considered one of the most effective tools in the fight against helminth parasites. It is well known that long-term use of anthelmintics causes some adverse reactions, such as drug resistance in parasites, drug residues in animal tissues consumed as food, and harmful effects of drug metabolites excreted in animal faeces on insects in nature. Due to these limiting factors, the search for alternatives to chemical compounds has recently increased. One of these alternatives is plant extracts [1]. The use of plants in the fight against diseases is almost as old as human history. Many traditional treatment methods have emerged because humans seek solutions for diseases using plants. Anthelmintic properties have been reported in many plant species [2].

The *Arum* genus is a flowering herbaceous perennial plant native to the Mediterranean region, North Africa, Western Asia, and Europe [3]. *Arum rupicola* Boiss var. *rupicola* is found in rocky areas and stony slopes at about 700–1500 m [4]. *Arum* species carry toxic chemicals that irritate the skin, mouth, tongue, and throat [5–7]. Raw consumption of this plant is harmful to animals and humans. After the plant is cooked, the poisonous effects of the leaves and rhizomes disappear [8].

Arum species contain flavonoids and phenolic compounds [9,10]. Isoflavones are effective against ageing and damaged cells and are used to prevent or treat atherosclerosis and cancer [11,12]. *Arum rupicola* contains isoflavonoid compounds such as genistein and genistein 8-C-glucoside [3]. Genistein (4',5,7-trihydroxyisoflavone) is a phytoestrogen with some anthelminthic properties [2].

Toxocara canis, also known as a nematode, is mainly parasitised in the intestine of dogs. The parasite's eggs are released into the environment with dog faeces. Larval development begins in the egg at temperatures above 10 °C [13,14]. Infectious larvae (L3) develop in the environment for about 4 to 7 weeks. Some animal species, including chickens, sheep, rabbits, and humans, act as paratenic hosts in parasite biology [13]. Infective stage larvae remain undeveloped in the paratenic host

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tissues. Eating the raw or uncooked tissues of the paratenic hosts containing L3 or accidental eating of *T. canis* eggs containing L3 causes human toxocariasis [14]. After humans ingest the eggs, L3 leaves the small intestines and passes into various tissues via the bloodstream [13]. The larvae do not become adults in humans as in dogs [15]. Toxocariasis is primarily asymptomatic in humans; however, it can result in serious clinical problems due to the organ damage caused by the number of larvae entering the body and the host's inflammatory reaction [16]. Toxocariasis clinical presentations are classified as visceral larva migrans, neurotoxocariasis, covert toxocariasis, and ocular toxocariasis in humans [15].

Human toxocariasis is a significant zoonotic disease in many countries, including Türkiye. [17]. Although some attempts have been made to treat toxocariasis in humans, no definitive treatment protocol exists. Several in vitro studies reporting the larvicidal and ovicidal effects of different plant extracts on *T. canis* eggs and larvae can be found [18–21]. Besides its use in Türkiye, *Arum rupicola* Boiss *rupicola* is used in the traditional medicine of Iran, Palestine, and Jordan [22–29]. The plant is considered a panacea for many in Türkiye [8] and is used to treat disorders such as abdominal pain, rheumatism, goitre, hypertension, and diabetes mellitus in Turkish traditional medicine [26–28]. In some regions of the country, people drink the juice from the *A. rupicola* Boiss var. *rupicola* leaves after boiling them or eat them after cooking them to cure intestinal parasites [29]. The current study aims to determine the antiparasitic activity of *A. rupicola* Boiss var. *rupicola* on *T. canis* eggs and larvae. The ovicidal and larvicidal effects of leaf and rhizome extracts were determined in vitro.

2. Material and methods

Permission for sampling plant specimens was obtained from the General Directorate of Nature Conservation and National Parks of the Ministry of Agriculture and Forestry (E–21264211–288.04–4138321).

2.1. Plant samples collection and extraction

The plant samples were collected with their leaves and rhizomes from the Güdül District of Ankara, Türkiye, in May 2022. The plant samples were identified as *A. rupicola* Boiss var. *rupicola* in Erciyes University's herbarium and recorded as CV6015. The rhizomes and leaves of the plant samples were extracted separately. The ethanol extraction of the leaf parts was obtained by modifying the protocol reported by Farahmandarfar et al. (2019) [30]. Ethanol-distilled water (50:50) was added to the leaves. The extraction process was accomplished at 50 °C with 150–200 millibar pressure for 1 h. Subsequently, the extract was filtered, and the liquid was removed using a vacuum evaporator. The aliquots were stored at −80 °C until their use was required.

