Natural chemotherapeutic alternatives for controlling of haemonchosis in sheep

Dominika Mravčáková1, Zora Váradyová1, Anna Kopčáková1, Klaudia Čobanová1, Lubomíra Grešáková1, Svetlana Kišidayová1, Michal Babják2, Michaela Urda Dolinská2, Emília Dvorozňáková2, Alžbeta Konigová2, Jaroslav Vadlejch3, Adam Cieslak4, Sylwester Ślusarczyk5 and Marián Várady2*

Abstract

Background: Parallel in vitro and in vivo experiments were designed to evaluate promising chemotherapeutic alternatives for controlling haemonchosis in ruminants. In vitro anthelmintic activities (egg hatch test – EHT; larval development test – LDT) of aqueous and methanolic herbal extracts Mix1 and Mix2 were investigated. The in vivo effects of dietary supplementation with Mix1 and Mix2 on the parasitological status, inflammatory response, antioxidant parameters and microbial community of the lambs infected experimentally with Haemonchus contortus were investigated. Lambs were divided into four groups for the in vivo study: uninfected control lambs (C), infected lambs (I), infected lambs supplemented with Mix1 (I + Mix1) and infected lambs supplemented with Mix2 (I + Mix2). The experimental period was 70 days.

Results: The number of eggs per gram (EPG) of feces was quantified 22, 30, 37, 44, 51, 58, 65 and 70 days post-infection, and mean abomasal worm counts were assessed 70 days post-infection. Quantitative analyses identified 57.3 and 22.2 mg/g phenolic acids, 41.5 and 29.5 mg/g flavonoids and 1.4 and 1.33 mg/g protoberberine-type alkaloids in Mix1 and Mix2, respectively. The methanolic extracts of the herbal mixtures in both in vitro tests had higher anthelmintic effects \( P<0.01 \) than the aqueous extracts, but the effects did not differ significantly between Mix1 and Mix2 \( P>0.05 \). I + Mix1 and I + Mix2 lowered mean EPGs between 44 and 70 d by 58.1 and 51.6%, respectively. The level of IgG antibodies against H. contortus increased significantly after infection in each infected group.

Conclusion: These results represent the first monitoring of the in vitro anthelmintic effects of herbal mixtures on H. contortus. The in vivo experiment indicated that the anthelmintic effect was not sufficient for the elimination of parasites, but this herbal treatment may affect the host over a longer term, reducing the parasitic infection in the host.

Keywords: 16S rRNA gene, Total antioxidant capacity, Haemonchus contortus antigen, Phytochemicals, UHRMS

Background

Modern production systems require the use of nutraceuticals for optimal production and health in ruminant nutrition. Gastrointestinal nematode (GIN) infections are the prevalent parasitic diseases contributing to the morbidity and mortality of various livestock species worldwide. Infection by the GIN Haemonchus contortus is mainly controlled by chemophrophylaxis by the repeated application of anthelmintics, which has led to the increased risk of anthelmintic resistance and multi-drug resistance [1, 2]. Resistance to anthelmintics also applies to the novel anthelmintic monepantel [3, 4]. Herbs with important biological activities are potential nutraceuticals for controlling GINs in ruminants [5–7]. A variety of herbs from traditional medicine with health-promoting properties have been used to treat various diseases in both humans and animals for centuries [8]. Alternative control approaches can therefore involve combinations of traditional herbal medicines [9, 10], the pharmacologically active plant compounds [11, 12] and the self-medication of grazing animals [13, 14]. Polyphenols, especially tannins, flavonoids and phenylpropanoids,
mainly have anthelmintic [15, 16] and antioxidative and anti-inflammatory [17, 18] properties. Hoste et al. [19] have demonstrated that the manipulation of host nutrition provides useful options for controlling GINs as a component of an integrated multidisciplinary strategy. The ability of the host to resist GIN infections also depends on the development of a protective acquired immune response [20]. Interactions between intestinal parasites, microbial communities and immune systems play a relevant role in modulating each other and in the maintenance of homeostasis [21].

Our previous trials found that the dry medicinal herbs can be used as chemotherapeutic alternatives for controlling haemonchosis in ruminants based on their beneficial effect [22–24]. The bioactive phytochemicals responsible for anthelmintic, antioxidant and anti-inflammatory activities in various herbal mixtures have not yet been characterized in vitro. Our goal was therefore to examine (1) the in vitro anthelmintic activities of extracts from two dry mixtures in both mixtures and tests. The patterns of egg shedding for I, I + Mix1 and I + Mix2 are shown in Fig. 1. Data from D44 were statistically compared and used to determine the reduction in egg output for I + Mix1 and I + Mix2 relative to I. Mean fecal eggs per gram (EPGs) for all groups increased until D44. The EPGs in the lambs treated with Mix1 and Mix2 decreased from D44 until the end of the experiment. Eggs per gram for I similarly decreased, but only to D58 and then remained stable. The egg-output data indicated that mean EPG decreased between D44 and D70 for I + Mix1 and I + Mix2, by 58.1 and 51.6%, respectively. EPGs after D58 were always lower for I + Mix1 and I + Mix2 than I. Eggs per gram on D70 was significantly lower for I + Mix1 than I (P < 0.05). Mean body weights and live-weight gains did not differ significantly (P > 0.05) between the experimental groups. The necropsy on D70 found a significant decrease at abomasal worm count for I + Mix1 compared to I (Table 5).

**Results**

**Bioactive compounds**

The herbal compositions (Table 1), fermentation parameters (Table 2) and bioactive compounds (Tables 3, 4) of Mix1 and Mix2 are presented. Quantitative analyses of Mix1 identified 57.3 mg/g phenolic acids (Nos. 1–9, 19, 21, 26 and 28) and 41.5 mg/g flavonoids (Nos. 10–18, 20, 22–25, 27 and 29–33) (Table 3). Quantitative analyses of Mix2 identified 22.2 mg/g phenolic acids (Nos. 1–6, 20, 23, 26 and 29) and 29.5 mg/g flavonoids (Nos. 7–19, 21, 22, 24, 25, 27, 28 and 30) (Table 4). Protoberberine-type alkaloids were also identified in Mix1 (1.4 mg/g) and Mix2 (1.33 mg/g) based on comparisons with chelidonine and protopine standards.

