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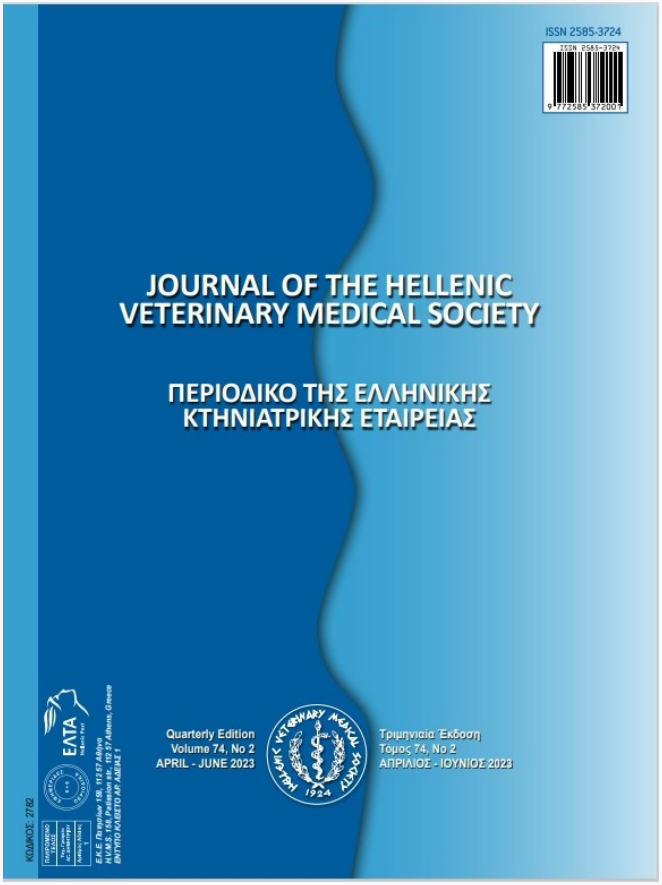

Effects of Antioxidants Supplemented Feed in Coccidiosis Treatment, Blood Antioxidative Status, and Enzymatic Activity of Domestic Cats

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Research article
Ερευνητικό άρθρο

Effects of Antioxidants Supplemented Feed in Coccidiosis Treatment, Blood Antioxidative Status, and Enzymatic Activity of Domestic Cats

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ABSTRACT: As coccidia become increasingly resistant to anticoccidial drugs, efforts have been made to find alternatives. In recent years, botanicals have been reported as potential alternatives to anticoccidials since they are effective against protozoa, arthropods, and helminths.In this study, different doses of dried pomegranate fruit (*Punica granatum* L.) were evaluated for their effectiveness in reducing the number of oocysts in domestic cats and their antioxidant properties.Under *in vivo* conditions, 24 six-month-old domestic cats of both genders naturally infected with *Cyto-isosporafelis* were tested. Four equal groups of infected cats were formed. Six cats made up each group. The control group (C) was on a basal diet, and one group received the chemical coccidiostat robenidine (CR) , supplemented in the amount of 0.5%. Two levels of whole dried pomegranate fruit as a natural antioxidant were applied in a concentration of 0.5% (P1) and 1.0% (P2) on top of the basic diet. With the McMaster technique, oocysts number and eggs per gram of feces were determined.From each cat, 6 per group, blood samples were taken from a jugular vein at the end of the experimental period to investigate the influence of dried pomegranate fruit on blood enzymatic activity and lipid oxidation. In conclusion, supplementing cats' diets with dried pomegranate fruit reduced the number of oocysts per gram of feces significantly, butit is important to carry out further and more detailed studies to prove the anticoccidial and antioxidant properties of dried pomegranate fruit in cats' diets.

Keywords: cats; coccidiosis; parasites; pomegranate, nutrition.

