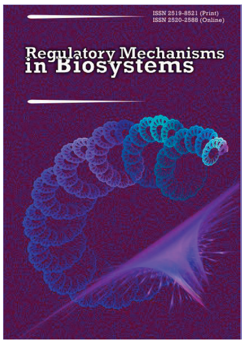

Regulatory Mechanisms in Biosystems

ISSN 2519-8521 (Print)
ISSN 2520-2588 (Online)
Regul. Mech. Biosyst., 9(3), 435–439
doi: 10.15421/021865

Influence of formic acid on the vitality of *Strongyloides papillosus*

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Article info

Received 14.06.2018
Received in revised form 27.07.2018
Accepted 29.07.2018

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Boyko, O. O., Gavrilina, O. G., Gavrilin, P. N., Gugosyan, Y. A., Brygadyrenko, V. V. (2018). Influence of formic acid on the vitality of Strongyloides papillosus. Regulatory Mechanisms in Biosystems, 9(3), 435–439. doi:10.15421/021865

Formic acid (methanoic acid, HCOOH) is an organic compound which belongs to saturated monobasic acids. In natural conditions, it is secreted from the glands of ants, and also extracted from the leaves of stinging nettles. It is soluble in water in any proportions, which makes it practical to use for making aquatic solutions. It is broadly used as a preservative in the food industry – E_{236} food additive (Codex Alimentarius), as a bactericide in medicine and veterinary medicine, and is also used against agricultural pest species of insects and mites. The *in vitro* and *in vivo* experiments revealed the anthelmintic properties of the acid against *Strongyloides papillosus* nematodes, parasites of the gastrointestinal tract of Ruminantia and rabbits. In the conditions of *in vitro*, 100% of (L_1, L_2, L_3) nematode larvae died from a 1% solution of formic acid (10 g/l) after 24 hours exposure. When exposed to less strong concentrations of the acid (1, 0.1, 0.01, 0.001 g/l), vital forms of L_3 *S. papillosus* were found. Non-invasive stages (L_1, L_2) are less resistant to the impact of the acid – death of 100% of the larvae was observed under the impact of 0.1% solution and up to 60% of larvae died at 0.01% solution of formic acid in the same conditions. LD_{50} for L_3 invasive larvae of *S. papillosus* equaled 0.47%, and 0.0076% for L_1, L_2 non-invasive larvae of *S. papillosus*. In the conditions of *in vivo* experiment (with guinea pigs), the effective dose of formic acid was 0.4% ml/kg of the animal`s body weight. The results of the coproscopy after the treatment demonstrated absence of the helminth larvae in the feces of the laboratory animals during 10 days and their occurrence only on days 15–20 with a low intensity (90 larvae/g of feces on average). During an external examination of the corpses of the animals of the experimental group, no pathological changes were found. The intestine, the heart, the lungs and the liver of the animals from this group had no macroscopic changes – they were of natural colour and size. The hepatocytes looked normal and the structure of the liver lobes was maintained. In the tissues of the liver of the animals from the experimental and control groups, we found processes of passive congestion, and an insignificant degree of signs of hepatic steatosis.

Keywords: nematodes; *Strongyloides*; antiparasitic activity; flavouring agents; formic acid.

Introduction

Currently, the requirements of society for quality products of animal origin is growing. Due to the popularizing of the data on the negative impact of the synthetic substances, most people more and more often demand ecologically pure products. During the production of these products, no substances of artificial origin are used. Due to the fact that the helminthiases regularly cause economic losses to livestock enterprises, manufacturers have begun searching for new methods against agricultural pests and parasites of animals, such as using agents of natural origin. The main property of such substances would be safety. They should not affect the quality of the production, therefore they should be safe for the consumer (Safiullin, 1997; Cabaret et al., 2002; Rahmann & Seip, 2006; Rinaldi et al., 2007; Burke et al., 2009; Charlier et al., 2009; Lu et al., 2010).

