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Volume 10 (2); June 25, 2020

Review

Surgical Treatment of Canine Femoral Fractures – a Review.

Lovrić L, Kreszinger M and Pećin M.

World Vet. J. 10(2): 137-145, 2020;

pii:S232245682000018-10

DOI: <https://dx.doi.org/10.36380/scil.2020.wvj18>



ABSTRACT

Femoral fractures in dogs and cats account for 20-25% of all fractures for which surgical treatment is a method of choice. Surgical treatment is based upon biological principle of open anatomic reduction and osteosynthesis. Arbeitsgemeinschaft für Osteosynthesefragen (AO) classification of fractures has a widespread use in general. Present study discusses different methods of osteosynthesis and healing process based on special cases managed in a certain small animal clinic in Hollabrunn, Austria, in 2016. The level of femoral fracture and the chosen method of osteosynthesis are shown respectively. According to available literature and author's personal observations during externship period, the best results have been achieved using minimally invasive surgery. The surgical method choice depends on type, level and complexity of fracture, surgical skills and equipment of the team providing care respectively.

Key words: Dog, Femur, Fracture, Osteosynthesis.

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Research Paper

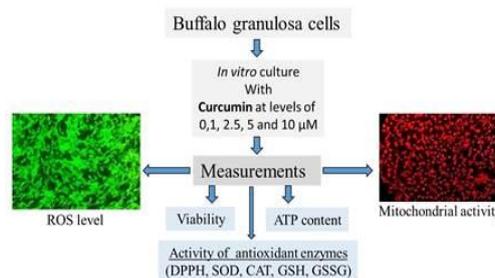
Effects of Curcumin Supplementation on Viability and Antioxidant Capacity of Buffalo Granulosa Cells under *In Vitro* Culture Conditions.

Ghanem N, Amin A, Saeed AM, Abdelhamid ShM, El-Sayed A, Farid OA, Dessouki ShM and Faheem MS.

World Vet. J. 10(2): 146-159, 2020;

pii:S232245682000019-10

DOI: <https://dx.doi.org/10.36380/scil.2020.wvj19>



Ghanem N, Amin A, Saeed AM, Abdelhamid ShM, El-Sayed A, Farid OA, Dessouki ShM and Faheem MS (2020). Effects of Curcumin Supplementation on Viability and Antioxidant Capacity of Buffalo Granulosa Cells under *In Vitro* Culture Conditions. *World Vet. J.*, 10 (2): 146-159.

ABSTRACT

The current study was conducted to investigate the possible protective effect of curcumin supplementation on buffalo granulosa cells (GCs) under *in vitro* culture condition. Buffalo ovaries were collected from local abattoir in physiological saline solution and transported directly to laboratory. Follicular fluid containing GCs and cumulus-oocyte-complexes were aspirated from antral follicles with diameter 2-8 mm. The collected GCs were seeded (Approximately 375,000 viable cells) in an 8-well culture plate containing tissue culture medium-199 (TCM-199) and kept at 37 °C in a humidified atmosphere of 5% CO₂. The curcumin was supplemented to TCM media at levels of 1, 2.5, 5 and 10 µM for 24 and 48 h at 37 °C or kept without treatment as control group. The viability of cells was determined using the trypan blue test. Intracellular reactive oxygen species (ROS) level was assessed by measuring the fluorescent intensity of 6-carboxy-2',7'-dichlorodihydro fluorescein diacetate (H₂DCFDA). In addition, mitochondrial activity of GCs was determined. The results of the present study indicated that the viability of GCs under culture conditions was significantly decreased in groups treated with 1, 2.5, 5 and 10 µM curcumin (86.0%, 86.26%, 83.0% and 74.0%, respectively) compared to control group (93.60 %). The two groups of granulosa cells cultured with 2.5 and 5 µM curcumin recorded greater level of mitochondrial activity than the groups cultured with 1 µM and 10 µM curcumin. Moreover, there was a significant increase in ROS level in group cultured with 10 µM curcumin, compared to control and other experimental groups. The enzyme activity of catalase (CAT), superoxide dismutase (SOD), glutathione (GSH) and 1, 1-diphenyl-2-picrylhydrazyl (DPPH) was increased after treating *in vitro* cultured granulosa cells with 5 µM of curcumin. However, the enzymatic activity of CAT, SOD, GSH and DPPH was declined significantly 48 h post-curcumin treatment. In conclusion, supplementation of curcumin at low concentration (2.5 µM) for 24 h to *in vitro* cultured GCs improved intracellular metabolic activity and antioxidant protective system, whereas it could not sustain this action for 48 h. Moreover, supplementation of curcumin at high concentration and for long duration may negatively affect viability of GCs under *in vitro* culture condition via induction of oxidative stress.

Key words: Antioxidant, Buffalo, Granulosa cells, Oxidative stress, Viability.

[Full text-[PDF](#)]

Research Paper

Efficacy of Ivermectin-Based Drugs against Ectoparasites in Broiler Chickens.

Arisova GB.

World Vet. J. 10(2): 160-164, 2020;

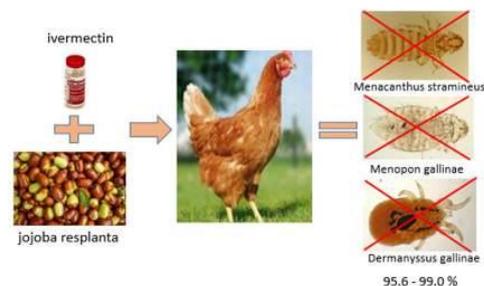
pii:S232245682000020-10

DOI: <https://dx.doi.org/10.36380/scil.2020.wvj20>

ABSTRACT

This research aimed to study the efficacy of two different ivermectin-based drugs against ectoparasites of chickens. In total 1200 Highsex brown chickens aged 1-1.5 years were examined to determine the prevalence of ectoparasites among chickens. The diagnosis of ectoparasites in chickens was established using clinical and entomological methods. For studying drug efficacy, 20 chickens were selected and divided into two groups (experimental and control) of 10 birds each according to the principle of analogs. A prepared ivermectin-based drug consisting of active substance ivermectin and the auxiliary substances including jojoba Resplanta, diethylene glycol monoethyl ether, Tween-80, benzyl alcohol, and purified water, was administered to the experimental group at a dose of 0.4 ml/L of drinking water (400 µg ivermectin per 1 kg of body weight) twice with a 24-hour interval. The treatment was repeated after 14 days. The control group was administered another drug based on ivermectin in the same dose and manner as the drug given in the experimental group. The efficacy of the drugs was determined by counting the number of ectoparasites per chicken before and after treatment. The clinical condition of the birds was monitored from day 1 to day 28 of the experiment. To evaluate the physiological state of chickens, blood and biochemical tests were performed on day 28 of the experiment. The results revealed that the prevalence of infection with *Menacanthus stramineus*, *Menopon gallinae*, and *Dermanyssus gallinae* in chickens was 34.5%, 21.5%, and 12%, respectively. The number of parasites/chicken after treatment between the experimental and the control group was significantly different. The efficacy of the drugs against ectoparasites in the experimental and control group was 95.6-99.0% and 85.1-91.1%, respectively. The blood tests showed that hematological and biochemical parameters were within physiological norms for both groups. Also, a pharmacokinetic study was performed on 18 ISA cross, 40-day-old chickens administered orally with the test drug at the same dose. The results revealed that ivermectin reached maximum concentration at 30-60 minutes after administration to the bird. After 1 hour, the concentration of the active substance of the drug in the blood serum of chickens decreased sharply and reached the limit of quantification by 12-24 hours. In conclusion, this drug can be recommended for use in poultry as an effective and safe drug for the treatment of arachnoentomosis in birds.

Key words: Chickens, Ectoparasites, Ivermectin.



Arisova GB (2020). Efficacy of Ivermectin-Based Drugs against Ectoparasites in Broiler Chickens. *World Vet. J.*, 10 (2): 160-164.

[Full text-[PDF](#)]

Research Paper

Sensitivity of Lateral Flow technique for Evaluation of Inactivated Rift Valley Fever Virus Vaccine in Comparison with Serum Neutralization Test.

Abousenna MS, Sayed RH, Darwish DM and Saad MA.

World Vet. J. 10(2): 165-169, 2020;

pii:S232245682000021-10

DOI: <https://dx.doi.org/10.36380/scil.20209.wvj21>

ABSTRACT

Rift Valley Fever (RVF) is a zoonotic mosquito-borne bunyaviral disease associated with high abortion rate, neonatal death, fetal malformations in ruminants, and mild to severe disease in human. The vaccination has significantly reduced the abortion of ewes and mortality of newborn lambs during an outbreak, and induced immunity in cattle. The evaluation of inactivated RVF vaccine required in vivo and in vitro techniques. The present research aimed to evaluate the sensitivity of the Lateral Flow Device (LFD) in comparison with Serum Neutralization Test (SNT) by reference sera to determine the humoral immune response of the sheep vaccinated with an inactivated RVF vaccine. Three batches of inactivated RVF vaccine were inoculated in three sheep groups. Then samples of their sera were collected weekly, and tested by SNT and LFD. It was found that the sensitivity of LFD at a serum dilution of 1:128 was 95%, while SNT carried out at the fourth week after the vaccination showed that antibody titers was 32,64 and 32. On the other hand, LFD had positive results at dilutions of 1:32, 1:128 and 1:64 for the vaccine batches 1, 2 and 3 respectively. These findings suggest the possibility of using LFD for detection of the immune response of vaccinated sheep to the inactivated Rift Valley Fever Virus vaccine, and it could be improved to be more quantitative in future.

Key words: Lateral flow device, Rift valley fever virus, RVFV inactivated vaccine, Vaccine evaluation



Abousenna MS, Sayed RH, Darwish DM and Saad MA (2020). Sensitivity of Lateral Flow technique for Evaluation of Inactivated Rift Valley Fever Virus Vaccine in Comparison with Serum Neutralization Test. *World Vet. J.*, 10 (2): 165-169.

[Full text-[PDF](#)]

Research Paper

Molecular Evidence of *Spirometra erinaceieuropaei* in Asian Wild Frogs (*Rana rugulosa*) from Banyuwangi City, Indonesia.

Yudhana A, Praja RN, Yunita MN and Wardhana DK.

World Vet. J. 10(2): 170-174, 2020;

pii:S232245682000022-10

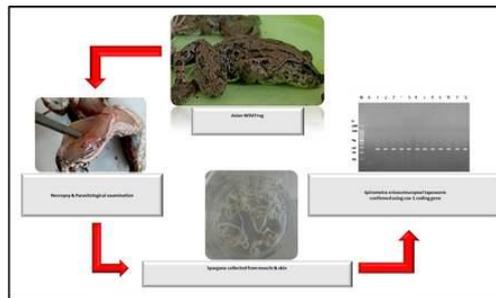
DOI: <https://dx.doi.org/10.36380/scil.2020.wvj22>

ABSTRACT

The tapeworm *Spirometra erinaceieuropaei* is the most frequently species which found in wild frog and causing a serious parasitic zoonosis known as sparganosis. This study aimed to provide molecular evidences of spargana collected from wild frogs which used as food and contribute to provide important implication for prevention and control of sparganosis. A total of 185 Asian wild frog (*Rana rugulosa*) samples were selected from food markets in Banyuwangi City, Indonesia. Molecular identification based on spargana that were collected and coding gene of mitochondrial cytochrome c oxidase 1 (*cox1*) using Polymerase Chain Reaction (PCR) method. Spargana were found in 9.1% (17/185) of the frogs and PCR analysis results identified all specimens belonging to the species *S. erinaceieuropaei*, therefore indicated that *S. erinaceieuropaei* is the major causative agent of sparganosis from frogs which sold as food in markets. These findings can be useful to the molecular diagnosis and control of *Spirometra* infections in humans and animals.