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INTRODUCTION

Cats' and dogs' feces have been harboring coccidian parasites for more than a century, but their clinical and public health significance was unknown until 1970 (Cox, 2002) .The feces of cats and dogs were thought to contain only one species of coccidia before Wenyon's report in 1923 (Wenyon, 1923) .It was determined that three coccidian parasites exist based on their oocyst sizes, the parasitewith large oocysts (*Cystoisosporafelis*) , medium-sized oocysts (*Cystoisosporarivolta*) , and small-sized oocysts (*Cystoisosporabigemina*) , and in addition, it was believed that they are non-host specific (Duszynski, 2021) . The parasites were later demonstrated to be host-specific with extraintestinal stages as well.Rodents, dogs, cattle, pigs, camels, albino mice, and rabbits can serve as paratenic hosts according to bioassay studies (Dubey et al., 2007; Gajadhar et al., 2015; Koutsoumanis et al., 2018) .Cats infected with *C. felis* excrete Toxoplasma gondii oocysts without showing any clinical signs, while*C. rivolta* was pathogenic for a newborn but not for weaned cats (Dubey, 2018) . Diarrhea usually begins 3-4 days after the newborn cat has been injected with 100,000 sporocysts or infected mice.Cats are reported to be at risk of infection with *C. felis*, but there is conflicting information regarding its pathogenicity (Shaw et al., 2004) .There have been reports of cats infected with enteric viruses experiencing severe diarrhea, especially before prophylactic antibiotics were available (Truyen et al., 2009) . The treatment of Cystoisospora infection in cats has been made possible by a variety of anticoccidial drugs.When cats are administered toltrazuril during the prepatent period, the number of oocysts excreted is significantly reduced (Litster et al., 2014; Petry et al., 2011) .

In the last decade, there have been questions raised about the use of antibiotics, such as sulfonamides as well as salinomycin and robenidine, in poultry nutrition for the treatment of coccidiosis.As avian coccidia (protozoa) become increasingly resistant to anticoccidial drugs, efforts have been made to find alternatives.

In recent years, botanicals have been reported as potential alternatives to anticoccidials since they are effective against protozoa, arthropods, and helminths (Puvača et al., 2022) . Pomegranate fruit (*Punica granatum* L.) is a nutrient-dense fruit rich in phytochemical compounds (Juhaimi et al., 2017) . As a mechanism of defense, plants produce low molecular weight compounds called phytochemicals.About 50% of the total fruit weight corresponds to the peel, which is an important source of bioactive compounds such as phenolics, flavonoids, ellagitannins, and proanthocyanidin compounds. Due to their biological and free radical scavenging activities, phenolic compounds, flavonoids, anthocyanins, and tannins are the most important antioxidant phytochemicals (Elfalleh et al., 2011) .Many studies have demonstrated that combinations of pomegranate extracts from different parts of the fruit are more effective than single extracts of the fruit because different parts of the fruit contain different phenolic acids, flavonoids, and tannins (Hussain et al., 2021) .

As a result of normal cellular metabolism, reactive oxygen species (ROS) are produced by living organisms, leading to cell oxidative destruction, also known as oxidative stress.Humans, as well as animals, may suffer from numerous pathological changes caused by oxidative stress, including membrane dysfunction, DNA damage, protein inactivation, cancer, arthritis, and neurodegenerative diseases (Islam, 2017) .In contrast, aerobic organisms possess highly effective antioxidants that are usually effective in blocking the negative effects of ROS. These antioxidants are both enzymatic and nonenzymatic,therefore, defensive processes involve a variety of protective mechanisms (Uttara et al., 2009) .

Activated oxygen is eliminated through the action of antioxidant enzymes.Several enzymes, such as superoxide dismutase (SOD) , convert superoxide anion radicals into H_2O_2 and reduce H_2O_2 to water, and glutathione peroxidase (GSHPx) reduces H_2O_2 and terminates lipid peroxidation.The enzyme glutathione reductase (GR) can also regenerate glutathione (GSH) , which plays an important role in the reduction of acute toxicity of xenobiotics and lipid peroxidation products (Bailly, 2004) .By stealing electrons from cell membrane lipids, free radicals cause lipid peroxidation, which damages the cells.Polyunsaturated fatty acids are particularly susceptible to this since they contain multiple double bonds, between which there are methylene bridges (-CH2-) with highly reactive hydrogens, resulting in lipid peroxides such as malondialdehyde (MDA) and hydroxyl, which damage tissues (Tsikas, 2017) .Consequently, MDA content is a significant indicator of the level of lipid peroxidation and an indirect indicator of cell damage (Bhattacharjee, 2014) .

In this study, different doses of dried pomegranate

fruit (*Punica granatum* L.) were evaluated for their effectiveness in reducing the number of oocysts in domestic cats and their antioxidant properties.

