Original Article

Safety study of polyunsaturated fatty acids extracted from New Zealand green lipped mussel and krill on hematology and blood chemistry profiles of healthy

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Abstract

New Zealand green-lipped mussel (*Perna canaliculus*) is a shellfish with a green shell containing many types of fatty acids, especially polyunsaturated fatty acids (PUFAs). The aim of the present study was to investigate the safety of dietary PUFAs supplementation on blood hematology, chemistry and coagulogram pancreatic enzymes in dogs and cats. Twelve healthy beagle dogs and healthy mature cats were used in these studies. Complete randomized block and triplicate 4×4 Latin square designs were used for these dog and cat safety studies, respectively. Experimental diets composed of sham control (0X) and 3 treatment groups, including 1X, 3X, and 10X of PUFAs capsules containing extracted mussel (60% or 30 mg/capsule) and krill (40% or 20 mg/capsule), were given consecutively for 56 days. Blood samples were collected every 28 days for hematology, chemistry and coagulogram, and pancreatic enzyme analysis. Hematology and blood chemistry results were in normal ranges and no animals appeared to reveal any abnormal signs. Dogs fed 3X had significantly (P<0.05) higher in creatine compared to 0X and 1X at D28. Likewise, there were statistically significant differences in the time of cat's ALT and lipase. Group of cats fed 10X significant reduction in ALT at D56 compared to D0 (P<0.01) and D28 (P<0.05). In the group of cats fed at 1X, there was a significant within-group difference between the values of ALT levels for baseline (D0) and D56 (P<0.05). At D56, cats in all groups had a significantly higher amount of lipase levels than those at D0 (P<0.05). The prothrombin time of cats in the 0X and 10X groups showed a significant within-group difference between the values for D0 and D56 (P<0.05). PLI of dogs and cats showed good pancreatic function based on precision accuracy. Overall, EAB-277 contains PUFAs and appears to be safe and improves liver function, especially in cats receiving 10 X administration of EAB-277.

Keywords: blood chemistry, cat, dog, hematology, New Zealand green lipped mussel, PUFA

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Introduction

Dietary lipids, also known as fats and oils (solid and liquid stages at room temperature, respectively), are a type of nutrient that serves as a source of energy, essential fatty acids, and fat-soluble vitamins in humans and animals. The major components of lipids are fatty acids, glycolipids, lipoproteins, phospholipids, sphingolipids, steroids (cholesterol), and triglycerides. Fatty acids are carboxylic acids with aliphatic chains which can be classified by many methods. One method is by saturation versus unsaturation. Saturated fatty acids have no carbon double bonds (C=C) with various numbers of carbons of aliphatic tails, for example, capric acid (10:0), lauric acid (12:0), stearic acid (18:0), etc. Unsaturated fatty acids have one or more carbon double bonds which are called mono or polyunsaturated fatty acids (PUFAs), respectively. On the carboxylic end, the last carbon of the chain is named omega. The position of the first double bond is named by number of carbons from the methyl end (CH3) (omega end) to the first carbon in the double bond closest to the methyl end. For example, omega-9 (n-9) fatty acids where the 1st double bond is located between the 9th and 10th carbon atoms from the omega end. PUFAs are classified into many groups, omega-3 (n-3) fatty acid and omega-6 (n-6) fatty acid are the two recognized families in animal health and nutrition. Examples of n-6 fatty acids are linoleic acid (LA, 18:2), gamma-linolenic acid (GLA, 18:3), calendic acid (18:3), eicosadienoic acid (20:2), arachidonic acid (AA, 20:4) etc. Only LA and AA are considered essential fatty acids for all life stages of mammals; the fatty acids those animals must consume from foods since their body is not capable of synthesizing or synthesizing but do not meet their requirements (Le et al., 2009). On the opposite, nonessential fatty acids refer to any of the various fatty acids that are required for normal health and can be synthesized within the body or derived in the body through various biochemical reactions. There are also conditioning essential fatty acids, the fatty acids those body required under specific circumstances such as aging and any abnormal health condition. The n-3 fatty acids, alpha-linolenic acids (ALA), docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) are examples of the conditioning essential fatty acids (Kaur *et al*., 2014).

Well known sources of n-3 fatty acids are deep sea fish. Although an interesting source of n-3 fatty acids is New Zealand green-lipped mussel, a marine mollusc *Perna canaliculus* endemic to New Zealand (Wakimoto *et al*., 2011). This mollusc is available for commercial use as either whole-frozen, live, fresh, or meat-only products. Furthermore, for the use as nutraceutical, it can be processed to powders or extracted products. In this current study, to preserve the functional properties such as anti-inflammation, the freeze-dried and patented superficial carbon dioxide method with no heat process has been used to extract lipids from the New Zealand green-lipped mussel (Wolyniak *et al*., 2005). As a result, this lipid extract is composed of a range of PLs, monounsaturated, and PUFAs. These bioactive compounds of lipid fraction possibly act as anti-inflammatory substances in combination with intestinal microbiota activity (Coulson *et al*., 2015).

Another source of n-3 fatty acids that have received attention is krill oil. Krill oil is a kind of novel food ingredient extracted from Antarctic krill, Euphausia superba. It is a unique lipid containing mainly highly concentrated phospholipids (PLs) associated with EPA and DHA. It also comprises of some bioactive components such as astaxanthin, sterols, tocopherols, vitamin A, flavonoids, and minerals (Xie *et al*., 2019). In vitro and in vivo animal studies have demonstrated the anti-inflammatory property of krill oil (Bonaterra *et al*., 2017; Costanzo *et al*., 2018). Other properties which had been studied are cardiovascular disease prevention, anti-obesity, anti-diabetic, neuroprotective, and anti-cancer effects (Xie *et al*., 2019).

A safety study is a study designed to provide information on the safety or adverse reaction of investigational active ingredients in the intended species under the proposed conditions of use and Veterinary International Conference on Harmonization, a trilateral (EU-Japan-USA) program aimed at harmonizing technical requirements for veterinary product registration (VICH Guideline, 2009). Since the n-3 fatty acids are nutrients with anti-inflammatory properties, they are used for inflammatory management of organ systems such as joint, skin, heart, kidney, and gastrointestinal tract (Treschow *et al*., 2007). These fatty acids are known as nutraceuticals, foods or some parts of foods that provide medical or health benefits including the prevention and/or treatment of diseases (Kalra, 2003; Mickleborough *et al*., 2015). Target dose of n-3 fatty acids from fish oil can vary quite widely for various conditions but often range between 50 to 220 mg/kg body weight (Lenox and Bauer, 2013). The dose of lipid extract from green-lipped mussel oil is much smaller as they provide only 50 mg of lipid extract per capsule. The recommended dose from the manufacturer for the lipid extract is 200 mg/day (Coulson *et al*., 2015). Whitehouse *et al*. (1997) reported a lipid-rich extract of New Zealand green-lipped mussel was non-gastrotoxic in disease-stressed rat at 300 mg/kg po and did not seem to affect platelet aggregation in human and rat. Adverse effects, if found, possibly relate not only to dosage used in animals, but also ratio of n-6: n-3 fatty acids within the animal. The dietary and blood n-6: n-3 fatty acid ratio is considered important, as a high n-6: n-3 ratio is thought to be proinflammatory (Gonzalez-Becerra *et al*., 2023). Usually, the concentration of n-6 fatty acids found in both humans and animals is greater than n-3 fatty acids due to dietary imbalances, and this is seen in both normal and abnormal conditions (Scaioli *et al*., 2017).

In veterinarian practices, aging and animal disorders with any inflamed or painful conditions can be supplemented with n-3 fatty acids continuously for a long period of time to improve or maintain the quality of life. Veterinarian's concerns and frequency client complaints however are weight gain, pancreatic function (pancreatitis), and lipid metabolism. Lenox and Bauer (2013) have reported likely adverse effects, abnormal signs or symptoms observed with use of n-3 fatty acids, especially DHA, and EPA, in dogs and cats e.g., altering platelet, gastrointestinal tract, pancreatic and immune functions, as well as decreasing wound

healing, increasing lipid peroxidation, and increased body weight gain.

While n-3 fatty acids play a significant role as anti-inflammation therapy in veterinarian practice, there is limited reports referring directly to adverse reaction study in cats. The aim of the present study was to investigate the safety of an extracted lipid preparation with New Zealand green-lipped mussel and krill on blood serum profile and pancreatic enzymes in dogs and cats.

Materials and Methods

Animal: Animal and research protocols were reviewed and approved by the Veterinary Faculty-Animal Care and Use Committee, Chulalongkorn university, Bangkok, Thailand. Experiment procedure was based on OECD Guidelines (1998). Healthy beagle dogs age between 1 to 3 years with an initial body weight range of 10-12 kg and healthy mature cats age between 8 months to 3 years with an initial body weight range of 3-4.5 kg were obtained from dog and cat farms in Thailand. All animals had body condition scores of 2 to 3 (on a 5-point scale) when entered the study. Health status of all animals was evaluated by physical examination and blood analyses. Prior to studying, all animals were bathed and treated for external parasites. Anti-helminthic drug and vaccination against distemper, parvovirus, hepatitis, and rabies were applied to the dogs and FeLV, feline combo (FVRPC; Feline distemper, Feline rhinotracheitis, and Feline calicivirus) and rabies were applied to the cats. Dogs and cats were housed individually in cages size W×L×H of 100× 120×100 cm3 and W×L×H of 100×150×180 cm3, respectively, under ambient temperature and natural light cycle. The Acclimatization period lasts for 14 days. During this period, animal caretakers and investigators were carefully monitored to ensure that the animals were free of obvious disease and capable of adapting to animal house life. Standard commercial dry dog and cat foods as recommended by the Association of American Feed Control Official (AAFCO) were given twice a day to the dogs and once a day to the cats. The amount to feed was calculated to meet an average daily energy requirement of 132×BW0.75 kcal/day in dogs and 100 kcal/kg0.67 in cats. Fresh-clean water was supplied ad libitum. All animals were provided with a comfortable, clean, and dry place to rest. Cages were cleaned once or twice a day. Dogs were released to run freely or exercise in the play area (at least 30 min/day).