One of the preparations used currently as an anthelminthic agent is gentian violet. This substance has antimicrobial and anthelmintic properties. By toxicity, gentian violet belongs to the group of substances which are insignificantly toxic for animals. During its usage, there is no need to expect removal of the preparation from the organism of animals. The products of such animals can be used without any limitations. However, during its usage, one can face its main disadvantage – the substance significantly colours the mucous membranes and tissues pink. This property is the main reason why this substance cannot be used for animals intended for slaughter (Mashkovskij, 2000). Currently, many studies are oriented towards the search of substances of natural origin, plants, different food additives, which could be used against pests. Also, phytopreparations are becoming used more and more often. This is related to the fact that they have the least negative effect on the organisms of animals in general, therefore on the products obtained from the animals (Burke et al., 2009; Belletti et al., 2010; Boyko & Brygadyrenko, 2016, 2018).

The results of the studies by Faye et al. (2003), which were performed on goats by adding food additives to their food demonstrated not only significant effect on weight gain during the goats' pregnancy and during lactation. The goats that received the additives were observed to have increased milk production. The animals which were fed using the additives demonstrated a decrease in the number of eggs of helminths in their feces. It is well known that formic acid is also used against insects in insecticide compositions. The organic acids cause no harm to the environment due to the fact that they are pure substances of natural origin (Okhanov, 2000).

The studies on the impact of flavourings on pathogenic microorganisms have already been conducted. These studies revealed that apigenin, linalool and ursolic acid is extracted from *Ocimum sanctum*, and have a broad range of antiviral properties. Also, a mixture of these substances was found to have the ability to inhibit the growth of pathogenic microflora (Chiang et al., 2005). Apart from the impact on microorganisms, scientists have proved the lethal effect of some of these substances on the larval forms of pathogens of nematode infections (Boyko & Brygadyrenko, 2016). Boyko & Brygadyrenko (2016) performed experiments on the comparative helminthocidal effect of the following flavourings: benzaldehyde, natural citral, D-limonene and B-iodine. Research on the larvae of *Strongyloides ransomi*, parasitic nematodes of swine, revealed that after a 24 hour exposure, these substances have an anthelmintic effect. Especially good results were obtained from experiments with benzaldehyde, which at 0.01–1% concentrations caused death of over 90% of the larvae. Currently, formic acid is becoming broadly used against parasitic organisms. Formic acid is a colourless substance with a distinctive odour. In natural conditions, it is secreted from the glands of ants, and it also can be extracted from some plants, for example leaves of stinging nettles. It can be dissolved in water in any proportions. This makes it good for preparing aquatic solutions. However, in concentrated form, this acid can leave burns on the skin. There are no data on using formic acid as an anthelmintic preparation. Therefore, it is important to study the impact of formic acid on helminths and analyze the possibility of including it in fodders as a food additive with anthelmintic properties.

Materials and methods

The study was performed in two stages during 2017–2018: determining a lethal dose of formic acid for *S. papillosus* larvae *in vitro*, determining the possibility of using this substance as a food additive with anthelmintic properties for laboratory animals (guinea pigs).

For the first stage of the experiment (*in vitro*), we used *S. papillosus* of small cattle and rabbits. Feces of these animals were examined for the eggs of strongyloidiasis pathogens using the McMaster method for further cultivation of the larvae (Zajac & Conboy, 2011). The cultivated material was centrifuged (by 4 ml in test tubes for 4 minutes, 1500 rotations/minute), the sediment with the larvae was uniformly mixed and put in 1.5 ml plastic test tubes by 0.1 ml. Then, 1 ml of the formic acid solution was added to each of the tubes. Five concentrations of aquatic solution of the substance were used in the experiment: 10, 1, 0.1, 0.01 and 0.001 g/l, and also the control (distilled water) in eight replications. The exposure time during the experiment was 24 hours at the temperature of 22–24 °C.