**Table 1. Compositions of the herbal mixtures**

| Species Family          | Part used | % in Mix1 | Species Family          | Part used | % in Mix2 |
|-------------------------|-----------|-----------|-------------------------|-----------|-----------|
| *Artemisia absinthium* L. Asteraceae | Stem | 1.0 | *Artemisia absinthium* L. Asteraceae | Stem | 1.0 |
| *Matricaria chamomilla* L. Asteraceae | Flower | 13.4 | *Achillea millefolium* L. Asteraceae | Stem | 12.4 |
| *Foeniculum vulgare* Mill. Apiaceae | Seed | 5.0 | *Calendula officinalis* L. Asteraceae | Flower | 12.4 |
| *Fumaria officinalis* L. Papaveraceae | Stem | 13.4 | *Matricaria chamomilla* L. Asteraceae | Flower | 12.4 |
| *Hyssopus officinalis* L. Lamiaceae | Stem | 13.4 | *Chichorium intybus* L. Asteraceae | Stem | 12.4 |
| *Malva sylvestris* L. Malvaceae | Flower | 13.4 | *Fumaria officinalis* L. Papaveraceae | Flower | 12.4 |
| *Melissa officinalis* L. Lamiaceae | Stem | 13.4 | *Hypericum perforatum* L. Hypericaceae | Stem | 12.4 |
| *Plantago lanceolata* L. Plantaginaceae | Leaf | 13.4 | *Malva sylvestris* L. Malvaceae | Stem | 12.4 |
| *Solidago virgaurea* L. Asteraceae | Stem | 13.4 | *Urtica dioica* L. Urticaceae | Stem | 12.4 |

Medicinal herbs (AGROKARPATY, Plavnica, Slovak Republic and BYLINY Mikeš s.r.o., Čičenice, Czech Republic)
4.207 ng/mL and differed significantly for I vs. I + Mix2 by D51 and D70 ($P < 0.001$ and 0.01, respectively).

Serum total antioxidant capacity (TAC), superoxide dismutase activity (SOD) and activity of blood glutathione peroxidase (GPx) (Table 7) were influenced by treatment ($P < 0.001$), and SOD and GPx activities were influenced by time ($P < 0.05$ and 0.001, respectively).

Serum Zn concentration was influenced by treatment ($P < 0.05$) and time ($P < 0.001$) (Table 8).

Serum Fe concentration was influenced by treatment ($P < 0.001$), time ($P < 0.001$) and the treatment × time interaction ($P < 0.05$). Serum Cu concentration was influenced by treatment ($P < 0.05$) and time ($P < 0.001$) (Table 8).

Serum Fe concentration was influenced by treatment ($P < 0.001$), time ($P < 0.001$) and the treatment × time interaction ($P < 0.05$). Serum Zn concentration was influenced by treatment ($P < 0.05$) and time ($P < 0.001$) (Table 8).

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Effect on ovine feces microbial community

Denaturing gradient gel electrophoresis (DGGE) was used to analyze changes in the fecal microbial communities of the infected lambs induced by supplementation with Mix1 and Mix2 (Fig. 2). A comparison of the DGGE banding patterns indicated that the communities were only weakly affected by supplementation with Mix1 and Mix2. Most of the samples had similar banding patterns, with similarity coefficients ranging from 0.20 to 0.58. The cluster analyses (UPGMA) did not identify any herbal-mixture or animal-dependent clustering. None of the herbal treatments significantly affected the eubacterial microflora of the lambs infected with *H. contortus*.

Discussion

The use of medicinal herbs containing bioactive compounds with important biological activities for preventing and treating GIN infections has its origin in traditional herbal medicine. Phenolic acids and flavonoids were the main bioactive compounds identified in both Mix1 and Mix2, but Mix1 contained more of these compounds than Mix2. Phenolic acids and flavonoids mitigate diseases associated with oxidative stress [25] and had excellent antioxidant activity in both our in vitro and in vivo experiments. Their anthelmintic activities also provide a potential option for treating nematode infections [26]. The phenolic acids 1,5-dicaffeoylquinic and 3-O-caffeoylquinic identified in both Mix1 (15.37 and 11.31 mg/g, respectively) and Mix2 (6.18 and 6.91 mg/g, respectively) possess antibacterial and anthelmintic activities [27]. Rosmarinic acid has well-known anti-inflammatory and antioxidant biological activities with beneficial health-promoting effects [28]. Its contents in Mix1 was 5.95 mg/g, two-fold higher than in a previously studied herbal mixture [23]. The presence of flavonol glycosides such as kaempferol, quercetin and myricetin contributes to the anthelmintic property of most browsed herbal species [29]. The herbal species in our study contained kaempferol (0.3–9.0 mg/g) and quercetin (0.5–7.7 mg/g), but the amount of myricetin was approximately three-fold higher in Mix1 than in another study [29]. Tannins and flavonoid glycosides may have similar mechanisms of action, because their chemical structures are similar [30]. All three groups of bioactive compounds also have antioxidant and anti-inflammatory properties [31, 32]. Another flavonoid with antioxidant activity, rutin, was also present in both Mix1 (2.96 mg/g) and Mix2 (5.73 mg/g), within the range of 2.3–10 mg/g reported for other herbal extract [33]. The flavonoid luteolin, with antioxidant and anti-inflammatory properties [34], was
the most abundant compound in both Mix1 (Luteolin O-diglucuronide - 0.26 mg/g, Table 3) and Mix2 (Luteolin-O-Hex-O-Dhex - 1.43 mg/g, Table 4). Luteolin and quercetin can inhibit the larval development [35, 36]. The ultra-high-resolution mass spectrometry (UHRMS) analysis of the bioactive compounds in Mix1 and Mix2 also identified protoberberine-type alkaloids. Alkaloids in herbal species are commonly poisonous [37], but protoberberine alkaloids have a large variety of biological and pharmacological [38] and anthelmintic [39, 40] activities. The anthelmintic activity of herbs has sometimes been ascribed to alkaloids, which can interfere with DNA synthesis in parasites [41]. Several examples indicated protoberberine type alkaloids as valuable substances with anti-parasitic activities. Among the alkaloids tested to find new anthelmintics against parasites living in host tissues, allocryptopine (protoberberine type alkaloid) showed significant nematocidal activity against the larvae of dog roundworm, Toxocara canis with low cytotoxicity, and was proposed as a potentially effective anthelmintic [42]. Fumaria indica closely related to Fumaria officinalis (presented in both Mix1 and Mix2) is used as an anthelmintic in the traditional veterinary practices in Pakistan. The main alkaloids identified in this species responsible for

