**In vitro** motility inhibition effect of Czech medicinal plant extracts on *Chabertia ovina* adults

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**Keywords:** chabertiasis, large-mouthed bowel worm, adult motility inhibition assay; plant extracts

Publication date 30/4/2014 [http://www.m.elewa.org/JAPS; ISSN 2071-7024](http://www.m.elewa.org/JAPS; ISSN 2071-7024)

1 ABSTRACT

Although chabertiasis causes great economic damage to sheep farms worldwide, a limited number of studies have focused on development of antihelmintic agents effectively inhibiting *Chabertia ovina* (*C. ovina*). In this study, ethanol extracts of 16 Czech medicinal plants were tested for their potential *in vitro* antihelmintic activity against *C. ovina* using adult motility inhibition assay. Values of half maximal inhibitory concentration (IC₅₀) were determined for 6, 24 and 48 hour exposure at extract concentrations 0.25, 0.5, 1, and 2 mg/mL. After 6 hours, extracts of *Daucus carota* (wild carrot), *Satureja hortensis* (summer savoury), *Valeriana officinalis* (valerian), *Dryopteris filix-mas* (male fern), *Artemisia absinthium* (absinthe wormwood), *Juglans regia* (common walnut), *Hedera helix* (common ivy) and *Inula helenium* (elecampane) were more effective than positive control albendazole, with IC₅₀ values 0.57, 1.15, 1.32, 1.34, 1.35, 1.60, 1.66 and 1.68 mg/mL, respectively. At 24-hour exposure IC₅₀ of all extracts had significantly decreased, however, only *A. sativum, D. carota, V. officinalis* and *Tanacetum vulgare* (tansy) possessed stronger motility inhibitory effect (IC₅₀ ranging from 0.30 to 0.65 mg/mL) than albendazole. All plants tested totally inhibited *C. ovina* motility at lower concentration tested (0.25 mg/mL) after 48 hours. Because of this test, the best antihelmintic activity against *C. ovina* was observed from *A. sativum, D. carota* and *V. officinalis*, which suggests these extracts as prospective materials for further development of novel plant-based antihelmints against *C. ovina*. However, detailed analysis of their chemical composition and *in vivo* activity should be carried out in order to validate their antihelmintic character and verify their possible practical use.

2 INTRODUCTION

The strongylid nematode infections are known to cause great economic damage to sheep farms, especially in lambs, where they are associated with lowered live weight, reduced growth rates, occurrence of diarrhoea and increased mortality (Sweeny et al., 2012). *Chabertia ovina* Fabricius (Chabertiidae) (large-mouthed bowel worm) belongs to the most
widely distributed nematodes worldwide causing severe damage to the mucosa of the colon with resulting congestion, ulceration, and small haemorrhages in sheep (Kahn and Line, 2010). Management and control of *C. ovina* in commercial sheep farms is critical because of income loss associated with reduced flock productivity. Synthetic drugs, such as benzimidazoles, levamisole and albendazole are commonly used for its elimination (Wagland et al., 1996; Waller et al., 1996; Sackett et al., 2006). In the recent time, the growing problem of anthelmintic resistance of nematodes to synthetic drugs their expenses and negative impact on both animals and environment has led to development of alternative classes of anthelmintics (Waller, 1994; Besier and Love, 2003; Githiori et al., 2006). Nowadays, several preparations based on plant-derived compounds or their semi-synthetic derivatives such as arecoline (*Areca catechu*), quisqualic acid (*Quisqualis indica*), santonin (*Artemisia maritima*) and artemisane (*Artemisia annua*) are used in veterinary medicine to treat nematode infections (Taylor, 2005; Dewick, 2009; Fathy, 2011). Taking into consideration relatively high incidence of *C. ovina* infections and the number of studies done in the field of anthelmintic properties of plants, only a small part is concerned with this *C. ovina*. With a few exceptions (Tariq et al., 2009; Silveira et al., 2012), most of these available studies were using egg hatch assay (Borgsteede et al., 1997; Al-Shaiban et al., 2009). With the aim of presenting more accurate data on effectiveness of plant-derived agents against *C. ovina*, this study evaluated anthelmintic activity of 16 extracts using adult motility inhibition assay (AMIA), which is the generally accepted as a standard method (Tritten et al., 2012).