Dietary treatments: Complete randomized block and triplicate 4×4 Latin square designs were used for dog and cat safety studies, respectively. Independent variable, PUFAs supplements (Antinol®, EAB 277TM) were provided by VetZ PetZ Antinol® Thailand. This PUFAs was a patent nutraceutical containing a mixture of marine oils fractionated from the New Zealand green-lipped mussel and krill. It was a mixture of the pure supercritical fluid extracted mussel (60% or 30 mg/capsule) and krill (40% or 20 mg/capsule) oils with an olive oil carrier that was used to assist encapsulation. The active ingredients compose of 91 fatty acids, including n-3 fatty acids, DHA, EPA, and eicosatetraenoic acid (ETA) from New Zealand green-lipped mussel blending with phospholipids from krill (Pharmalink International Co., Ltd.,). The capsules of PUFAs were orally administered once a day as soft gel capsules in the morning. Animals of sham group received empty gelatin capsules equal in number to those given to the high-dose group for each study. In the complete randomized block design, sixty beagle dogs were blocked (by time) and randomly divided into 4 groups (sham control and 3 treatment groups). The recommended maximum label dose of the n-3 fatty acid supplement is 2 soft gel capsules for dogs up to 23 kgs once a day is the reference dose (equivalent to 1X). The three treatment groups were:1) 1X (2 capsules/dog/day); 2) 3X (6 capsules/dog/day); and 3) 10X (20 capsules/dog/day) given consecutively for 56 days. For the triplicate 4×4 Latin square designs of cat safety study, each period composed of adaptation or washout time for 28 days follow by 56 days of sample collection. The animals were randomly assigned to 4 treatment groups, the same for the dogs. However, the doses for the cats were 1) 1X (1 capsule/cat/day);2) 3X (3 capsules/cat/day); and 3) 10X (10 capsules/cat/day) given consecutively for 56 days. Every morning during the safety study, general physical examination of the animals such as ocular, nervous, skin, and musculoskeletal system, signs of illness and behavior change were performed and recorded if any abnormality was found. Stool characters, food and water consumption were also observed.

Blood sample collection and analysis: Blood samples were collected every 28 days, as on day 0 (D0), day 28 (D28), and day 56 (D56), for a total of 3 times. Five and two milliliters of blood were collected from the dogs and the cats, respectively. These samples were collected by aseptic venipuncture and subjected into 3 fractions. The blood first fraction was put in tubes containing potassium ethylene diamine tetra-acetic acid (EDTA) for complete blood count (CBC) analysis. The blood second fraction was put in tubes with silica dioxide (a clot activator) for blood chemistry analysis [alanine aminotransferase (ALT), aspartate aminotransferase (AST), creatinine, blood urea nitrogen (BUN), alkaline phosphatase (ALP), albumin, total protein, cholesterol, triglyceride, amylase, and lipase]. The third fraction was put in tubes with sodium citrate for determination of blood coagulation profile including thrombin time (TT), prothrombin (PT), and activated partial thromboplastin (aPTT). Blood smears were performed to analyze the blood parasite. Routinely, place a drop of blood sample on a clean glass slide. Smear very thinly onto a clean slide and cover with coverslip. Use less light on the microscope for examination. For dogs, electrolytes (sodium, potassium, and chloride) at D0, D28, and D56 were also determined. Pancreatic Lipase Immunoreactivity (PLI) (Vcheck cPL and fPL, Bionote) were used for evaluation of pancreatic function at D0, D28 and D56 for the dogs and D0 and D56 for the cats. All blood fractions were kept cool at 4°C and transported to the standard central lab for further analysis.

Statistical analysis: The data was assessed for normality using the Kolmogorov–Smirnov test. Since the Kolmogorov–Smirnov showed significant differences within the study period for almost variables, we decided to use nonparametric tests for all variables. Hence, data were expressed as descriptive statistics and interquartile range (IQR). To determine an appropriate sample size for present study, a post-hoc power analysis of previous study (Jamikorn and Yibchok-anun, 2014) was conducted using Minitab 17 (Minitab Statistical Software for Windows; Minitab, State College, Pennsylvania, USA). Wilcoxon non-parametric test was used to analyze the effect of the treatment and time on blood parameters. Kruskal-Wallis test was used to analyze the differences between treatment. For those that showed statistically significant differences over time the Friedman test was computed for comparison of the individual time points within the group. Statistical analysis was conducted with SAS 9.4 (SAS Institute, NC, USA) and P-value ≤ 0.05 was considered to be statistically significant. Box plots were created using GraphPad Prism (v10.2 GraphPad Software, La Jolla, CA, USA). For diagnosis of pancreatitis, this safety study used Vcheck cPL and fPL test by Bionote, USA Inc. Regarding diagnostic guidelines of Vcheck cPL, PLI testing for value as <200, 200-400, and >400 ng/mL are considered normal, equivocal, and pancreatitis, respectively. Diagnostic guidelines of Vcheck fPL indicating value as <3.5, 3.6-5.3, and >5.4 ng/mL are considered normal, equivocal, and pancreatitis, respectively. Polynomial contrast was conducted to evaluate the potential of collinearity between blood chemistry values and dose treatments.

Results

Animals used in the current study composed of twenty healthy mature beagle dogs (12 female and 8 male), body weight 10.83±2.08 kg (Table 1) and twelve healthy mature domestic cats (8 female and 4 male), body weight 3.25±0.50 kg (Table 2). All animals had body condition scores of 2 to 3 (on a 5-point scale) at the beginning of study. Physical examination of all studied population composing of body temperature (100.00-103.80°F), capillary refill time (CRT, 1 to 2 second) and fecal score (2 to 4) (on a 5-point scale) were all normal. No animals had neither constipation nor diarrhea. Studied dogs and cats maintained their initial body condition without significant weight loss or gain. No abnormalities and illness sign of any system (ocular, nervous, musculoskeletal and integumentary systems) were observed. Blood parasites were not found for both dogs and cats. Both dogs and cats of all four treatment groups preferred consumption of the PUFAs supplement capsules, even the empty ones. No association was detected between the dose treatments and the dependent variables including blood hematology, blood chemistry, blood clotting factors, and PLI values.

Blood hematology and chemistry analyses: Before the study, hematology and blood chemistry of both dogs and cats in each of the four treatment groups were not significantly different. Hematology and blood chemistry of 0X, 1X, 3X, and 10X of D0, D28, and D56 are shown as IQR.

No significant difference was observed between treatment groups (P>0.05) nor date of sample collection (P>0.05) and determination for blood hematology parameters for both dogs (Fig 1.) and cats (Fig. 2). There was also no difference between treatment groups (P>0.05) nor date of sample collection (P>0.05) for serum chemistry parameters for both dogs (Fig. 3) and cats (Fig. 4) except for dog's creatine at D28 when the level of creatine in 3X was significantly higher than 0X and 1X (P<0.05). Likewise, there were statistically significant differences in the time of cat's ALT and lipase. Group of cats fed 10X significant reduction in ALT at D56 compared to D0 (P<0.01) and D28 (P<0.05). In the group of cats fed at 1X, there was a significant within-group difference between the mean values of ALT levels for baseline (D0) and D56 (P<0.05). At D56, cat in all groups had significantly higher amount of lipase level than those at D0 (P<0.05).

Blood coagulation factors analyze: Dependent variables for blood coagulation factors including thrombin time (TT), prothrombin time (PT) and activated partial thromboplastin time (aPTT) are shown in Figure 5. and Figure 6. for dogs and cats, respectively. Blood values of D0 were considered as baseline for the current study dog and cat colonies. Data are expressed as IQR. For dogs, the mean values of the TT were greater than the normal range of Chulalongkorn university lab. There were no significant differences between-group differences in TT, PT and aPTT in dogs (P>0.05). There was also no significant within-group or between-group differences in TT and aPTT in cats (P>0.05) except PT which the 0X and 10X group showed a significant within-group difference between the mean values for D0 and D56 (P<0.05).

Blood analyses for pancreatic function: PLI values of all treatment groups for both dogs and cats demonstrate no pancreatitis status at the beginning, during the study, and the finishing times. Numeric results of PLI determined on D0, D28 and D56 for dogs and on D0 and D56 for cats are shown in Table 3 and Table 4, respectively. Almost all dogs had PLI values in the normal status. Only 2 out of 13 dogs in the 3X and 10X groups on D56 were classified as equivocal status. However, no animals appeared to reveal any abnormal signs. Aside from the dogs, all cats in every treatment group of the current study had normal PLI.

Physical examination: The results of physical examination in dogs are shown in Figure 7. There were no significant within-group or between-group differences in body weight, body temperature, capillary refill time and fecal score in dogs (P>0.05).

Table 1 Descriptive data on blood hematology, morphology, chemistry, coagulogram and physical examination of experimental dogs. (n=60).