During the second stage of the experiment, 6 guinea pigs were used. The animals were kept in separate cages. The animals were fed regularly and fully. The diet consisted of fresh vegetables, fruits, greens, and also meadow hay. The animals had free access to water in individual water bowls. As litter, sawdust was used and changed daily. Normal saline solution with *S. papillosus* larvae (by 0.5 ml) of rabbits was introduced to the guinea pigs subcutaneously and orally. Then, monitoring of the infestation of the laboratory animals was performed by periodic selection of the feces and coprological study of it once every 10 days.

After the infestation, the guinea pigs were divided into two groups: experimental and control. The solution of formic acid was introduced to the guinea pigs orally using a laboratory pipette. To prepare the solution, a concentrated formic acid and distilled water was used. The first group of animals received 1% solution of formic acid in 15 ml dose twice in 10 and 5 ml doses at 2 hours interval. The control group of animals did not receive the substance. The litter was changed every 24 hours. Initially, examination of the feces for nematode larvae was made every three days until the pathogen was found, and then once in 10 days . 80 days after the beginning of the second stage of the experiment, a pathoanatomical autopsy was performed on the laboratory animals, which had been euthanized using ether. An external examination of the corpses was performed. During the pathoanatomical autopsy, the condition of the liver, the intestine, and the heart was determined.

To study the liver, we used the classical method of preparing paraffin sections using a rotary microscope and staining the sections with hematoxylin and eosin. The histological study consisted of the following stages: selecting the samples, fixating the material in 10% solution of neutral buffered formalin, dehydration of the samples, incorporation of paraffin over the samples, preparing histological sections of 3–5 µm thickness, with subsequent staining of them with hematoxylin and eosin, incorporating the sections in balsam, and microscopic examination of the preparations (Bancroft & Stevensens, 1990).

Results

All (100%) of the larvae of *S. papillosus* nematodes died during exposure to 1% solution of the acid. First and second stage larvae of the studied nematode species were exposed to the acidic solution of 0.1% concentration. After 24 hours in the formic acid solution, about 10% of the nematode larvae remained viable. Almost 100% of the invasive larvae survived in 0.1% concentration of formic acid. The next level of decrease in the concentration of the studied substance also had no positive result against the invasive larvae of the studied species of helminth. In 0.01% concentration, 100% of the invasive larvae remained vital. Up to 60% of the L_1 and L_2 died in 0.01% concentration. Only up to 30% of these larvae died in 0.001% acidic solution (Fig. 1).