Key words: Asian wild frog, *Rana rugulosa*, Sparganosis, *Spirometra erinaceieuropaei*.

[Full text-[PDF](#)]



Yudhana A, Praja RN, Yunita MN and Wardhana DK (2020). Molecular Evidence of *Spirometra erinaceieuropaei* in Asian Wild Frogs (*Rana rugulosa*) from Banyuwangi City, Indonesia. *World Vet. J.*, 10 (2): 170-174.

Research Paper

Influence of *Panicum maximum* Replacement of Clover Hay on the Performance of Growing Rabbits.

Refaie AM, Salama WA, Shams El-deen AE, Beshara MM, Khalil FS, and Alazab AM.

World Vet. J. 10(2): 175-182, 2020;

pii:S232245682000023-10

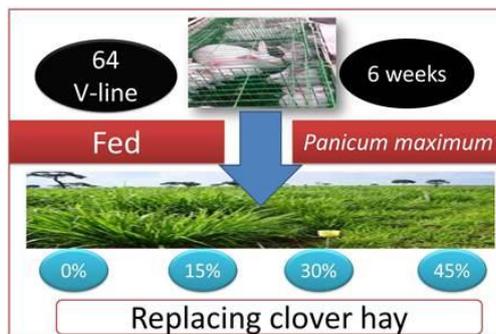
DOI: <https://dx.doi.org/10.36380/scil.2020.wvj23>

ABSTRACT

Two experiments were performed to evaluate *Panicum maximum* (Pm) and its effect on rabbits' growth performance. In the first experiment, six adult V-line male rabbits were used to determine the digestible energy in Pm by continuously feeding these 120 gram (g) Pm and 120 g clover hay for 3 days, and then the digestible energy was recorded 1959 kcal /kg. In second experiment, a total of 64 rabbits of V-line, 6 weeks old with average weight of 702 g, were divided into 4 groups, each in 4 replicates (4 rabbits/replicate), the first fed basic diet; control (T1), the 3 groups fed on the diet contained Pm to replace clover hay as a percentage of 15%, 30% and 45%, which corresponds to 4.5%, 9% and 13.5% of the total diet; which represent T2, T3, and T4, respectively. Rabbits were fed ad libitum with pellet feed until the end of growth attempt (14 weeks). The results indicated that the proximate analysis of Pm was 11.65% crude protein, 2.67% crude fat, and 30.66% crude fiber. Rabbits in T4 group significantly had the best final weight, daily weight gain, and Feed Conversion Ratio FCR. All groups had high crude protein digestibility except the group fed T3 diet. The total number of cecum bacterial count was improved in all tested groups. In conclusion, feeding growing rabbits with Pm up to 45% instead of clover hay achieved higher growth performance and lower cecum coliform bacteria.

Key words: Cecum bacteria, Growth performance, *Panicum maximum*, Rabbits.

[Full text-[PDF](#)]



Refaie AM, Salama WA, Shams El-deen AE, Beshara MM, Khalil FS, and Alazab AM (2020). Influence of *Panicum maximum* Replacement of Clover Hay on the Performance of Growing Rabbits. *World Vet. J.*, 10 (2): 175-182.

Research Paper

Detecting intestinal parasitic infections in laboratory mice.

Alkhashab FMB, Alnuri AIJ, and Al_Juwari RSA.

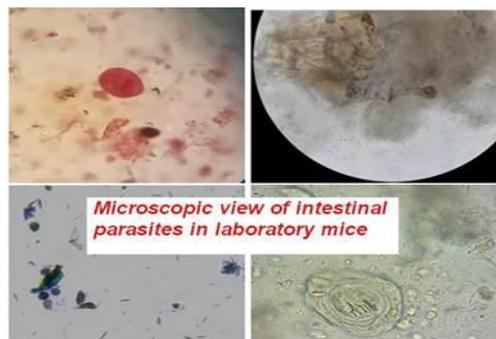
World Vet. J. 10(2): 183-189, 2020;

pii:S232245682000024-10

DOI: <https://dx.doi.org/10.36380/scil.2020.wvj24>

ABSTRACT

A total of 150 Laboratory mice divided into four age groups consisted of 4, 6, 8 and 10 weeks old were used in this study by placing each animal



Alkhashab FMB, Alnuri AIJ, and Al_Juwari RSA (2020). Detecting intestinal parasitic infections in laboratory mice. *World Vet. J.*, 10 (2): 183-189.

individually in a special cage within the period between October 2019 to the end of February 2020 at the Research and Graduate Studies Laboratory University of Mosul, Iraq. This study aimed to investigate intestinal parasitic infections in laboratory mice, stool samples were collected for 150 laboratory mice and periodically to perform laboratory tests that included direct slide examination and using the concentration method to detect eggs of worms and cysts of protozoa parasites, the culture of parasites also was used by prepared manufactured culture media to develop parasites. The infection was diagnosed in 136 (90.66%) mice while the rest 14 (9.33%) mice did not record any parasitic infection (clean). The higher rate of infection 58% was reported for *Trichomonas muris* followed by *Entamoeba muris* and *Giardia muris* which found in 22%, 15.3% respectively. In the other hand the infection with *Hymenolepis diminuta* was recorded in 16% from infected cases by identifying the eggs of this worm in stool samples. This study shows the high rate of parasites infection in laboratory mice which might have negative effects on the result of previous scientific researches, in addition to wasting effort, time, and materials.

Key words: *Entamoeba muris*, *Giardia muris*, Laboratory mice, *Trichomonas muris*

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Research Paper

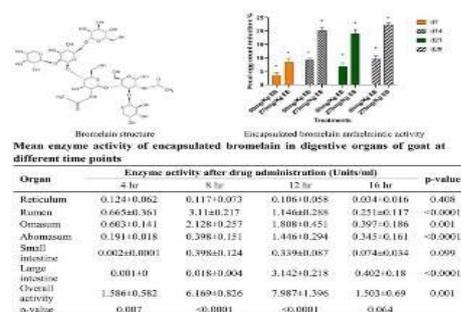
Toxicity, Anthelmintic Efficacy and Proteolytic Activity of Chitosan-Encapsulated Bromelain within the Gastrointestinal Tract of Small East African Goats.

Wasso S, Kagira J and Maina N.

World Vet. J. 10(2): 190-198, 2020;

pii:S23224568200002510

DOI: <https://dx.doi.org/10.36380/scil.2020.vwj25>



Wasso S, Kagira J and Maina N (2020). Toxicity, Anthelmintic Efficacy and Proteolytic Activity of Chitosan-Encapsulated Bromelain within the Gastrointestinal Tract of Small East African Goats. *World Vet. J.*, 10 (2): 190-198.

ABSTRACT

The development of resistance to anthelmintic drugs has prompted researches into alternative methods for controlling intestinal nematodes in ruminants. This study aimed to evaluate the anthelmintic efficacy, proteolytic activity, and toxicity of bromelain encapsulated in chitosan within the gastrointestinal tract (GIT) of Small East African goats in Kenya. Twelve healthy indigenous male goats were divided into four groups contained three goats in each group. Treatment groups included: G1, chitosan-encapsulated bromelain (90 mg/kg); G2, chitosan-encapsulated bromelain (270 mg/kg); G3, positive control (albendazole 7.5 mg/kg); and G4, negative control. The animals were orally treated with the drugs in a single dose. The hematological and serum biochemical parameters were determined using standard methods. The strongyle fecal egg count was evaluated weekly using a modified McMaster technique. To determine the proteolytic activity of nanoencapsulated bromelain within the GIT, another set of twelve goats was used and administered 270 mg/kg of encapsulated bromelain. Every four hours, three goats were sacrificed and the proteolytic activity of the drug was determined in the different organs of the GIT. Significant differences were observed between the mean PCV of goats treated with 270 mg/kg encapsulated bromelain and non-treated goats on days 21 and 28 post-treatment. The mean aspartate aminotransferase, urea, and creatinine levels of treated and control goats did not significantly differ during the experiment period. Also, no significant difference was observed between the mean alanine aminotransferase level of treated and untreated goats 28 days post-treatment. The administration of encapsulated bromelain was not associated with any clinical sign and mortality. The reduction in fecal egg count in G1 and G2 at 28 days post-treatment was 9.5% and 22.6%, respectively. The encapsulated bromelain remained proteolytically active along the goat GIT but its protease activity varied according to the type of GIT organ and time elapsed since administration. In conclusion, chitosan-encapsulated bromelain is safe, but have low efficacy against GIT strongyle nematodes when given as a single dose. Future studies should evaluate higher and repeated doses of encapsulated bromelain for controlling GIT nematodes.

Key words: Bromelain, Chitosan, Efficacy, Goats, Nanoencapsulation, Proteolytic activity.

[Full text-[PDF](#)]

Research Paper

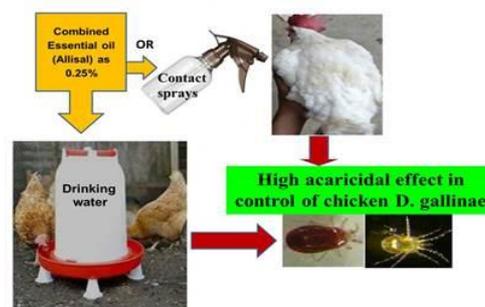
Effect of Combined Plant Essential Oils on *Dermanyssus gallinae*: In vitro and in vivo study.

Amer AMM, Amer MM, Mekky HM and Fedawy HS.

World Vet. J. 10(2): 199-206, 2020;

pii:S232245682000026-10

DOI: <https://dx.doi.org/10.36380/scil.2020.vwj26>



Amer AMM, Amer MM, Mekky HM and Fedawy HS (2020). Effect of Combined Plant Essential Oils on *Dermanyssus gallinae*: In vitro and in vivo study. *World Vet. J.*, 10 (2): 199-206.

ABSTRACT

The present study was carried out to evaluate the effect of plant essential oils on *Dermanyssus gallinae* (*D. gallinae*). In vitro six groups of red mites, 20 mites in each group were exposed to direct spray of combined plant essential oils (Allisal) in rate of 0.25% on mites. activity and changes under stereomicroscope showed that sprayed mites completely stopped movements at both 1- and 2- h after treatment with completely stretched legs and white bead-like spots of oils accumulation on legs and bodies at 1- and 2- h. while, the non-treated mites were active with pale light brown colour. In vivo effect of Allisal to control red mite infestation in laying hens was investigated. In case of drinking water method mite count reduction rate on the bird was 60 %, 10%, and 0% as well as 0%, 0% and 10% in their traps at 4,7, and 12 days respectively, from the

start of treatment in drinking water. While, in spray method mite reduction was 40%, 20%, and 10% on birds and 0%, 0% and 30% in the traps. On the other hand, water intake, feed intake, general health condition, skin health, and feather condition scores were improved at 4th day post treatment (DPT). Total lesion score at 12 DPT was improved. The present study concluded that in vitro combined plant essential oils have rapid and strong acaricide effect in contact sprays. *In vivo*, there was obvious improvement in groups treated with plant oils than non-treated group. Drinking water treated birds showed good results than spray treated group. Therefore, it is recommended to use combined plant essential oils in *D. gallinae* control strategies in poultry.

Key words: Acaricides, Chickens mite, *Dermanyssus gallinae*, Plant essential oils, Red mite.