Parameter (s)	Unit	Merck (2020) Range	Range
Blood hematology			
RBC	($x10^6$ cells/mm^3)	4.95-7.87	4.90-8.40
Hemoglobin	g/dl	11.9-18.9	10.10-18.50
Hematocrit	%	35-57	30.00-52.00
WBC	10^6 cell/mm^3	5.0-14.1	5.50-18.90
Neutrophils	10^6 cell/mm^3 (%)	3-12 (58-85)	51.00-87.00
Bands	10^6 cell/mm^3 (%)	0-0.1 (0-0.5)	0.00-1.00
Eosinophils	10^6 cell/mm^3 (%)	0-3 (0-9)	0.00-9.00
Lymphocytes	10^6 cell/mm^3 (%)	0.5-3 (8-21)	10.00-30.00
Monocytes	10^6 cell/mm^3 (%)	1-2 (2-10)	1.00-7.00
Blood morphology			
Indices MCV	fL	66-77	56.40-78.00
MCH	pg	21.0-26.2	19.80-25.90
MCHC	g/dl	32.0-36.3	32.40-39.20
RDW	%	11-14	12.00-17.50
Platelet count	10^3 cell/mm^3	211-621	168.00-684.00
Serum chemistry			
ALT (SGPT)	U/L	10-109	20.00-103.00
AST (SGOT)	U/L	18-56	24.00-89.00
Creatinine	mg%	0.5-1.5	0.70-1.20
BUN	mg%	8-28	7.00-28.00
Alk.P/tase	U/L	1-114	19.00-122.00
Glucose	mg/dl	76-119	76.00-129.00
Albumin	g/dl	2.3-3.1	2.60-3.80
Total Protein	g/dl	5.4-7.5	5.20-7.90
Cholesterol	mg/dl	135-278	120.00-279.00
Triglyceride	mg%		
Amylase	U/L	322-1310	345.00-1330.00
Lipase	U/L	15-228	23.00-77.00
Sodium	mEq/L	142-152	134.10-159.00
Potassium	mEq/L	3.9-5.1	3.93-6.00
Chloride	mEq/L	110-124	101.90-130.00
Coagulogram			
Thrombin Time (TT)	Sec(s)	10-21	11.50-30.60
Prothrombin Time (PT)	Sec(s)	6.7-16.6	5.80-15.60
Activated Partial Thromboplastin (aPTT)	Sec(s)	15-20	10.00-21.80
Physical examination			
Body weight	kg		7.00-15.10
Rectal temperature	°F		100.00-103.80
Capillary refill time	Sec(s)		1.00-2.00
Fecal score		1-5	2.00-4.00

Results are presented as range. RBC, red blood cell count; WBC = white blood cell count; MCV, Mean Corpuscular Volume; MCH, Mean Corpuscular Hemoglobin; MCHC, Mean Corpuscular Hemoglobin Concentration; RDW, Red Cell Distribution Width; ALT = Alanine transaminase; AST = aspartate aminotransferase; BUN = blood urea nitrogen; ALK = Alkaline phosphatase.

Table 2 Descriptive data on blood hematology, morphology, chemistry, coagulogram, and physical examination of experimental cats. (n=36).

Parameter (s)	Unit	Merck (2020) Range	Range
Blood hematology			
RBC	($x10^6$ cells/mm^3)	5-10	6.90-14.30
Hemoglobin	g/dl	10-15	8.60-16.20
Hematocrit	%	30-45	26.00-49.00
WBC	10^6 cell/mm^3	5.5-19	7.60-19.40
Neutrophils	10^6 cell/mm^3 (%)	4-10 (35-75)	4.01-14.36
Bands	10^6 cell/mm^3 (%)	0-3 (0-2)	0.00-0.19
Eosinophils	10^6 cell/mm^3 (%)	0-5 (0-4)	0.00-2.27
Lymphocytes	10^6 cell/mm^3 (%)	3-5 (27-36)	1.14-7.67
Monocytes	10^6 cell/mm^3 (%)	0-6 (0-5)	0.08-0.60
Blood morphology			
Indices MCV	fL	39-55	30.00-49.70
MCH	pg	12.5-17.5	11.20-15.80
MCHC	g/dl	30-36	28.70-39.70
RDW	%	300-600	214.00-642.00
Platelet count	10^3 cell/mm^3	14-18	15.70-18.80
Serum chemistry			
ALT (SGPT)	U/L	25-97	29.00-97.00
AST (SGOT)	U/L	10-60	21.00-61.00
Creatinine	mg%	0.9-2.2	0.70-1.80
BUN	mg%	19-34	11.00-32.00
Alk.P/tase	U/L	10-80	10.00-85.00
Glucose	mg/dl		
Albumin	g/dl		
Total Protein	g/dl		
Cholesterol	mg/dl	70-130	18.00-130.00
Triglyceride	mg%	8-105	9-109
Amylase	U/L	550-1,460	346-1,458
Lipase	U/L	0-600	13-108
Sodium	mEq/L		
Potassium	mEq/L		
Chloride	mEq/L		
Coagulogram			
Thrombin Time (TT)	Sec(s)		15.80-28.30
Prothrombin Time (PT)	Sec(s)		8.30-26.80
Activated Partial Thromboplastin (aPTT)	Sec(s)		11.40-26.30

Results are presented as range. RBC, red blood cell count; WBC = white blood cell count; MCV, Mean Corpuscular Volume; MCH, Mean Corpuscular Hemoglobin; MCHC, Mean Corpuscular Hemoglobin Concentration; RDW, Red Cell Distribution Width; ALT = Alanine transaminase; AST = aspartate aminotransferase; BUN = blood urea nitrogen; ALK = Alkaline phosphatase.

Table 3 Number of the dogs in each treatment groups subjected by PLI values into 3 groups as indicated by the analyzer leaflet; <=200 ng/mL = Normal, 200-400 ng/mL = Equivocal, and >=400 ng/mL= Consistent with pancreatitis.

	0X[1]			1X[1]			3X[2]			10X[2]		
	D0	D28	D56	D0	D28	D56	D0	D28	D56	D0	D28	D56
Normal	14	14	14	14	14	14	13	13	11	13	13	11
Equivocal	0	0	0	0	0	0	0	0	2	0	0	2
Pancreatitis	0	0	0	0	0	0	0	0	0	0	0	0

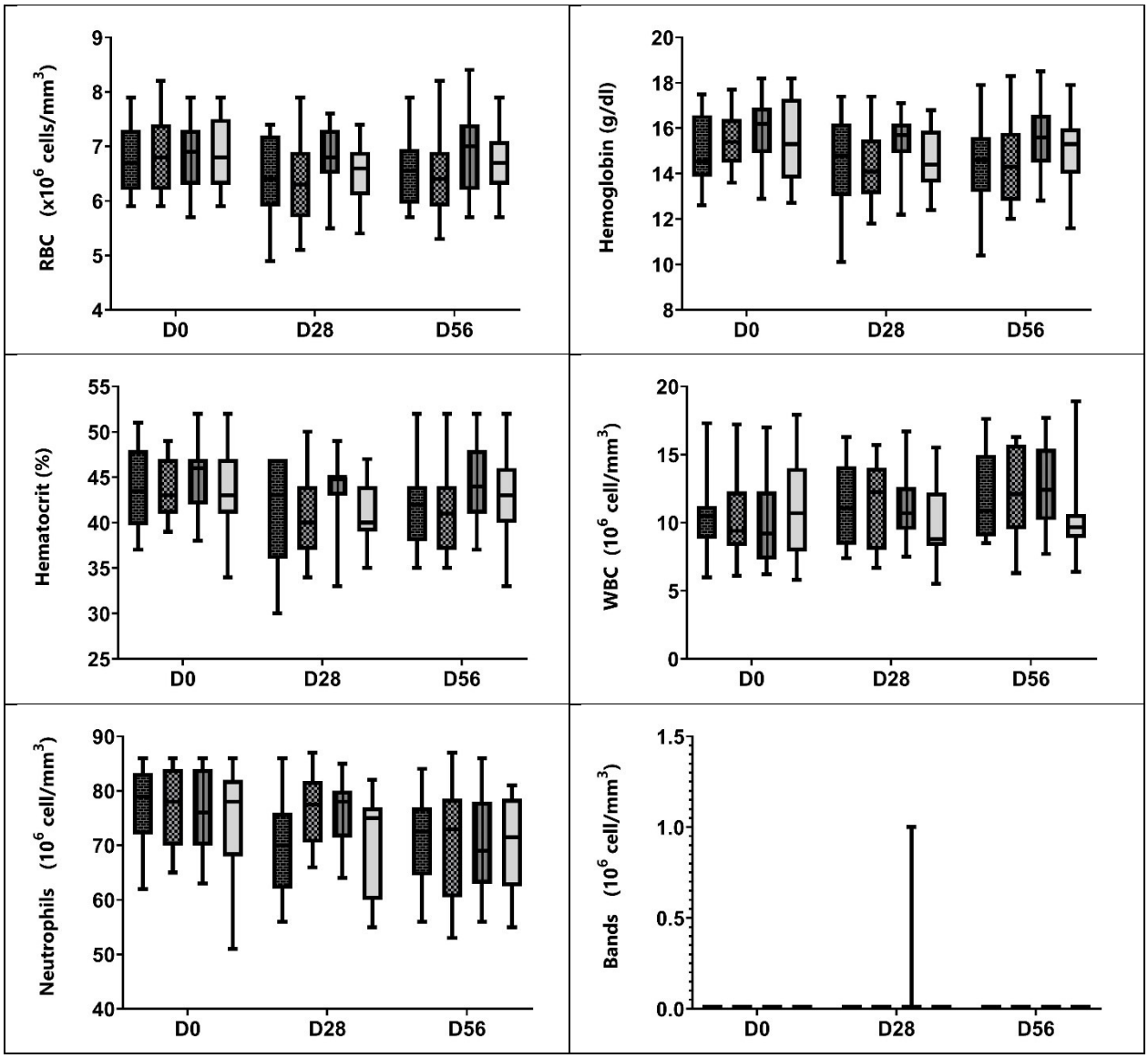
[1]n = 14

[2]n = 13

Table 4 Number of the cats in each treatment group subjected by PLI values into 3 groups as indicated by the analyzer leaflet; ≤ 3.5 ng/mL = Normal, 3.6-5.3 ng/ml = Equivocal, and ≥ 5.4 ng/mL = Consistent with pancreatitis.