The rhizome parts of the plant samples were extracted by modifying the protocol reported by Ynalvez et al. (2015) [31]. The rhizomes were washed, weighed, and homogenised in sterile distilled water using a tissue homogeniser (OMNI Tips). They were mixed with a shaker (Boeco TS-100C) at 4 °C for 60 min. The extract was filtered using a cheesecloth. The aliquots were stored at −80 °C until they were required for use.

2.2. Extract dilutions

The ethanolic extracts of the leaves (0.15, 0.31, 0.62, 1.25, 2.5, 5, and 10 mg/mL) and the distilled water extraction of the rhizome (undiluted, 1:2, 1:4, 1:8, 1:16, 1:32, and 1:64) diluted by DMEM were used for MTT (3–(4,5–dimethylthiazol–2–yl)-2,5-diphenyltetrazolium bromide) assay and haemolytic activity. After detecting the cytotoxic and haemolytic activities, 3 leaf extracts (10, 20, and 40 mg/mL) and rhizome extracts (undiluted, 1:2 and 1:4) diluted by RPMI-1640 were used to detect ovicidal and larvicidal activities.

2.3. MTT assay

The MTT assay and haemolytic activity analysis were performed at Kırıkkale University's Scientific and Technological Research and Application Centre (KUBTUAM). To detect the cytotoxicity effects of the plant extracts, the leaf and rhizome extracts were added to an L929 mouse fibroblast cell line and kept for 24 h (37 °C, 5% CO_2). After adding the MTT solution, the wells were incubated for 2 h under the same conditions. The absorbance was observed using an ELISA reader (absorbance at 570 nm). The results were evaluated according to the International Organization for Standardization 1099–5:2019.

2.4. Haemolytic activities of the plant extracts

Drabkin's colourimetric assay was performed to detect the haemolytic activity of the extracts. The leaf and rhizome extracts were added to the rabbits' venous blood samples and incubated for 3 h at 37 °C. After adding Drabkin's haemoglobin reagent, the absorbance was observed using the ELISA reader with a wavelength of 540 nm. The haemolytic index (HI) was calculated. The results were evaluated according to the American Society for Testing and Materials (ASTM) F576-00 standard.

2.5. In Vitro anthelmintic activity assay

2.5.1. Obtaining *T. canis* eggs

Adult female *T. canis* specimens were obtained from veterinary clinics. The worms were washed in sterile distilled water, and the distal part of the uterus was removed after dissection. The eggs were twice centrifuged with sterile distilled water and then once with RPMI-1640 (×2000 g, 3 min). After counting them with a light microscope (Leica DM200), the egg suspension was reconstituted in RPMI-1640 as 1000 eggs per mL.

2.5.2. Determination of the effects on embryogenesis in the *T. canis* eggs

The egg suspension (100 µL) and 100 µL of each extract dilution were put into sterile microtubes. The eggs and extract co-cultures were mixed homogeneously into the tubes by gentle pipetting. The tubes were kept at 32 °C for 6, 12, and 24 h (3 incubation periods). The supernatant was then removed gently. The tubes were centrifuged 3 times with sterile distilled water (×300 g, 5 min) and then once with formalin solution (0.5%). The eggs were transferred into sterile polystyrene cell culture plates (Costar 24-well plate) and gently incubated at 32 °C with a daily mix. A licenced drug containing 35 mg of pyrantel pamoate was a positive control. The nontreated *T. canis* eggs in RPMI-1640 served as a negative control. At the end of the 28-day incubation period, larvae development was examined using a light microscope.

