PHYTOTHERAPEUTIC EXTRACTS PHARMACODYNAMICS IN EQUINE STRONGYLES

FARMACODINAMIA EXTRACTELOR FITOTERAPEUTICE ÎN STRONGILIDOZE LA CABALINE

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ABSTRACT | **REZUMAT**

Equine strongylidosis represent an actual problem by the economic and animal health implications they can cause. In the last decade, complex anthelmintic resistance against specific medication is reported worldwide. This imposes the research and use of alternative fighting methods against this parasitosis, such as phytotherapy (1, 3, 8, 10, 13). For this purpose we tested the eficacy of nine aqueous plant extracts. Eight were individual extracts, containing: anise, black walnut, cloves, dog rose, ginger, summer savory, sea buckthorn, wormwood, and the ninth was a combination of alder buckthorn*,* common centaury*,* chamomile*,* dog rose, elecampane*,* tansy*,* wild thyme*,* willow gentian and wormwood, called anthelmintic tea extract. The assays were conducted *in vitro,* consisting in Egg Hatch Assay (EHA) and Larval Development Assay (LDA), between October 2016 and April 2017.

The classification of the tested extracts acording to their efficacy and the median lethal dose values (LC_{50}), obtained in the EHA test, placed the anthelmintic tea extract in the first place, followed by sea buckthorn and thyme extracts, these plants also posing a limited risk of inducing adaptive phenomena in the future. Comparative MIC value analysis obtained in the LDA test revealed the maximum efficacy for anise extract (-0.2623%) followed by cloves, ginger and anthelmintic tea extract.

Keywords: phytotherapy, strongylidosis, equine, anthelmintic resistance

Strongilidozele la cabaline reprezintă o problemă actuală prin implicațiile pe care le pot avea atât din punct de vedere economic, cât și asupra sănătății animalelor. În ultimul deceniu a fost semnalat un amplu fenomen de rezistență al helminților asupra medicației specifice la nivel mondial, ceea ce impune căutarea și utilizarea unor metode alternative de combatere a acestor parazitoze, cum ar fi fitoterapia (1, 3, 8, 10, 13). În acest scop, a fost testată eficacitatea a nouă extracte apoase, opt dintre ele fiind extracte individuale și conținând: cuişoare, anason, pelin, măceş, cimbru, cătină, ghimbir, nucă neagră, iar cel de-al nouălea extract a fost o combinație de cimbrișor*,* crușin*,* gențiană*,* iarbă mare*,* mușețel*,* țintaură și vetrice, combinație numită ceai antihelmintic.

Determinările s-au realizat în perioada octombrie 2016 - aprilie 2017, efectuându-se in vitro testul de inhibare a eclozionării ouălor (EHA) și testul de inhibare a dezvoltării larvare (LDA), pe probe de fecale de cabaline. Clasificarea extractelor testate în funcție de eficacitate și valorile și valorile și valorile CL_50 obținute în cadrul testului EHA situează pe primul loc extractul ceai antihelmintic, urmat de extractele de cătină și cimbru, aceste plante prezentând și un risc limitat de a induce fenomene adaptative pe viitor. Analiza comparativă a valorii CMI obținute în cadrul testului LDA a relevat eficacitatea maximă în cazul extractului din anason (-0,2623%) urmat la egalitate de extractele de cuișoare și ghimbir.

Cuvinte cheie: fitoterapie, strongilidoze, ecvine, rezistență antihelmintică

Digestive parasites resistance against antiparasitic active substances is manifested by a decrease in initial efficacy. It occurs and propagates due to refractory individuals that are not affected by the recommended therapeutic doses, survive the treatment, and transmit their ability to their offsprings, resulting in environmental contamination with resistant strains. In this way, the number of resistant parasites increases, the active substance no longer being effective (2, 3, 8). Due to the lack of new antiparasitic molecules to be used in short or medium-term treatments, with the global spread of anti-parasitic resistance, there will no longer be options to control these common infestations (10, 12) and phytotherapy may represent a the-

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rapeutic solution in reducing propagation of this resistance (1, 4, 11, 13, 14).