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Research Paper

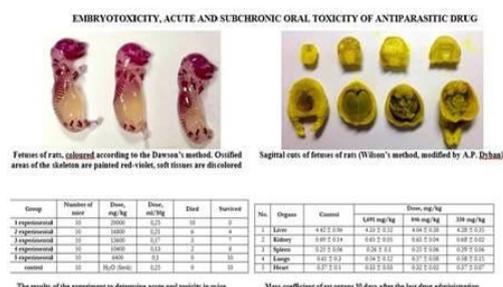
Toxicity Assessment of a Multicomponent Antiparasitic Drug in Animals.

Stepanova IA, Arisov MV and Arisova GB.

World Vet. J. 10(2): 207-215, 2020;

pii:S232245682000027-10

DOI: <https://dx.doi.org/10.36380/scil.2020.wvj27>



ABSTRACT

The important aspect of the high quality new pharmaceuticals is safety assessment in animals in practical conditions. Toxicity assessment of the new antiparasitic multicomponent drug (Inspector Quadro Tabs) composed of lufenuron, praziquantel and moxidectin in the form of tablets for cats and dogs was carried out. The parameters of acute oral toxicity were determined on white mice and white rats and subchronic toxicity was observed after repeated oral administration to rats. Embryotoxicity and teratogenicity of the drug were also evaluated. As a result of toxicological studies, median lethal doses (LD₅₀) of the drug during oral administration to the 60 white mice were established which were equal to the following: LD₅₀ = 14800 mg/kg (Karber's method), 13800 mg/kg (Miller and Tatener's method); to the white rats LD₅₀ > 16912 mg/kg; according to the generally accepted classification, the drug belongs to the fourth class of hazard (low hazard substances). It was established that doses of 1691 mg/kg, 846 mg/kg and 338 mg/kg were threshold in a subchronic experiment on the rats. Moreover, it was found that the drug did not possess embryotoxic and teratogenic properties in pregnant female rats. Experimental results have confirmed the low toxicity of a new antiparasitic multicomponent drug.

Key words: Acute toxicity, Antiparasitic Drug, Embryotoxicity, Mice, Rats, Subchronic Toxicity

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Research Paper

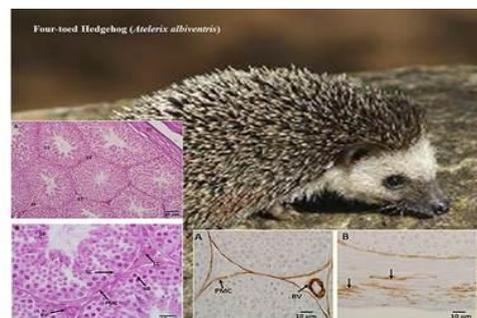
Immunoreactivities to α-SMA and S-100 Proteins in the Testis of the African Four-toed Hedgehog (*Atelerix albiventris*).

Olukole SG, Coker OM and Oke BO.

World Vet. J. 10(2): 216-222, 2020;

pii:S232245682000028-10

DOI: <https://dx.doi.org/10.36380/scil.2020.wvj28>



Olukole SG, Coker OM and Oke BO (2020). Immunoreactivities to α-SMA and S-100 Proteins in the Testis of the African Four-toed Hedgehog (*Atelerix albiventris*). *World Vet. J.*, 10 (2): 216-222.

ABSTRACT

The African four-toed hedgehog is a small nocturnal mammal, characterized by a short-grooved brown or grey spine covering the dorsum of the body with a band of whitish fur running across their forehead, little is known about the reproductive biology of this animal. The present study aimed to evaluating the validity of immunohistochemistry in the differential labelling of the different cellular components of the testis of the African four-toed hedgehog. Paraffin-embedded testicular sections were stained by conventional histological technique using ten male African four-toed hedgehogs captured from the wild animals in Ibadan, Nigeria. Primary antibodies against alpha smooth muscle actin (α-SMA) and S-100 were applied on paraffin sections. The peritubular myoid cells, the testicular capsule and vascular endothelium expressed strong immunostaining for α-SMA. The spermatogenic cells, Sertoli and Leydig cells, peritubular myoid cells, the testicular capsule, straight tubules as well as rete testis and vascular endothelium all expressed positive immunostaining for S-100. α-SMA and S-100 proteins play active roles in cytoskeletal physiology of testis of the African four-toed hedgehog while S-100 protein plays additional role in the structural formation and maintenance of the blood-testis barrier during the process of spermatogenesis in the animal. It is concluded that α-SMA and S-100 proteins has active roles in the cytoskeletal structure of testis and physiology of the African four-toed hedgehogs.

Keywords: African four-toed hedgehog, Immunoreactivities, Spermatogenic cells, Sertoli cells, Testis.

[Full text-[PDF](#)]

Research Paper

The Labial and Zygomatic Salivary Glands in Mixed Breed Dogs in Trinidad: Anatomical Location, Histological Features and Histochemical Characteristics.

Mohamed R.

World Vet. J. 10(2): 223-230, 2020;
pii:S232245682000029-10

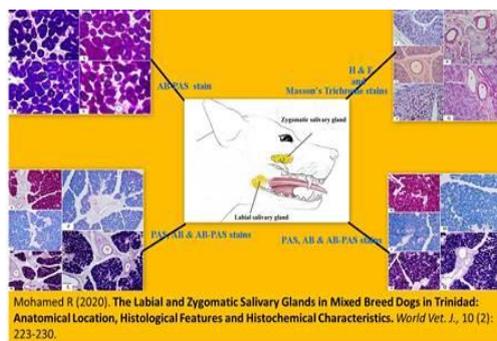
DOI: <https://dx.doi.org/10.36380/scil.2020.wvj29>

ABSTRACT

The objective of this investigation was to give detailed descriptions of the anatomical location, histological features and histochemical characteristics of the labial and zygomatic salivary glands in mixed breed dogs. This study was performed on five heads of adult mixed breed dogs of both sexes. The heads were dissected to detect in situ position of the labial and zygomatic salivary glands. The glands were dissected and examined grossly. Samples of the glands were taken, processed and stained using hematoxylin and eosin and Masson's Trichrome for histological examination as well as Periodic Acid-Schiff, Alcian Blue (pH 2.5 and 1.0) and a combination of Periodic Acid-Schiff and Alcian Blue (pH 2.5 and 1.0) techniques for histochemical examination. The labial and zygomatic salivary glands were located in the lower lip and in the orbit respectively and they were surrounded by fibrous capsules containing collagen fibers. They were minor, compound, mixed tubuloalveolar glands. They composed of mucous acini, mucous acini with serous demilunes and isolated serous acini. The secretion of the glands (chiefly mucous) consisted of neutral mucins, acid carboxylated mucins and acid sulphated mucins. The duct system of the glands was intralobular (intercalated and striated ducts) and interlobular ducts. The anatomical location as well histological and histochemical structures of the labial and zygomatic salivary glands were important to classify the glands and their secretion as well as to give veterinarians knowledge during clinical examination of the oral and orbital regions, and to recognize normal and pathological conditions.

Keywords: Anatomy, Dog, Labial, Salivary glands, Zygomatic

[Full text-[PDF](#)]



Research Paper

Pharmacokinetic Characteristics of the Drug Based on Moxidectin for Young Stock and Small Breed of Domestic Animals.

Belykh IP.

World Vet. J. 10(2): 231-236, 2020;
pii:S232245682000030-10

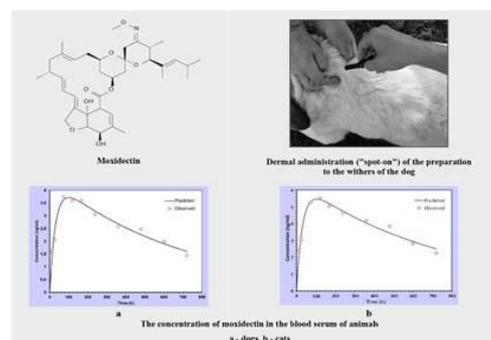
DOI: <https://dx.doi.org/10.36380/scil.2020.wvj30>

ABSTRACT

The pharmacokinetic characteristics of moxidectin in the blood serum of dogs and cats after a single cutaneous (spot-on) application of drug for veterinary use "Inspector Mini" to prevent and treat arachnoses, entomoses and intestinal nematodes in kittens and puppies as well as in small breed dogs and cats were investigated. Twelve outbred dogs and cats of different ages and weights were involved in present study. All the animals were weighed to determine the exact dosage of the drug. The determination of moxidectin in blood serum was carried out by high performance liquid chromatography with pre-column modification of N-methylimidazole and trifluoroacetic anhydride followed by fluorescence detection. According to the results of the study, it was found that moxidectin was well absorbed into the systemic circulation and reached to maximum concentration in the blood serum of dogs and cats after 4-10 days. After treatment with the drug, moxidectin was determined in the blood serum of animals after 12 hours at concentration of 2 ng/ml. Significant concentrations of moxidectin in the blood serum of animals remained for 28 days after topical application (spot-on). Moxidectin was detected in the blood serum of animals at the end of the experiment (after 30 days) which indicates its therapeutic effect for at least one month after the application of the drug.

Key words: Blood Serum, Cats, Dogs, Moxidectin, Pharmacokinetics

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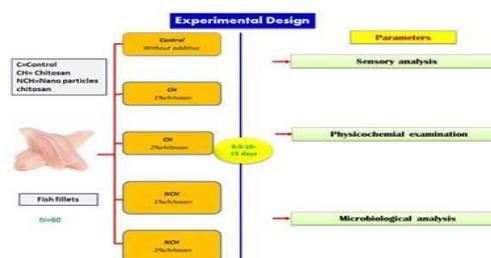
Research Paper

Quality Evaluation of Nile Tilapia Fish (*Oreochromis niloticus*) Fillets by Using Chitosan and Nanochitosan Coating during Refrigerated Storage.

Walaa ME, Shereen AY and Mohamed NS.

World Vet. J. 10(2): 237-245, 2020;
pii:S232245682000031-10

DOI: <https://dx.doi.org/10.36380/scil.2020.wvj31>



ABSTRACT

Using natural preservatives has a probability to improve the quality and integrity of fish products. Such research investigated the antimicrobial and antioxidant effects of chitosan and chitosan nanoparticles casing on the quality of tilapia (*Oreochromis niloticus*) fish fillets through refrigerated storage. In the present investigation solutions of chitosan (1 and 2%) and nanochitosan (1 and 2%) were applied for the casing of tilapia fish slices thereafter stored at 4°C for 15 days. Uncoated (control) and coated fish fillets pieces were examined intermittently for bacteriological parameters (Total bacterial count, Proteolytic bacterial count, Lipolytic bacterial count, and *Staphylococcus aureus* count), quality parameters (pH, total volatile basic nitrogen (TVB-N), and thiobarbituric acid reactive substances, TBARS) and sensory features. Results showed that 2% chitosan and 2% chitosan nanoparticle solutions were the optimal concentrations for improving the quality of tilapia fish fillets until 10 days of refrigerated storage period compared to the control group. However, using 2% chitosan nanoparticles showed higher antimicrobial activity, strong ability in preventing protein degradation, retarding lipid oxidation, accepted pH values and delay in declining of sensory score more than 2% chitosan solution during the storage period. Therefore, 2% chitosan nanoparticles as a natural preservative can be utilized for the conservation of quality properties and expanding the shelf life of tilapia fish slices through chilled storage.

Key words: Bacteriological and quality parameters, Chitosan, Nanochitosan, Tilapia fish fillets

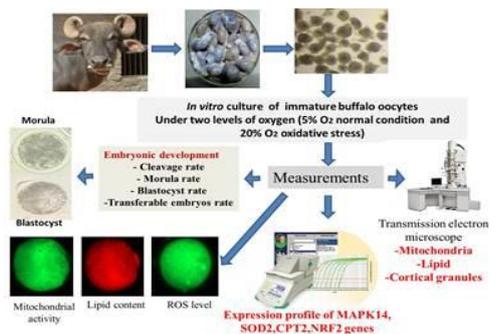
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Research Paper

Developmental Competence of Buffalo Oocytes Cultured Under Different Oxygen Tensions after Selection with Brilliant Cresyl Blue.

