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Research paper

Thymus vulgaris L. essential oil and its main component thymol: Anthelmintic effects against *Haemonchus contortus* from sheep

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ABSTRACT

Haemonchus contortus is an important gastrointestinal parasite on sheep farms in tropical regions. The resistance of the parasite against most anthelmintic drugs represents a great economic problem to sheep farming and is a major challenge that needs to be overcome. The searches for new anthelmintic agents that act on different stages of the parasite's life cycle are necessary for the development of new therapeutic options. The aim of this study was to evaluate the *in vitro* and *in vivo* anthelmintic activity of *Thymus vulgaris* essential oil against *H. contortus* and of its main component, the monoterpene thymol. Despite the relative ineffectiveness of the oil in the *in vivo* test, which may be corrected in the future after technical improvements to increase the oil's bioavailability, the *in vitro* results validated the popular use of *T. vulgaris* oil as an anthelmintic agent, at least against *H. contortus*. In fact, both the essential oil and thymol, which accounts for 50.22% of the oil composition, were effective against the three main stages of *H. contortus*. The oil and thymol were able to inhibit egg hatching by 96.4–100%, larval development by 90.8–100%, and larval motility by 97–100%. Similar to the positive control (levamisole 20 mg/mL), the oil and thymol completely inhibited the motility of *H. contortus* adults within the first 8 h of the experiment. Since thymol reproduces the anthelmintic effects of the oil and because it is the main component of the oil, it is reasonable to assume that thymol is the most important compound responsible for the anthelmintic effect of *T. vulgaris*. These results are of ethnopharmacological importance and may contribute to the development of new drugs and even herbal medicines, increasing treatment options for the farm breeding.

1. Introduction

Diseases caused by gastrointestinal parasites in sheep generate significant losses in productivity worldwide (Roeber et al., 2013). *Haemonchus contortus* is one of the most important parasite species of sheep due to its widespread occurrence and high virulence (Rose et al., 2014). The pathological damage caused by the parasite results in the loss of large amounts of blood in affected animals, causing severe anemia, weakness, nutritional deficiency, weight loss and, in many cases, death (Roeber et al., 2013). In an attempt to control the infection, the inappropriate use of commercial anthelmintic drugs contributed to the selection of resistant strains of this parasite, which resulted in the partial or total inefficacy of most classes of available anthelmintic drugs (Roeber et al., 2013). The economic impacts caused by this increasing resistance have rendered the discovery and development of new anthelmintic agents a great challenge for sheep farming (Coles et al., 2006; Kotze et al., 2012a; Buckingham et al., 2014).

Within this context, the search for botanical anthelmintics has emerged as an attractive alternative. In addition to the traditional use of plants as phytotherapeutics, the isolation and study of derivative molecules, whose chemical structures are well defined, have contributed to the development of conventional medicines and to the understanding of the mode of action of phytotherapeutics or plant extracts with anthelmintic activity (Buttle et al., 2011; Domingues et al., 2013; Ibrahim et al., 2014; Klongsiriwet et al., 2015). Plant essential oils are a complex mixture of different compounds, most characteristically terpenes and terpenoids (Hashmi et al., 2013), which are biomolecules of great biotechnological and pharmaceutical interest, including those with anthelmintic activity (Gupta et al., 2005; Staniek et al., 2013). Indeed, sev-

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eral terpenes and terpenoids commonly found in essential oils have been identified as promising anthelmintics. These compounds include linalool, citronellal, eugenol, geraniol, caryophyllene, menthol, menthofuran, menthone, thymol, ◯-cymene, limonene, citral, myrcene, β-pinene, α-terpinene, and carvacrol, among others (Echeverrigaray et al., 2010; Carvalho et al., 2012; Katiki et al., 2012).

Thymus vulgaris L. is a shrub in the Lamiaceae family, which is cultivated in different parts of the world, mainly in Mediterranean climates. Due to its strong aromatic characteristics, *T. vulgaris* has been traditionally used in food and cosmetic products. Phytochemical analysis has shown the presence of different compounds in the plant, such as flavonoids, tannins, saponins, phenolic compounds, and especially an essential oil that is rich in the monoterpene thymol and its isomer carvacrol (Queiroz et al., 2012; Walentowska and Flaczyk, 2013). The plant is used in traditional medicine for a variety of purposes, including as an anthelmintic agent (Behnia et al., 2008). The fact that *T. vulgaris* is used as food indicates good toxicological tolerability of the plant in mammals. This fact and the proven pharmacological activities of *T. vulgaris* suggest a high nutraceutical potential of the plant that permits the development of more diverse products such as medications and food supplements (Behnia et al., 2008; Hashmi et al., 2013).

Considering the anthelmintic potential of essential oils in general and of *T. vulgaris* in particular, the aim of this study was to evaluate the anthelmintic activity of *T. vulgaris* essential oil against the different life cycle stages of *H. contortus* using *in vitro* and *in vivo* assays. Additionally, an extensive phytochemical characterization of the oil was performed, investigating the presence of thymol as the responsible for the pharmacological activity observed.