MATERIALS AND METHODS

The tests were performed between October 2016 and April 2017. For the purpose of testing the anthelmintic action of aqueous plant extracts against equine strongyles exogenous forms, 15 species of plants were included. Eight of these planta were individually tested: cloves (*Syzygium aromaticum*), anise *(Pimpinella anisum*), wormwood (*Artemisia absinthium*), dog rose (*Rosa canina*), summer savory (*Satureja hortensis*), sea buckthorn (*Hippophae rhamnoides*), ginger (*Zingiber officinalis*), black walnut (*Juglans nigra*) and the others were used in a combination called anthelmintic tea extract, having the following composition: wild thyme (*Thymus serpyllum),* alder buckthorn *(Rhamnus frangula),* willow gentian *(Gentiana asclepiadea),* elecampane *(Inulae radix),* chamomile *(Matricharia chamomilla),* common centaury *(Centarium erithrea),* tansy *(Tanacetum vulgare),*wormwood *(Artemisia absinthium)* and dog rose *(Rosa canina*). Of these plants, 4 decoctions and 5 infusions were made, in a concentration of 6%, according to the Romanian Pharmacopoeia, X-th edition.

The strongil eggs used for the tests were obtained from the faecal samples of six horses housed in the Equine Hospital of the Cluj-Napoca Veterinary Medicine Faculty. These were collected by flotation method and then concentrated by centrifugation at 4100 rpm, for five minutes.

The aqueous extracts obtained were considered the "mother solution" regarded as having a concentration of 100%. From each mother solution 11 serial dilutions were obtained, from the concentration of 50% up to 0.04%. Twelve wells were prepared for each tested plant extract. Each well contained 1 ml of diluted extract and 1 ml of aqueous solution containing strongyl eggs, thus, the concentrations in the wells were from 25% up to 0.02%. For each plant extract, the twelfth well contained a control sample formed by one ml of aqueous solution with strongyl eggs and 1 ml of distilled water. Plates were kept at thermostat (27° C, relative humidity 80%).

The in vitro tests performed were Egg Hatch Assay (EHA), performed after 24 hours of incubation and Larval Development Assay (LDA), performed after 14 days of incubation. The data was statistically interpreted by determining hatching percentage, larval development percentage (L3), the prediction line equation, lethal concentration for 50% (LC_{50}) and minimum inhibitory concentration (MIC), both expressed as plant extract percentage (5, 6, 7). For accuracy, correct correlation and data interpretation, the statistical calculus program: Anthelminitic Resistance Program (ARP) was used (5, 9, 8).

RESULTS AND DISCUSSIONS

The comparative analysis of the results obtained in testing the action of nine plant extracts over the exogenous forms of equine strongiles showed different results depending on the plant species and the performed test.

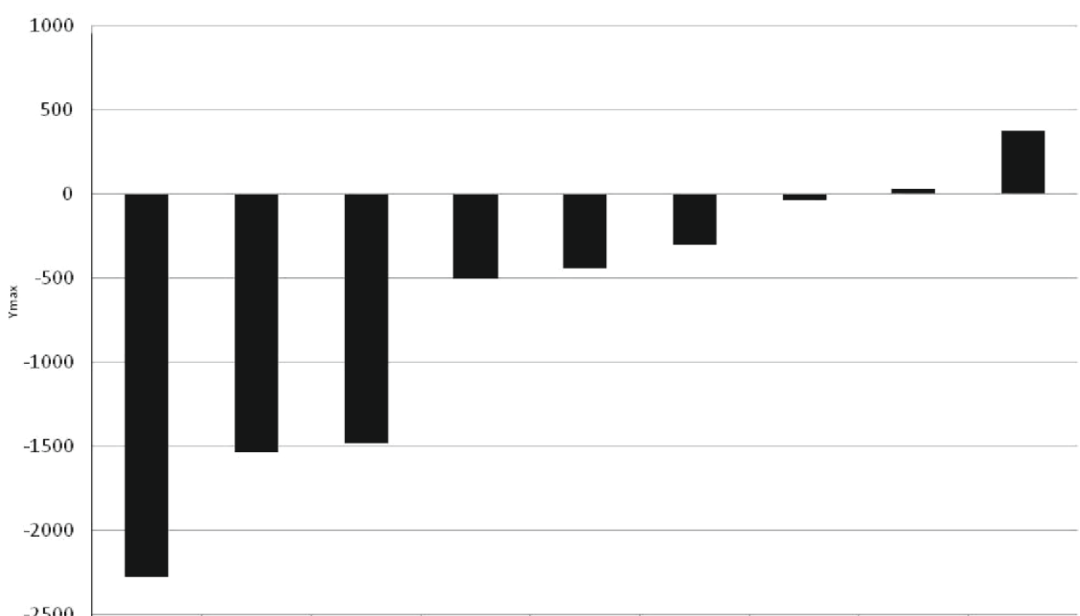
In EHA, for the cloves extract, the hatching percentage varied between 33.33% and 100%, average hatching percentage being 73.53%. Statistical interpretation revealed a negative prediction line, reaching the maximum value of - 446.91. LC_{50} had a negative value of -83.0685%, highlighting that the active ingredients from the cloves extracts are capable to inhibit hatching even at lower concentrations than those under study, respectively 0.02%.