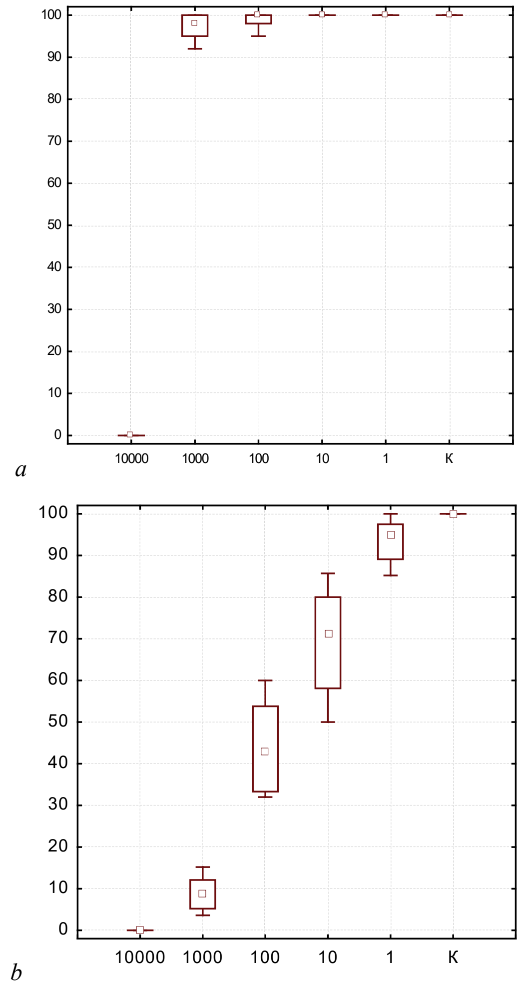

Fig. 1. The effect of formic acid on the viability of larvae of nematodes of Ruminantia: *a* – L_3 invasive larvae of *S. papillosus*; *b* – L_1, L_2 non-invasive larvae of *S. papillosus*; the ordinate axis indicates the percentage of living nematode larvae in the course of the 24-hour experiment; the abscissa axis indicates the concentration of the solution's active substance (%); *K* – control, where the concentration of the active substance is 0%; small square in the centre corresponds to the median, lower and upper edge of the large rectangle correspond to first and third quartiles, respectively, the vertical segments, directed upward and downward from the rectangles, correspond to minimum and maximum values (n = 8)

LD_{50} L_3 invasive larvae of *S. papillosus* on average does not exceed 0.47%, for L_1, L_2 non-invasive larvae of *S. papillosus* – 0.0076% (Table 1).

Table 1
LD_{50} (%, x ± SD) for the experiment on determining the impact of the chemical substance on the viability of nematode larvae in the laboratory experiment after one day

Substance	L_3 invasive larvae of *S. papillosus*	L_1, L_2 non-invasive larvae of *S. papillosus*
Formic acid	0.47 ± 0.29	0.0076 ± 0.0065

Thus, minimal concentration of the formic acid solution, in which 100% of invasive nematode larvae of *S. papillosus* died *in vitro* – 10 g/l.

Pathogens of strongyloidiasis among the guinea pigs were found in the coprological examination on days 25–50. We observed fluctuations of the infestation intensity from 283 to 1244 larvae/g of feces. On the 35th day of the experiment, the number of larvae increased on average by 592 per 1 g of excrement compared to the 25th day. On the 45th day, the infestation was reduced on average by 398 larvae/g of feces compared to the 35th day. However, these indicators were higher compared to the 25th day on average by 195 larvae/g of feces. The next examination was performed after 5 days. It revealed a significant increase in the invasion. On the 45th day, the infestation increased on average by 767 larvae/g of feces. Therefore, on the 50th day, the highest level of infestation of the guinea pigs was recorded – 1244 larvae/g of feces.

On the 56th day, the animals received treatment. The experimental group received 1% solution of formic acid in the dose of 0.40 ml/kg of body weight. The results of coprological examinations after the treatment indicated absence of the helminths in the animals over 10 days. On the 15th day after the treatment, the helminths were found in the guinea pigs' feces again in the amount of 13 larvae/g of feces. However, throughout the monitoring, their intensity was no higher than 90 larvae/g of feces on average.

External examination of the corpses of the experimental group animals revealled no pathological changes . The coat was dense, fur was glossy and fixed well in the skin, and no alopecia was observed. During the autopsy, no pathomorphological changes were found in the internal organs of the guinea pigs. The lungs were of regular form, pink colour, elastic consistency, moderately filled with blood. The pleura were smooth, moist, glossy. The small and large intestine of the animals were in the correct positions in the abdominal cavity. The mucous membrane of the intestine was pale-pink, moist, with no hyperemia (Fig. 2). The liver of all of the animals in the experimental group was of normal dark-brown colour, not enlarged, had a smooth and glossy surface, elastic consistency and was moderately filled with blood.

Fig. 2. The organs of the abdominal cavity of the experimental group animals (after peroral introduction of 1% solution of formic acid)

According to the results of the histological analysis of the liver of the experimental group animals , the organ had a typical lobed structure with insignificantly developed interlobular connective tissue. The hepatic tubules were clearly seen in the form of radial bands, sinusoid capillaries contained insignificant amount of erythrocytes. The hepatocytes had a many-sided form, with no clearly manifested signs of grainy texture and no fat droplets in the hepatocytes' cytoplasma. There were singular perivascular lymph nodes on the periphery of the lobes and no polymerphocellular accumulations (Fig. 3).