Abd-El Rahman Ahmed D, Ghanem N, Dessouki ShM, Faheem MS, Gad AY and Barkawi AH.

World Vet. J. 10(2): 246-253, 2020;
pii:S232245682000032-10
DOI: <https://dx.doi.org/10.36380/scil.2020.wvj32>



Abd-El Rahman Ahmed D, Ghanem N, Dessouki ShM, Faheem MS, Gad AY and Barkawi AH (2020). Developmental Competence of Buffalo Oocytes Cultured Under Different Oxygen Tensions after Selection with Brilliant Cresyl Blue. *World Vet. J.*, 10 (2): 246-253.

ABSTRACT

The aim of this investigation was to follow up *in vitro* preimplantation development of buffalo cumulus-oocyte complexes (COCs) after BCB test and followed by *in vitro* maturation under two different levels of oxygen tension. Cumulus-oocyte complexes (n=1045) were selected with BCB staining (oocytes with any degree of blue color in cytoplasm was defined as BCB+, oocytes without any degree of blue color in cytoplasm was defined as BCB-) in addition to a third control group. The previous experimental groups (BCB+, BCB-, control) were matured *in vitro* under low (5%) and high oxygen tension (20%), followed by *in vitro* fertilization and *in vitro* culture of presumptive zygotes. There were no differences ($P \leq 0.05$) in cleavage, morula and transferable embryos rates among BCB+, BCB- and control group. However, blastocyst rate was greater significantly in control group (14.4 ± 2.0) than BCB- COCs (8.4 ± 1.9). According to the oxygen tension effect, the rate of morula and transferable embryos was increased ($P \leq 0.05$) in buffalo COCs developed under low oxygen tension (11.6 ± 1.4 and 23.8 ± 1.9) compared to high oxygen tension group (7.4 ± 1.4 and 17.9 ± 2.1). In addition, cleavage, morula, blastocyst and transferable embryos rates were greater in BCB+ under low (43.6 ± 3.9 , 14.9 ± 2.5 , 14.1 ± 2.9 and 28.4 ± 3.6) than high oxygen tension group (33.5 ± 3.9 , 7.1 ± 2.5 , 11.6 ± 2.9 and 18.8 ± 3.6) which may reflect enhanced biological processes controlling early development. Moreover, blastocyst rate was significantly higher in control group cultured under high (12.0 ± 2.9) and low (16.9 ± 2.8) oxygen level than their counterparts of BCB- group (9.3 ± 2.9 and 7.6 ± 2.6 , respectively). In conclusion, there was no differences in embryo development between BCB+ and BCB-, COCs; therefore, oocyte selection based on BCB staining is not an effective tool to select developmental competent buffalo COCs. Buffalo morula and transferable embryos prefer low oxygen tension for early development, which should be applied during *in vitro* embryo production of this species.

Keywords: Brilliant cresyl-blue staining, Cumulus-oocyte complex, Morula, Preimplantation.

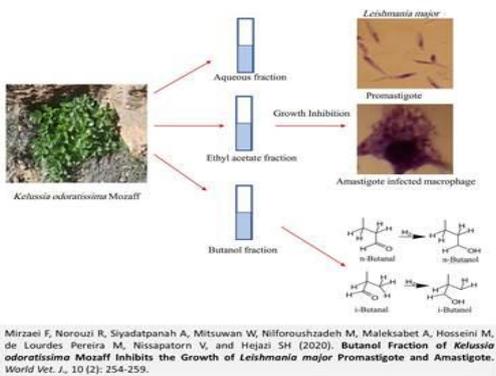
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Research Paper

Butanol Fraction of *Kelussia odoratissima* Mozaff Inhibits the Growth of *Leishmania major* Promastigote and Amastigote.

Mirzaei F, Norouzi R, Siyadatpanah A, Mitsuwan W, Nilforoushzadeh M, Maleksabet A, Hosseini M, de Lourdes Pereira M, Nissapatorn V, and Hejazi SH.

World Vet. J. 10(2): 254-259, 2020;
pii:S232245682000033-10
DOI: <https://dx.doi.org/10.36380/scil.2020.wvj33>



Mirzaei F, Norouzi R, Siyadatpanah A, Mitsuwan W, Nilforoushzadeh M, Maleksabet A, Hosseini M, de Lourdes Pereira M, Nissapatorn V, and Hejazi SH (2020). Butanol Fraction of *Kelussia odoratissima* Mozaff Inhibits the Growth of *Leishmania major* Promastigote and Amastigote. *World Vet. J.*, 10 (2): 254-259.

ABSTRACT

Naturally derived compounds have been used to treat several infectious diseases including leishmaniasis. The study aimed to investigate the *in vitro* effects of *Kelussia odoratissima* Mozaff extract on *Leishmania major* promastigote and amastigote. Dried leaves of *K. odoratissima* were fractionated by 3 solvents including aqueous, butanol, and ethyl

acetate. The results showed that the butanol fraction of *K. odoratissima* showed the highest anti-*Leishmania* effects against *L. major* promastigotes. Ninety four percent growth inhibition of the promastigote was observed when cells were treated with the 1,280 µg/mL butanol fractions. Moreover, 100% inhibition of amastigotes was detected after treatment with the butanol fraction. Half maximal inhibitory concentration (IC₅₀) of the butanol fraction in promastigotes and amastigotes was 264.1 and 154.1 µg/mL, respectively. The obtained results suggested the potential medicinal benefits of *K. odoratissima* butanol fraction as an alternative treatment for leishmaniasis caused by *L. major* infections.
Key words: Amastigotes, Butanol fraction, *Leishmania major*, *Kelussia odoratissima* Mozaff, Promastigotes

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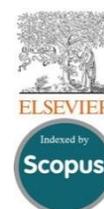
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Surgical Treatment of Canine Femoral Fractures – a Review

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ABSTRACT

Femoral fractures in dogs and cats account for 20-25% of all fractures for which surgical treatment is a method of choice. Surgical treatment is based upon biological principle of open anatomic reduction and osteosynthesis. Arbeitsgemeinschaft für Osteosynthesefragen (AO) classification of fractures has a widespread use in general. Present study discusses different methods of osteosynthesis and healing process based on special cases managed in a certain small animal clinic in Hollabrunn, Austria, in 2016. The level of femoral fracture and the chosen method of osteosynthesis are shown respectively. According to available literature and author's personal observations during externship period, the best results have been achieved using minimally invasive surgery. The surgical method choice depends on type, level and complexity of fracture, surgical skills and equipment of the team providing care respectively.

Keywords: Dog, Femur, Fracture, Osteosynthesis.

INTRODUCTION

Femoral fractures in dogs and cats include 20%-25% of all fractures. It is represented 45% of all long bone fractures, the percentage is doubled regarding all other bones individually (DeCamp et al., 2016). Traffic accident is the most common cause. Fractures may be also caused by shot injuries and bone disorders, mostly due to primary or metastatic tumors with consequent pathological fracture. Almost 86% of femoral fractures occur in animals younger than 5 years, whereas 48% appear in animals under the age of one year. Proximal and distal epiphyseal fractures happen in younger animals, whereas diaphyseal and metaphyseal fractures are more frequent in older animals (Guiot et al., 2012, Abd El Raouf, 2017). Fracture healing is influenced by many factors: biomechanical factors (postoperative fragment instability), the bone physiology (metabolic factors, importance of fracture hematoma) and anatomical factors (the type of fracture and deficient vascularization with possible vascular variations) (Perren, 2002; Kolar et al., 2010 Pan et al., 2019). Anatomy of the fracture is very well described in AO classification which is presented further in this text.

The significance of biological potential of the fracture hematoma is well known and important in the use of intramedullary nailing of the long bone fractures (Horstman et al., 2004; Schell et al., 2017). Due to inability to retain the fragments in anatomical position after fracture reduction, internal or external fixation is indicated, the choice of method depending on the fracture type respectively (DeCamp et al., 2016).

The goal of fracture treatment is to achieve final physiological function of the affected limb. To accomplish this aim the method of internal fixation can vary from intramedullary nailing/pinning (where the cellular potential of fracture hematoma has its importance) to plate application, thus including biological and mechanical benefit of the method chosen (Déjardin, 2020).

The objective of present retrospective study was to show cases of osteosynthesis of various types of femoral fractures in dogs and also results of osteosynthesis by 4-8 weeks post-surgical radiography. The data and radiographic images were used with special permission from the veterinary clinic, Tierklinik Hollabrunn, Austria.

MATERIALS AND METHODS

In this retrospective study, medical records of eight dogs who sustained traumatic femoral fracture clinically managed and operated in 2016 at veterinary clinic "Tierklinik Hollabrunn", Austria were used. Clinical examination was followed by radiological imaging in two projections: Medio-lateral and cranio-caudal, with mandatory imaging of contralateral limb. All patients were submitted to radiological imaging by digital x-ray machine preoperatively, immediately postoperatively, 4 weeks postoperatively and some of them 8 weeks after surgery. Radiological technique was designated for each patient (60-70 kV and 4-6 mAs) respectively.

Selected cases were divided according to fracture type into three groups consecutively: proximal epiphyseal (A); diaphyseal (B) and distal epiphyseal (C). AO classification was applied, and Salter-Harris classification the same for epiphyseal fractures. AO classification uses alpha-numeric system to assess location and degree of the fracture. Number 3 denotes femur and is divided further numerically: number 1: for proximal epiphysis, number 2; for diaphysis, and number 3: for distal epiphysis. The letters denote fracture type: letter A: for single fracture, letter B: for fracture with free fragment, and letter C: for compound fracture. Each fracture type is denoted with number suffix from 1 to 3 describing the comminution (Rüedi and Murphy, 2000).

Ethical approval

This article is not experimental investigation, but retrospective review of management of accidentally injured patients with purpose of companion animals' welfare.

RESULTS

Group A

Case 1. AO: 31-B3

A 6-month-old Border Collie bitch, with 15.4 kg of body weight was injured in a car incursion and sustained Salter-Harris type I femoral head fracture. Through cranio-lateral incision, the hip capsule was opened and two parallel 1.2 mm Kirschner wires were positioned. 4 and 8 weeks post-operative radiographic follow up revealed good bone healing and restitution of full function. Pre- and postoperative radiographs are shown on [figure 1](#).

Case 2. AO: 31-B3

A 5-month-old bitch of Pyrenean mountain dog with body weight of 30 kg was injured in a car incursion and sustained Salter-Harris type I femoral head fracture with three free fragments. Through cranio-lateral incision the cranial segment reduction was performed followed by fixation with three 0.8 mm Kirschner wires, remaining fragments stabilized with three 1.4 mm Kirschner wires. The remains of *ligamentum capitis ossis femoris* were removed. Ehmer bandage was applied postoperatively. After four weeks all wires were removed except 1.4 mm wire. Femoral head and acetabulum arthrosis are visible radiographically. Eight weeks post-surgical radiography showed hip displacement with clinical finding of limping, pain and crepitation on palpation of the hip joint. In this case, femoral head and neck ostectomy was indicated. Pre- and postoperative radiographs are shown on [figure 2](#).

Group B

Case 3. AO: 32-A3

An 8-month-old bitch German hunting Terrier bitch was presented with a transverse fracture in femoral shaft. MIPO approach was performed for application of intramedullary Kirschner wire and string of pearls (SOP) plate and 5 screws. In a 4 weeks post-operative radiographic examination a good callus formation was found. Clinically the dog manifested limping (¼). Pre- and post-operative radiographs are shown on [figure 3](#).