In the case of anise decoction the hatching percentage varied between 0% and 100%, having an average of 80.56%. "a" and "b" parameter values were -2.6289 and 90.8492. LC_{50} presented a negative value, as in case of cloves extract, being -940.5961%. Y parameter, which defines the prediction line, had positive values, between 25.13 and 90.80, showing a reduced possibility for the use of this decoction in the treatment of equine strongylidosis. In the case of testing the wormwood extract active principles, the hatching percentages were higher for most of the tested concentrations, average hatching percentage being 79.17%, with a minimum at 0.04%. Positive values for a and b parameters and a prediction line with an obvious tendency towards $+\infty$. The maximum value of the Y parameter was 374.37, and the minimum one was 63.64. LC_{50} calculation pointed out that, *in vitro,* in the case of wormwood extracts, for the tested strongyle population, would be required concentrations higher than 25%. LC_{50} value was 685.3521% (Fig. 1, 2).

The dog rose extract EHA testing showed an average hatching percentage of 83.33% with null values at 12.5%, and 3.12%. For the rest of the tested concentrations, hatching percentage was 100%. The mathematical analysis of these values has highlighted this extract's limited ability to inhibit strongyl eggs hatching.

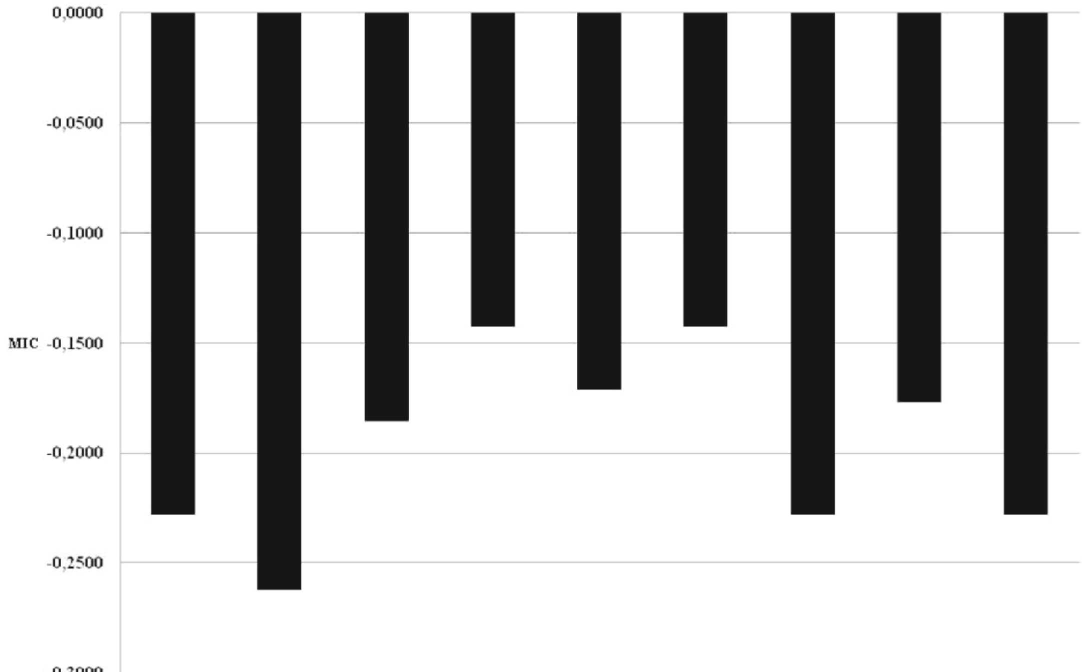
	Cloves	Anise	Wormwood	Dog rose	Summer savory	Sea buckthorn	Ginger	Black walnut	Anthelmintic tea
LC50	-983.0685	-940.5961	-685.3521	-166.3407	-3161.1082	-3503.5699	-692.5099	-1097.5688	-4624.6057

Fig. 1. Comparative statistical results obtained at EHA tests through CL_{50} analysis (% of plant extract)



	Anthelmintic tea	Summer savory	Sea buckthorn	Black walnut	Cloves	Ginger	Dog rose	Anise	Wormwood
Maximum Y EHA Test	-2278.33	-1537.69	-1487.07	-503.96	-446.91	-303.04	-35.66	28.13	374.37