MATERIALS AND METHODS

The animal study protocol was approved by the Ethics Committee of University BA in Novi Sad, Serbia (EC-01/1/22) .

Experimental design and pre-experimental investigation

Domestic cats were observed and documented daily for natural infection before the experimental trial. Allcats were found to be infected with *Cystoisosporafelis*given the mild bloody diarrhea which was confirmed based onfecal examination.The direct smear and the saturated sugar floatation technique were used fordetermining the presence and identification of oocysts.Using the McMaster technique, coccidia oocysts per gram (OPG) of feces were determined.

Under *in vivo* conditions, 24 six-month-old domestic cats of both genders naturally infected with *C.felis* were tested.All pet cats were observed in their home environment; therefore, four equal groups of infected cats were formed.Six cats made up each group. Thecontrol group (C) was on a basal diet,and one group receivedthe chemical coccidiostat robenidine (CR) , supplemented in theamount of 0.5%.Two levels of whole dried pomegranatefruitas a natural antioxidant were applied in a concentration of 0.5% (P1) and 1.0% (P2) on top of the basic diet (Tables1 and 2) . From 6 to 8 months of age, the cats were monitored.A standard basal diet was fed to the cats, with access to water and food as needed.

Table 1. Ingredient composition of domestic cats' diets.

Ingredients, %	C	CR	P1	P2
Boneless chicken	30.0	30.0	30.0	30.0
Dehydrated chicken protein	28.0	28.0	28.0	28.0
Sweet potato	4.3	4.3	4.3	4.3
Chicken fat	3.0	3.0	3.0	3.0
Dehydrated eggs	9.0	9.0	9.0	9.0
Dehydrated herring protein	9.0	9.0	9.0	9.0
Fish oil	8.0	8.0	8.0	8.0
Alfalfa flour	2.5	2.5	2.5	2.5
Sodium chloride	0.2	0.2	0.2	0.2
Dry beer yeast	6.0	6.0	6.0	6.0
Coccidiostat robenidine	0.0	0.5	0.0	0.0
Dried pomegranate fruit	0.0	0.0	0.5	1.0

C - control group; CR - group received chemical coccidiostat robenidine; P1 -group received dried pomegranate fruit in the concentration of 0.5%; P2 - group received dried pomegranate fruit in the concentration of 1.0%.

Table 2. Chemical composition of domestic cats' diets.

Nutrients	%
Crude protein	44.0
Crude fat	20.0
Crude fiber	1.8
Crude moisture	8.0
Crude ash	8.5
Calcium	1.1
Phosphorus	0.9
Magnesium	0.08
Omega 6 fatty acids	3.3
Omega 3 fatty acids	0.9
ME, MJ/kg	17.59

Additives per 1kg of feed: Vitamin A 18000IU; vitamin D3 1200IU; vitamin E 600mg; vitamin C 300mg; niacin 150mg; calcium D-pantothenate 50mg; vitamin B2 20mg; vitamin B6 8.1mg; vitamin B1 10mg; biotin 1.5mg; folic acid 1.5mg; vitamin B12 0.1mg; choline chloride 250mg; beta-carotene 1.5 mg; zinc (zinc chelate methionine hydrate) : 163.8mg; manganese (manganese chelate methionine hydrate) : 64.6mg; iron (iron- (II) -chelate glycine hydrate) : 58.3mg; copper (copper chelate of methionine hydrate) : 15.8mg; DL-methionine 5000mg; taurine 4000 mg; L-carnitine 300 mg.

Oocyst count (OPG index)

A fecal sample was taken from each cat before natural antioxidants and chemical coccidiostat were introduced into the diets.An oocyst count per gram of feces was measured at the end of six, seven, and eight months of the cat's life.The number of oocysts in ten samples per gram of feces from each group was determined by collecting fecal samples of around 30 g from each group using mosquito net mesh.Refrigeration was used to keep the samples before analysis.With the McMaster technique, OPG and eggs per gram of feces were determined.

