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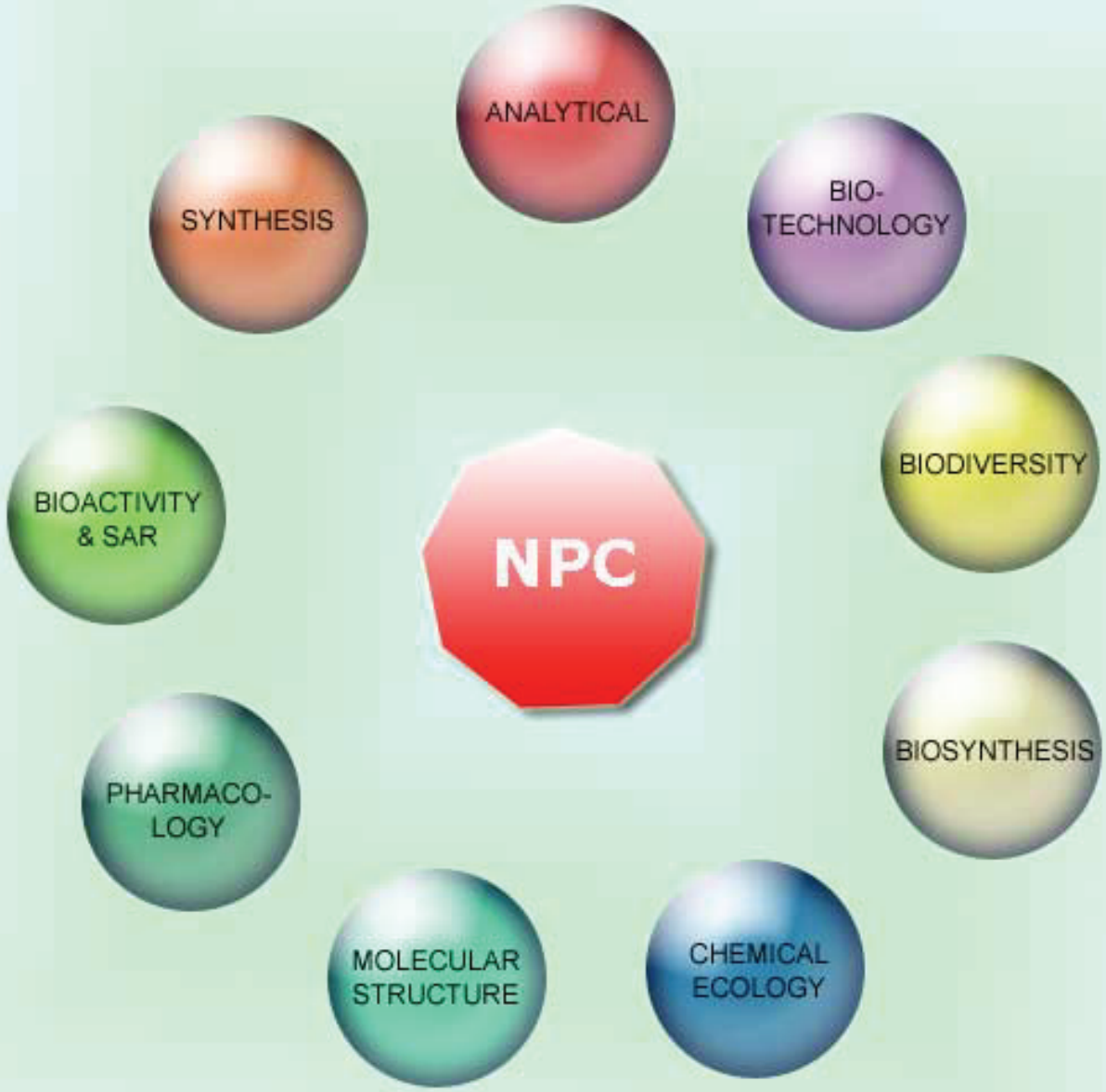

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In vitro Anthelmintic Activity of Two Aloe-derived Active Principles against Sheep Gastrointestinal Nematodes