| No. | Compound | RT (min) | UV | m/z [M-H]− | Formula | MS2 main ion | MS2 fragments | mg/g DM |
|-----|----------|----------|----|------------|---------|-------------|--------------|--------|
| 1   | 4-O-Caffeoylquinic acid | 3.8      | 215/325 | 353.0880 C16H18O9 | 191.0553 | 0.71        |
| 2   | 3-O-Caffeoylquinic acid | 5.2      | 215/325 | 353.0880 C16H18O9 | 191.0556 | 11.3        |
| 3   | 5-O-Caffeoylquinic acid | 5.5      | 215/325 | 353.0880 C16H18O9 | 191.0557 | 0.73        |
| 4   | Caffeic acid | 6.9      | 215/324 | 179.0349 C6H4O4 | 135.0435 | 0.81        |
| 5   | 1-O-Feruloylglucose | 8.0      | 215/324 | 355.1041 C16H20O9 | 191.0187 | 3.45        |
| 6   | 3-O-p-Coumaroylquinic acid | 8.7 | 215/325 | 337.0933 C16H18O8 | 191.0187 | 0.65        |
| 7   | Quercetin O-Pen-Hex | 12.3     | 271/345 | 479.0828 C21H20O13 | 317.0297 | 20.2        |
| 8   | Quercetin O-Hex-Hex-Dhex | 12.4 | 252/351 | 609.1442 C27H30O16 | 300.0273 | 2.63        |
| 9   | Rutin | 13.5     | 255/340 | 609.1451 C27H30O16 | 191.0187 | 6.40        |
| 10  | Myricetin 3-O-galactosid | 12.2     | 271/345 | 479.0828 C21H20O13 | 317.0297 | 20.2        |
| 11  | Quercetin O-Pen-Hex | 12.3     | 252/351 | 595.1298 C21H22O12 | 173/191/135 | 149/161/135  | 6.40        |
| 12  | Quercetin O-Hex-O-Dhex | 12.4 | 252/351 | 667.1029 C27H30O16 | 300.0273 | 2.63        |
| 13  | Quercetin O-Hex-Dhex | 12.5     | 255/340 | 637.1049 C27H30O16 | 461.0707 | 285/04      | 1.42        |
| 14  | Quercetin O-Hex-Dhex | 12.5     | 255/340 | 637.1029 C27H30O16 | 461.0707 | 285/04      | 1.42        |
| 15  | Quercetin O-Hex-Dhex | 12.5     | 255/340 | 637.1029 C27H30O16 | 461.0707 | 285/04      | 1.42        |
| 16  | Quercetin O-Hex-Dhex | 12.5     | 255/340 | 637.1029 C27H30O16 | 461.0707 | 285/04      | 1.42        |
| 17  | Quercetin O-Hex-Dhex | 12.5     | 255/340 | 637.1029 C27H30O16 | 461.0707 | 285/04      | 1.42        |
| 18  | Quercetin O-Hex-Dhex | 12.5     | 255/340 | 637.1029 C27H30O16 | 461.0707 | 285/04      | 1.42        |
| 19  | Quercetin O-Hex-Dhex | 12.5     | 255/340 | 637.1029 C27H30O16 | 461.0707 | 285/04      | 1.42        |
| 20  | Quercetin O-Hex-Dhex | 12.5     | 255/340 | 637.1029 C27H30O16 | 461.0707 | 285/04      | 1.42        |
| 21  | Quercetin O-Hex-Dhex | 12.5     | 255/340 | 637.1029 C27H30O16 | 461.0707 | 285/04      | 1.42        |
| 22  | Quercetin O-Hex-Dhex | 12.5     | 255/340 | 637.1029 C27H30O16 | 461.0707 | 285/04      | 1.42        |
| 23  | Quercetin O-Hex-Dhex | 12.5     | 255/340 | 637.1029 C27H30O16 | 461.0707 | 285/04      | 1.42        |
| 24  | Quercetin O-Hex-Dhex | 12.5     | 255/340 | 637.1029 C27H30O16 | 461.0707 | 285/04      | 1.42        |
| 25  | Quercetin O-Hex-Dhex | 12.5     | 255/340 | 637.1029 C27H30O16 | 461.0707 | 285/04      | 1.42        |
| 26  | Quercetin O-Hex-Dhex | 12.5     | 255/340 | 637.1029 C27H30O16 | 461.0707 | 285/04      | 1.42        |
| 27  | Quercetin O-Hex-Dhex | 12.5     | 255/340 | 637.1029 C27H30O16 | 461.0707 | 285/04      | 1.42        |
| 28  | Quercetin O-Hex-Dhex | 12.5     | 255/340 | 637.1029 C27H30O16 | 461.0707 | 285/04      | 1.42        |
| 29  | Quercetin O-Hex-Dhex | 12.5     | 255/340 | 637.1029 C27H30O16 | 461.0707 | 285/04      | 1.42        |
| 30  | Quercetin O-Hex-Dhex | 12.5     | 255/340 | 637.1029 C27H30O16 | 461.0707 | 285/04      | 1.42        |
| 31  | Quercetin O-Hex-Dhex | 12.5     | 255/340 | 637.1029 C27H30O16 | 461.0707 | 285/04      | 1.42        |
| 32  | Quercetin O-Hex-Dhex | 12.5     | 255/340 | 637.1029 C27H30O16 | 461.0707 | 285/04      | 1.42        |
| 33  | Quercetin O-Hex-Dhex | 12.5     | 255/340 | 637.1029 C27H30O16 | 461.0707 | 285/04      | 1.42        |

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those activities are protopine, fumarizine, papraine, papracine as well as flavonoids, glycosides, tannins and saponins [43]. In both herb mixtures was similar content of protoberberine-type alkaloids. Therefore it seems that better in vivo anthelmintic potential of Mix1 was probably mediated by content of phenolic acids and flavonoids. This is consistent with recent results reported limited anthelmintic potential of some alkaloids [44]. However, potent alkaloids probably act as antagonist of parasitic cholinergic receptors and lead to the development of novel drugs or may be used in combination with current anthelmintics to improve their efficacy [44].

The use of medicinal herbs containing bioactive compounds as an alternative treatment to chemical drugs is one approach that could reduce the development of resistance to antiparasitic drugs. One of our objectives was therefore to evaluate and compare the in vitro ovicidal and larvicidal efficacies of the aqueous and methanolic extracts of two herb mixtures against H. contortus. We previously evaluated the in vitro anthelmintic effects of aqueous and methanolic extracts from 13 species of medicinal herbs [45]. The results indicated that most herbs had a minimal effect on the hatching and larval development of H. contortus at the highest concentration used in the EHT and LDT. However, herbal extracts of four herbs (i.e., Artemisia absinthium, Matricaria chamomilla, Fumaria officinalis and Malva sylvestris) had strong anthelmintic activities in vitro [45]. We also previously showed that the wider spectrum of aqueous extracts from herbs (i.e., Althaea officinalis, A.