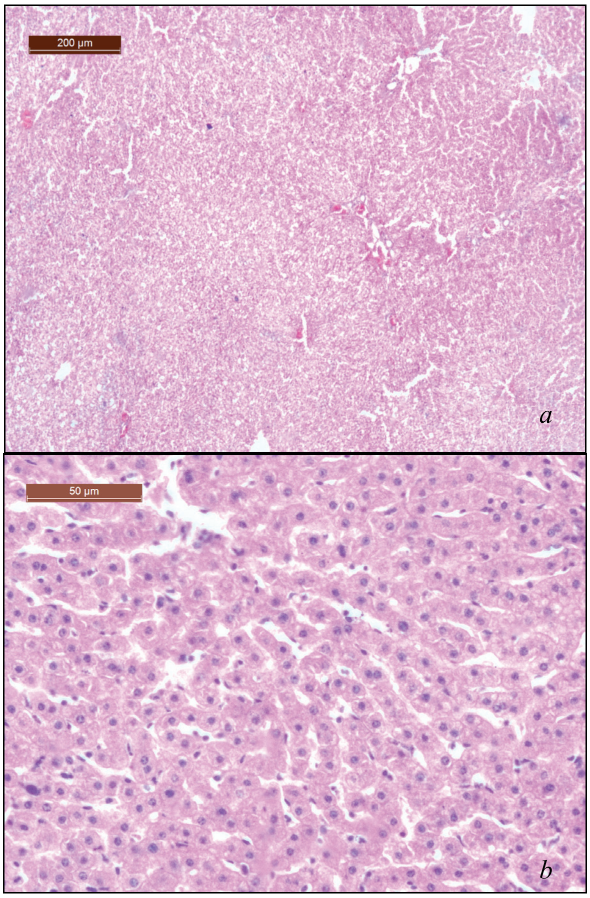

Fig. 3. Microscopic image of the liver of the animals from the experimental group: *a* – clear lobular structure of the liver parenchyma, *b* – hepactic tubules in the form of radial bands; hematoxylin and eosin

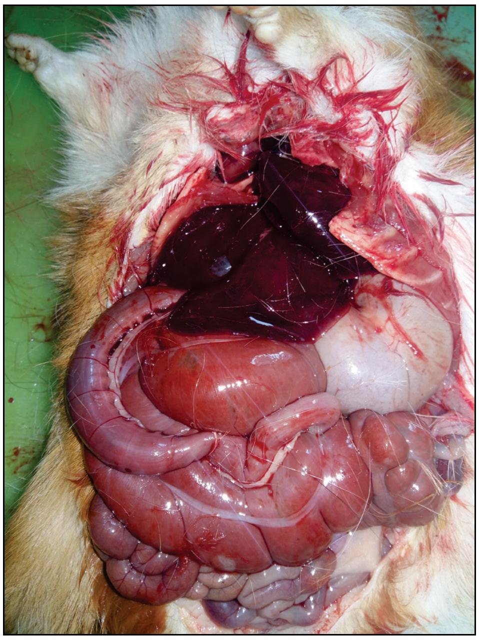

Fig. 4. The organs of the abdominal cavity of the animals from the control group

During an external examination of the corpses of the animals from the control group, we observed moderately manifested pathological changes in the coat. The hair was observed to be tarnished, mussed, not closely attached to the skin, thinned-out in the upper and lower spine. During the autopsy, we determined that the organs and serous membranes were not changed. In the small and large intestine, we observed signs of severe catarrhal inflammation (catarrh). The mucous membrane of the intestine was swollen, had areas with hyperemia of spotted or striped forms, which was especially clearly seen on the tops of its folds. In the mucous membrane of the small intestine, we found petechial hemorrhaging. The surface of the mucous membrane was covered with viscous, half-liquid slime. The aggregates of the lymph nodes of the mucous membrane were swollen and appear in lumen of intestine. The liver was insignificantly increased in size, pale-yellow flabby areas of different size were observed under the organ's capsula and on the section (Fig. 4).