	0X[1]		1X[1]		3X[1]		10X[1]	
	D0	D56	D0	D56	D0	D56	D0	D56
Normal	12	12	12	12	12	12	12	12
Equivocal	0	0	0	0	0	0	0	0
Pancreatitis	0	0	0	0	0	0	0	0

[1]n = 12



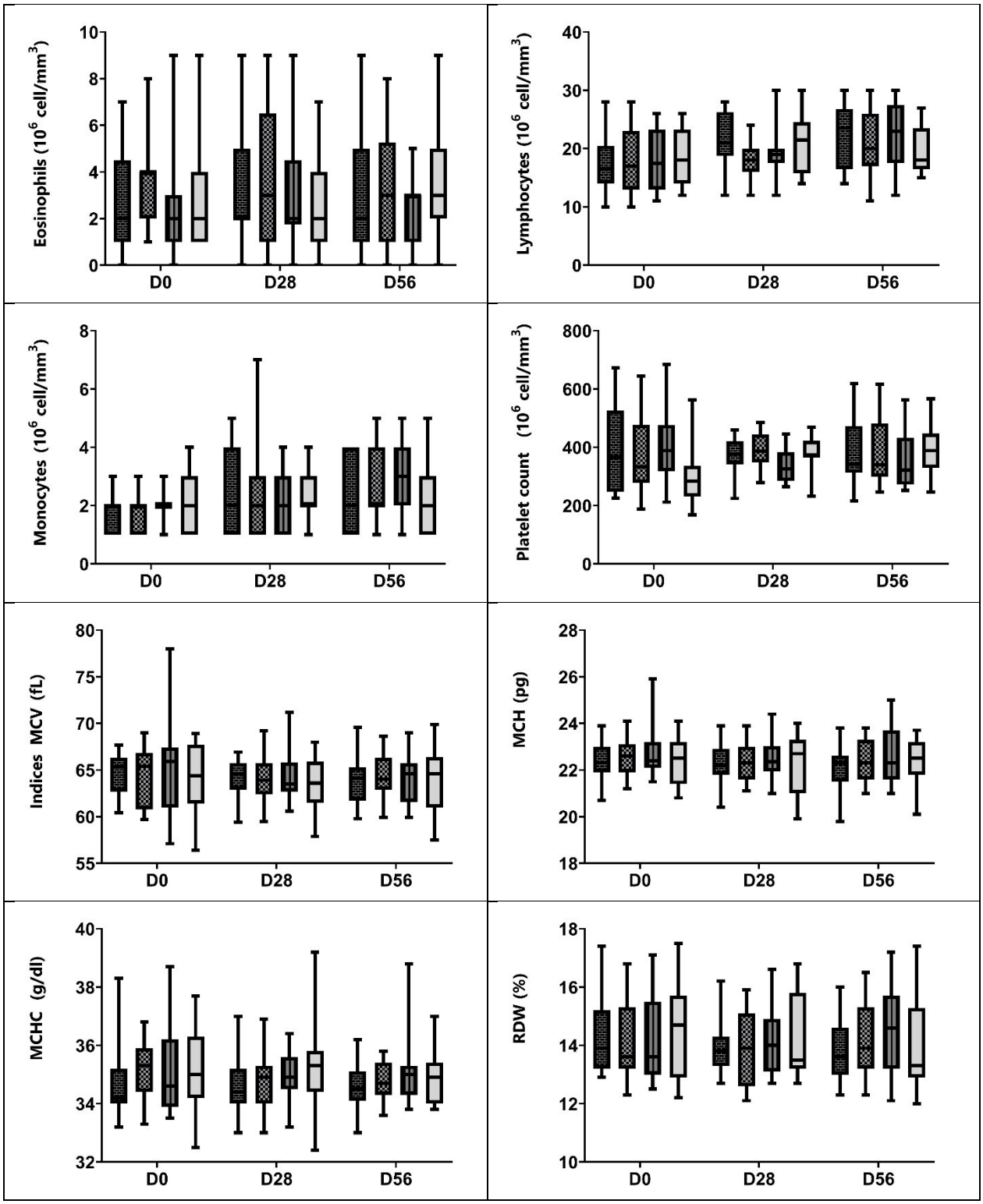
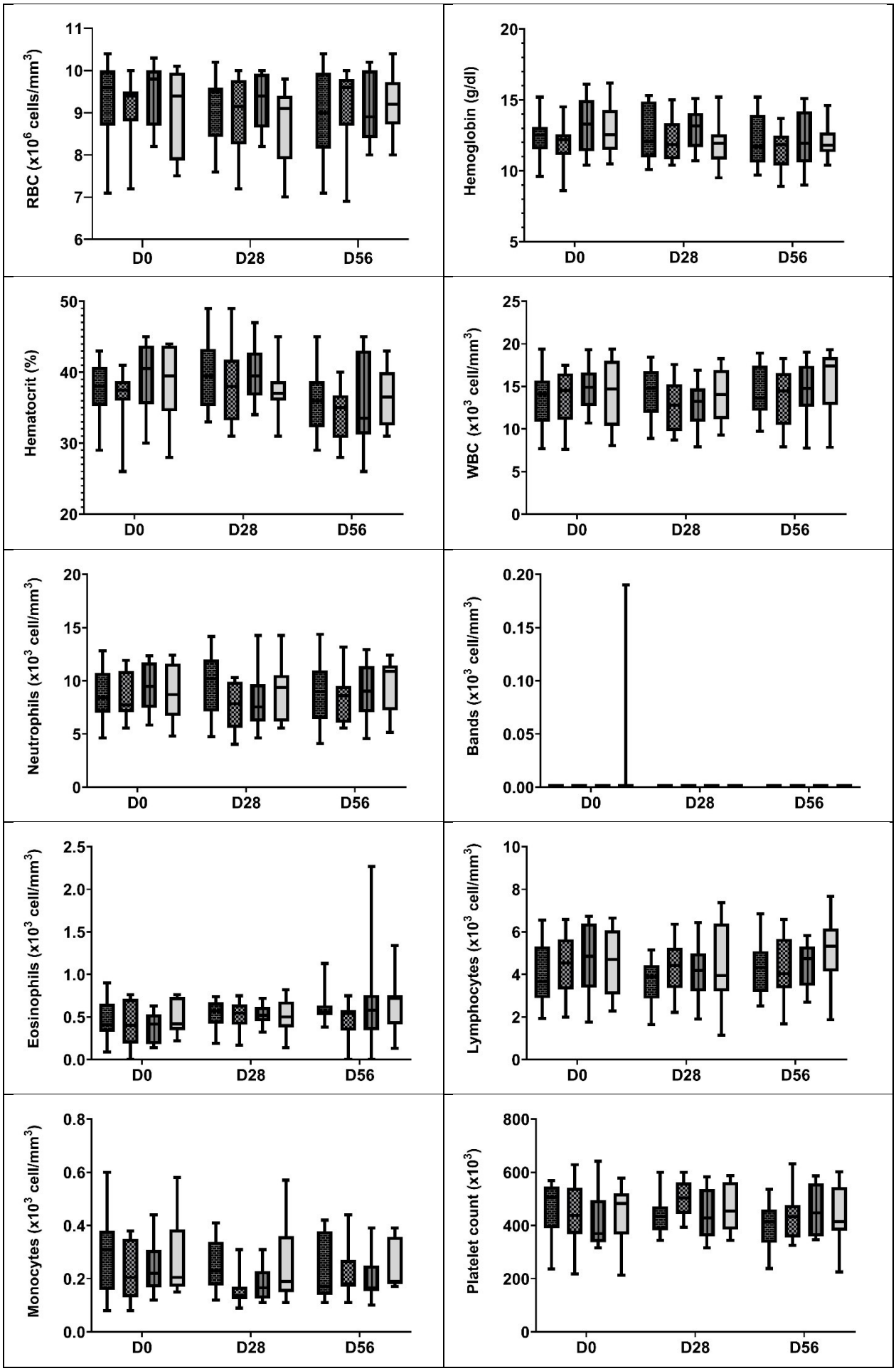


Figure 1 Effect of different levels of EAB-277® administration on blood hematology at D0, D28 and D56 in dogs. (= 0X; = 1X; = 3X and = 10X). All values are presented as IQR. Upper and lower boxes represent the 25th and 75th percentiles (lower whiskers = minimum, upper whiskers = maximum values) and the line represents the median. Values were significantly different between the groups or time (* = P<0.05; ** = P<0.01).