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The *in vitro* anthelmintic activity on sheep gastrointestinal strongyle (GIS) eggs and larvae of 0.5% aloin and 0.1% aloe-emodin was investigated. From fresh faecal samples collected by ewes naturally infected by *Haemonchus*, *Trichostrongylus* and *Teladorsagia* nematodes, GIS eggs were isolated and cultivated in Petri dishes (100 eggs/dish). For the *in vitro* evaluation of the anthelmintic activity of tested compounds, the Egg hatch test (**EHT**), the Larval development test (**LDT**) and the Larval mortality/paralysis test (**LMT**) were used. In each assay, the activity of tested compounds was compared to untreated and treated (0.1% thiabendazole, TBZ) controls. Six repetitions were made through the experiment. Obtained data were statistically elaborated using the X^2 test. In **EHT**, 0.5% aloin gave highly significantly different ($P<0.01$) results from the untreated controls. In **LDT**, both 0.1% aloe-emodin and 0.5% aloin almost completely prevented the larval development from L_1 to L_3, showing no significant differences ($P<0.01$) when compared to TBZ. In **LMT**, larval mortality observed in 0.5% aloin treated plates was significantly higher ($P<0.01$) than that observed in TBZ treated controls. These results show the *in vitro* anthelmintic properties on sheep GIS of the examined plant secondary metabolites. In **LDT** and/or **LMT**, the activity of 0.5% aloin and 0.1% aloe-emodin was comparable to or higher than that of the reference drug.

Keywords: Aloin, Aloe-emodin, Anthelmintic activity, Gastrointestinal nematodes, Sheep.

Worldwide, disease caused by gastrointestinal strongyles (GIS) is considered one of the most important health constraints affecting productivity in small ruminants [1]. GIS may limit sheep production by causing retarded growth, weight loss, reduced food consumption, lower milk production, impaired fertility and even mortality in heavy parasite burden [2]. Sheep GIS control programs rely mostly on the use of synthetic anthelminthics [3]. However, the anthelmintic resistance developed by these nematodes worldwide, the risk of residues in sheep products and the cost of commercial anthelmintic drugs, have led to the need to find alternative treatments and methods for sheep nematode control [1, 4, 5]. In particular, there is a clear desire to create systems of sheep production that rely less heavily on chemotherapy [1]. Among alternative strategies, there has been considerable and expanding interest in the search for effective and safe herbal dewormers [2]. In fact, the use of plant-derived compounds with anthelmintic properties seems to be an effective alternative from the standpoint of parasite control and their low environmental impact from residues in relation to commercial anthelmintics. Herbal medicine can increase profits, by reducing the use of conventional anthelmintics and extending the useful life of the limited number of available anthelmintics [1, 6].

Aloin and aloe-emodin are two active principles mainly found in the leaves of several *Aloe* species [7, 8]. In ethnoveterinary studies, the antiparasite activity of *Aloe* species against some helminths of farm animals, including the anthelmintic efficacy of *Aloe rupestris* and *Aloe ferox* against the sheep GIS species *Haemonchus contortus*, has been shown [9-11]. Moreover, the antiprotozoal activity of aloin and aloe-emodin has been demonstrated [12-14].

In this context, this study was aimed to evaluate the *in vitro* anthelmintic activity on GIS of 0.5% aloin and 0.1% aloe-emodin. More specifically, the efficacy of tested compounds was evaluated on GIS eggs obtained by naturally infected ewes and on GIS larvae obtained from the cultivation of eggs. For the in vitro evaluation of the anthelmintic activity of tested compounds, the Egg hatch test (EHT), the Larval development test (LDT) and the Larval mortality/paralysis test (LMT) were used. Furthermore, GIS larvae were identified at the genus level. In each test, plates treated with tested compounds were compared to untreated plates (untreated controls) and plates treated with 0.1% thiabendazole (TBZ) (treated controls).

Identified GIS larvae were found to belong mostly to the genus *Haemonchus* and to a lesser extent to the genera *Trichostrongylus* and *Teladorsagia* (Table 1).

Table 1: Mean percentage of each gastrointestinal strongyle genus identified in positive faecal samples from a naturally infested ewe living in an organic sheep flock located in Tuscany (Italy).