During the histological examination of the liver of the animals of the control group, we determined that in the changed pale yellow areas of parenchyma of the organ, the tubular structure of the liver lobes was disrupted, hepatocytes were increased in size, and were mostly of rounded shape. The cytoplasm of the hepatocytes was alveolar. The alveoles were different in size. The hepatocytes' nuclei were positioned in the centers of the cells, had clear signs of karyopyknosis, some nuclei had become lysed. As a result of lysis, fat necrosis areas were formed. We observed perivascular proliferates of macrophages, fibroblasts and lymphocytes. In general, the pathomorphological condition of the liver in the animals of the control corresponds to the focal form of degenerative obesity (focal fat deposition) (Fig. 5).

Thus, at the macrolevel, formic acid in the tested concentrations had no negative effect on the internal organs of the studied animals. During the histological examination, the parameters of the liver of animals from the experimental group were better compared to the parameters of the control. The processes of fatty degeneration were possibly caused by helminths. Passive hyperemia which was found in all samples of the experimental group, was, probably, a consequence of using ether for euthanizing the animals.

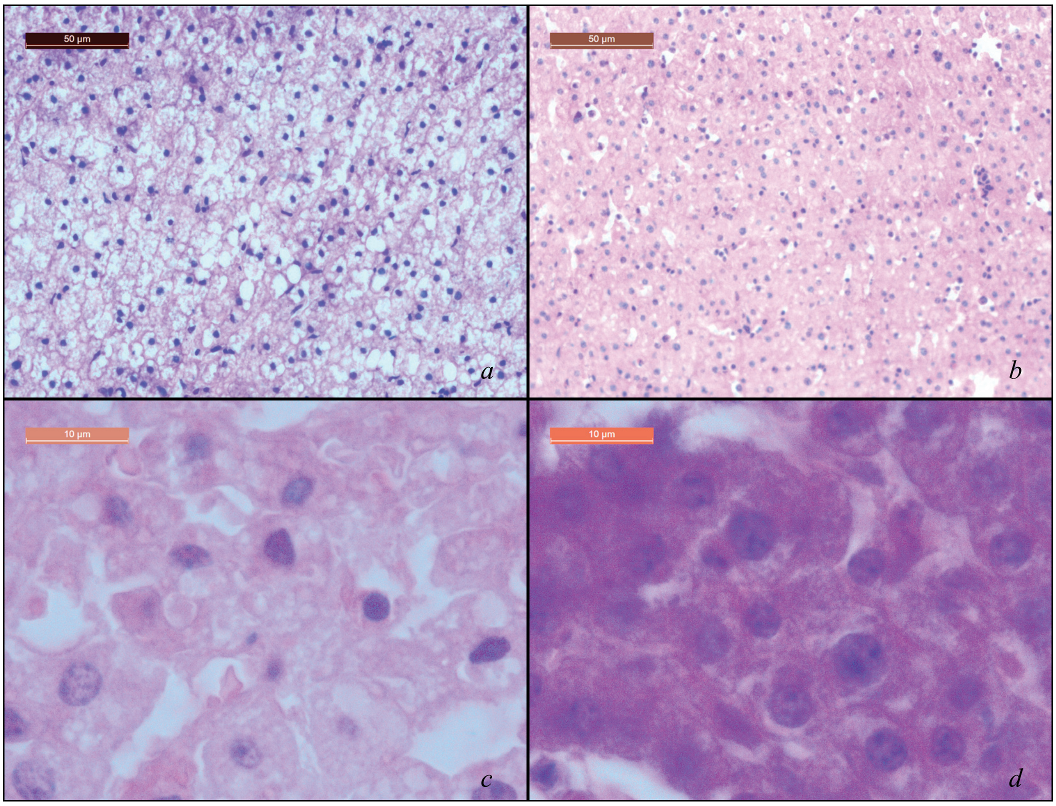

Fig. 5. Microscopic image of the liver of the animals in the control group: *a* – degenerative obesity of the hepatocytes, *b* – disorders in the tubular structure of liver lobes, *c* – the nuclei of the hepatocytes with signs of karyopyknosis and karyolysis, *d* – non-uniform structure of the cytoplasm of the hepatocytes; hematoxylin and eosin