Table 4 Contents of the main bioactive compounds identified in the Mix2 analyzed in negative-ionization mode

| No. | Compound                        | RT (min) | UV | m/z [M-H] | Formula          | MS² main ion | MS² fragments | mg/g DM |
|-----|---------------------------------|----------|----|-----------|------------------|--------------|---------------|---------|
| 1   | 3-O-Caffeoylquinic acid         | 4.7      | 215/325 | 353.0880  | C₁₀H₁₂O₉         | 191.0553     | 179/151/135  | 6.91    |
| 2   | 2-O-Caffeoylhydroxycitric acid  | 5.2      | 215/324 | 369.0464  | C₁₀H₁₄O₁₀        | 189.0039     | 127/207/179  | 0.78    |
| 3   | 1-O-Feruloylgucose              | 5.7      | 215/324 | 355.1046  | C₁₀H₁₂O₉         | 193.0498     | 149/0589/134 | 0.50    |
| 4   | Caffeic acid                    | 6.1      | 179.0342 | C₆H₈O₇    | 135.0436         |              |              | 1.09    |
| 5   | Caffeoylmalic acid              | 6.2      | 250/324 | 295.046   | C₁₀H₁₄O₁₀        | 179.0036     | 133/207      | 1.27    |
| 6   | 2-O-Feruloylhydroxycitric acid  | 8.2      | 215/324 | 383.0623  | C₁₀H₁₄O₁₁        | 189.0039     | 191/337/127  | 3.64    |
| 7   | N-Malonyl-D-phenylalanine       | 8.3      | 250.0723 | C₁₂H₁₃NO₅ | 165.055          | 207          |              | 1.88    |
| 8   | Astilbin                        | 9.2      | 449.1089 | C₁₁H₁₂O₁₁ | 287.0561         | 259/243      |              | 1.07    |
| 9   | Nigellicine                     | 9.4      | 245.0934 | C₁₁H₁₄N₂O₅ | 203.0829        |              |              | 0.69    |
| 10  | Quercetin O-Pen-Hex             | 10.1     | 255/352 | 595.1298  | C₁₅H₁₄O₁₆       | 300.0273     |              | 0.48    |
| 11  | Isoquercitrin O-Dhex            | 10.2     | 252/351 | 609.1459  | C₁₅H₁₄O₁₆       | 300.0279     |              | 0.54    |
| 12  | Quercetin O-Hex                 | 10.5     | 252/351 | 463.0874  | C₁₅H₁₂O₁₁       | 301.0366     | 141/151      | 2.25    |
| 13  | Typhaneoside                    | 10.8     | 255/354 | 769.2207  | C₁₅H₁₂O₁₀       | 314.0436     | 595          | 2.71    |
| 14  | Rutin                           | 11.0     | 255/352 | 609.1451  | C₁₅H₁₂O₁₆       | 300.0275     |              | 5.73    |
| 15  | 6-Hydroxykaempferol 7-glucoside isoquercitin | 11.5     | 264/341 | 463.089   | C₁₁H₁₂O₁₂       | 300.0284     |              | 1.64    |
| 16  | Quercetin-O-glucuronide         | 12.2     | 255/352 | 477.0681  | C₁₁H₁₂O₁₃       | 301.0359     |              | 0.85    |
| 17  | Kaempferol O-Hex                | 12.3     | 265/343 | 447.0937  | C₁₁H₁₂O₁₁       | 285.0407     |              | 1.0     |
| 18  | Sorhamnetin 3-O-Dhex-Hex        | 12.9     | 623.1631 | C₂₀H₁₄O₁₆ | 314.0436         | 461/161      |              | 0.25    |
| 19  | Patuletin O-Hex                 | 13.0     | 256/351 | 493.0999  | C₁₅H₁₂O₁₃       | 331.0469     | 447/285/151  | 0.41    |
| 20  | 3,5-Dicaffeoylquinic acid       | 13.2     | 215/325 | 515.1208  | C₁₅H₁₂O₁₂       | 353.0885     | 173/179/191  | 0.92    |
| 21  | Luteolin-O-Hex-Dhex             | 13.9     | 265/343 | 593.1518  | C₁₅H₁₂O₁₅       | 285.0409     |              | 1.43    |
| 22  | Calendoflavoside                | 14.1     | 351/000 | 623.1627  | C₁₅H₁₂O₁₆       | 315.0515     | 269          | 0.71    |
| 23  | 3,4-Dicaffeoylquinic acid       | 14.5     | 215/326 | 515.1208  | C₁₅H₁₂O₁₂       | 353.0885     | 173/179/191  | 0.44    |
| 24  | Apigenin O-Hex                  | 14.7     | 266/339 | 431.0994  | C₁₁H₁₂O₁₀       | 268.0382     |              | 2.82    |
| 25  | Isohamnetin-O-Hex               | 14.9     | 267/338 | 447.0298  | C₁₁H₁₂O₁₁       | 284.0332     | 300/0279/327/255 | 1.11    |
| 26  | 1,5-Dicaffeoylquinic acid       | 15.2     | 214/325 | 515.1206  | C₁₅H₁₂O₁₂       | 353.0885     | 173/179/191  | 6.18    |
| 27  | Isohamnetin O-(Hex-Ac)          | 15.3     | 260/000 | 519.1141  | C₁₅H₁₂O₁₃       | 314.0436     | 331/299      | 1.75    |
| 28  | Quercetin O-(Hex-Ac)            | 15.4     | 255/355 | 505.0963  | C₁₅H₁₂O₁₁       | 300.0275     | 271          | 0.25    |
| 29  | 3-Caffeoyl-4-dihydrocaffeoyl quinic acid | 15.9     | 215/325 | 517.1358  | C₁₁H₁₂O₁₂       | 323.0779     | 193/161/179/149/221 | 0.49    |
| 30  | Apigenin O-(Hex-Ac)             | 16.5     | 265/325 | 473.1101  | C₁₁H₁₂O₁₁       | 268.0385     |              | 1.94    |
absinthium, M. chamomilla, F. officinalis and M. sylvestris) exhibited stronger ovicidal activity and larvicidal activity in comparison to methanolic extracts of herbs; however methanolic extract of *A. absinthium* was the most efficient in both EHT and LDT [45]. These results suggest that herbal mixtures should be enriched with herbs with the highest anthelmintic potencies. Previous in vitro studies [46, 47] have only addressed the effects of individual herbal extracts on *H. contortus*. Our study is the first to monitor the ovicidal and larvicidal effects of herbal mixtures. The relatively high values (i.e., weak effects of extracts) in our in vitro tests suggested that the herbal mixtures could have an indirect antiparasitic effect in vivo and may promote the resistance of the host to parasitic infection only in the longer term. The effects were significantly stronger for the methanolic than the aqueous extracts in both the in vitro tests, suggesting a higher potency of the methanolic extracts, in accordance with some previous studies [48, 49] when aqueous and methanolic extracts did not produce the same anthelmintic response.