Case 4. AO: 32-C1

A 3-year-old male German Shepard was referred with open spiral fracture in left femoral diaphysis associated with a free fragment. Using lateral approach 8 mm x 185 mm intramedullary interlocking nail was introduced with one screw proximal and two screws distal locking. Drainage was also established. Four weeks post-operative radiography revealed the beginning of bone healing. Eight weeks postoperatively radiography showed poor callus formation with complete restitution of function. Pre- and post-operative radiographs are shown on [figure 4](#).

Case 5. AO: 32-A3

A 4-month-old male bitch of mongrel breed with body weight of 17.4 kg sustained a transverse fracture of right femoral diaphysis due to being hit by a horse. Using a lateral approach on diaphysis the 3.5 mm dynamic compression plate (DCP) with 8 screws was placed. Four weeks post-operative X-ray examination revealed moderate amount of callus formation, and clinical status showed clinical union. Pre- and post-operative radiographs are shown on [figure 5](#).

Group C

Case 6. AO: 33-C1

A 3-year-old castrated American Stafford Terrier bitch with body weight of 23 kg was presented with transverse supracondylar, and spiral intracondylar metaphyseal fracture in right femur (Salter-Harris type II). Using a lateral approach to diaphysis and distal part of the femur, 3.5 mm leg screw was placed transcondylar and polyaxial locking system (PAX) 3.5 plate with 4 screws proximal and 3 distal from the fracture site. Four weeks postoperative radiography indicated poor amount of callus formation. The dog was actively putting pressure on the leg and the wound healing was completed in this time. Pre- and post-operative radiographs are shown on [figure 6](#).

Case 7. AO: 33-A1

A 1.5-year-old mongrel bitch with body weight of 7 kg sustained Salter-Harris type I fracture in distal epiphysis of left femur consequent to fall over suspended wire during running. Using lateral approach to distal femoral diaphysis, two Kirschner wires were introduced. Four weeks postoperative radiography showed good bone healing, and clinically complete function restitution after 8 weeks. Pre- and post-operative radiographs are shown on figure 7.

Case 8. AO: 33-A1

A 7-year-old male Yorkshire terrier with body weight of 1.5 kg was referred with Salter-Harris type II fracture in right distal femoral epiphysis due to fall from first floor balcony. Using lateral approach to distal femoral epiphysis two cross Kirschner wires 1.0 mm and 1.2 mm were introduced. Four weeks postoperative radiography revealed normal location of implants and the beginning of bone healing associated with no signs of limping clinically. Pre- and post-operative radiographs are shown on figure 8.



Figure 1. Pre- and postoperative radiographs of Salter-Harris type I femoral head fracture in dog.

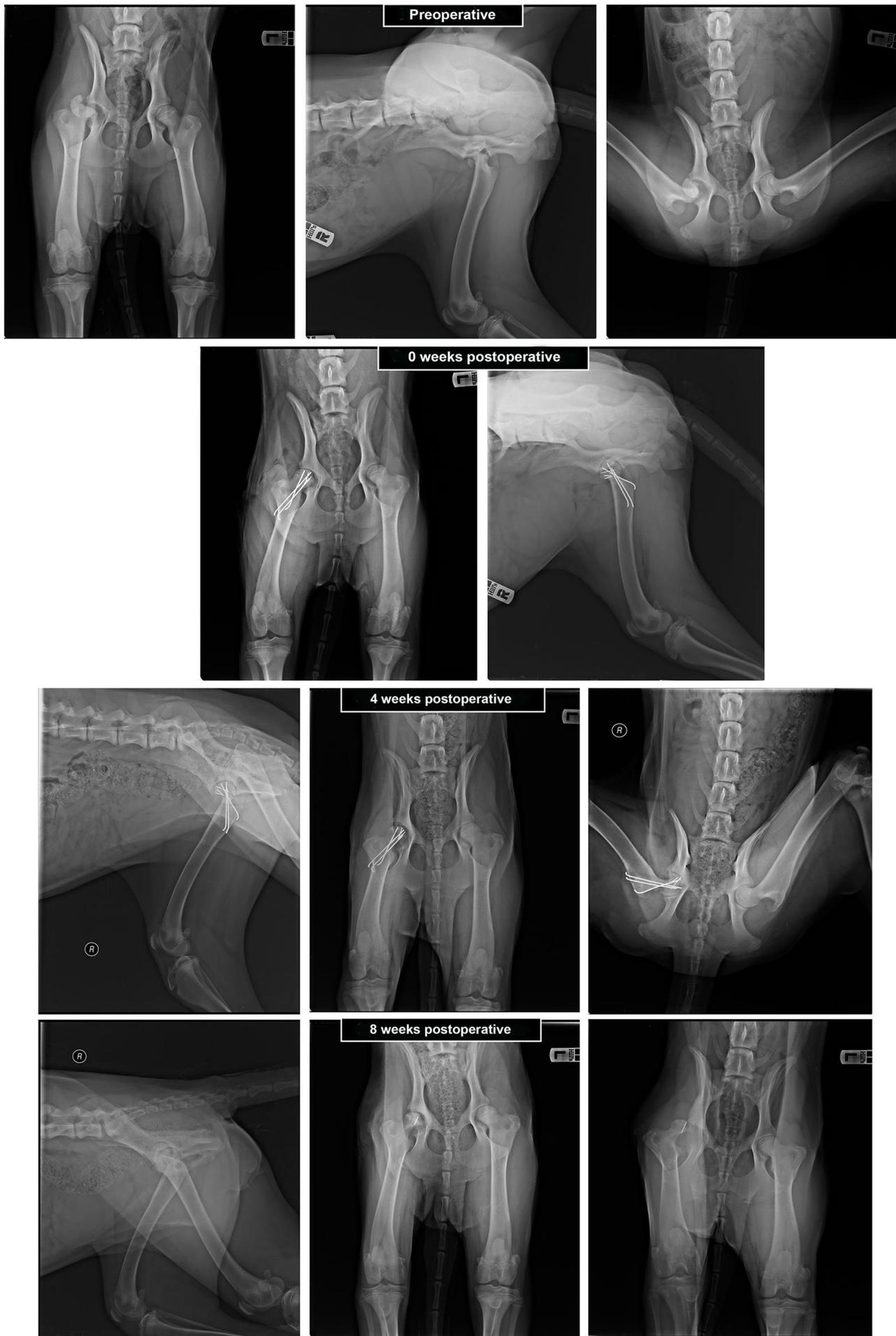


Figure 2. Pre- and postoperative radiographs of Salter-Harris type I femoral head fracture associated with three free fragments in dog.

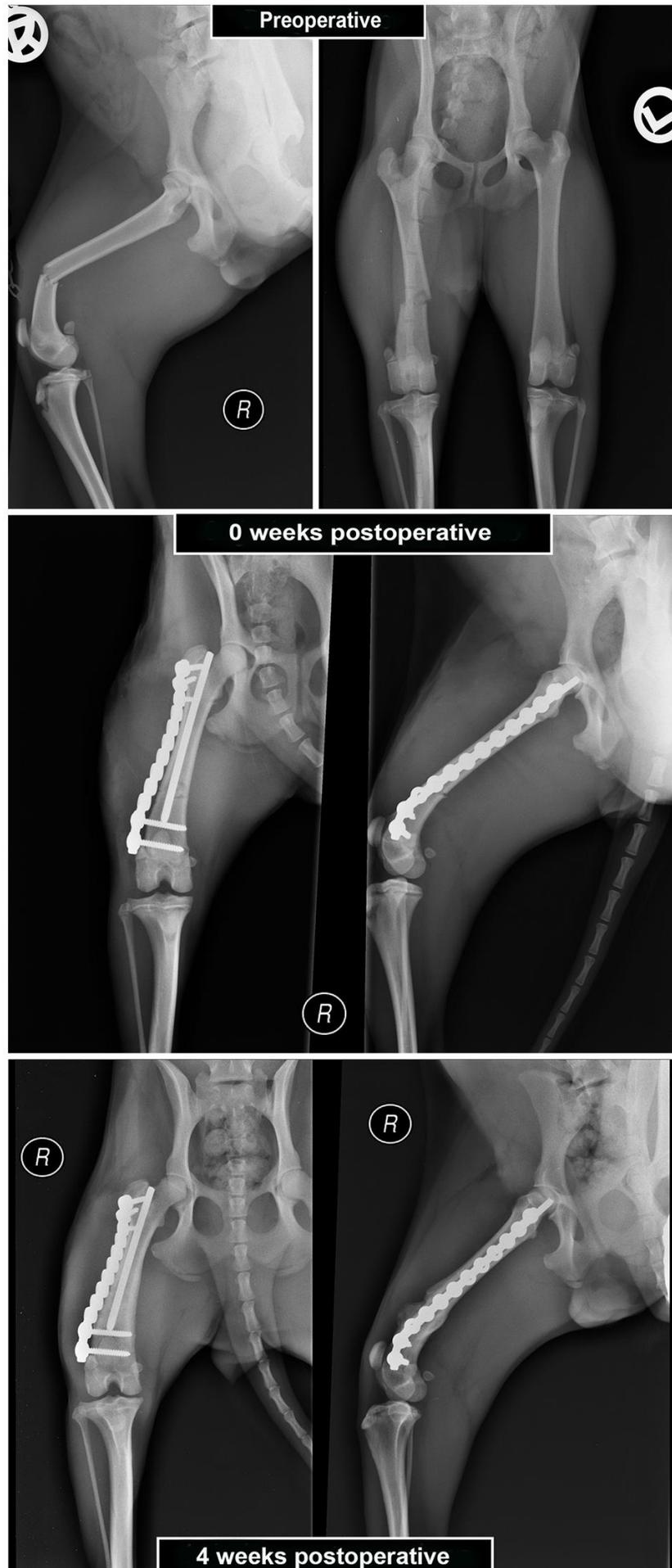


Figure 3. Pre- and postoperative radiographs of transverse right femoral diaphysis fracture in dog.



Figure 4. Pre- and postoperative radiographs of open spiral left femoral shaft fracture in dog.

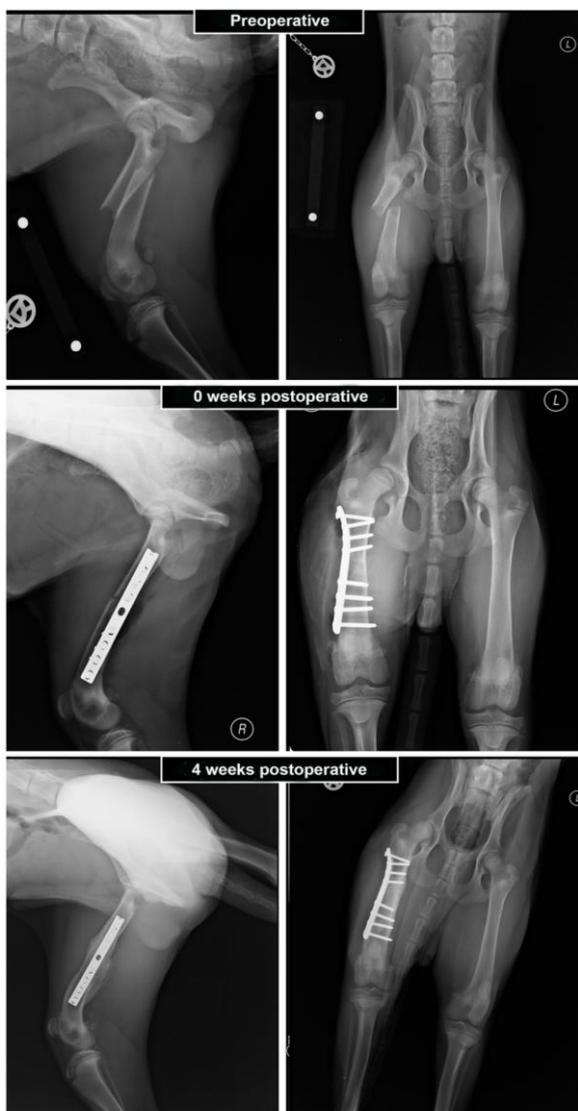


Figure 5. Pre- and postoperative radiographs of transverse right femoral shaft fracture in dog.