Preparation of blood hemolysates

From each cat, 6 per group, blood samples were taken from a jugular vein after fasting overnight at the end of the eighth month and the end of the experimental period. The plasma was separated by centrifugation (3500 g, 10 min) and stored at -70 ºC for analysis of biochemical parameters. Theremaining red blood cells (RBCs) were washed three times with isotonic NaCl (0.15 M) before use in the biochemical assay. The RBC hemolysates were prepared by diluting RBCs1:10 with ice-cold double distilled water, shaken vigorously to force hemolysis, and storedat -70 ºC (Fébel et al., 2008) .

Determination of enzymatic activity

The glutathioneperoxidase (GSHPx) activity was determined by spectrophotometric measurementof absorbance at 412 nm with cumene hydroperoxide as the substrate (Chiu et al., 1976) , while malondialdehyde (MDA) concentration in the serum was measured by the 2-thiobarbituric acid assay. Further on, superoxide-dismutase (SOD) activitywas determined by the spectrophotometric method based on the inhibition of adrenalinereduction to adrenochrome at pH 10.2 (L. M. Kostadinović et al., 2011) , andactivity of the glutathione-reductase (GR) was determined from the rateof NADPH oxidation and it was monitored by measuring the absorbance at 340 nm (Łukaszewicz-Hussain & Moniuszko-Jakoniuk, 2004) .

Statistical analysis

Significant effects were evaluated using ANOVA. Fisher's l.s.d. post-hoc multiple-range test was used to ascertain differences among groups. A significance level of P = 0.05 was used.Statistical analyses were conducted using the statistical software program Statistica 13 for Windows (StatSoft, Inc., Tulsa, OK, USA) .

RESULTS AND DISCUSSION

The addition of dried pomegranate fruit to the diet of domestic cats led to statisticallysignificant ($P<0.05$) differences in the number of oocysts per gram of feces (Figure 1) .A statistically significant difference in OPG between the cats was not observed at the beginning of the study ($P>0.05$) . Later, the level of contamination-by oocysts in the C group was systematically higher compared to the other groups ($P<0.05$) , while there was no significant difference between groupsP1 and P2.Finally, at the end of the experimental period, the lowest number of oocysts achievedwas with groupP1 (36998 oocysts per g of feces) followed by groups P2 (37510 oocysts per g of feces) and CR (38123 oocysts per g of feces) .

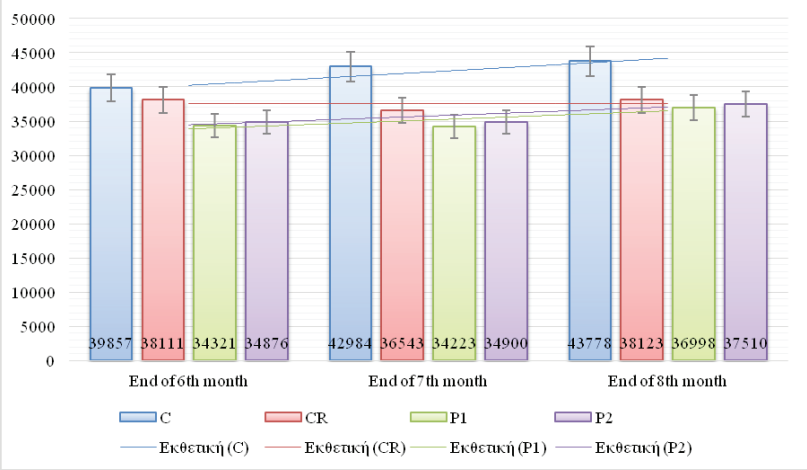

Figure 1. Coccidiosis oocyst counts in domestic cats' fecal samples (number of oocyst per gram of feces) .C - control group; CR - group received chemical coccidiostat robenidine; P1 - group received dried pomegranate fruit atthe concentration of 0.5%; P2 - group received dried pomegranate fruit atthe concentration of 1.0%.

In the present study the antioxidant defense system (both enzymatic and nonenzymatic) in blood hemolysates (Table 3) of the cats, was investigated.As can be seen from Table 3 and Figure 2, the content of glutathione (GSH) and malondialdehyde (MDA) in the blood hemolysates ofthe cats treated with dried pomegranate fruit showed a decrease in comparison with the control (C) and robenidine (CR) groups ($P<0.05$) , while glutathione peroxidase,superoxidedismutase, and glutathione reductase activities significantly increased in the P1group compared to the other diets.