Discussion

Mature *S. papillosus* helminths parasitise the small intestine, their larvae migrate through the body of animals. They cause mechanical, allergic and inoculative effects (Thamsborg et al., 2016). The results of experimental infestation of rabbits with *S. papillosus* larvae caused disorders in the gastrointestinal tract and led to development of anorexia, loss of weight and ultimately the death of the animals. Decrease in the body weight and daily food consumption were observed in a combination with heightened EPG in the infested animals (Kobayashi & Horii, 2008). The researchers have determined that infestation of non-typical hosts – Mongolian gerbils (*Meriones unguiculatus*) with mature individuals of *S. papillosus* led to increase in mortality in the experimental group and decrease in their life expectancy. At the same time, the main cause of death was disorders in the peristalsis – paralytic disruption of the normal propulsive ability of the gastrointestinal tract (Paralytic ileus) (Kobayashi et al., 2009). Apart from the infestation of the intestine with mature individuals, larvae of *S. papillosus* migrate in the organs and tissues, causing allergic processes with release of biologically active substances and development of eosinophilia of the infested animals (Nakanishi et al., 1993). Kváč & Vítovec (2007) determined that the main cause of death of calves infested with *S. papillosus* was pathological changes in the lungs, caused by the migration of larvae. They histologically determined the development of purulent granulomatous inflammation with the new connective tissue and predominance of eosinophils in the inflammatory infiltrates (Kváč & Vítovec, 2007). Therefore, parasitism of *S. papillosus* worsens the quality of life of animals and even causes their death. Therefore the development of effective methods against them is relevant.

Formic acid is widely used in the food industry as a preservative. This substance affects the taste and the aroma of food products and is used for making semi-finished fruit products, and also for preservation of vegetables and fruits. Currently, formic acid (E_{236}, Codex Alimentarius) and its salts (E_{237} sodium formate and E_{238} calcium formate) are used as a salt substitute (food additives). Formic acid is quickly metabolized and removed from the organism. Therefore, in the solutions with low concentration, E_{236} additive has local irritant, anesthetic and anti-inflammatory effects. Currently, formic acid is used for disinfection. Its impact mostly targets yeasts and some bacteria. Mold and lactic bacteria are resistant to it. In Europe, formic acid is used mostly as a preservative of cattle fodder due to its strong bactericidal property. It is used as a spray for hay, therefore inhibiting the decomposition processes. Fodder maintains its nutrient qualities (Waldo et al., 1969; Castle & Watson, 1970). Also, there are data on using this substance in the compounds used against coccidiosis (Muzi & Rahman, 2005). One way of using formic acid is using it against *Varroa destructor* parasitic mites of bees. According to the researchers, effective destruction of these parasites was reached after treating the infested bees with formic acid in 0.08 and 0.16 mg/l concentrations as a fumigate (Underwood & Currie, 2003). Larvae L_3 *S. papillosus* and *H. contortus* were more resistant to the impact of formic acid. Our studies revealed the effectiveness of this substance in vitro only at 10 g/l concentration.

Conclusions

At the current stage, one can presume that systematic treatment of animals with formic acid (once in 10 days) in a dose of 0.40 ml/kg of body weight can achieve anthelmintic effect against the strongyloidiasis pathogen without any damage to the health of animals. This property of the studied substance is especially relevant in organic animal husbandry. Therefore, the anthelmintic properties of formic acid as a food additive need to be studied further.

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