RBC (x10^6 cells/mm^3)
Hemoglobin (g/dl)
Hematocrit (%)
WBC (x10^3 cell/mm^3)
Neutrophils (x10^3 cell/mm^3)
Bands (x10^3 cell/mm^3)
Eosinophils (x10^3 cell/mm^3)
Lymphocytes (x10^3 cell/mm^3)
Monocytes (x10^3 cell/mm^3)
Platelet count (x10^3)
D0
D28
D56

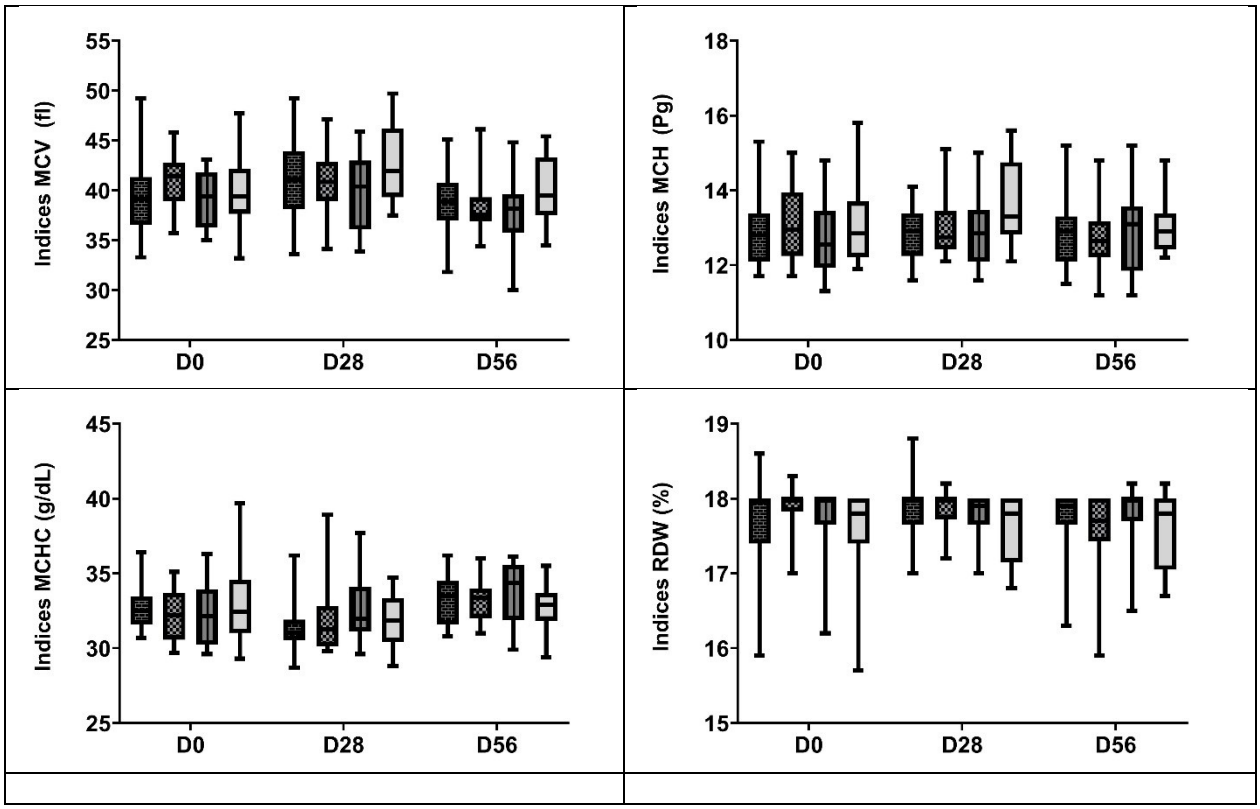
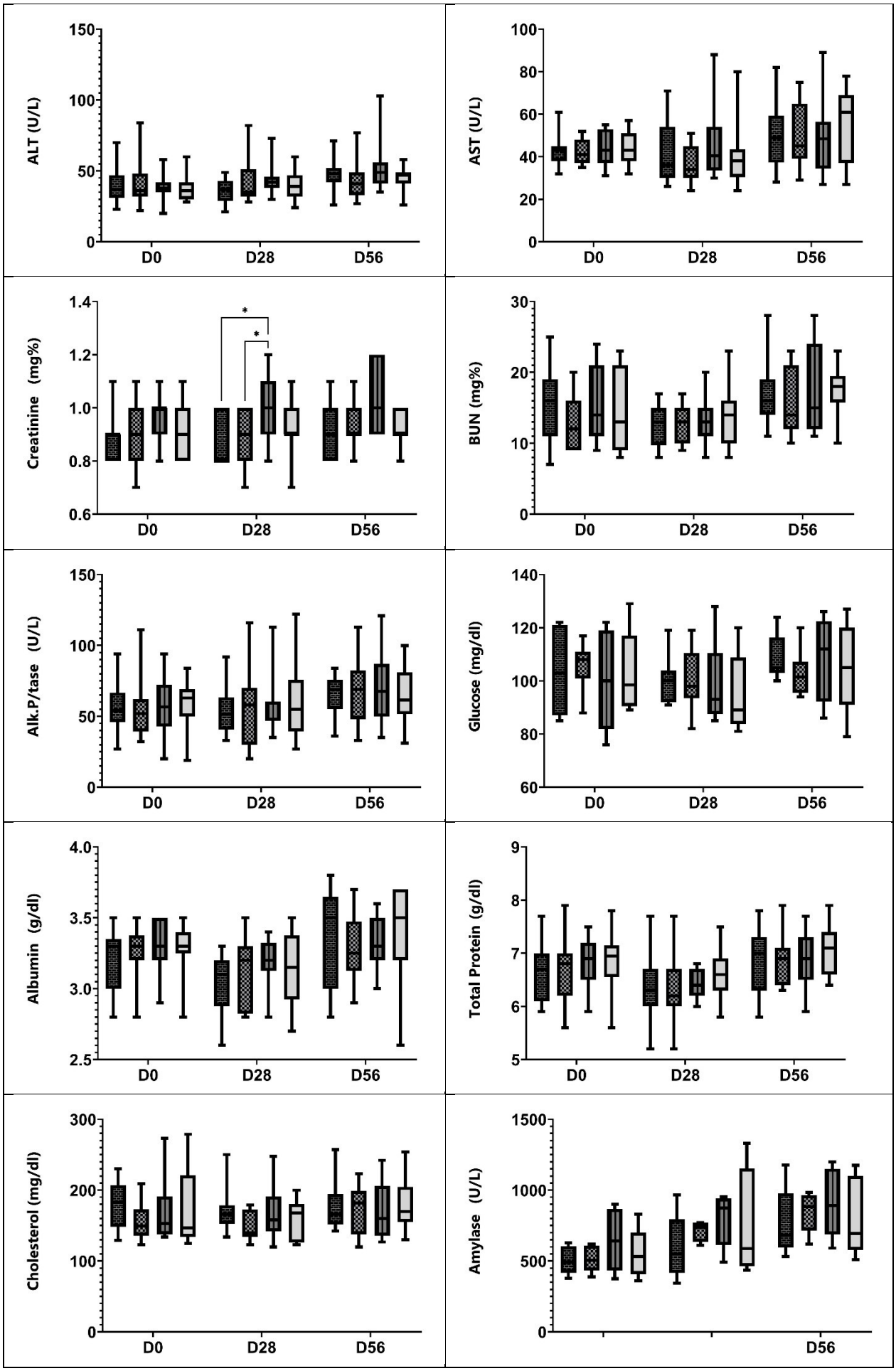


Figure 2 Effect of different levels of EAB-277® administration on blood hematology at D0, D28 and D56 in cats. (= 0X; = 1X; = 3X and = 10X). All values are presented as IQR. Upper and lower boxes represent the 25th and 75th percentiles (lower whiskers = minimum, upper whiskers = maximum values) and the line represents the median. Values were significantly different between the groups or time (* = $P<0.05$; ** = $P<0.01$).


ALT (U/L)
AST (U/L)
Creatinine (mg%)
BUN (mg%)
Alk.P/tase (U/L)
Glucose (mg/dl)
Albumin (g/dl)
Total Protein (g/dl)
Cholesterol (mg/dl)
Amylase (U/L)
D0
D28
D56
*
*