2.5.3. Determination of the effects on *T. canis* larvae

Hatchling *T. canis* larvae were obtained according to Sena-Lopes et al. (2020) with some modifications [32]. The nontreated *T. canis* eggs were incubated in the formalin solution (0.5%) for 28 days, and larva development was followed daily. After incubation, the eggs containing larvae were centrifuged 3 times with sterile distilled water (×300 g, 5 min). Sodium hypochlorite solution (5%) was added to the eggs to remove the protein coat on the shell. After 20-min incubation at room temperature, the hatchling larvae were centrifuged twice with sterile distilled water and then once with RPMI-1640. The number of larvae in the suspension was counted and reconstituted as 500 per mL in RPMI-1640. The extracted dilutions, incubation times, and the positive control were the same as indicated in the embryogenesis assay mentioned above. The nontreated larvae in RPMI-1640 were used as a negative control. At the end of each incubation period, a trypan blue dye test (1:1 ratio) was carried out to establish the effect of the extracts on larva viability. Larvae moving with their whole body or only part of their body were considered living specimens [33].

2.6. Statistical analysis

SPSS 14.01 (SPSS Inc., Chicago, IL, USA) was used for data analysis. Dose and time factor analysis were assessed with Multiway contingency analysis, and $p > 0.05$ was considered a significant difference.

3. Results

3.1. MTT assay and haemolytic activities

Table 1 shows the cytotoxicity activities of the *A. rupicola* Boiss var. *rupicola* extracts. The extract dilutions that reduced cell viability below 70% in the cell line were accepted as cytotoxic. The cytotoxic potential was observed at a 10 mg/mL dilution of the leaf extract (69.7%). In contrast, the rhizome extract without diluting had no cytotoxic potential on the L929 mouse fibroblast cell line.

According to the ASTM F576-00 standard, compounds with a HI between 0%–2% are accepted as nonhaemolytic, whereas those with an HI greater than 5% are considered haemolytic. In the present study, the leaf extract diluted at 10 mg/mL had a haemolytic activity with an HI of 25.1%. However, the rhizome extract did not exhibit haemolytic activity (1.9% HI) even if added undilutedly.

Table. Paralysed larvae rate of the different incubation periods.

Extract dilutions	**Paralysed larvae rate (%)**		
Leaf extract (mg/mL)	**6 h**	**12 h**	**24 h**
10	$0^{a,x}$	$9^{a,y}$	$15^{a,z}$
20	$20^{b,x}$	$22^{b,y}$	$32^{b,z}$
40	$30^{c,x}$	$37^{c,y}$	$42^{c,z}$
Rhizome extract			
Direct	10	12	17
1:2	5	7	9
1:4	5	13	11
Positive control	100	100	100
Negative control	0	0	0

Dose and time factor analysis were assessed with multiway contingency analysis.
There is statistical difference between data appointed with letters in the same row and column ($p < 0.001$).
^{a,b,c}: difference between the incubation times in the same row is statistically significant ($p < 0.001$).
^{x,y,z}: difference between the extract doses in the same column is statistically significant ($p < 0.001$).

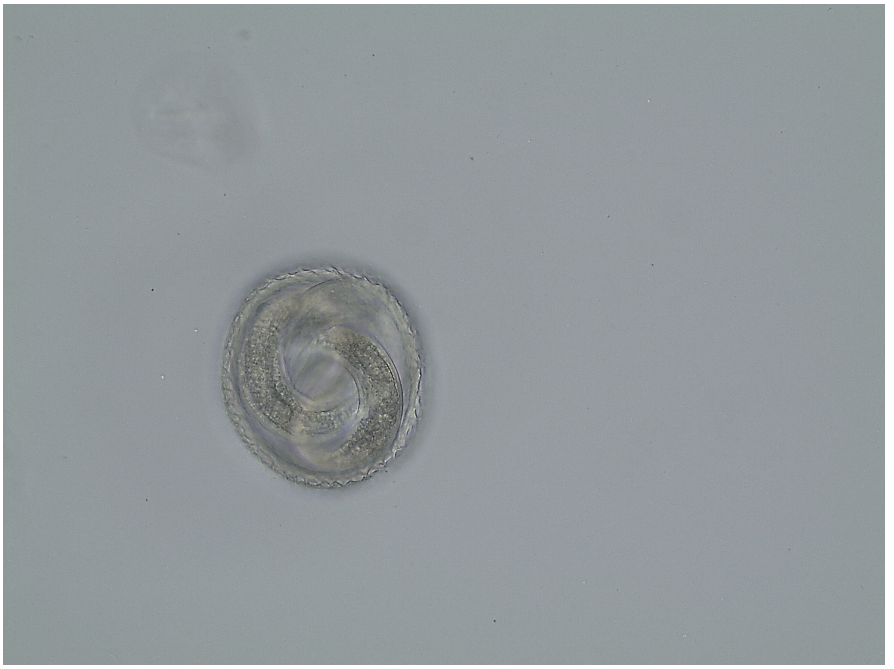

Figure 1. Larval development in the *Toxocara canis* egg. The egg suspension and undiluted rhizome extract were incubated for 24 h. After being centrifuged with sterile distilled water, the treated eggs were gently incubated for 28 days with a daily mix.