Figure 6. Pre- and postoperative radiographs of supracondylar transverse and metaphyseal fracture (Salter-Harris type II) of right femur in dog.



Figure 7. Pre- and postoperative radiographs of distal femoral epiphysis fracture (Salter-Harris type II) in dog.

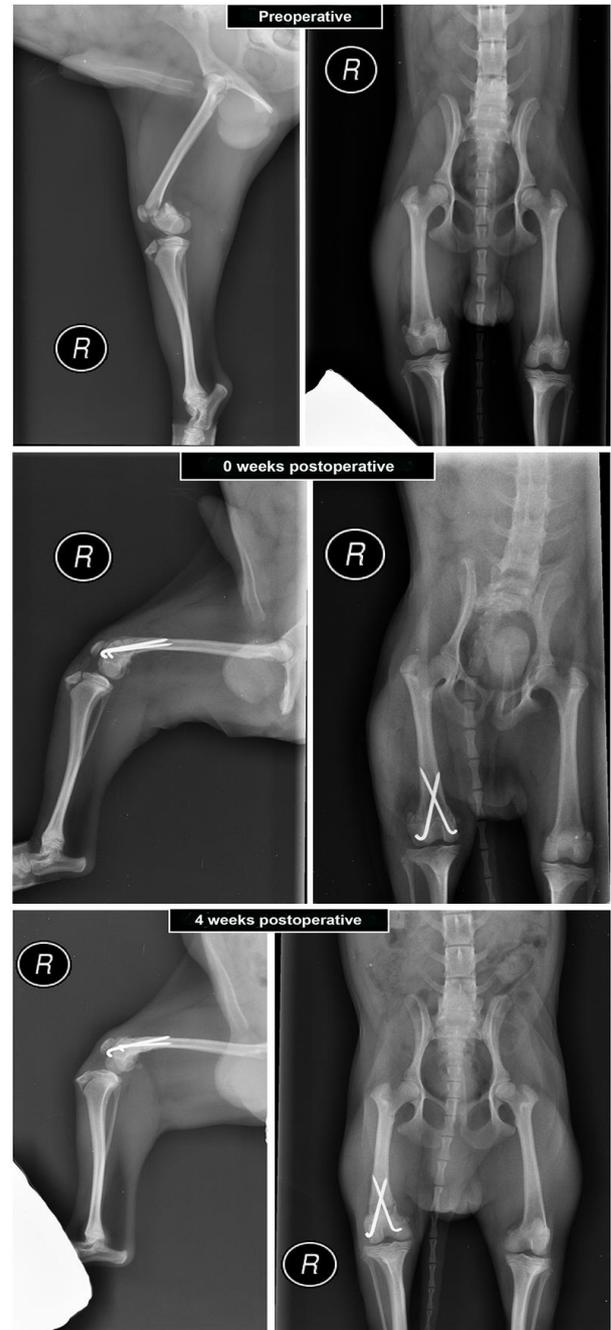


Figure 8. Pre- and postoperative radiographs of distal femoral epiphysis fracture (Salter-Harris type II) in dog.

DISCUSSION

The significance of femoral fractures in dogs and cats is high in comparison with other long bone fractures because 45% of fractures relates to femoral fractures. Closed fractures are the most frequent because of direct effect on the femur. Clinical examinations have been completing with radiography. Conservative management is practically abandoned because of poor results. Therefore, surgical treatment is a method of choice. Selection of surgical method depends on the fracture type, level and complexity, competence and available equipment as well. There are some advantages and disadvantages for each treatment method.

The most frequent of all proximal epiphyseal femoral fractures is metaphyseal fracture Salter-Harris type I (Guiot et al., 2012). In operating proximal epiphyseal femoral fractures, the most common method of choice is combination of Kirschner wires with leg screw or 2-3 Kirschner wires application. Femoral diaphyseal fractures can be stabilized by surgical application of external or internal fixation. Internal fixation can be performed by applying intramedullary wire, interlocking nail, various plates with screws, or cerclage rings and combining of all these methods (Beale, 2004). Bridging veterinary cuttable plates (VCP), minimally invasive plate osteosynthesis (MIPO) or open but do not touch (OBDNT) approach revealed to be the best choice in management of femoral shaft fractures in young dogs (Cabassu, 2019; Déjardin and Cabassu, 2004; Kowaleski, 2020; Sarran et al., 2007). Interlocking nail is another efficient implant

for biological or anatomic osteosynthesis of comminuted femoral shaft fractures (Bellon and Mulon, 2011). Biological advantage and efficacy of this implant in the management of comminuted femoral shaft fractures had been evidenced (Horstman et al., 2004; Piorék et al., 2012; Déjardin et al., 2020). It is modified Küntscher nail with transverse openings designed for insertion of transcortical screws. Those screws increase strength against bending, rotation and compression forces. This system is cheaper than intramedullary wires combined with plate, but owning similar biomechanical ability coincidentally, this is an alternative to plates as mentioned before. (Piorék et al., 2012).

The complication rate is low, even 83%-96% of femoral fractures reductions have no complications (Guiot et al., 2012; Déjardin et al., 2020). The technique of interlocking nail application produces minimal disruption of surrounding soft tissue, due to small incision, thus diminishing the possibility of retarded or delayed bone union and fracture site infections. All specified advantages make interlocking nail a plausible method of choice (Horstman et al., 2004; Piorék et al., 2012; Déjardin et al., 2020). Epiphyseal fractures are often in young animals between 4 and 11 months of age, the most frequent is Salter-Harris type II respectively. The most common method of stabilization is application of Kirschner wires, slim Steinmann nails or Rush wires in cross mode. These fractures result as much as 83% of premature closure of the epiphysis. Supracondylar fractures are considered to be rare representing only 6% of all femoral fractures. These fractures can be treated by application of plates, leg screws, Kirschner wires, Rush wires, interlocking nails or external fixators. In elder dogs, the most common method for this type of fractures is plate application. Intraarticular unicondylar or bicondylar fractures represent 10%-15% of all distal epiphyseal femoral fractures. The stabilization in these cases can be accomplished using more Kirschner wires, leg screws or combination of these methods (Guiot et al., 2012).

The complications in femoral fracture operations include wound rupture, seroma formation, retreated or delayed bone union, incision or bone infection, and hip and stifle joint arthritis or arthrosis in cases of epiphyseal fractures. According to experiences of the surgical team of veterinary clinic "Tierklinik Hollabrunn", the method preferred is intramedullary wires application associated with locking plate in cases of femoral shaft fractures. In this study, group B includes three cases of femoral shaft fractures managed by three different techniques. Radiography after 4 weeks in case number 5 (application of dynamic compression plate with 8 screws) revealed moderate amount of callus formation associated with complete restituted function. Comparing to Asma et al. (2014) study results showed discrepancy. The present study and Asma et al. (2014) study, have a lack of statistical significance due to small number of cases. According to available literature, numerous authors prefer intramedullary wires with the addition of minimally invasive plate osteosynthesis (Cabassu, 2019). This approach includes indirect method of fracture reduction by minimal incisions with intact periosteum. Some authors assume the use of intramedullary locking nail to be a method of choice providing the best results respectively (Brückner et al., 2016; Déjardin et al., 2020). Human medical studies support this attitude (Jain et al., 2004; Kesemenli et al., 2012; Ouyang et al., 2015; Duymus et al., 2019; Grubor et al., 2019). Each surgical procedure has a burden of certain percentage of complications. Taking into consideration biomechanical attributes of particular methods of bone fixation and predictable percentage of complications, it depends on the decision of every team dealing with operative treatment of femoral fractures to choose the method in which the team is the most competent and sufficiently equipped.

DECLARATIONS

Competing interests

The authors have declared that no competing interest exists.

Author's contribution

All authors participated equally in study design, data collection, data analysis, writing, and approving the final manuscript.

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Effects of Curcumin Supplementation on Viability and Antioxidant Capacity of Buffalo Granulosa Cells under *In Vitro* Culture Conditions

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ABSTRACT

The current study was conducted to investigate the possible protective effect of curcumin supplementation on buffalo granulosa cells (GCs) under *in vitro* culture condition. Buffalo ovaries were collected from local abattoir in physiological saline solution and transported directly to laboratory. Follicular fluid containing GCs and cumulus-oocyte-complexes were aspirated from antral follicles with diameter 2-8 mm. The collected GCs were seeded (Approximately 375,000 viable cells) in an 8-well culture plate containing tissue culture medium-199 (TCM-199) and kept at 37 °C in a humidified atmosphere of 5% CO₂. The curcumin was supplemented to TCM media at levels of 1, 2.5, 5 and 10 µM for 24 and 48 h at 37 °C or kept without treatment as control group. The viability of cells was determined using the trypan blue test. Intracellular reactive oxygen species (ROS) level was assessed by measuring the fluorescent intensity of 6-carboxy-2',7'-dichlorodihydro fluorescein diacetate (H₂DCFDA). In addition, mitochondrial activity of GCs was determined. The results of the present study indicated that the viability of GCs under culture conditions was significantly decreased in groups treated with 1, 2.5, 5 and 10 µM curcumin (86.0%, 86.26%, 83.0% and 74.0%, respectively) compared to control group (93.60 %). The two groups of granulosa cells cultured with 2.5 and 5 µM curcumin recorded greater level of mitochondrial activity than the groups cultured with 1 µM and 10 µM curcumin. Moreover, there was a significant increase in ROS level in group cultured with 10 µM curcumin, compared to control and other experimental groups. The enzyme activity of catalase (CAT), superoxide dismutase (SOD), glutathione (GSH) and 1, 1-diphenyl-2-picrylhydrazyl (DPPH) was increased after treating *in vitro* cultured granulosa cells with 5 µM of curcumin. However, the enzymatic activity of CAT, SOD, GSH and DPPH was declined significantly 48 h post-curcumin treatment. In conclusion, supplementation of curcumin at low concentration (2.5 µM) for 24 h to *in vitro* cultured GCs improved intracellular metabolic activity and antioxidant protective system, whereas it could not sustain this action for 48 h. Moreover, supplementation of curcumin at high concentration and for long duration may negatively affect viability of GCs under *in vitro* culture condition via induction of oxidative stress.

Key words: Antioxidant, Buffalo, Granulosa cells, Oxidative stress, Viability.

INTRODUCTION

Oxidative stress, mediated by oxygen-derived free radicals (also known as reactive oxygen species, ROS) is a frequent state affecting nearly all living organisms because of suboptimal environmental conditions. In homeostatic situation, there is stability between the production of ROS and scavenging power of cells through the cellular antioxidant system (Panieri et al., 2016). Nevertheless, when the production of ROS overcomes the cellular antioxidant capability, it perhaps contributes to a problem referred to oxidative stress (Agarwal et al., 2005). The ROS level could be elevated endogenously during many physiological and reproductive procedures including ovulation (Agarwal et al., 2005; Gupta et al., 2010). Moreover, the use of oxygen as a respiratory substrate was reported to produce oxidative stress throughout the aerobic metabolic process and energy production (Frisard and Ravussin, 2006). Furthermore, other endogenous sources (mitochondria, inflammatory cell activations, plasma membrane nicotinamide adenine dinucleotide phosphate, oxidase, lysosomes, and peroxisomes) may affect the production of ROS in mammalian cells (Klaunig et al., 2009).