The egg outputs of our three parasitized groups (I, I + Mix1 and I + Mix2) indicated that egg reduction was largest in the I + Mix1 lambs. Egg production by *H. contortus* females has been reported to remain high to D50 post-inoculation and then to decrease [50]. We found the same pattern, where mean EPGs peaked from D44 to D51. The rapid reduction in egg excretion in the treated groups after D44, however, may have been due to the beneficial effects of the herbal treatments, supported by the dissections where the number of adult *H. contortus* worms was significantly lower for I + Mix1. The chemical composition of both herbal mixtures had optimal nutritional value and digestibility. Some herbs in

| Table 5 | In vitro and in vivo anthelmintic activity against *Haemonchus contortus* |
|---------|-------------------------------------------------|
| Part A: In vitro | Concentration (µg/mL) |
| Egg hatch test ED₅₀ ± SD | Methanolic extract | Aqueous extract |
| Mix1 | 539.2 ± 174.4 | 940.0 ± 73.0** |
| Mix2 | 486.9 ± 228.9 | 880.0 ± 67.8** |
| Larval development test LD₅₀ ± SD | | |
| Mix1 | 266.6 ± 107.2 | 922.7 ± 232.1** |
| Mix2 | 826.4 ± 370.5 | 1256.5 ± 465.0** |
| Part B: In vivo | Numbers of adult worms |
| Mean number of adult worms ± SD | Infected animals | Infected animals with Mix1 | Infected animals with Mix2 |
| | | | |
| | 1523 ± 187 | 1113 ± 274* | 1327 ± 351 |

In vitro activity: data are means ± SDs of three independent assays with duplicate samples. Ovicidal and larvicidal activities are expressed as the concentration (µg/mL) of a median lethal dose (ED₅₀ and LD₅₀, respectively; the concentration of a methanolic or aqueous extract that prevents 50% of the eggs from hatching or larvae from developing to the infective L3 stage) using the egg hatch test or larval development test.

In vivo activity: *Reduction of adult worms relative to infected animals 70 days after infection (P < 0.05)*

**Means within a row are significantly different at P < 0.01**

![Fig. 1](image-url) Mean fecal egg counts for the groups of lambs infected with *Haemonchus contortus*
Mix2 have yet to be evaluated for their anthelmintic properties under experimental conditions. Our previous studies, however, found no adverse effects on the patterns of ruminal fermentation when the diet was substituted up to 10% by a mix of some of the medicinal herbs used in Mix1 and Mix2 [22, 51]. Mix1 and Mix2 did not significantly affect the body weights or live-weight gains of the infected lambs, in contrast to the previous results [22]. Meta-analysis reported significant negative effect of parasitism on production in 58.3% of the trials [52]. The level of IgG antibodies against *H. contortus* increased significantly after infection in each infected group. This activity was apparent throughout the experiment. The necropsy confirmed the significant reduction in worm burden in the group fed Mix1, which was probably due to an immune response in the infected lambs. Associations of serum IgG levels with GIN infection and resistance against GIN infection in sheep have been reported [53, 54]. The ability to resist GIN infection, however, depends on the development of a protective acquired immune response, although the level of immunity depends on age, nutritional status and host genotype [20]. Calprotectin is a non-specific serum marker of neutrophil activation that directly correlates with chemical mediators of intestinal inflammation and with macroscopic and microscopic signs of disease [55]. The serum levels of calprotectin in our study were influenced by the herbal treatments. The response of serum calprotectin, however, was inconsistent with previous results [22, 23]. Some infectious and inflammatory diseases increase the serum levels of calprotectin [56], but calprotectin is mainly an early biomarker of bacterial infections [57].

Infected lambs generally have a reduced ability to absorb nutrients from the gastrointestinal tract, leading to morbidity and occasional mortality. Our results

| Table 6 | Inflammatory responses of the experimental lambs |
|---------|-----------------------------------------------|
| Parameter | Day | C | I | I + Mix1 | I + Mix2 | SD | Significance of effect |
|-----------|-----|---|---|--------|--------|----|------------------------|
| IgG (mg/mL) | 22 | 0.073 | 0.321 | 0.544 | 0.525 | 0.0713 | ** | ** |
| | 37 | 0.074 | 0.352 | 0.425 | 0.482 | 0.0858 | NS | NS |
| | 51 | 0.115 | 0.506 | 0.314 | 0.418 | 0.0931 | ** | NS |
| | 70 | 0.091 | 0.484 | 0.244 | 0.351 | 0.0566 | ** | NS |
| Calpro (ng/mL) | 22 | 5.290 | 4.820 | 4.860 | 4.207 | 2.2650 | ** | NS | ** |
| | 37 | 3.505 | 1.900 | 2.997 | 2.777 | 0.9263 | NS | NS |
| | 51 | 5.050 | 4.178 | 4.642 | 1.833 | 0.9642 | NS | ** |
| | 70 | 3.665 | 4.877 | 5.882 | 2.178 | 1.3114 | NS | ** |

C control uninfected animals, I infected animals, I + Mix1 infected animals with Mix1, I + Mix2 infected animals with Mix2, Calpro calprotectin, NS not significant

| Table 7 | Antioxidant status in the experimental lambs |
|---------|-------------------------------------------|
| Parameter | Day | C | I | I + Mix1 | I + Mix2 | SD | Significance of effect |
|-----------|-----|---|---|--------|--------|----|------------------------|
| TAC (mmol/L) | 22 | 0.552 | 0.537 | 0.538 | 0.543 | 0.055 | *** | NS | NS | NS | NS |
| | 37 | 0.593 | 0.508 | 0.562 | 0.568 | 0.060 | NS | NS | NS |
| | 51 | 0.667 | 0.502 | 0.517 | 0.535 | 0.102 | NS | NS | NS |
| | 70 | 0.560 | 0.458 | 0.550 | 0.548 | 0.075 | NS | NS | NS |
| SOD (U/g Hb) | 22 | 2.202 | 3.139 | 2.705 | 3.048 | 73.19 | *** | NS | NS | * | NS |
| | 37 | 2.566 | 3.572 | 3.510 | 3.768 | 1271 | NS | NS | NS |
| | 51 | 2.288 | 3.592 | 3.510 | 4.206 | 1002 | NS | NS | NS |
| | 70 | 2.865 | 3.859 | 3.895 | 3.764 | 974.4 | NS | NS | NS |
| GPx (U/mL) | 22 | 0.114 | 0.109 | 0.108 | 0.101 | 0.038 | *** | NS | NS | *** | NS |
| | 37 | 0.101 | 0.052 | 0.064 | 0.055 | 0.030 | NS | NS | NS |
| | 51 | 0.091 | 0.075 | 0.051 | 0.053 | 0.022 | NS | NS | NS |
| | 70 | 0.092 | 0.076 | 0.044 | 0.055 | 0.030 | NS | NS | NS |