### 3 MATERIALS AND METHODS

#### 3.1 Plant materials

Plant species were selected according to their traditional use for treatment of parasitical infections recorded in the literature (Korbelar et al., 1978) and their promising anthelmintic activity against nematodes *Ascaris suum* and *Trichostrongylus colubriformis* confirmed in our previous studies (Urban et al., 2007, 2008). Different plant parts of *Allium sativum* L. (bulb), *Artemisia absinthium* L. (areal part), *Artemisia vulgaris* L. (areal part), *Carum carvi* L. (fruit), *Consolida regalis* Gray (flower), *Cucurbita pepo* L. (seed), *Daucus carota* L. (root), *Dryopteris filix-mas* (L.)Schott (rhizome), *Erigeron canadensis* L. (areal part), *Hedera helix* L. (leaf), *Inula helenium* L. (rhizome and root), *Juglandis regia* L. (pericarp), *Satureja hortensis* L. (areal part), *Tanacetum vulgare* L. (areal part), *Thymus vulgaris* L. (areal part), and *Valeriana officinalis* L. (rhizome). These were collected from various areas of Pilsen (Domazlice and Tachov districts) and Prague regions in the Czech Republic from April to October 2005. Voucher specimens were authenticated and deposited at the Faculty of Tropical AgriSciences, Czech University of Life Sciences Prague. Detailed description of tested plants (voucher specimen number, families and ethnobotanical data) is presented in Urban et al. (2008).

#### 3.2 Preparation of extracts

The plants were dried at a room temperature (20–25°C). Appropriate plant parts were grounded (15 g) and then macerated with 80% ethanol (450 mL) for 5 days. The extracts were subsequently filtered and concentrated in vacuo at 40°C. The residue was dissolved in 20 μL dimethyl-sulfoxide (DMSO) and in 980 μL phosphate-buffered saline (PBS; pH 7.2, 0.15 M), creating concentration 2 mg/mL. All samples were stored at -20 °C until tested.

#### 3.3 Adult motility inhibition assay

The anthelmintic effect of 16 plant extracts against *C. ovina* adults was measured using motility inhibition assay previously described by Hounzangbe-Adote et al. (2005). Three specimens of adult *C. ovina* worms per well were inserted into 24-well microtitration plates. The worms were first washed in PBS buffer.
solution (pH 7.2, 0.15 M) and incubated at room temperature for one hour. Washing solution was discarded and 1 mL of plant extract was added to each well at concentrations 0.25, 0.5, 1 and 2 mg/mL. Positive (albendazole) and negative (2% DMSO in PBS solution) controls were included on each plate. Samples were assayed in four independent experiments each performed in duplicate. The motility of adult worms was controlled and recorded after 6, 24 and 48 hours optically using binocular lens. After each observation motility inhibition index (MII) was calculated using following the formula: MII (%) = [(T-M)/T] x 100, where M refers to mobile (living) worms and T to total worm count. Values of MIIs were further used to calculate half-maximal inhibitory concentration (IC_{50}). Results are therefore expressed as minimal concentration of plant extract needed to inhibit motility of 50% adult *C. ovina* worm population tested.

4 RESULTS

In this study performed with 16 plants whose selection was based on data suggesting their possible anthelmintic effect, ethanol extracts of eight species possessed significant activity against *C. ovina* adult worms using motility inhibition *in vitro* assay. Complete results are shown in Table 1.