3.2. Effect of the plant extracts on embryogenesis in *T. canis* eggs

Concerning embryogenesis in the *T. canis* eggs, no difference between the extract groups (leaf and rhizome) and the control groups was observed during the incubation periods. The larvae developed in all experiment groups, including the positive control (Figure 1).

3.3. Effect of leaf extracts on the viability of *T. canis* larvae

Table shows the effect of the extracts on larvae viability after hatching. All *T. canis* larvae were motile after being incubated for 6 h with the leaf extract at 10 mg/mL. However, after 12 and 24-h incubation with the leaf extract at 10 mg/mL, the paralysed larvae rate was 9% and 15%, respectively. After 6, 12, and 24-h incubation with the leaf extract at 20 mg/mL, the paralysed larvae rate was 20%, 22%, and 32%, respectively. After 6, 12, and 24-h incubation with the leaf extract at 40 mg/mL, the paralysed larvae rate was 30%, 37%, and 42%, respectively (Figure 2). Dose and time factor analysis results were statistically significant ($p < 0.001$). Some paralysed larvae were straight, and others displayed a C-shape form. The paralysed larvae rate was 1.45 times higher in the leaf extracts than in the rhizome extracts ($p < 0.001$).

After 6, 12, and 24-h incubation with the undiluted rhizome extract, the paralysed larvae rate was 10%, 12%, and 17%, respectively. After 6, 12, and 24-h incubation with the rhizome extract diluted at 1:2, the paralysed larvae rate was 5%, 7%, and 9%, respectively. After 6, 12, and 24-h incubation with the rhizome extract diluted at 1:4, the paralysed larvae rate was 5%, 6%, and 13%, respectively. Dose and time factor analysis results were not statistically significant ($p > 0.05$). All larvae were paralysed in the positive control group.

4. Discussion

Interest in traditional and complementary medicine is increasing. Various plant extracts have been investigated for their anthelminthic properties on *T. canis* eggs [18,19,21]. Ovicidal activity of the *Zingiber officinale* ethanolic extract (100 mg/mL) was reported as 98.2% on *T. canis* eggs, and the activities were 82.5% and 59.22% at 50 mg/mL and 25 mg/mL doses, respectively [19]. The two different ethanolic extracts of *Allium cepa* (10.000 and 1.250 µg/mL) inhibited larvae development in *T. canis* eggs. However, this effect was not observed in the ethanolic extract of *Allium sativum* [18]. The aqueous extracts of *Allium sativum* and *Jatropha curcas* did not affect larva development in *T. canis* eggs [18]. Several ovicidal and larvicidal effects of the ethanolic extracts of *Euterpe edulis* and *Mikania laevigata* were reported on *T. canis* eggs [21]. In the current study, ovicidal activity was not observed in the ethanolic extract of the leaves and distilled water extract of the rhizome of *A.*