Fig. 2. Comparative statistical results obtained by analyzing the maximum Y parameter at EHA tests



MIC LDA Test	Cloves	Anise	Wormwood	Dog rose	Summer savory	Sea buckthorn	Ginger	Black walnut	Anthelmintic tea
	-0.2280	-0.2623	-0.1853	-0.1425	-0.1710	-0.1425	-0.2280	-0.1767	-0.2280

Fig. 3. Comparative analysis of MIC statistical results (% of plant extract) obtained at LDA tests

Maximum Y LDA Test	Sea buckthorn	Dog rose	Summer savory	Black walnut	Wormwood	Cloves	Ginger	Anthelmintic tea	Anise
	-2424.92	-2424.92	-2182.32	-2139.40	-2078.29	-1818.60	-1818.60	-1818.60	-1653.11

Fig. 4. Comparative statistical results obtained by analyzing the maximum Y parameter at LDA tests

LC_{50} presented a negative value (-166.3407) and by correlating its value with the prediction line equation we can conclude that dog rose extract can be recommended for its anthelmintic effects, but subject to the need of more detailed studies. By testing EHA efficacy of active principles in the summer savory extract, maximal hatching percentage was observed for all tested concentrations, except 0.04%. the prediction line had a obvious tendency towards -∞ (-1537.69), denoting the fact that *Satureja hortensis* extract active substances will keep their ovicidal effect in time LC_{50} -3161.1082%, as a result, in the future, even concentrations lower than 0.02% should be used. Similar results have been obtained with sea buckthorn extract, for which average hatching percentage was 77.78 %. A strongly negative prediction line, maximum Y parameter being -1487,07. LC_{50} was -3053.5699%, highlighting the increased ability of this plant extract to inhibit strongyl eggs hatching (Fig. 1, 2). The EHA test on the ginger extract serial dilutions, showed hatching percentage results between 33.33% at 6.25% extract concentration and maximum starting at 0.39% extract concentration. Average hatching percentage was 73.31%, the maximum value being in control sample. The prediction line had negative values (maximum Y paramether = -303.04), but not as obvious as for sea buckthorn and summer savory extracts. LC_{50} was -692.5099%. Similar effects to those obtained for ginger extract were seen for black walnut extact. Average hatching percentage was 82.69%, a paramether being -23.7443, b paramether 89.6464, and LC_{50} -1097.5688. Global analysis of these results determined a negative tendency prediction line, maximum Y paramether being -503.96. For anthelmintic tea extract, hatching was completely inhibited at 1.56%, 0.78% and 0.04% extract concentrations, average hatching percentage being 63.89%. For this extract, compared to the other tested extracts, the lowest value of the "a" parameter was obtained (-93.8508). Calculating maximum Y paramether based on "a" and "b" paramether values lead to the drawing of a prediction line with the most obvious negative prediction (Y maxim = -2278.83). LC_{50} was -4624.6057 being the lowest of the tested concentrations (Fig. 1, 2).

The comparative statistical results of EHA obtained by analysis of LC_{50} of the nine tested extracts revealed that the highest egg hatching inhibiting concentration was for wormwood (685.3521%). For the other extracts the LC_{50} values were negative, which, in conjunction with the line prediction line, denotes that tested active herbal principles have a strong ability inhibit strongyle egg hatching. As a result, for a more accurate determination of LC_{50} we recommend to lower series of concentrations below 0.02% in subsequent research work. In the classification of tested extracts according to the LC_{50} values obtained in the EHA test places the the plant mixture extract (anthelmintic tea extract) as first (LC_{50} = - 4624.6057) followed by sea buckthorn, summer savory, black walnut, cloves, anise, ginger and dog rose (Fig. 1). The comparative results obtained by analyzing the maximum Y parameter, respectively the prediction line, highlighted that for the exogenous forms of horse strongyles there is a limited risk of inducing adaptive phenomena in the future against the active principles found in the tested extracts. In descending order, the most limited risk was obtained for antihelmintic tea extract (maximal Y = -2278.33), followed by summer savory, sea buckthorn, black walnut, cloves, ginger and dog rose. The maximum risk for induction of these adaptive phenomena was obtained in anise extract (maximal Y=28.13), respectively wormwood (maximal Y = 374.37) (Fig. 2).