**Table 1: In vitro motility inhibition effect of Czech medicinal plant extracts on adult *Chabertia ovina***

<table>
<thead>
<tr>
<th>Plant species</th>
<th>IC_{50} (mg/mL)/time exposure (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>6</td>
</tr>
<tr>
<td><em>Allium sativum</em></td>
<td>&gt; 2</td>
</tr>
<tr>
<td><em>Artemisia absinthium</em></td>
<td>1.35 ± 0.36</td>
</tr>
<tr>
<td><em>Artemisia vulgaris</em></td>
<td>&gt; 2</td>
</tr>
<tr>
<td><em>Carum carvi</em></td>
<td>&gt; 2</td>
</tr>
<tr>
<td><em>Consolida regalis</em></td>
<td>&gt; 2</td>
</tr>
<tr>
<td><em>Caucurlita pepo</em></td>
<td>&gt; 2</td>
</tr>
<tr>
<td><em>Daucus carota</em></td>
<td>0.57 ± 0.09</td>
</tr>
<tr>
<td><em>Dryopteris filix-mas</em></td>
<td>1.34 ± 0.31</td>
</tr>
<tr>
<td><em>Erigeron canadensis</em></td>
<td>&gt; 2</td>
</tr>
<tr>
<td><em>Hedera helix</em></td>
<td>1.66 ± 0.38</td>
</tr>
<tr>
<td><em>Inula heliannum</em></td>
<td>1.68 ± 0.39</td>
</tr>
<tr>
<td><em>Juglans regia</em></td>
<td>1.60 ± 0.41</td>
</tr>
<tr>
<td><em>Salvia officinalis</em></td>
<td>1.15 ± 0.26</td>
</tr>
<tr>
<td><em>Tanacetum vulgare</em></td>
<td>&gt; 2</td>
</tr>
<tr>
<td><em>Thymus vulgaris</em></td>
<td>&gt; 2</td>
</tr>
<tr>
<td><em>Valeriana officinalis</em></td>
<td>1.32 ± 0.36</td>
</tr>
<tr>
<td><em>Albendazole</em></td>
<td>1.92 ± 0.38</td>
</tr>
</tbody>
</table>

*half maximal inhibitory concentration expressed as mean value ± standard deviation

"positive control

At 6 hour exposure, extracts of *D. carota, S. bortensis, V. officinalis, D. filix-max, A. absinthium, J. regia, H. helix* and *I. heliannum* were more effective than positive control albendazole, with IC_{50} values 0.57, 1.15, 1.32, 1.34, 1.35, 1.60, 1.66 and 1.68 mg/mL, respectively. The rest of the plants exhibited no inhibitory activity (IC_{50} > 2 mg/mL). The motility inhibition effect of all extracts had significantly increased after 24 hours, however, only *A. sativum, D. carota, V. officinalis* and *T. vulgare* achieved stronger activity than positive control with IC_{50} values ranging
from 0.30 to 0.65 mg/ml. The rest of the plant species had considerable weaker effect (IC_{50} ≥ 1.01 mg/mL). All plants tested totally inhibited *C. ovina* motility at lower concentration tested (0.25 mg/mL) after 48 hours. When IC_{50} values for all time exposures were compared to those of albendazole, the stronger activity was observed for extracts of *D. carota*, and *V. officinalis*, which were more effective than positive control at both 6 and 24 hours of exposure. The significant anti-chabertial effect of both plants is also illustrated in detail by comparison of their MIIs at concentration of 1 mg/mL with those of positive and negative controls (Figure 1.).

![Motility Inhibition Indexes](image)

**Figure 1:** Motility inhibition indexes of plants (A and B) with the best anti-chabertial efficacy at concentration of 1 mg/mL in comparison to positive (C) and negative (D) controls.

The stronger anti-chabertial effect than positive control has also been observed for extracts of *A. sativum*, *A. absinthium*, *C. carvi*, *D. filix-mas*, *H. helix*, *I. belemium*, *J. regia*, *S. bortensis* and *T. vulgare* for at least one of exposure times used. All plants tested totally inhibited motility of *C. ovina* at exposure of 48 hours; however, viability of tested nematodes decrease to 45.83% that significantly influenced observed antihelmintic effect of extracts tested.