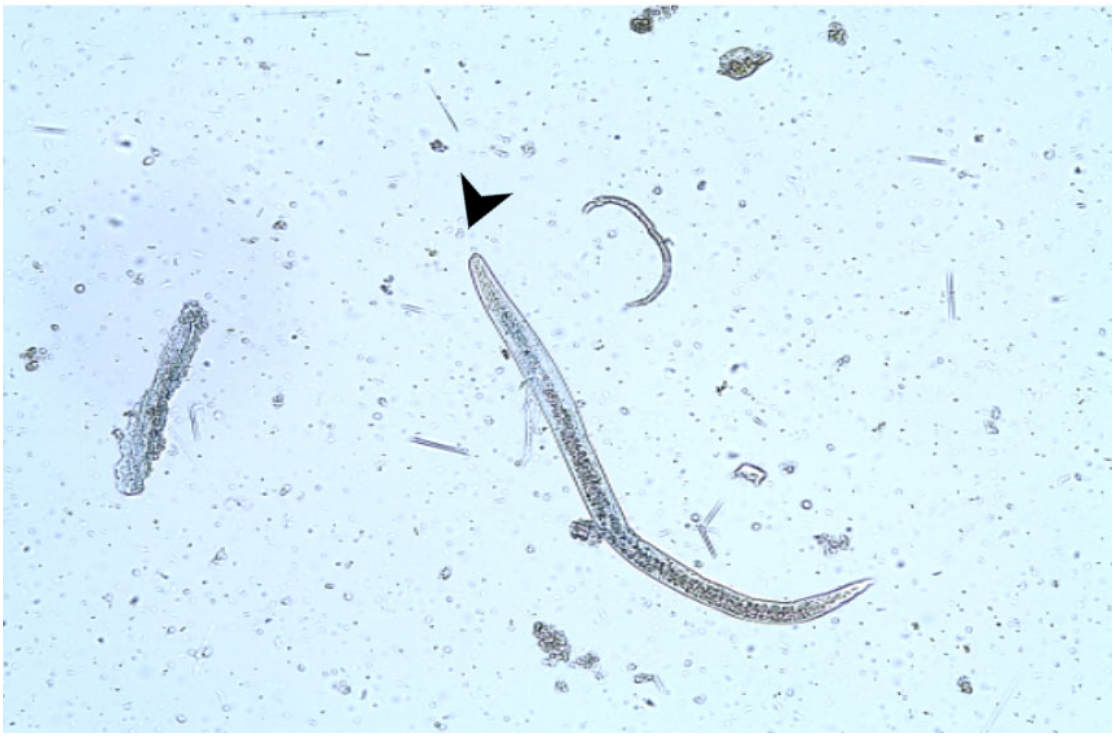

Figure 2. Paralysed *Toxocara canis* larva. The nontreated eggs were incubated for 28 days. The sodium hypochlorite solution was added to the larvae-developed eggs. The hatchling larvae were incubated for 24 h with the leaf extract at 40 mg/mL (arrowhead: anterior end of the larva).

rupicola Boiss var. *rupicola*. This difference may be related to the shell structure of the *T. canis* egg, which consists of 5 layers (from inside to outside of the lamellar, granular, chitinous, vitelline, and thin uterine layer) [34]. Due to their shell structure, *T. canis* eggs resist many disinfectants and natural conditions [35]. The protective property of the eggshell may have prevented the potential effects of the extracts on embryogenesis in the *T. canis* eggs in this study.

Few in vitro studies on the larvicidal effect of plant extracts on *T. canis* larvae exist [20]. The viability of *T. canis* larvae was reported as 89.4% after incubation with the decoction of *Vernonia cinerea* for 18 h [20]. The present study observed the dose and time-dependent larvicidal activity of the ethanolic extracts of *A. rupicola* Boiss var. *rupicola* leaves on the *T. canis* larvae. The paralysed larvae rate was higher in the leaf extracts than in the rhizome extracts ($p < 0.001$). El-Sayed et al. (2017) suggested that the anti-*T canis* effect may be related to the phytochemical composition of *Z. officinale* extract, including flavonoids [19]. The leaves of the *Arum* species consist of different flavonoids and phenolic compounds [9,10]. *Arum rupicola* leaves have isoflavonoids such as genistein and genistein 8–C-glucoside [3]. Genistein acts on helminth parasites in different ways and is primarily responsible for helminth paralysis [2]. Due to its collagen-rich fibrosis surface interface, paralysis caused by phytoestrogen develops for a longer time in nematode parasites than in cestodes [2]. In this study, the genistein in the leaf extract of *A. rupicola* Boiss var. *rupicola* could be the reason for larvae paralysis. Additionally, the paralysis rate increased in parallel with leaf extract concentration and time.