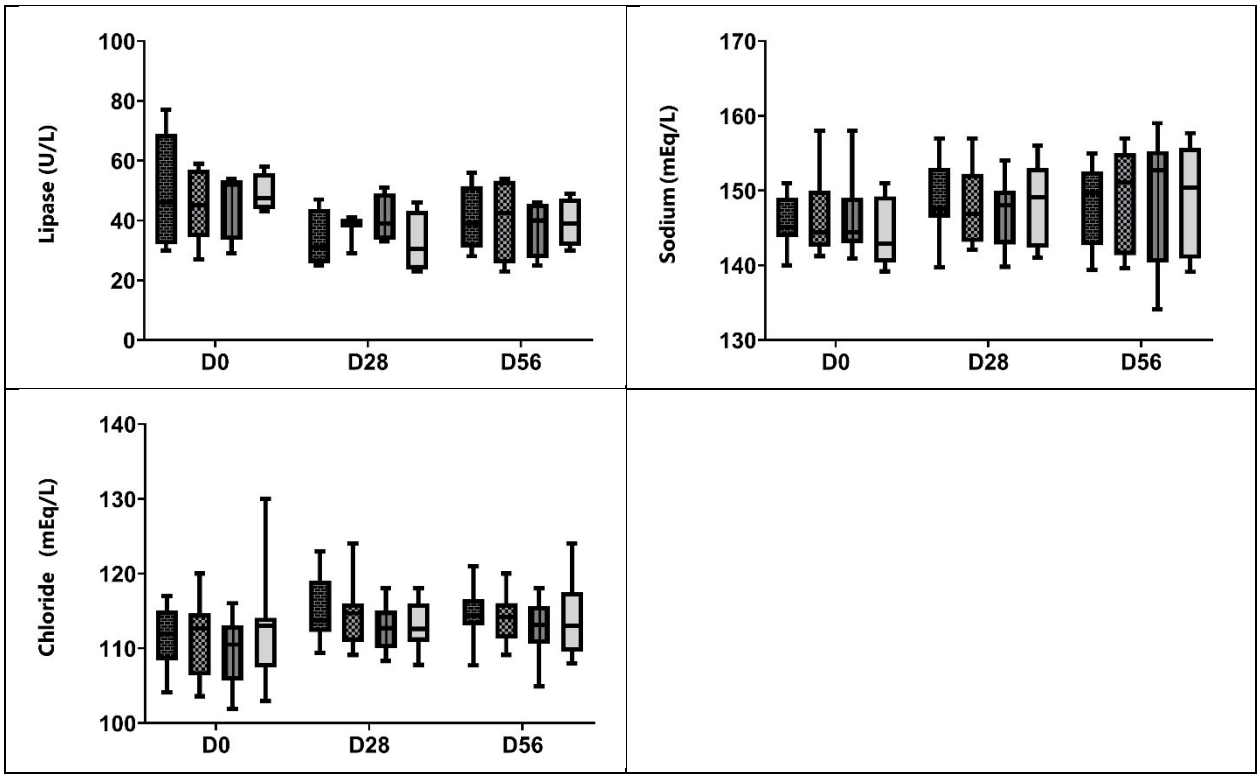


Figure 3 Effect of different levels of EAB-277® administration on serum chemistry at D0, D28 and D56 in dogs. (= 0X; = 1X; = 3X and = 10X). All values are presented as IQR. Upper and lower boxes represent the 25th and 75th percentiles (lower whiskers = minimum, upper whiskers = maximum values) and the line represents the median. Values were significantly different between the groups or time (* = $P<0.05$; ** = $P<0.01$).

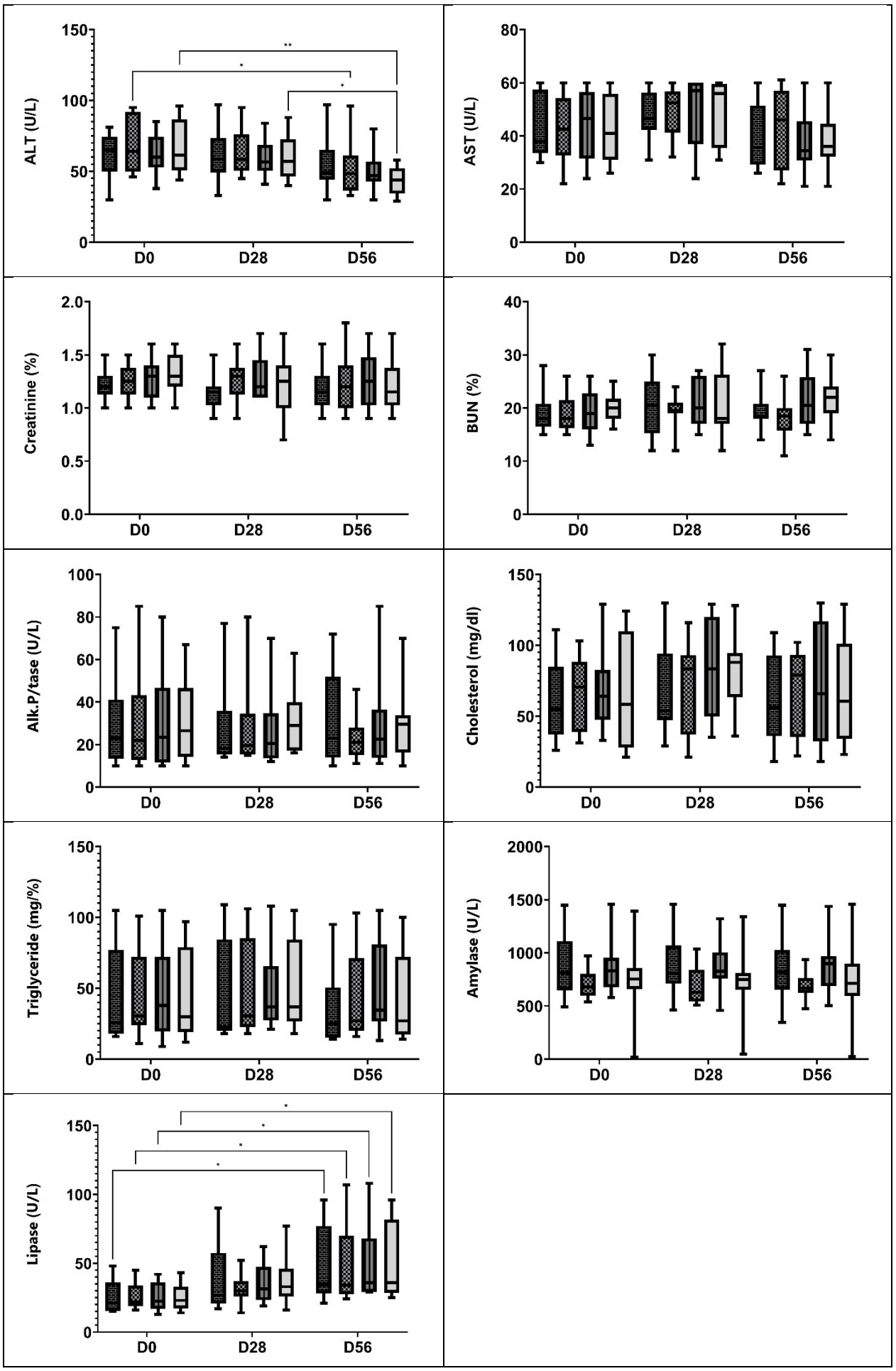

ALT (U/L)
AST (U/L)
Creatinine (%)
BUN (%)
Alk.P/tase (U/L)
Cholesterol (mg/dl)
Triglyceride (mg/%)
Amylase (U/L)
Lipase (U/L)
D0
D28
D56

Figure 4 Effect of different levels of EAB-277® administration on serum chemistry at D0, D28 and D56 in cats. ( = 0X;  = 1X;  = 3X and  = 10X). All values are presented as IQR. Upper and lower boxes represent the 25th and 75th percentiles (lower whiskers = minimum, upper whiskers = maximum values) and the line represents the median. Values were significantly different between the groups or time (* = $P<0.05$; **= $P<0.01$).

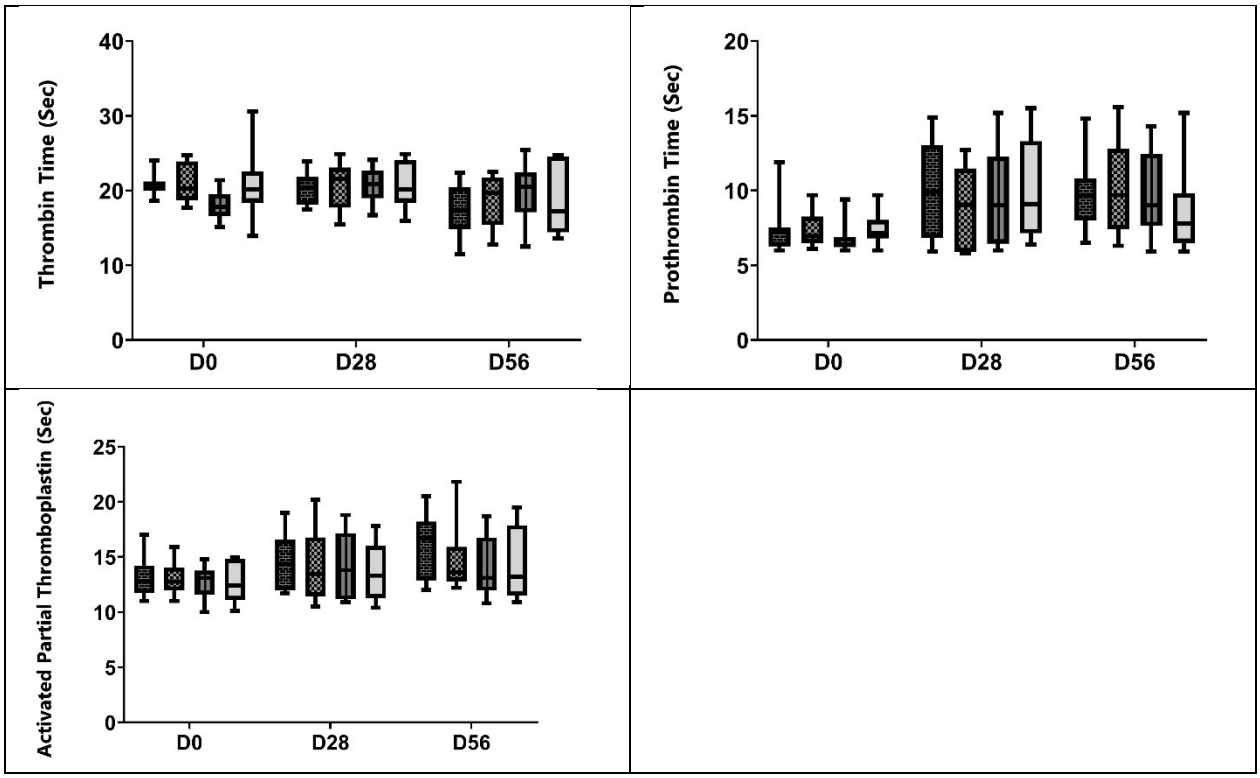


Figure 5 Effect of different levels of EAB-277® administration on blood coagulation factors at D0, D28 and D56 in dogs. (= 0X; = 1X; = 3X and = 10X). All values are presented as IQR. Upper and lower boxes represent the 25th and 75th percentiles (lower whiskers = minimum, upper whiskers = maximum values) and the line represents the median. Values were significantly different between the groups or time (* = $P<0.05$; ** = $P<0.01$).