Genus	Percentage
Haemonchus	93%
Trichostrongylus	4%
Teladorsagia	3%

Haemonchus contortus is the species of the genus *Haemonchus* infecting sheep and other livestock and it is considered one of the main parasites of small ruminants all over the world [15].

From the evaluation of the *in vitro* anthelmintic activity of tested compounds, in EHT aloin gave highly significantly different results ($P<0.01$) from the untreated controls (Table 2). In particular, in this test 0.5% aloin showed 39.7% inhibition of egg hatch, while only 13.4% of eggs were found un-hatched in control plates (Table 2). However, the inhibition of the egg hatch of 0.5% aloin and, mainly, 0.1% aloe-emodin (16.6%) was significantly lower than that observed in 0.1% TBZ treated plates (94.8%) ($P<0.01$) (Table 2).

In LDT, the two examined compounds showed significant differences ($P<0.01$) from the untreated controls (Table 2).

Table 2: *In vitro* inhibition (in %) of sheep gastrointestinal strongyle egg hatch (EHT), larval development (LDT) and larval motility/vitality (LMT) by 0.5% aloin and 0.1% aloe-emodin when compared to untreated and treated (0.1% thiabendazole) controls.

Compounds	EHT % Inhibition (Confidence Interval)	LDT % Inhibition (Confidence Interval)	LMT % Inhibition (Confidence Interval)
0.5% Aloin	39.7^{B} (27.6-51.8)	96.9^{A} (94.6-99.2)	65.4^{A} (59.3-71.5)
0.1% Aloe-emodin	16.6^{Cb} (12.1-21.0)	97.7^{A} (95.8-99.6)	-
Untreated Controls	13.4^{Cc} (8.8-17.9)	21.3^{C} (17.1-25.5)	4.2^{C} (1.5-6.9)
0.1% Thiabendazole	94.9^{A} (94.1-100)	100.00^{A} (100-100)	27.7^{B} (19.9-35.6)

A, B, C P<0.01; a, b, c P<0.05.

Moreover, in this test 0.5% aloin (96.9%) and 0.1% aloe-emodin (97.7%) almost completely prevented the larval development from L_1 to L_3, showing no significant differences (P<0.01) when compared to 0.1% TBZ (Table 2). In LMT, 0.5% aloin (65.4%) gave the best results, since larval mortality observed in such treated plates was found significantly higher (P<0.01) than in the untreated controls (4.2%) but also in 0.1% TBZ treated controls (27.7%) (Table 2).

The aim of the present study was to evaluate the *in vitro* anthelmintic potential efficacy of two aloe-derived active principles, aloin and aloe-emodin, in comparison to the reference drug thiabendazole against gastrointestinal strongyles of sheep. Results obtained in this study revealed that 0.5% aloin and 0.1% aloe-emodin effectively targeted the larval developmental stages of these parasites. Although both these compounds were not as effective as the reference drug in reducing the hatching of the eggs, their effects on GIS larvae resulted in significantly reduced larval development. Especially aloe-emodin showed the same efficacy of the reference drug when used at the same concentration (0.1%). Moreover, 0.5% aloin also caused increased paralysis and/or death of GIS larvae. Though aloin was tested at a higher concentration (0.5%) than the reference drug, the activity of this compound was higher in causing paralysis and/or death of the larvae. Results here obtained confirm the anthelmintic efficacy previously reported for some aloe-derived extracts, as the inhibition of *Haemonchus contortus* egg hatching observed for *A. rupestris* and *A. ferox* [9, 10]. The anthelmintic activity of these *Aloe* species has been related to their content in amino acids, saponins and sterols [9]. Results from this study show for the first time that aloin and aloe-emodin are endowed with *in vitro* anthelmintic properties against sheep gastrointestinal strongyles. Results are also indicative that the anthelmintic properties previously reported for several aloe species may possibly depend on their content in aloin and aloe-emodin. Therefore, it would be interesting to proceed with further studies on the effectiveness and toxicity of these natural active principles, especially with regard to the evaluation of their efficacy as de-wormers and as a mean to control the environmental development of gastrointestinal strongyles when administered to infected animals as dietary supplement.