Incidence of oxidative stress mediated by ROS was found to be having a negative effect on female reproductive system and finally causes infertility (Agarwal et al., 2012). Ovarian granulosa cells (GCs), the major cellular constituent in a follicle, have two vital functions in female reproduction: steroid production and defend the oocyte throughout ovulation (Yada et al., 1999; Soheli et al., 2013; Cinar and Soheli, 2015). At the end of follicular growth, GCs in the dominant follicle are differentiated into luteal cells by an ovulatory luteinizing hormone (LH) surge (Duffy and Stouffer,

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2003). This method is crucial for successful ovulation and formation of corpus luteum to keep the pregnancy. On the other hand, all through ovulation after the pre-ovulatory rise of LH, inflammatory cells particularly neutrophils and macrophages are vastly hired to produce ROS to facilitate follicular rupture and the release of the oocyte (Shkolnik et al., 2011), indicating exposure of GCs to some sort of oxidative stress during ovulation. In addition to an endogenous source, environmental sources of ROS could make the situation more complex.

One of the plants efficiently used in folk medicine is *Curcuma longa* Linn (Hatcher et al., 2008). In this herb, curcumin component has the highest proportion (Aggarwal et al., 2007). Curcumin is a yellow polyphenol compound found in turmeric (Esatbeyoglu et al., 2012), and its chemical structure is *1, 7-bis (4-hydroxy-3-methoxyphenyl)-1, 6-epitadiene-3, 5-dione* (Nadkarni, 2007; Kádasi et al., 2012). It has proven to be a highly effective anti-carcinogenic, antiviral, antioxidant (Steward et al., 2008; Correa et al., 2013; Tapia et al., 2013), and anti-inflammatory substance in human and animal models (Epstein et al., 2010; Sung et al., 2012).

Curcumin acts as an antioxidant since it scavenges reactive oxygen and nitrogen species (Barzegar and Moosavi-Movahedi, 2011; Trujillo et al., 2013; Mohebbati et al. 2017) and induces cytoprotective enzymes such as glutathione-S-transferase (GST), γ -glutamyl cysteine ligase (γ -GCL) and heme oxygenase-1 (HO-1) (Dinkova-Kostova et al., 2008; Reyes-Fermín et al., 2012). It is able to scavenge hydrogen peroxide, peroxy radicals, superoxide anion, hydroxyl radicals, singlet oxygen, nitric oxide, and peroxynitrite anion (Trujillo et al., 2013). It has been revealed that curcumin causes endogenous antioxidant defense systems by modulating transcription factors such as nuclear factor (erythroid-derived 2)-like 2 (Nrf2) (Tapia et al., 2012; Liu et al. 2016; Zhang et al., 2019a; Zhang et al., 2019b; Zhu et al., 2020), activator protein-1 (AP-1), and nuclear factor kappa B (NF κ B) (Pinkus et al., 1996). Therefore, the aim of this study was to investigate the effects of curcumin supplementation to *in vitro* culture media of buffalo GCs on their viability and enzymatic defense system.

MATERIALS AND METHODS

Experimental groups

A primary culture of GCs was used as a basic technique to study the effects of curcumin supplementation on buffalo GCs cultured *in vitro* in TCM-199 medium. Primary cultures of GCs were grown in six groups. The groups were divided as the following: group 1: untreated (control), group 2: untreated control and add dimethyl sulfoxide (DMSO) (it is the dissolving solution for curcumin), group 3: treated only with 1 μ M curcumin, group 4: treated only with 2.5 μ M curcumin, group 5: treated only with 5 μ M curcumin and group 6: treated only with 10 μ M curcumin. The recovered cells were grown until they reached up to 40–50% confluency before being allocated into the different treatment groups. A minimum of 10 ovaries were used in each biological replicate. Three biological replicates of GCs were used for each experimental assay done in this study.

Collection of ovaries and granulosa cells

Granulosa cells were collected and cultured according to the procedure described by Sohel et al. (2017). A total of 120 buffalo ovaries were obtained from a local slaughterhouse, and transported in 0.9% saline solution at 37 °C within 2 h of collection. A minimum of 10 ovaries were used in each replicate in order to obtain a sufficient number of GCs for different assays. Ovaries were washed twice with 0.9% saline solution and then washed once with 70% ethanol. The follicular contents (follicular fluid containing GCs and cumulus-oocyte complexes) were aspirated from antral follicles of 2–8 mm in diameter by an 18-gauge needle attached to a 5-mL syringe and placed in a 50-mL sterile falcon tube containing 10-mL TCM-199 medium (Sigma- Aldrich, M5017, Steinheim, Germany). After collection, tubes were left for 15 min at 37 °C to allow the oocyte-cumulus complexes and cellular debris to settle at the bottom of the tube. The upper liquid containing GCs was then collected in a 15-mL falcon tube, and centrifuged at 1800 rpm for 5 min to obtain the GCs. The collected GCs were washed with 5 ml of phosphate buffer saline (PBS) that is free from calcium magnesium by repeat pipetting followed by centrifugation at 1500 rpm for 10 min. Finally, 3 ml of trypsin was added and the tube incubated at 37 °C for 3 min, then 5 ml of TCM-199 was added to inactivate trypsin by repeat pipetting of GCs followed by centrifugation at 1500 rpm for 10 min.

In vitro culture and treatment of granulosa cells

Approximately 375,000 viable cells were seeded in an 8-well culture plate (Corning Incorporated, Kennebunk, ME, USA), *in vitro* culture in medium containing TCM-199 medium (Sigma-Aldrich, D6046, Steinheim, Germany) supplemented with 10% fetal bovine serum (vol/vol), penicillin (100 U/mL) and streptomycin (100 μ g/mL) (Sigma Aldrich, P4333, Steinheim, Germany) and kept at 37 °C in a humidified atmosphere of 5% CO₂ until reached 40–50% confluency. The curcumin was added to the TCM-199 medium at the following levels (Control, DMSO, 1 μ M, 2.5 μ M, 5 μ M and 10 μ M) for 48 h with change the medium once after 24 h.

Cell morphology and viability

After treatment, GCs from different treatment groups were observed using inverted microscopy for confluency and changes in morphology. The viability of cells was determined using the trypan blue exclusion test as described by Strober (2015) with some modifications. Briefly, after the treatment, both adherent and floating cells from each treatment group were collected and resuspended in one mL of *in vitro* culture medium. Following that, 100 μ L of cell suspension and 100 μ L of 0.4% trypan blue were mixed into a micro-centrifuge tube and incubated for 1–2 min at room temperature. Ten microliters of cell mixture/ trypan blue were applied to the hemocytometer and placed under a microscope (Inverted Microscope, Leica DMI 3000B, Wentzler, Germany) at magnification of 20X for counting live and dead cells. GC viability was calculated as a percentage of viable cells from total cell count.

Cytotoxicity assay

In the present study, the neutral red uptake assay supplies a quantitative estimation of how many feasible cells in culture. It is one of the most applied cytotoxicity tests with many biomedical and environmental applications. It is based on the ability of viable cells to incorporate and bind the supravital dye neutral red in the lysosomes. The granulosa cells were seeded in 96-well tissue culture plates and were treated for the appropriate period. The plates were incubated for 2 h with a medium containing neutral red. The cells were subsequently rinsed with media, the dye was removed properly and the absorbance was read using a spectrophotometer. Once the cells have been treated, the assay can be completed in <3 h (Repetto et al., 2008).

Intracellular reactive oxygen species detection

Intracellular ROS accumulation in different treatments and control group was assessed by 6-carboxy-2', 7'-dichlorodihydro fluorescein diacetate (H₂DCFDA, Sigma-Aldrich, USA) according to the protocol described by Soh el al. (2017). The GCs from each group were incubated with 400 μ L of 15 μ M H₂DCFDA for 20 min in the dark at 37 °C. Cells were then washed twice with PBS and images were immediately captured with a Nikon Eclipse Ti-S microscope (Nikon Instruments Inc., Tokyo, Japan) using a green-fluorescence filter at excitation/emission: ~492–495/517–527 nm and images were acquired by NIS Elements software. For quantitative analysis, the mean fluorescence intensity of five non-overlapping fields in each well was measured using Image J software (Rueden et al., 2017). Data are presented as mean \pm standard deviation.

Mitochondrial activity

Mitochondrial activity of buffalo GCs was determined using MitoTracker Red CMXRos (M7512, Invitrogen, Karlsruhe, Germany) according to the previous published protocol (Prastowo et al., 2017) with small modifications. The GCs from each group were incubated with 15 μ L of 200-nM MitoTracker red dye for 45 min, followed by two washings with PBS and were then fixed overnight at 4 °C with 4% formaldehyde. The mitochondrial activity of GC samples was visualized under a laser scanning confocal microscope (LSM 710; Carl Zeiss, Germany) using specific excitation lasers at 579– 599 nm. A constant level of laser gain (master gain = 700), pinhole (1 μ m) and pixel size (1024 \times 1024) were applied during image acquisition aim to allow image fluorescence signal comparison. Resulted images were then processed using ZEN 2011 software (Carl Zeiss, Germany). For quantitative analysis, the mean fluorescence intensity of five non-overlapping fields in each well was measured using Image J software. Data are presented as mean \pm SD.

Enzyme activity

1, 1-Diphenyl-2-picrylhydrazyl (DPPH[•]) radical scavenging assay

The ability of different extracts to act as hydrogen donors was measured by DPPH radical scavenger activity. The assay was carried out according to method of Blois (1958). The DPPH, a stable free radical, contains an odd electron, which is responsible for the absorbance at 515-517 nm and for a visible deep purple color. When DPPH accepts an electron from an antioxidant compound, it is reduced to 1,1-diphenyl-2-picrylhydrazine (decolorized non-radical, DPPH₂).

Determination of superoxide dismutase (SOD) activity

SOD activity was assayed in the liver tissue by the method of Marklund and Marklund,(1974) at 420 nm for 1 min on a Shimadzu UV-2450 spectrophotometer (Shimadzu, Kyoto, Japan). Activity was expressed as the amount of enzyme that inhibits the autoxidation of pyrogallol by 50%, which is equal to 1 U/mg protein.

Determination of catalase (CAT) activity

The activity of CAT was measured by spectrophotometric method based on the decomposition of H₂O₂ as described by Aebi (1984).

Determination the profile of GSSG and GSH by HPLC

The thiols compounds of oxidized and reduced glutathione were detected by HPLC using the method of Jayatilleke and Shaw (1993). Glutathione (oxidized and reduced) reference standard purchased from Sigma-Aldrich Chemical Co (G4376, USA). The powder of glutathione was dissolved in 75% methanol in a stock of 1 mg/ml and diluted before application to HPLC. The HPLC system of Agilent (Santa Clara, USA) consisted of quaternary pump, a column oven,

Rheodine injector and 20µl loop, UV variable wavelength detector. The report and chromatogram taken from Chemstation program purchased from Agilent. Synerji RP Max column 3.9 at wavelength 210 nm with flow rate 2ml/min was used. Pot. Phosphate buffer - acetonitrile at pH 2.7 was used as an isocratic mobile phase.