5 DISCUSSION

As far as previously described, antihelmintic activity of the most effective plants tested in this study is considered, *A. sativum*, *A. absinthium*, *I. belemium* *J. regia* and *T. vulgare* was found to be effective against various parasites such as *Haemonchus contortus* (Squires et al., 2011; Ahmed et al., 2013), *Ascaris lumbricoides* (El Garhy and Mahmoud, 2002), *Eicinia foetida* (Kale et al., 2011) and *Oesophagostomum* spp. (Magi et al., 2005). In addition, plant extracts of *D. carota*, *V. officinalis* and *C. carvi* showed *in vitro* antihelmintic effects against *Ascaris suum* and
Trichostrongylus colubriformis in our previous study (Urban et al., 2008). Despite the existence of above-mentioned reports on anthelmintic activity of A. sativum, A. absinthium, C. carvi, D. carota, I. bidentatum, J. regia, T. vulgar and V. officinalis, according to our best knowledge, this is the first report on in vitro motility inhibition effect of these plants against C. ovina. This study hypothesized which bioactive chemical constituents are present in the tested plant extracts and could be responsible for anthelmintic activity. A. sativum contains several sulfur based aliphatic compounds, whereas allicin is considered the most important. This compound has been reported to influence growth of various nematodes including Ascaridia galli and Schistosoma mansoni both in vitro and in vivo (Lima et al., 2011; Velkers et al., 2011). Anthelmintic activity of artemunate (water-soluble semi-synthetic drug derived from artemisinin, a compound present in A. absinthium) was previously described by Fathy et al. (2011). Its detected high in vivo activity together with reported safety has been recommended for clinical use in humans for treatment of some nematode infections such as Clonorchis sinensis, Fasciola hepatica and Schistosoma japonicum. Limonene, major component of C. carvi essential oil (Dewick, 2009), has previously demonstrated significant anthelmintic activity against Ascaridia galli both in vitro and in vivo (Abdelqader et al., 2012). According to this result, it is assumable that limonene can significantly contribute to the anthelmintic effect of C. carvi observed in this study. Fetterer and Fleming (1991) had previously described anthelmintic activity of juglone, a quinone compound of J. regia pericarp, against Haemonchus contortus and Ascaris suum in vitro. Therefore, it is highly probable that juglone is accountable for anthelmintic activity of J. regia extract. Several studies have discussed alantolactone and isosalantolactone, terpenoids of I. bidentatum root, as compounds responsible for anthelmintic activity of the plant, however, it was discovered that their effect is relatively weak (Bruneton, 1999). Kaempferol, quercetin and their glycosylated derivatives, which have been detected in I. bidentatum roots (Spiridon et al., 2013) have previously exhibited considerable anthelmintic activity against Schistosoma mansoni adult worms in vitro (Braguine et al., 2012). This finding suggests that phenolic compounds together with presented lactones contribute to overall anthelmintic activity of I. bidentatum (Azaizhe et al., 2013). Root of V. officinalis contains alkaloids, epoxy-iridoid esters (valepotriates) and various sesquiterpenoids (Letchamo et al., 2004; Dewick 2009; Parveen et al., 2012). Since sesquiterpene structures have previously showed to be very potent anthelmintic agents in vitro (Li et al., 2008), sesquiterpenes can significantly contribute to anthelmintic efficacy of V. officinalis. Thujone, monoterpenoid naturally occurring in aerial parts of T. vulgar (Ramasubramaniaraja and Nirajan Babu, 2010), has previously possessed anthelmintic activity against Ascaris lumbricoides and Fasciola hepatica in vitro (Mackie et al., 1955). Therefore, this compound can be responsible for detected anthelmintic activity also in this test. One of the most interesting outcomes of this study is seen in relatively high D. carota anthelmintic efficacy. Medicarpin and 4-hydroxymedecarpin (phytoalexins) have previously demonstrated substantial anthelmintic effect towards Caenorhabditis elegans in vitro (Stadler et al., 1994). Because of presence of structurally related compound (6-methoxymellein) in root of D. carota (Dewick, 2009) it is presumable that this compound could be responsible for relatively high anthelmintic activity of this plant. However, further analysis will be needed for identification of the main anti-chabertial agent of D. carota extract. The practical usability of plants as anthelmintic agents primarily depends on their toxicity and this fact should be considered in potential application of their extracts as veterinary pharmaceutics. There have been some indices that contented sulfuric compounds in A. sativum may be potentially toxic to sheep individuals, causing allergic reactions, flatulence, nausea, and abdominal
discomfort (Balasinska and Kulasek, 2004; Barceloux, 2008). However, studies performed with *A. sativum* extracts showed that it can be safely fed to sheep in large quantities (Fredrickson *et al.*, 1995; Nowroozi-Asl *et al.*, 2010). Due to lack of relevant data dealing with potential toxicity of *A. absinthium, C. carvi, D. carota, I. bellowium, J. regia* and *V. officinalis* to sheep, further toxicological information for these species is presented for organisms other than small ruminants. *A. absinthium* essential oil is sold as a dietary supplement in some countries over the world to treat various human diseases including digestive disorders (Wojcikowski *et al.*, 2004). Several cases of acute liver and kidney failure (together with nausea, vomiting, stomach pain, headache, dizziness, seizures, numbness of the legs and arms, delirium, and paralysis) have been reported after intake of *A. absinthium* essential oil (Weisbord *et al.*, 1997; Luyckx and Naicker, 2008). Symptoms of acute toxicity were also observed after ingestion of *T. vulgare* (Foster *et al.*, 1999; Barceloux, 2008) in humans, even though it has commonly been used as herbal medicine in several countries (Lahlou *et al.*, 2008; Alvarez *et al.*, 2011). Thujone is considered as main toxic component of both *A. absinthium* and *T. vulgare* (Pelkonen *et al.*, 2013). Alqasoumi *et al.* (2012) determined a maximum tolerance dose and genotoxicity of *C. carvi* water suspension on mice and suggest that oral administration is safe. It was discovered that extract of *D. carota* root contains carotatoxin. Upon injection to mice, compound was found to possess neurotoxic symptoms and its LD$_{50}$ was settled to 100 mg/kg (Crosby and Aharonson, 1967). There have been some rare reports of *D. carota* causing mild intoxications to horses and cattle upon feeding. This phenomenon was predicated to carotatoxin content, however, its concentration in fresh *D. carota* material was found to be very low (10-20 ppm). A fatal toxicological effect is seen only if large quantities are eaten. *D. carota* is therefore considered being safe to its potential consumer (Tavares *et al.*, 2008). Lactones presented in *I. bellowium* can cause allergic reactions and in higher doses can induce vomiting, diarrhoea and other problems in humans (Warshaw and Zug, 1996). Crude extract exhibited cytotoxic effect *in vitro* (Dorn *et al.*, 2006). Several authors discourage prolonged use; however, extracts of rhizomes and roots of *I. bellowium* are accepted as preparations of choice to treat various human illnesses (Bruneton, 1999). The chief constituent of *J. regia* leaves and pericarp is juglone, which had previously demonstrated *in vitro* cytotoxicity (Inbaraj and Chignell, 2004; Spiridonov *et al.*, 2005). The registry of toxic effects of chemical substances describes juglone as potential mutagenic and carcinogenic agent (Thakur, 2011). Furthermore, Van den Berg et al. (1990) described dermal allergy localized at various parts of body after application of juglone to skin. However, *J. regia* is an ingredient of plant-based medications and acute toxicity in humans has not been reported after oral application of the drug (Bruneton, 1999). Constituents presented in *V. officinalis* root (baldrinals, valepotriates) are believed to possess cytotoxic, mutagenic and teratogenic properties (Bos *et al.*, 1998). Nevertheless, until now, these effects have only been observed *in vitro*. *V. officinalis* is widely accepted as dietary supplement and tranquilizer both in human and veterinary medicine (Dewick, 2009). Health risk after prolonged use is therefore negligible (Bruneton, 1999).