Genistein's other effects are changes in the tegument and tegumental enzymes of some platyhelminthes [2]. After being incubated with genistein, some effects have been reported on the tegument of cestodes, such as *Echinococcus multilocularis*, *Echinococcus granulosus* [36], *Raillietina echinobothrida*, [37] and trematodes like *Fasciolopsis buski* [38] and *Schistosoma mansoni* [39]. In the present study, the larvicidal effect may have occurred due to alterations that depended on the leaf extract in the tegument and tegumental enzymes of *T. canis* larvae. *Toxocara canis* larvae are known to be strong. After hatching, they can live long periods in cell culture mediums, such as RPMI-1640, which is rich in essential ingredients, including salt, amino acids, vitamins, and D-glucose [40]. In this study, all nontreated larvae (the negative control) were observed as alive in RPMI-1640 after completing the experiment.

Temperature, pressure, and solvent determine extract quality [40]. Farahmandarfar et al. (2019) reported that total flavonoid contents are higher in the extract of *Arum maculatum* leaves with a mixture of ethanol and distilled water (50:50) extraction than with extracts using only distilled water or only ethanol [30]. In this study, an ethanol and distilled water mixture (50:50) was used for the leaf extraction of *A. rupicola* Boiss var. *rupicola*.

The rhizome of *A. maculatum* includes lectins [41]. Majumder et al. (2005) reported that *A. maculatum* rhizome had an insecticidal effect on the *Lipaphis erysimi* and *Aphis craccivora* lice species in plants. Lectins show their insecticidal effect by binding to the intestinal epithelium of the lice [42]. In addition, lectins have various anthelmintic effects [43]. The larvicidal and ovicidal activities of some plant-derived lectins have been reported on the gastrointestinal nematodes of ruminants [44–46]. Another plant-derived organic chemical is saponin, found in the rhizome of *A. rupicola* Boiss var. *rupicola* [8]. Saponins cause vacuolisation and fragmentation in the tegument and affect cell permeability in some parasites [47]. The current study observed the larvicidal effect on *T. canis* larvae after incubation with *A. rupicola* Boiss var. *rupicola* rhizome extract. This activity may have occurred due to the lectins and partly because of the saponins, which were contained. Saponins serve as triggers for haemolysis in erythrocytes [48]. In the present study, the saponin rate was low in the rhizome extract because the haemolytic effect was not observed – even when the rhizome extract was added to rabbit erythrocytes without dilution.

Some reports exist on the cytotoxicity of *A. rupicola* [3, 49]. Hexane fraction of *Arum rupicola* Boiss leaves shows maximum cytotoxic activity on MCF-7 breast cancer cells [3]. Cytotoxic activity occurred from the distilled water extract of *Arum rupicola* Boiss var. *rupicola* on SK–HEP-1 cells [49]. The cytotoxic effect was observed only in the leaf extract (10 mg/mL) on the L929 mouse fibroblast cells in this study. The same activity was not detected on the rhizome extract.

Although the primary goal of using plants is to benefit from their therapeutic qualities, some plant species are toxic. For instance, several metabolites produced during plant synthesis are dangerous to humans and animals [50]. The *Arum* species contain some toxic compounds, including oxalate, glycosidic saponins, lignan, and neolignan, which can irritate the skin, mouth, tongue, and throat [5–7]. Raw consumption of these plants' leaves and rhizomes harms living beings; however, their toxic effect disappears after cooking them [8]. The harmful features of *A. rupicola* Boiss *rupicola* is well known, and it is traditionally consumed after being cooked by individuals living in the region observed in the present study. Some interesting findings were observed while the research was carried out. The plant's rhizome parts may be eaten by some animals in nature. While the researchers sampled for specimens, the plant and the rhizome were removed from the soil, and only the leaf parts were left behind in some areas. A wild pig population lived in the sampling area, and the researchers determined that the animals might only eat the rhizome parts of the plant. The current study results may partly support this observation. Cytotoxic or haemolytic effects were not detected after incubation with the rhizome extracts; however, these effects were only evident in the leaf extract in this study.

Arum rupicola Boiss var. *rupicola* leaves are consumed by humans in different geographical regions, including Türkiye. The ethanolic extract of its leaves and the distilled water extract of its rhizome have no activity-related effect on embryogenesis in *T. canis* eggs. However, both extracts demonstrate larvicidal activity on *T. canis* larvae in vitro. Future research might clarify how *A. rupicola* Boiss var. *rupicola* extracts affect visceral larva migrans in experiments in vivo and ascertain whether these plant extracts are effective against more parasites.

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