Finally, it was noticed that the content of GSH in the blood samples of cats treated with dried pomegranate fruit showed a significant decrease ($P<0.05$) in comparisonwith the control and robenidine groups. The same tendency was recorded for MDAcontent. The GSHPx activityinsamples was the highest in the P1group andwas statistically significantly different ($P<0.05$) from the other groups. A similartendency was observed in the activities of SOD and GR in the blood samplesof cats, where the highest values were recorded in the group receiving 0.5% of dried pomegranate fruit with a significant difference ($P<0.05$) in comparison with the control androbenidine groups.

To our knowledge, there are no relevant studies in the literature on the effects of pomegranate fruit (*Punica granatum* L.) on cats in the fight against coccidiosis. Consequently, our findings will be compared with other published studies about coccidiosis in companion and food animals, respectively. The results obtained with other animal species with the addition of medicinal plants as alternatives to coccidiosis indicate numerous positive effects on animals' health (Hashemi & Davoodi, 2011; L. Kostadinović & Lević, 2018) .When it comes to cats, animal shelter kittens are often afflicted with intestinal coccidiosis, and its pathogenesis remains unclear (Dubey et al., 2009) .In mammals, coccidia regularly inhabits the intestinal tracts as obligate intracellular parasites (Mundt et al., 2006) .The most common species of coccidia in cats, *C. felis* and *C. rivolta*, appear to be influenced by age and living conditions (Dubey, 2018) .The rate of nat-

Table 3. The effect of dried pomegranate fruit on the antioxidative systems of cats' blood.

Parameters	C	CR	P1	P2
GSH (nmol/mg protein)	7.24±0.6^{a}	7.02±0.2^{a}	5.98±0.2^{b}	5.64±0.4^{b}
MDA (nmol/mL)	0.81±0.5^{b}	0.69±0.11^{a}	0.57±0.3^{a}	0.58±0.8^{a}
GSHPx (nmol/mg protein min)	8.51±0.4^{c}	9.31±0.1^{b}	9.66±0.1^{a}	9.54±0.2^{a}
SOD (nmol/mg protein min)	33.24±1.2^{c}	78.70±1.9^{b}	93.61±0.8^{a}	81.00±2.4^{b}
GR (nmol/mg protein min)	15.87±0.4^{c}	18.20±0.3^{b}	21.96±0.1^{a}	18.28±0.2^{b}

Means within a row followed by the different letters are significantly different ($P<0.05$) . C - control group; CR - group received chemical coccidiostat robenidine; P1 - group received dried pomegranate fruit in a concentration of 0.5%; P2 - group received dried pomegranate fruit in a concentration of 1.0%.

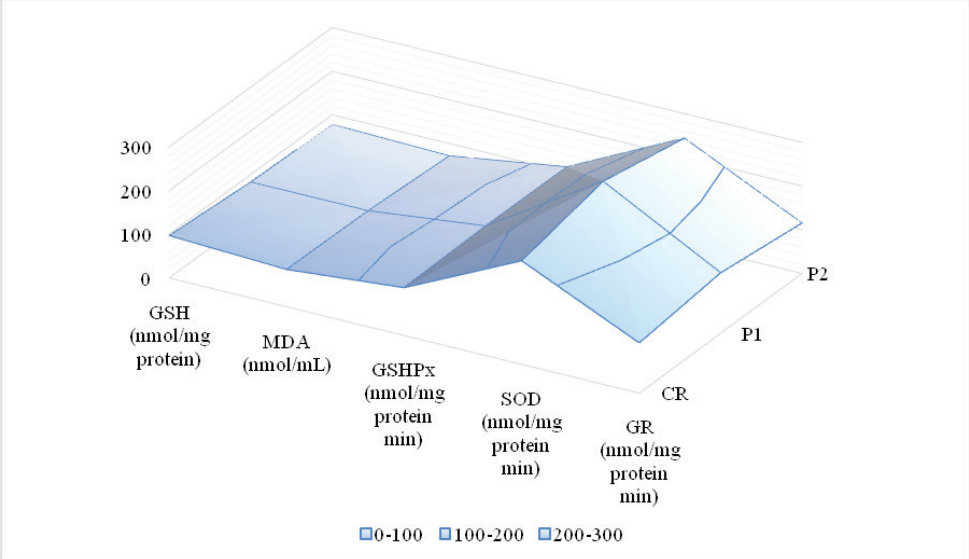

Figure 2. Positive differences of dried pomegranate fruit on the antioxidative systems of cats' blood compared to control group of cats, %.