TAC total antioxidant capacity of serum, SOD superoxide dismutase activity in erythrocytes, GPx glutathione peroxidase activity in serum, C control uninfected animals, I infected animals, I + Mix1 infected animals with Mix1, I + Mix2 infected animals with Mix2, NS not significant

* P < 0.05, *** P < 0.001
indicated that nematode infection in lambs was associated with oxidative stress and affected the antioxidant status of the animals. Both herbal mixtures containing bioactive compounds had strong antioxidant properties but did not have a beneficial effect on the serum antioxidant parameters in the infected lambs. The antioxidant potential of the mixtures or their bioactive compounds may have been influenced by several factors in vivo, including gut absorption, metabolism, bioavailability and the presence or absence of co-antioxidants and ions of transition metals [25]. The significantly lower serum Zn and Fe levels of the infected lambs relative to the control suggest a disturbance in their metabolism caused by *H. contortus*. Both mixtures had high iron contents, so differences between unsupplemented infected lambs and infected lambs supplemented with a herbal mixture

Table 8 Mineral status in the sera of the experimental lambs

| Element | Day | C      | I      | I + Mix1 | I + Mix2 | SD | Significance of effect |
|---------|-----|--------|--------|---------|---------|    | Treatment              |
|         |     |        |        |         |         |    | I vs. I + Mix1 | I vs. I + Mix2 | Time | Treatment × time |
| Zn (mg/L) | 22  | 0.476  | 0.462  | 0.567   | 0.591   | 0.07| *                | NS             | NS   | NS               |
|         | 37  | 0.548  | 0.477  | 0.512   | 0.519   | 0.03| NS             | NS             | NS   | NS               |
|         | 51  | 0.590  | 0.512  | 0.542   | 0.582   | 0.04| NS             | NS             | NS   | NS               |
|         | 70  | 0.687  | 0.590  | 0.548   | 0.682   | 0.07| NS             | NS             | NS   | NS               |
| Fe (mg/L) | 22  | 1.317  | 0.510  | 1.089   | 0.789   | 0.35| ***           | *              | NS   | ***             |
|         | 37  | 1.575  | 0.724  | 0.975   | 0.935   | 0.37| NS             | NS             | NS   | NS               |
|         | 51  | 2.120  | 0.998  | 1.180   | 1.536   | 0.49| NS             | NS             | NS   | NS               |
|         | 70  | 2.188  | 0.618  | 1.665   | 1.585   | 0.66| ***           | ***            | NS   | ***             |
| Cu (mg/L) | 22  | 0.774  | 0.651  | 0.834   | 0.841   | 0.09| NS             | **            | NS   | ***             |
|         | 37  | 0.773  | 0.696  | 0.724   | 0.732   | 0.03| NS             | NS             | NS   | NS               |
|         | 51  | 0.817  | 0.730  | 0.810   | 0.795   | 0.04| NS             | NS             | NS   | NS               |
|         | 70  | 0.835  | 0.876  | 0.810   | 0.873   | 0.03| NS             | NS             | NS   | NS               |

C control uninfected animals, I infected animals, I + Mix1 infected animals with Mix1, I + Mix2 infected animals with Mix2, NS not significant

* P < 0.05, ** P < 0.01, *** P < 0.001

Fig. 2 Unweighted pair group method with arithmetic mean algorithms (UPGMA) analysis of the DGGE profiles of the microbial communities from fecal samples of the lambs infected with *Haemonchus contortus* collected on days 0, 22, 37, 51 and 70 post-infection and treated with Mix1 and Mix 2.
could influence the serum Fe status during treatment [58]. Some medicinal herbs from central Europe containing essential microelements have been reported to possess antioxidant capacity which is correlated with the content of total phenolic compounds [59].

Adding the herbal supplement to the diets of infected lambs did not affect the composition of the fecal eubacterial communities in a substrate-specific manner. *Haemonchus contortus* manipulates the ovine gastrointestinal microbiome, modifying the balance between host and gastric microbiota [60]. Denaturing gradient gel electrophoresis can evaluate just the most dominant members of the bacterial community accounting for at least 3% of total population. We can assume that the main constituents of fecal microbial community were not influenced by the treatments. The diversity of the gastrointestinal microbiome is also extremely high, and the microbiome of adult *H. contortus* worms and L3 larvae differ between abomasa and feces [61]. Finding supplements that influence parasites, bacteria and the host is therefore important for the long-term maintenance of sheep health.

**Conclusion**

The Mix1 had better in vivo anthelmintic potential than Mix2, probably due to its higher contents of phenolic acids and flavonoids. The in vitro results demonstrated an anthelmintic effect of the mixtures on *H. contortus*, but the in vivo experiments indicated that this effect was not sufficient for the primary elimination of parasites. Mix1, however, may affect the host over the longer term, leading to a reduction in parasitic infection intensity in the host. Multidisciplinary approach to control infection is important and provides a more complex view without overestimating partial results.

**Methods**

**Experimental design, nutrition and animal management**

Animal use and experimental design were approved by the Ethics Committee of the Institute of Parasitology of the Slovak Academy of Sciences in accordance with the national legislation in Slovakia, Animal Welfare Act (No. 23/2009). Permission to collect samples and to carry out the experiment was granted by the participating sheep farmers. Twenty-four female lambs (Improved Valachian) 3–4 months of age with initial body weights of 11.7 ± 1.23 kg were housed in common stalls for 15 d to acclimatize to the feeding treatments, with free access to water. The lambs were obtained from a commercial farm (Agricultural farm, Kluknava, Slovakia) and were maintained in their productive system during the experiment. All parasite-free lambs were then randomly divided on their live-weight into four groups (six lambs/group, one stall/group): uninfected control animals (C), animals infected with *H. contortus* (I), infected animals supplemented with herbal mixture 1 (I + Mix1) and infected animals supplemented with herbal mixture 2 (I + Mix2). The number of animals used in the experiment was assigned following VICH GL13 guidelines proposed by European Medicines Agency. Lambs were infected orally with approximately 5000 third-stage (L3) larvae of the MHco1 strain of *H. contortus*, which is susceptible to all main classes of anthelmintics. The experimental period was 70 d (during summer), and the animals were housed on a sheep farm. Lambs were fed the Mix1 and Mix2 (100 g DM/d/animal) during the experimental period.