Another way to test *in vitro* the efficacy of an antihelmintic product is to determine its ability to inhibit larval development and thus prevent the occurrence of Stage 3 larvae (L3). Unlike the EHA test, where the results were different depending on the tested plant, in the case of LDA test, the results were similar for all the tested extracts with a maximum capacity of larval growth inhibition for all concentrations. For cloves extract, average development percentage was 4.17%, being due only to the L3 hatching of the control sample (50%). as a result, the prediction line was negative, maximum Y being -18.1860. The minimum inhibitory concentration was -0.2280, demonstrating the fact that in future concentration lower than 0.02% should be used to obtain positive MIC values. The anise extract had a MIC of -0.2623%, maximum Y = -1653.11. Larval inhibition was observed at all concentrations, except for the control where it was 45.45% (Fig. 3, 4).

Similar results have been obtained for wormwood infusion, to which the MIC was -0.1853. Prediction line had negative tendency due to maximum Y parameter -2078.29. The dog rose decoction had a maximum larval hatching inhibition capacity, similar to those of cloves, anise and wormwood. Instead, it had one of the most obvious negative tendencies of the prediction line (Y max = -2424.92). Medium hatching percentage was 5.56%, and MIC -0.1425%. Comparable results were also obtained for summer savory and sea buckthorn extracts. In the case of summer savory extract, MIC was -0.1710%, and for sea buckthorn was 0.1425%.

Maximum Y parameter showed a negative trend for the two extracts, summer savory being -2182.32 and for sea buckthorn -2424.92. When testing the ginger decoction prnciples effect on the ability to inhibit the development of L3 larvae MIC was -0.2280, and prediction line had a negative tendency, maximum Y being -1818.60 (Fig. 3, 4). In descending order, after dog rose of and sea buckthorn extracts, the most significant values of the maximum Y parameter were obtained for black walnut (-2139.40). The average hatching percentage was 4.9% and MIC -0.1767%. For the anthelmintic tea extract in LDA, the positive results were not so obvious compared to the other eight extracts made from single compositions. Average percentage of larval development was 4.17%, MIC -0.2280% and maximum Y has reached -1818.60. Comparative analysis of MIC values obtained in the LDA test revealed maximum efficacy in the anise decoction (-0.2623%) followed by cloves, ginger and anthelmintic tea extract (-0.2280 %), all with equal results. In decreasing order, the ability of the active principles to inhibit larval development according to the percentage expression of MIC was as follows: wormwood (-0.1853%), black walnut (-0.1763 %), summer savory (-0.1710%) sea buckthorn and dog rose (-0.1425%) (Fig. 3). Analyzing the maximum Y parameter for LDA tests, a limited risk of inducing the adaptive phenomena of the strongils to the active principles was found for sea buckthorn and dog rose (maximum Y =-2424.92%) was, followed by summer savory, black walnut, wormwood, cloves, ginger, anthelmintic tea extract and anise (Fig. 4). Analyzing the results obtained in the two tests as a whole, it was observed that with the prolongation of the contact time between the parasitic forms and the plant extracts, a stronger ability to inhibit the development of the infesting strongil forms. Extrapolating these results to further *in vivo* testing, we recommend using these herbal extracts for several consecutive days to increase the chances of destroying larval and adult endogeneous forms.

CONCLUSIONS

The comparative analysis of the data obtained revealed different results depending on the plant species and the test (EHA or LDA).

1. The tested extracts classification according to their EHA test efficacy and the LC_{50} values placed in first the anthelmintic tea extract (LC_{50}=-4624.6057) followed by sea buckthorn, summer savory, black walnut, cloves, anise, ginger and dog rose.

2. In EHA, maximum Y parameter highlighted the most limited risk of inducing adaptive phenomena for the anthelmintic tea extract (max. Y = -2278.33), followed by summer savory and sea buckthorn extracts.

3. The maximum risk of induction of adaptive phenomena was obtained at anise (max. Y = 28,13) and wormwood (max. Y = 374.37).

4. MIC values obtained in the LDA test revealed maximum efficacy in the anise decoction (-0,2623%) followed by equal results for cloves, ginger and anthelmintic tea extracts (-0.2280%).

5. In descending order, the ability of the other extracts to inhibit larval development according to the MIC: wormwood(-0.1853%),black walnut (-0.1763%), summer savory (-0.1710), dog rose and sea buckthorn (-0.1425).

6. Y parameter in the LDA test revealed a limited risk of inducing adaptive phenomena to sea buckthorn and dog rose extracts (max. Y = -2424.92%), followed by summer savory (max. Y = -2182) and black walnut (max. Y = -2139).

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