ural infection is highest in neonates and kittens less than three months old. Synthetic drugs are common in the treatment of coccidiosis in cats. Different drugs such as sulfadimethoxine, toltrazuril, and ponazuril have been regularly used. Dosing recommendations have ranged from only one dose, one dose followed by a second dose five days later, to daily doses for three or five days, also depending on drug availability in certain countries and on the approval of drug usage by the Food and Drug Administration (FDA) .Resistance of coccidia to currently used coccidiostats to treat coccidiosis represents a serious problem in veterinary practice (Zhang et al., 2013) . The use of whole plants or plant extracts to treat coccidiosis is not a new approach (Abbas et al., 2012; Naidoo et al., 2008) . When searching for the best natural extract to treat coccidiosis, it is necessary to take into account that the extract needs to be at least partially soluble in lipids to penetrate the cellular membrane, because coccidia is located inside the cells. Besides, herbs and spices can protect the feed against oxidative deterioration during storage. This is a widely used practice in the pet food and human food industry (VapaTankosić et al., 2022) .Naidoo et al. (2008) studied the capacity of four African plants which would be appropriate to treat coccidiosis: leaves of *Combretum woodii*, leaves and stem of *Artemisia afra*, a whole plant, and seeds of *Vitis vinifera*. Extracts of all chosen plants improved the feed conversion in poultry to the same extent as coccidiostats toltrazuril used in cats' treatment against coccidiosis. The best effect was seen with *Tulbaghiaviolacea*, which also partially lowered the shedding of oocysts. Furthermore, Popović et al. (2017) have investigated wormwood (*Artemisia absinthium*) as apotential anticoccidial drug in the rabbit diet, as well as a growth promoter and antioxidant promoter, and obtained similar positive results as in our present study. In the experiment with tea tree essential oil Puvača et al. (2020) showed that this essential oil can be usefully used in the treatment of coccidiosis of mature laying hens.Nowadays, significantly higher attention is being paid to coccidia in companion and food animals than earlier (Pritt et al., 2012) .A variety of parasites have been successfully treated with medicinal herbs in a variety of studies, and enviable success has been achieved against a variety of parasites (Culliney, 2005) .The mostactive components in such as trans-sabinene, α- and β-thujon, trans-sabinylacetate, linalyl acetate, and cis-chrysanthenyl acetate, have been responsible for the anticoccidial activity of plants in animals (Ismail et al., 2021; Puvača et al., 2022) .

On the other side, in a chain reaction, antioxidant enzymes like glutathione peroxidase and superoxide dismutase can break down free radicals.As a substrate for glutathione peroxidase, glutathione serves as an antioxidant protector, reducing the acute toxic effects of xenobiotics and lipid peroxidation products (Agarwal et al., 2012; Ighodaro & Akinloye, 2018) .Lipid oxidation contributes to the deterioration of physiological functions such as growth, reproduction, and immunity, making individuals more susceptible to infectious diseases (Sultana et al., 2017) .To detect the level of oxidative stress in the body, malondialdehyde is used as a sensitive indicator (Beaulieu & Costantini, 2014) . As a simple way to assess lipid peroxidation in biological materials, determining malondialdehyde has attracted widespread interest.Thus, the increased activity of allantioxidant enzymes in our study may be attributed to the environmentalpathogens of *C. felis* in domestic cats. The results indicate that pomegranate fruit (*Punica granatum* L.) couldimprove antioxidant activities and reduce the damage caused by free radicals in domestic cats.

CONCLUSION

According to the results obtained, cats can benefit greatly from supplementing their diet with dried pomegranate fruit, without experiencing any adverse effects.Due to its ability to activate the enzymes responsible for antioxidant protection in animals, dried pomegranate fruit may be beneficial in terms of antioxidant protection in cats.In addition, supplementing cats' diets with dried pomegranate fruit reduced the number of oocysts per gram of feces significantly.It is important to carry out further and more detailed studies to prove the anticoccidial and antioxidant properties of dried pomegranate fruit in cats' diets.

CONFLICT OF INTEREST

The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

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