**Parasitological techniques**

Aqueous and methanolic extracts were prepared from both mixtures for EHTs and LDTs as was previously described [45]. Ovicidal activity was expressed as the concentration (μg/mL) of median effective dose (ED$_{50}$, the concentration of a methanolic or aqueous extract that prevented 50% of the eggs from hatching). Larvicidal activity was expressed as the concentration (μg/mL) of median lethal dose (LD$_{50}$, the concentration of a methanolic or aqueous extract that prevented 50% of the larvae from developing to the infective L3 stage).

Fecal samples from lambs were collected on D0, D22, D30, D37, D44, D51, D58, D65 and D70 post-infection and stored at 5 °C until laboratory examination. The detection of strongylid eggs was performed as was previously described [62]. All animals were humanely killed on D70 (abattoir of the Centre of Biosciences of SAS, Institute of Animal Physiology, Košice, Slovakia, No. SK U 06018), and helminthological dissections were performed [23]. The carcasses of animals were sent at the Department of Pathological Anatomy and Pathological Physiology, University of Veterinary Medicine and Pharmacy in Košice in Slovak Republic.

**Chemical analysis and fermentation parameters of the dietary substrates**

The dietary substrates were analyzed in triplicate by standard procedures [63, 64]. The chemical composition of the Mix1 and Mix2, respectively, was (mean values, g/ kg DM): DM: 898 (895); NDF: 500 (460); ADF: 360 (350); CP: 160 (180); N: 26 (29); ash: 110 (110); IV/DM: 600 (670); (mg/kg DM): Zn: 32.4 (36.7); Fe: 380 (396); Cu: 11.3 (13.8). IV/DM and in vitro measurements (Table 2) followed the procedures [22]. Ciliated protists in the fermentation ruminal fluid were counted microscopically and identified [65].

**Analysis of bioactive compounds**

Phenolic acids and flavonoids in the samples of Mix1 and Mix2 were analysed as was previously described.
separated using CHCl₃. The organic layer was collected, combined, and adjusted to pH 9. The procedure was then repeated, and the filtrates were evaporated to dryness under reduced pressure and then dissolved in 80% MeOH for further analysis. The bioactive compounds were analyzed by UHRMS on a Dionex UltraMate 3000RS system (Thermo Scientific, Darmstadt, Germany) with a charged aerosol detector connected to a high-resolution quadrupole time-of-flight mass spectrometer (Compact, Bruker Daltonik GmbH, Bremen, Germany) as was previously described [23]. The total content of protoberberine-type alkaloids was determined as a chelidonine (CAS 476-32-4) equivalent from seven concentration points of chelidonine (from 200 to 1.2 μg/mL). Alkaloids were separated using the same chromatographic conditions as for the phenolic compounds, except the gradient was from 7 to 70% phase B in phase A over 20 min. All analyses were performed in triplicate.

Antioxidant and mineral parameters
Activity of blood glutathione peroxidase (GPx) and total antioxidant capacity (TAC) of the sera were determined as previously described [22, 23]. Superoxide dismutase (SOD) activity in erythrocytes was analyzed in fresh blood using a commercial kit (RANSOD, Randox Laboratories, Ltd., London, UK). Enzymatic activity was evaluated at 37 °C and a wavelength of 505 nm using an UV-VIS Spectrophotometer (UV-2550, Shimadzu Co., Kyoto, Japan) and the results are expressed in units/g hemoglobin. The serum mineral content was determined by flame atomic absorption spectrometry in an air-acetylene flame, with deuterium background correction, using an AA-7000 atomic absorption spectrophotometer (Hitachi, Tokyo, Japan) and the results are expressed in units/g.

Inflammatory response
Haemonchus contortus antigen (HcAg) was obtained from ca. 50,000 L3. The larvae were washed twice in phosphate buffered saline (PBS, pH 7.4), diluted to a volume 15 mL and homogenized in an ice-cold glass (Sonopuls ultrasonic homogenizer HD3100, Bandelin, Germany). The homogenate was centrifuged (Heraeus Megafuge 16R, Thermo Fisher Scientific, Waltham, USA) at 4500 g for 5 min at 4 °C, and the supernatant was concentrated in 3000 MWCO VIVASPIN tubes (Sartorius, Goettingen, Germany) at 4000 g for 100 min at 4 °C. The protein concentration of the larval antigen was measured using the Bradford protein assay (Bio-Rad Laboratories, Munchen, Germany). Anti-H. contortus antibodies in the sera of the experimental lambs were detected using the HcAg somatic antigen and an indirect enzyme-linked immunosorbent assay (ELISA). Haemonchus contortus antigen was diluted to 5 μg/mL in carbonate buffer (pH 9.6) and then bound to microtiter plates (Nunc, Thermo Fisher Scientific, Roskilde, Denmark) at 4 °C overnight. The wells were washed three times with 0.5% Tween 20 in PBS (pH 7.4, PBS-T), and non-specific bonds were blocked with PBS containing 0.5% skimmed milk after 1 h of incubation at room temperature. The wells were again washed three times with PBS-T, and the serum samples were diluted to 1:100 and incubated at 37 °C for 1 h. All samples were examined in duplicate. The wells were washed again as above and bound antibodies were detected by incubating at 37 °C for 1 h with horseradish peroxidase-conjugated rabbit anti-sheep IgG (Sigma-Aldrich, Hamburg, Germany) diluted to 1:10000. The wells were washed again as above and 0.05 mol/L of the substrate o-phenylene diamine (Sigma-Aldrich, Hamburg, Germany) in citrate buffer (pH 4.7) with 0.005% H₂O₂ was used to induce a color reaction. The reaction was stopped by 1 M H₂SO₄ after a 15-min incubation at room temperature in the dark. The optical density was measured at 492 nm (Multiskan Reader, Thermo Fisher Scientific, Vantaa, Finland). The concentration of serum calprotectin was determined using commercial sheep ELISA kits (MyBioSource, Inc., San Diego, USA).