Experimental

Plant Material: Two commercial products, aloin (Sigma-Aldrich S.r.l., Milan, Italy) and aloe-emodin (Sigma-Aldrich S.r.l., Milan, Italy), were used. Aloin was used at 0.5% and aloe-emodin was used at the concentration of 0.1% and was not tested in the Larval Mortality/ Paralysis Test. The needed concentrations of tested compounds were obtained by dilute them directly in the medium used for GIS cultivation.

Egg recovery, suspension and cultivation of eggs and larvae: Individual fresh faecal samples collected from naturally infested ewes living in an organic sheep flock located in Tuscany (Italy) found positive for about 2000 GIS nematode eggs per gram of faeces (EPG) after analysis with a McMaster method [16], were used in the study.

Recovery, suspension and cultivation of GIS eggs were performed by using previous reported methods [17, 18]. Briefly, for the egg concentration and purification about 10 g of selected faecal samples were mixed and homogenized with distilled water. The mixture was strained and centrifuged for 2 min at 2300 rpm. The sediment was collected, suspended in saturated NaCl solution and centrifuged for 2 min at 1000 rpm. The superficial layer of the supernatant was collected, diluted in distilled water and centrifuged at 800 rpm for 2 min to collect the sediment containing the eggs. GI nematode eggs were counted and a batch of about 100 eggs was cultivated in Petri dishes. Each dish (100 eggs/ dish) contained 3ml of a culture medium composed by 0.54 ml of saline solution, 0.06 ml of Earl Balanced Salt Solution (Sigma-Aldrich S.r.l., Milan, Italy), 12 µL of lyophilized *Escherichia coli* (*E. coli* strain W, lyophilized cells, Sigma-Aldrich S.r.l., Milan, Italy), suspended in water and sterilized for an hour at 100°C, 12 µg of amphotericin B (Amphotericin B from *Streptomyces* approx 80% HPLC, Sigma-Aldrich S.r.l., Milan, Italy), 60 mg of yeast extract (Sigma-Aldrich S.r.l., Milan, Italy) and about 2.4 ml of distilled water. Dishes were incubated in the dark at 22°C. The same culture medium and environmental conditions were used to cultivate larvae obtained by egg cultures, by placing about 100 larvae/dish.

In vitro evaluation of the anthelmintic activity of tested compounds: For the *in vitro* evaluation of the anthelmintic activity of tested compounds, the Egg hatch test (**EHT**), the Larval development test (**LDT**) and the Larval mortality/paralysis test (**LMT**), were used [19-21]. In EHT, culture plates containing about 100 GIS nematode eggs/plate were prepared. All plates were checked after 48 hours. Hatched first stage larvae (L1) and unhatched eggs were then counted under the microscope and the percentages of hatched eggs were calculated.

In LDT, live L1 larvae obtained from additional untreated GIS nematode egg cultures after 48 hours of cultivation were isolated, counted and about 100 L1/Petri dish were used. Plates were checked after **7** days in order to evaluate the development from first stage larvae (L_1) to third stage larvae (L_3). L_3 larvae were counted under the microscope after adding a drop of Lugol's iodine solution in each plate and the percentages of L3 on the total number of larvae of each plate were calculated.

In LMT, about 100 motile L3/plate, derived from additional untreated larval cultures, were used. After 24 hours, the percentage of L3 that was found motionless for at least 6 seconds at microscopical examination was calculated for each plate. In each test, plates treated with each tested compound were compared to untreated plates (untreated controls) and plates treated (treated controls) with 0.1% thiabendazole (TBZ) (2-(4-Thiazoly) Benzimidazole minimum 99%, Sigma S.r.l., Milan, Italy). Six repetitions were made through the experiment.

Identification of GI nematode genera: L_3 recovered from further additional plates were identified at the genus level based on their morphological and metric features [22] and the mean percentage of each identified genus on the total number of larvae was calculated.

Statistical Analysis: Data from EHT, LDT and LMT obtained from untreated and treated control cultures and from cultures treated with the tested compounds, were statistically elaborated with X^2 with $P<0.01$ or $P<0.05$ significance level [23]. The confidence intervals were also calculated.

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