2. Materials and methods

2.1. Thymus vulgaris essential oil and thymol

In vitro and *in vivo* assays were performed using *T. vulgaris* essential oil purchased from Ferquima Ind e Com Ltda (Vargem Grande Paulista, SP, Brazil) (Lot No. 181, density (d) = 0.918). The essential oil from leaves of *T. vulgaris* was extracted by hydrodistillation. Thymol in its pure organic form was purchased from Neon Comercial Ltda (São Paulo, Brazil) (ref. No. 3692, Lot No. 18467) and was used in the *in vitro* tests to establish the relationship between oil composition and the pharmacological activity observed.

2.2. Phytochemical analysis of the essential oil of T. vulgaris by GC–MS

A sample of the oil was subjected to gas chromatography coupled with mass spectrometry (GC–MS) (Varian, model Saturn 2100T). The following conditions were used: capillary column: DB-5 (30 m × 0.25 mm × 0.25 μm); gun: 240 °C; detector: 230 °C; electron impact: 70 eV; carrier gas: He; flow rate: 1.0 mL/min; split: 1/20; temperature program: 60 °C–240 °C, 3 °C/min; injection volume: 1 μL. The compounds were identified by comparison of their mass spectra with the database of the GC–MS system (Nist 62 lib.) and Kovats retention index (Adams, 1995).

2.3. Experimental animals and artificial infection

Male Santa Ines sheep aged 4–6 months and weighing 20–30 kg were purchased from farms located in the region of Ribeirão Preto (SP, Brazil). The animals were kept on the experimental farm of the Ribeirão Preto University (Unaerp) and received food (ration and hay) and water *ad libitum* two times per day. All experimental procedures were approved by the Ethics Committee of Unaerp (Protocol number 042/2012).

After purchase, all animals were dewormed with Zolvix® (Monepantel, Novartis Animal Health, 2.5 mg/kg body weight, BW) and were maintained in clean pens until complete reduction in the number of eggs per gram of feces (EPG) (Ueno and Gonçalves, 1998) to zero (7–14 days). After this period, the animals were submitted to monospecific infection with a resistant isolate of *H. contortus* (Embrapa2010) by oral administration of approximately 4000 L_3 larvae of the parasite. Infection was confirmed by determining EPG after 28–30 days of contamination. Resistant isolate of *H. contortus* (Embrapa2010) is well known to be resistant to benzimidazoles, macrocyclic lactones and imidazothiazoles (Chagas et al., 2013).

2.4. In vitro anthelmintic assays

The *in vitro* anthelmintic activity of the essential oil of *T. vulgaris* and of thymol was evaluated by the egg hatch test (EHT), larval motility test (LMT), larval development test (LDT), and adult worm motility test (AWMT).

2.4.1. Egg hatch test

The EHT was performed according to Coles et al. (1992) as follow: 20 g of feces collected rectally from a donor animal monoparasitized with *H. contortus* (EPG > 3000) was macerated and filtered, under running water (37 °C), through a series of overlapping sieves (500, 150, 90, and 20 μm). The eggs retained in the last sieve were recovered with NaCl supersaturated solution and centrifuged for 5 min at 1810 × g for simple flotation, and then washed with distilled water in the 20-μm sieve. Aqueous suspensions containing approximately 100 eggs were distributed into the 24-well plates (TPP ref. no. 92024) containing the different treatments solubilized in distilled water plus Tween 80 (3%, v/v) in a final volume of 500 μL/well. The final concentrations were 50, 25, 12.5, 6.25, 3.125, 1.562, 0.781, 0.390, 0.195 and 0.097 mg/mL for the essential oil of *T. vulgaris*, and 25, 12.5, 6.25, 3.125, 1.562, 0.781, 0.390, 0.195, 0.097 and 0.048 mg/mL for thymol.

Thiabendazole (0.025 mg/mL; aqueous solution) and Tween 80 (3%, v/v) in distilled water were used as positive and negative controls, respectively. The results were assessed under an inverted microscope after 24 h of incubation at 27 °C by differential counting of eggs and L_1 larvae. The results are the average of three replicates and are expressed as the percentage of egg hatching inhibition of three independent experiments.

2.4.2. Larval motility test

The assay for the evaluation of motility of third stage (L_3) larvae was performed as described by Ferreira et al. (2013). Approximately 20 g of feces rectally collected from the donor animal was cultured according to Ueno and Gonçalves (1998). After 7 days under optimal conditions of incubation, L_3 larvae were recovered by spontaneous migration using warm water (37 °C).

Aliquots containing about 100 L_3 larvae were distributed into the 96-well plates (TPP ref. no. 92024) containing the different treatments (solubilized in 3% (v/v) Tween 80 in distilled water) in a final volume of 200 μL/well. The final concentrations of the *T. vulgaris* essential oil and thymol were the same as in the EHT. Levamisole (20 mg/mL; aqueous solution) and Tween 80 (3%, v/v) in distilled water were used as positive and negative controls, respectively. The results were assessed by light microscopy after 24 h of incubation at 27 °C. The number of motile and non-motile larvae was counted by observing the presence or absence, respectively, of smooth sinusoidal movement stimulated by light (Ferreira et al., 2013). The results are expressed as% inhibition of larval motility of three independent experiments, each performed in triplicate.