DNA isolation and PCR amplification
Total DNA was extracted from frozen fecal samples using a QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany). Isolated DNA was used as a template for the PCR amplification of 16S rRNA gene fragments. All PCRs were performed in 50-μL volumes containing 1 μL of DNA, 1 × PCR buffer, 2 mmol/L MgCl₂, 1 μL of 200 μmol/L each dNTP, 1.25 U of Platinum Taq DNA polymerase (Invitrogen, New York, USA) and 25 pmol each primer using a C1000™ Thermal Cycler (Bio-Rad Laboratories, New York, USA). Universal primers fD1 (5'-AGA GTT TGA TCC TGG CTC AG-3) and rP2 (5'-ACG GCT ACC TTG TTA CGA CTT-3') [66] were used to amplify a 1500-bp region of the 16S rRNA gene in the first round of PCR. PCR conditions were: 94 °C for 5 min and then 35 cycles of 94 °C for 1 min, 54 °C for 1 min and 72 °C for 1 min 30 s, followed by 72 °C for 5 min. The 16S rRNA gene fragments were subsequently used as a template for the second round of PCR using the specific bacterial primers GC-clamp-968f (5'-CGC CCG GCC CGC GCC GCC GCC GCC GCC GCA GGG GAA CGC GAA GAA GAA GAA CCT TAC-3') and 1401r (5'-CGG TGT GTA CAA GAC CC-3') [67]. The cycling conditions were 94 °C for 5 min; 9 cycles of 94 °C for 1 min, 45 °C for 1 min and 72 °C for 1 min; 14
cycles of 94 °C for 1 min, 60 °C for 1 min and 72 °C for 1 min and a final extension at 72 °C for 10 min. The PCR products were detected by electrophoresis on a 0.8–1% agarose gel containing ethidium bromide and were photographed using a Gel Logic 212 PRO imaging system (Carestream, New York, USA). The second-round PCR products were subjected to DGGE. Denaturing gradient gel electrophoresis was performed using the DCode™ Universal Mutation Detection System (Bio-Rad Laboratories, Hercules, USA). The PCR products in a total volume of 45 μL were loaded onto an 8% (w/v) polyacrylamide gel (37.5:1 acrylamide-bis-acrylamide) in 1 × TAE (40 mM Tris, 20 mM acetate, 1 mM EDTA) containing a linear denaturing gradient ranging from 30 to 60% denaturant (100% denaturant solution consisted of 7 M urea and 40% formamide). The electrophoresis was run for 17 h at a constant voltage of 51 V and a temperature of 60 °C. The gel was then incubated for 20 min in ethidium bromide (0.5 μg/mL), rinsed for 20 min in distilled water and photographed with UV transillumination using a Gel Logic 212 Pro Imaging System (Carestream, New York, USA).

Calculations and statistical analysis
Analyses of variance (ANOVAs) (GraphPad Prism, GraphPad Software, Inc., San Diego, USA) were used for analyzing the inflammatory responses, antioxidant and mineral statuses as repeated-measures mixed models representing the four animal groups (C, I, I + Mix1 and I + Mix2) and sampling days. Effects included in the model were treatment, time and their interaction. Differences between the infected group (I) and both treated groups (I + Mix1 and I + Mix2) were analyzed by a two-way ANOVA with a Bonferroni post hoc test. Student’s t-tests were applied to assess the differences between the arithmetic EPG means on different sampling days from D44 for I, I + Mix1 and I + Mix2 and between worm counts at dissection. All EHT and LDT data were analyzed by Student’s t-tests to assess the differences between the arithmetic means of ED50/LD50 for Mix1 and Mix2 and between the aqueous and methanolic extracts. A probit model of regression analysis was applied to the data to express a median lethal dose (ED50 and LD50). Differences were determined using Tukey’s multiple comparison post hoc test. The significance level for all tests was set at P < 0.05.

The gel images were processed using CLIQS 1D Pro software (Total Lab Ltd., Newcastle, UK). A dendrogram representing band-pattern similarities was constructed using the unweighted pair group method with arithmetic mean (UPGMA) algorithms. UPGMA uses a sequential clustering algorithm, in which local topological relationships are identified in order of similarity, and a phylogenetic tree is sequentially built.

Abbreviations
ADF: Acidic-detergent fiber; ANOVA: analysis of variance; C: control; Calpro: calprotectin; Concentrate: commercial concentrate composed of barley, soybean meal, wheat bran, bicarbonate and vitamin premix; CP: Crude-protein; D: day of sample collection; DGGE: denaturing gradient gel electrophoresis; DM: dry matter; DMSO: Dimethyl sulfoxide; EHT: hatch test; EPG: eggs per gram; GN: gastrointestinal nematode; GPx: glutathione peroxidase; HAcg: Haemonchus contortus antigen; I + Mix1: infected animals with herbal mixture 1; I + Mix2: infected animals with herbal mixture 2; I: infected animals; IgG: immunoglobulin G; IVMD: in vitro dry DM digestibility; LDT: larval development test; MH: meadow hay; MS5: tandem mass spectrometry; N: nitrogen; NDF: neutral-detergent fiber; NS: not significant; SOD: superoxide dismutase activity in erythrocytes; TAC: total antioxidant capacity; UHRMS: ultra-high resolution mass spectrometry; UPGMA: unweighted pair group method with arithmetic mean algorithms

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Authors' contributions
DM performed the laboratory and statistical analyses and helped with wrote the manuscript. ZV interpreted the data and wrote the manuscript. AKop performed microbial community analyses. KC and LG reviewed the ethical application dossier and provided laboratory analysis. SK and AC helped with laboratory analyses and reviewed the manuscript. MB, MUD and AKon performed the in vivo and in vitro trials and the post-mortem examinations. ED conducted immunological analyses. JV assisted with in vitro experiments and reviewed the manuscript. SS provided analysis of the bioactive compounds in the herbal mixtures. MV designed the study protocol and supervised the research and reviewed the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials
The data sets used and/or analyzed are available from the corresponding author on reasonable request.

Ethics approval and consent to participate
Animal use and study design were approved by the Ethics Committee of the Institute of Parasitology of the Slovak Academy of Sciences in accordance with the national legislation in Slovakia - Animal Welfare Act No. 23/2009. Permission to collect study samples was granted by participating sheep farmers. The owner of the animals gave verbal consent to carry out of the experiment.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

Author details
1Centre of Biosciences, Institute of Animal Physiology, Slovak Academy of Sciences, Košice, Slovak Republic. 2Institute of Parasitology, Slovak Academy of Sciences, Hlínka 3, 040 01 Košice, Slovak Republic. 3Department of Zoology and Fisheries, Czech University of Life Sciences Prague, Faculty of Agrobiology, Food and Natural Resources, Suchdol, Prague, Czech Republic. 4Department of Animal Nutrition, Poznan University of Life Sciences, Poznan, Poland. 5Department of Pharmaceutical Biology with Botanical Garden of Medicinal Plants, Medical University of Wrocław, Wrocław, Poland.
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