6 CONCLUSION

In summary, the current study proved *in vitro* anthelmintic activity of ethanol extracts of eight plant species, namely *A. sativum, A. absinthium, C. carvi, D. carota, I. bellowium, J. regia, T. vulgare* and *V. officinalis*. Even though, *A. absinthium, I. bellowium, J. regia* and *T. vulgare* has been shown to significantly inhibit motility of *C. ovina*, their possible toxicity should be considered when employing these in veterinary medicine. Therefore, extracts of *A. sativum, D.
carota and V. officinalis seems to be the prospective materials for further development of novel plant-based anthelmintics against Chabertia ovina. Especially extracts of D. carota and V. officinalis, which produced stronger or equal effect than albendazole at all times of experimental exposure, deserve more research attention. However, detailed analysis of their chemical composition and in vivo anthelmintic activity should be carried out in order to verify their possible practical use.

7 CONFLICT OF INTEREST
All authors disclose that they have no financial and personal relationships with other people or organization that could inappropriately influence (bias) their work, including employment, consultancies, stock ownership, honoraria, paid expert testimony, patent applications/registration, and grants or other funding.

8 ACKNOWLEDGEMENTS
This work was supported by the Internal Grant Agency of the Faculty of Tropical AgriSciences of the Czech University of Life Sciences Prague (project no. IGA 20145021). The review of the manuscript by the ELEWA biosciences publications office is greatly acknowledged.

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