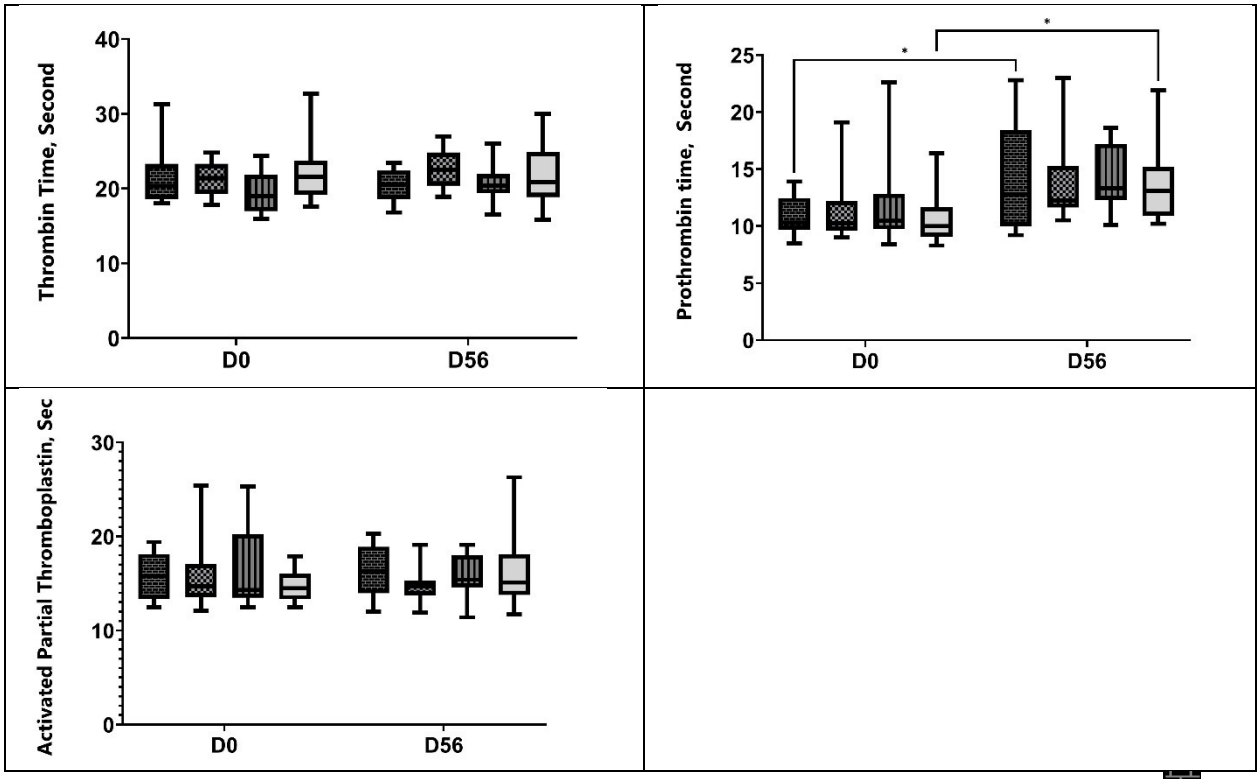


Figure 6 Effect of different levels of EAB-277® administration on blood coagulation factors at D0, D28 and D56 in cats. (= 0X; = 1X; = 3X and = 10X). All values are presented as IQR. Upper and lower boxes represent the 25th and 75th percentiles (lower whiskers = minimum, upper whiskers = maximum values) and the line represents the median. Values were significantly different between the groups or time (* = $P<0.05$; ** = $P<0.01$).

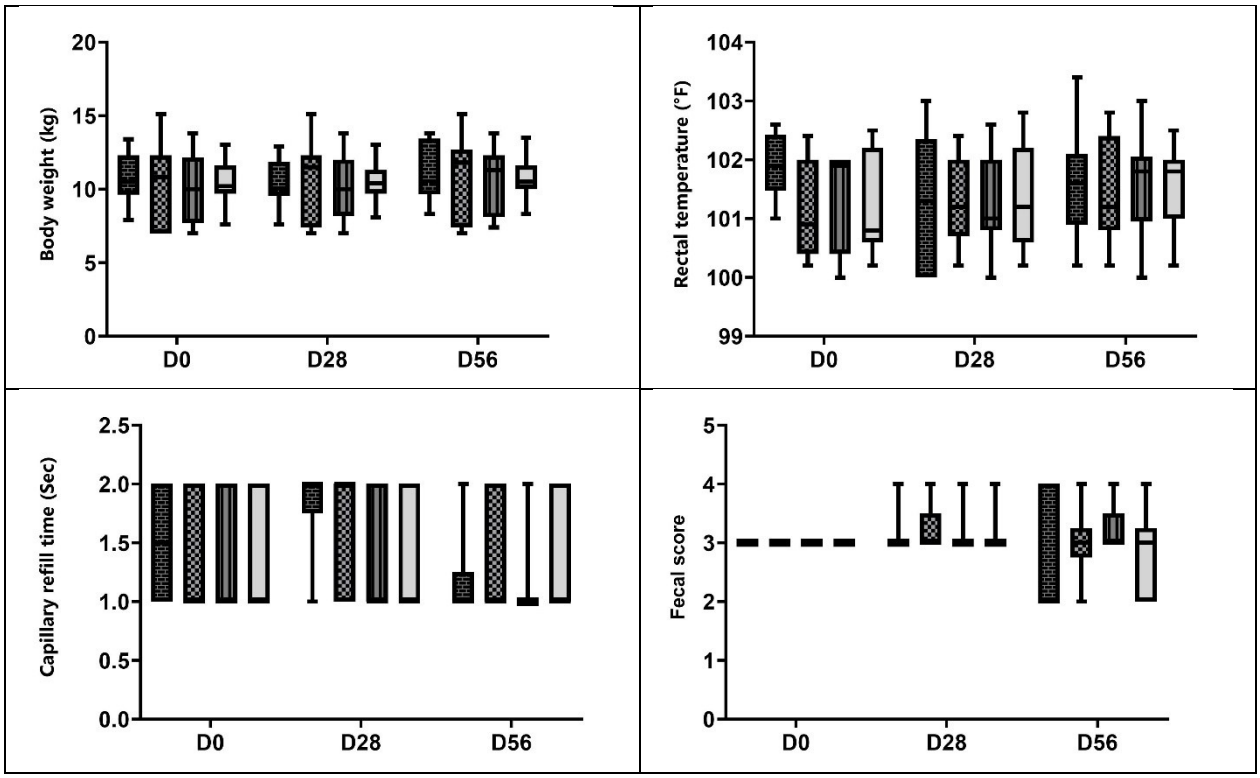


Figure 7 Effect of different levels of EAB-277® administration on physical examination at D0, D28 and D56 in dogs. (= 0X; = 1X; = 3X and = 10X). All values are presented as IQR. Upper and lower boxes represent the 25th and 75th percentiles (lower whiskers = minimum, upper whiskers = maximum values) and the line represents the median. Values were significantly different between the groups or time (* = $P<0.05$; ** = $P<0.01$).

Discussion

This safety study demonstrated the various dose effects of EAB-277 supplementation on blood hematology, chemistry and coagulogram in healthy dogs and cats. EAB-277 has been developed from PCSO-524, which is composed of 91 fatty acids, including omega 3 fatty acid, DHA, EPA, DPA, ETA as the key components. These components have been used as an anti-inflammatory treatment in many disorders, especially arthritis in dogs and cats, during the past decade (Kampa *et al*., 2023). The omega-3 fatty acids from marine sources typically reduce inflammatory processes and result in several other desirable effects (such as gastrointestinal bleeding); they are also considered good fats for dog and cat (Bauer, 2006). Adverse reactions which concern both veterinarians and pet owners mostly involve physiological function, e.g., weight gain, serum cholesterol and triglyceride, tendency to bleed, and pancreatic function e.g., pancreatitis (Xenoulis *et al*., 2020). Recently, Mektrirat *et al*. (2022) found that PUFA EAB-277 supplementation improves clinical signs and attenuates Human rhinovirus (HRV) impairment by reducing oxidative stress and inflammation in tracheal collapse dogs. This might increase the satisfaction of pet ownership. Additionally, EAB-277 shows promise as a substitute for current immune-mediated hemolytic anemia (IMHA) medications (Mektrirat *et al*., 2023). It also contains sterol esters, polar lipids, and carotenoids, which play roles in cellular functions and possess anti-inflammatory properties, cell membrane integrity, and antioxidant properties that contribute to the extract's health benefits (Tsiantas *et al*., 2022). As nutraceutical are prescribed by many veterinarians, not only efficacy, but also safety is important to confirm when used with companion animals. Dogs and cats are classified as carnivores even though only cats are obligatory carnivores. This means that cats may respond differently to PUFAs supplement than dogs.

The aim of this study was to evaluate any possible adverse reaction regarding the concern in dogs and cats. One concern for use of PUFAs supplement is the possibility of body weight gain. All animals demonstrated neither weight gain nor loss, with body condition scores being in ideal range (score 2/5 to 3/5). The US National Research Council (2006) had the recommended allowance levels of EPA and DHA for adult dogs and cats at 0.11 and 0.025 g/1000 kcal, respectively. Association of American Feed Control Official (AAFCO) have not determined the requirement of EPA and DHA for adult dog and cat (Lenox, 2016). Suggested supplemental doses of EPA and DHA for dogs' weights 10 kg and cats' weights 5 kg were 500-750 (50-75 mg/kg×10 kg) and 150-250 (30-50 mg/kg×5 kg) mg, respectively, with the safe upper limit (dog) at 2080 mg (0.37×100.75) (Lenox, 2016). Each capsule of PUFAs supplement (Antinol®, EAB 277TM) used in this study contains 50 mg of active ingredients. Even though the 20 capsules (5X of a maximum dose or 10X groups) of the supplement provided a total of 1,000 mg PUFAs (about 9 kilocalories, kcal). Approximate daily energy requirement (DER) of dog weights 10 kg and cat weights 3 kg are 742 and 209 kcal/day, respectively. Therefore, the PUFAs supplement provides only 1.21 and 4.31% of DER for dog and cat, respectively. In addition, serum cholesterol and triglyceride concentration of all treatment groups for both dog and cat did not differ significantly when compared between time or treatment.

Hematology and blood chemistry values of all dose treatment groups of dogs and cats obtained from the current study were all in the reference range (Merck, 2023). For dog blood creatinine analyses, significant differences were observed in dogs receiving 3X, which had higher creatinine levels than other treatments at D28. Administration of 3X of dietary PUFAs could be appropriate to build up muscle mass and health conditions, while low level blood creatinine might indicate malnutrition or lower muscle mass (Ruaux *et al*., 2012). Excessive blood creatine level (over than 1.4 mg/dl) can be used as a marker of kidney disease as acute kidney injury (AKI) and chronic kidney disease (CKD) in dogs (Babyak *et al*., 2017). Even so, the 3X dog's rising creatine level (less than 1.4 mg/dL) is still within the standard range and indicates no health risks.

For cat blood chemistry analyses, significant differences were observed between times within treatments for concentration of ALT (1X and 10X) and lipase (3X and 10X). During the period of safety study, many extraneous factors such as photoperiod, humidity, ventilation, ambient temperature (Fox, 1986) and even routine procedures such as removing test animals from their cage (Reinhardt, 2004) may possibly affect animal physiology including the dependent variable, blood hematology and serum chemistry values. In addition, a slight change in blood chemistry that remains within reference interval could be due to clinical variation. Aside from these, hemolysis could also interfere with the analysis of some serum chemistry such as creatinine kinase, aspartate dehydrogenase, lipase, and albumin (O'Neill and Feldman, 1989). However, there was no blood hemolysis detected in this study.

Prolonged bleeding time with PUFAs supplements is another adverse reaction which concerns veterinarian and pet owners. This condition can possibly increase risk (for example, trauma, fracture, burn) and affect many practical procedures such as any surgery, scratch of tartar etc. Blood coagulation factors of the studied dogs and cats mostly were not significantly difference for comparison neither between treatment nor time. Thin blood condition was not observed on any of the studied animals. The range of blood coagulation varies between Merck (2023) and Chulalongkorn university laboratory (CU lab) index. Regarding Merck (2023) reference, the range of TT, PT, and aPTT are 10-21, 6.7-16.6, and 15-20 seconds while CU lab values are 11.5-30.6, 5.8-15.6, and 10.0-21.8seconds, respectively. However, Prihirunkij *et al*. (2003) reported the mean +SD of TT of cats at 22.2+2.9. Therefore, using Merck as the baseline for the comparison seem to be appropriate. Cats fed 1X and 10X of dietary PUFA had higher PT level during day 56 compared to day 1. An increase in PT alone may be interpreted as an extrinsic pathway issue (e.g., Factor VII deficiency, early rodenticide poisoning), but

prolongation of both aPTT and PT mostly occurs in cases of severe liver disease, vitamin K deficiency or antagonism and is typically caused by an absence of multiple variables (Parry, 1989). In associated with our ALT result, as expected, the ALT level at day 56 of cat fed 1X and 10X was significantly lower than day 1. This result has also confirmed the negative correlation between blood PT and ALT, which is associated with hepatic dysfunction (increased ALT or ALP, and in most cases other possible biomarkers of hepatic dysfunction of damage). A coagulogram's variation may be triggered by an array of factors, including sample time and technique, anticoagulant transfer delays, and insufficient anticoagulant mixing in the glass tube (Mischeke *et al*., 2005). Other possible causes of the prolongation include a deterioration of factors because of the delay between blood collection and analysis, inadvertent thawing and incorrect plasma to anticoagulant ratio. Alternatively, factor VII deficiency may be responsible for prolongation of the PT. Factor VII is the coagulation protease responsible for starting a cascade of proteolytic events that lead to thrombin generation, fibrin deposition, and platelet activation (Eigenbrot, 2002).

ALT is important in the metabolism of nitrogen and is most often associated with the liver function. High levels of ALT indicate either liver disorders, damage, or toxin ingestion. In the present study, cat's ALT level was significantly decreased when receiving 10X of dietary PUFAs at D56 compared to D0 and D28. This possibly concluded that not only as an energy source but also helps to carry out various biological reactions involved in the development and maintenance of normal renal functions (Katakura *et al*., 2015). Dietary PUFAs can influence the immune response through changes in metabolic status and coagulation (Ferroni *et al*., 2012). In contrast, PUFAs can decrease the levels of several coagulation factors (V, VII, and X) and reduce thrombin generation and does not improve coagulation, metabolic, and inflammatory status (Macchia *et al*., 2012; Poreba *et al*., 2017). The possible mechanism could relate to the anti-inflammatory and antioxidant properties and may have beneficial effects on liver health. The role of dietary PUFAs in inhibiting proliferation, inducing apoptosis, and promoting differentiation in antioxidant enzymes has been recently studied (Thérien *et al*., 2021). Generally, oxidative damage may be the primary cause of lipid peroxidation and cellular damage. Negative correlation between malondialdehyde (MDA) and glutathione (GSH) indicated the occurrence of an oxidative insult that caused hepatic and renal damage (Abdou and Hassan, 2014).

Our study showed that cats at D56 had higher serum lipase than D0 even in control group. Regarding digestive physiology, cats could adjust to dietary fat better than dogs, as a result, their serum lipase remained stable no matter what doses ware applied. Additionally, supplementation of PUFAs in continuous for two weeks up could possibly enhance pancreatic lipase secretion although after terminating the supplement. Furthermore, serum lipase and fPLI observed in the current study were all in normal range as serum lipase range was 13-108 U/L and fPLI was below 3.5 ng/mL.

Pancreatitis is a potential problem in animals fed with high-fat diets or high doses of fatty acid supplementation (Lenox and Bauer, 2013). Variables indicating pancreatic function of the animals include serum amylase and lipase. These two enzyme concentrations were found in the normal range for dogs, and no significant difference was observed overtreatment or time. Polynomial contrast found neither linear, cubic nor quadratic relationships between dose treatments and any parameters. Evaluation of pancreatic function of dogs using PLI values mainly resulted in normal status at every point of time. PLI values of all studied cats demonstrated the normal status of pancreas, and no difference was observed over the dose, treatment or time. Furthermore, no clinical sign of pancreatitis was observed on any animals in this study. Bauer (2011) reported the dosages of EPA and DHA when use in clinical treatment are approximate from 120 up to 310 mg/kg0.75. The maximum dose of the supplement used in this safety study (10X groups) was total of PUFAs 1,000 mg for 10 kg BW dog or 500 mg for 3-4 kg BW cat. The PUFAs supplement extracted from New Zealand green-lipped mussels, *Perna canaliculus*, contained not only omega 3 fatty acids, EPA and DHA, but also some other omega 6, monounsaturated and saturated fatty acids (Taylor and Candida, 2006). These inconsistent effects demonstrate the effective dose of the lipid extract, EPA and DHA, have not been clearly identified. However, the data from the current safety study verify that the fatty acids composition and dose of the PUFAs supplement used in the current study showed no influence on pancreatic function.

Current results are supported by the findings of our previous safety study with healthy beagle dogs, where the blood hematology, chemistry and coagulogram were not significant among the various groups considered when administered with 0X, 1X, 3X and 10X dose of PCSO-524 (Antinol®) (Jamikorn and Yibchok-anun, 2014). This was also demonstrated by no change in food intake and behavior, which suggested that this supplement did not have a harmful effect. However, the current data could not exclude that there were other factors such as previous dietary fatty acid composition and source or formulation of the basal diet that might influence the results. To the best of our knowledge, this is the first study evaluating 10 times overdose administration as safety study in healthy dogs and cats with dietary PUFAs (EAB-277). All dependent variables were in normal ranges referenced by Merck (2023). Therefore, interpreting the results of blood hematology, chemistry and coagulogram should be approached with recommendation. This is because they may fluctuate greatly due to factors such as age, nutrition, and environmental conditions, as well as regulations of technology and analytical methodologies utilized, all of which must be addressed before drafting final results.

In conclusion, supplementation of the PUFA containing EAB 277 extract from New Zealand green lipped mussel to the dogs and the cats at 0X, 1X, 3X and 10X resulted in the normal ranges for most of the blood hematology and blood chemistry values. No abnormal signs were observed during the safety study period

including amylase, lipase and PLI values for all animals. For blood coagulation factors, only the PT of all treatment groups were in the normal range of Chulalongkorn university lab. However, the TT, PT and aPTT of all treatment groups demonstrated similar patterns with no significant difference except between the 1X and 10X at D56 (P<0.05). These could indicate the normal ranges of the TT, PT and aPTT of animals at the kennels used in the current study. Overall, this safety study found that PUFA supplementation using EAB-277 derived from New Zealand green lipped mussel appeared to be safe for use in dogs and cats.

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