Determination of adenosine tri-phosphate content in media by HPLC

The detection of adenosine tri-phosphate (ATP) by HPLC was done according to the method of Teerlink et al. (1993)

Statistical analysis

A minimum of three biological replicates was used in each experiment per each essay (number of each replicate = 3n and total number of replicates = 72n). Statistical differences of means were compared between different experimental groups and were analyzed by applying one-way ANOVA, followed by Duncan's multiple range test that was used to detect differences among means. Differences in values of means were considered significant at $P \leq 0.05$. The General Linear Model (GLM) procedure on SAS Software (SAS, 2004) was used for statistical analysis. Data are supposed to be normally distributed and were expressed as mean \pm SD of three biological replicates.

The parameters were analyzed according to the following model:

$$Y_{ijk} = \mu + A_i + e_{ij}$$

Y_{ijk} = the measured trait.

μ = Overall means.

A_i = The Effect of different levels of curcumin.

e_{ij} = Experimental error.

RESULTS

Viability

The viability of *in vitro* cultured granulosa cells (figure 3) was significantly decreased ($p \leq 0.05$) on groups treated with DMSO (88.0 \pm 1.6%), 1 µM curcumin (86.0 \pm 1.6%), 2.5 µM curcumin (86.26 \pm 1.6%), 5 µM curcumin (83.0 \pm 1.6%) and 10 µM curcumin (74.0 \pm 1.6%) compared to control group (93.60 \pm 1.6 %).

Mitochondrial activity

There were no significant differences in mitochondrial activity of granulosa cells cultured with DMSO, 2.5 µM curcumin and control group (Figures 2 and 4). In addition, there was no significant difference in the activity of mitochondria between the group cultured with 2.5 µM curcumin and 5 µM curcumin. Moreover, the two groups cultured with 1 µM curcumin and 10 µM curcumin did not show differences on mitochondrial activity. However, the two groups of granulosa cells cultured with 2.5 µM and 5 µM curcumin recorded higher level of mitochondrial activity than the groups cultured with 1 µM and 10 µM curcumin.

Reactive oxygen species (ROS) level

There were no significant differences on ROS level of granulosa cells cultured with DMSO, 1 µM and 2.5µM curcumin group (Figures 1 and 5). However, there was a significant ($p \leq 0.05$) increase in ROS level in group cultured with 10µM curcumin compared to control and other treatments. In addition, the group of granulosa cells cultured with 5µM curcumin recorded higher level of ROS than the groups cultured with 1µM, 2.5µM and 10µM curcumin.

Activity of 1, 1-diphenyl-2-picrylhydrazyl (DPPH) in *in vitro* cultured granulosa cells

There were no significant differences on DPPH radical scavenger activity of granulosa cells cultured with DMSO, 2.5µM after 48 h, 5µM after 48 h, 10µM after 48 h and 1µM after 24 h cultured of curcumin (Figure 6). In addition, there was no significant difference between granulosa cells cultured with 1µM after 48 h, 10 µM after 24 h and 2.5 µM after 24 h cultured of curcumin. In addition, there was a significant difference between the granulosa cells cultured as a control group and 5µM curcumin group for 24 h. However, the two groups of granulosa cells cultured with 5µM curcumin for 24 hours and 1 µM curcumin for 48 h recorded high level of DPPH radical scavenger activity in compared with other groups.

Activity of superoxide dismutase (SOD) in *in vitro* cultured granulosa

There were significant differences on SOD activity between the granulosa cells cultured in control group and experimental groups supplemented with curcumin (Figure 7). The maximum activity of SOD was recorded in granulosa cells cultured with 5 µM curcumin for 24 h compared to control group that had the lowest activity of this enzyme. In addition, there were no significant differences on SOD activity of granulosa cells cultured with DMSO, 1µM curcumin for 24 h, 2.5 µM, 5 µM, and 10 µM curcumin for 48 h. In addition, too no significant difference of granulosa cells cultured 2.5 µM curcumin for 24 h, 5 µM curcumin for 24 h and 1 µM curcumin for 48 h, As noted the group of granulosa cells cultured with 5 µM curcumin for 24 h recorded higher level of SOD activity than other experimental groups.

Activity of catalase in in vitro cultured granulosa cells

There were significant differences on CAT activity between the granulosa cells cultured as a control group, DMSO and 5 μ M curcumin groups for 24 h (Figure 8). In addition, no significant differences on CAT activity of granulosa cells cultured DMSO, 1 μ M curcumin for 24 h, 2.5 μ M curcumin for 24 h, 10 μ M curcumin for 24 h, 1 μ M, 2.5 μ M, 5 μ M, and 10 μ M curcumin for 48 h. As noted, the group of granulosa cells cultured with 5 μ M curcumin for 24 h recorded higher level of SOD activity than the others groups.

Activity of glutathione in in vitro cultured granulosa cells

There were significant differences ($P < 0.05$) on GSH activity between the granulosa cells cultured control group, DMSO and 5 μ M curcumin for 24 h (Figure 9). As noted, the group of granulosa cells cultured with 5 μ M curcumin for 24 h recorded higher level of SOD activity than the others groups.

Activity of oxidized glutathione in in vitro cultured granulosa cells

There was a significant ($P < 0.05$) decline in GSSG level on groups treated with curcumin at concentration of 5 μ M and 10 μ M after 24 h of culture in addition the same trend was observed in granulosa cells treated with curcumin at concentration of 1 μ M after 48 h compared to all experimental groups (Figure 10). However, the highest level of this enzyme was recorded in control group.

Intracellular adenosine triphosphate content in in vitro cultured granulosa cells

There were significant differences ($P < 0.05$) on CAT activity between the granulosa cells cultured in control group, DMSO and 5 μ M curcumin for 24 h (Figure 11). The content of ATP was increased gradually and significantly ($P < 0.05$) in ascending pattern in GCs and reached the maximum profile after 24 H in the group cultured with 5 μ M. After that, the profile of ATP was decreased ($P < 0.05$) in GCs cultured with curcumin at concentration of 5 and 10 μ M for 48 h. The lowest profile of ATP was recorded in control group.

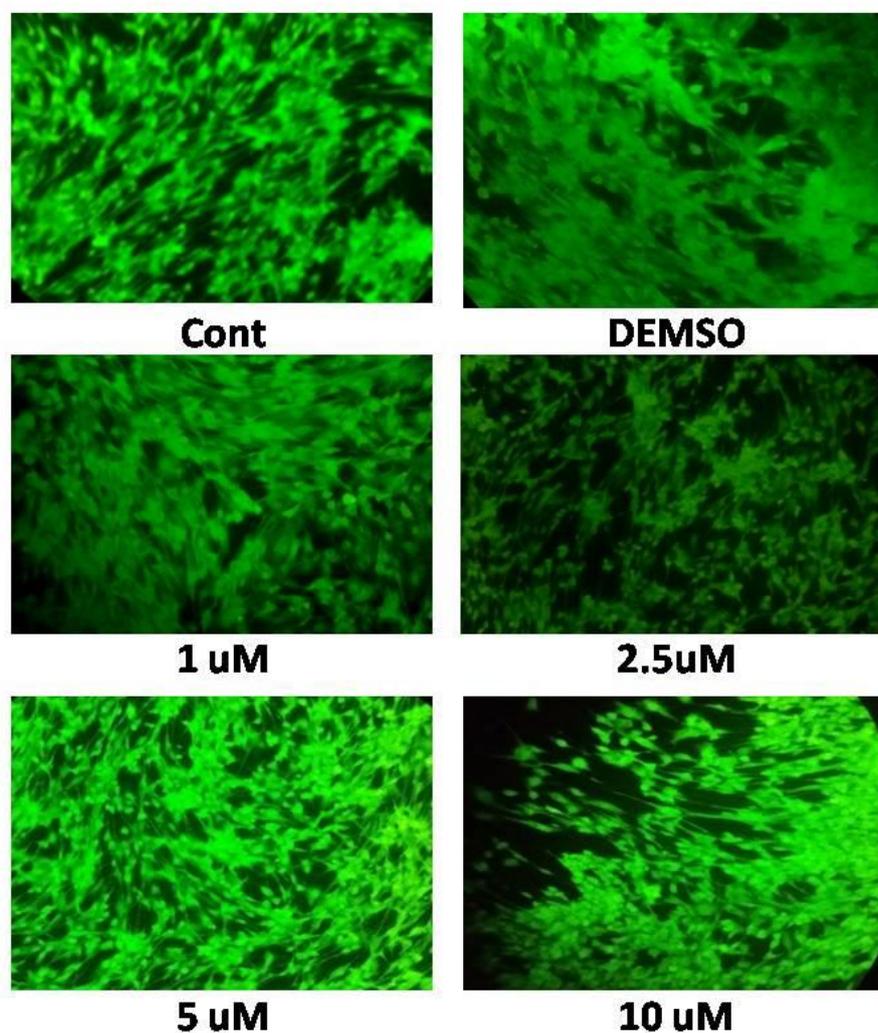


Figure 1. Image of *in vitro* cultured granulosa cells stained with H₂DCFDA measuring the level of reactive oxygen species (ROS) after supplementation with different concentrations of curcumin (1, 2.5, 5 and 10 μ M) after 24h. The image was taken by inverted microscope (Leica DMI 3000B, Wentzler, Germany) at magnification of 20X.

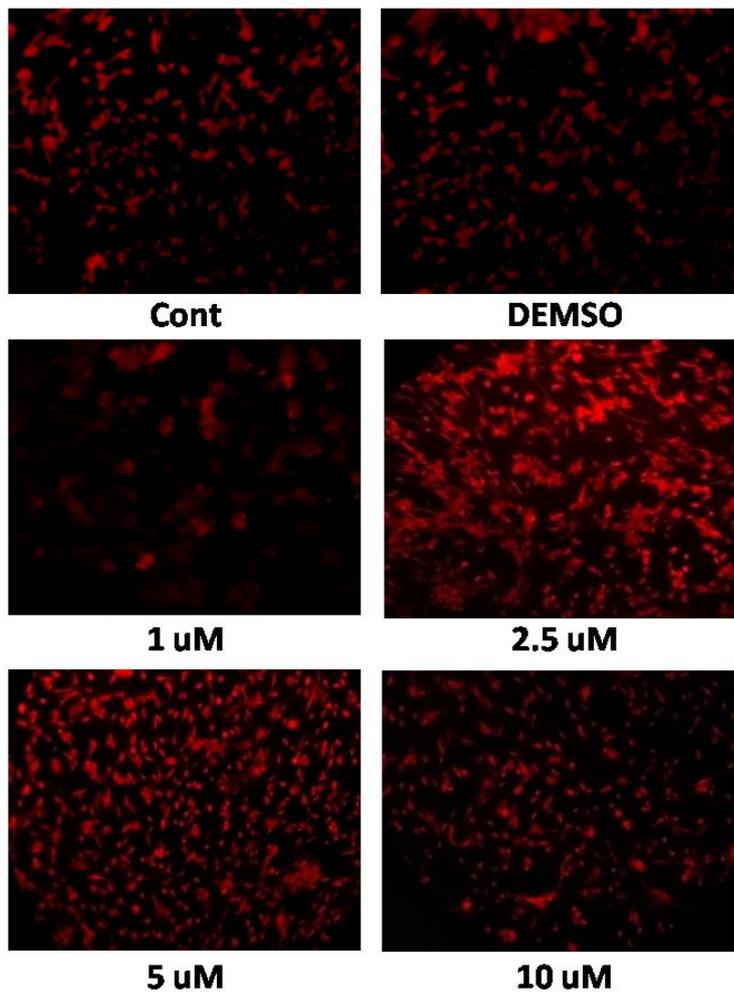


Figure 2. Image of *in vitro* cultured granulosa cells stained with mitotraker red measuring mitochondrial activity after supplementation with different concentrations of curcumin (1, 2.5, 5 and 10 μ M) after 24h. The image was taken by inverted microscope (Leica DMI 3000B, Wentzler, Germany) a magnification of 20X.

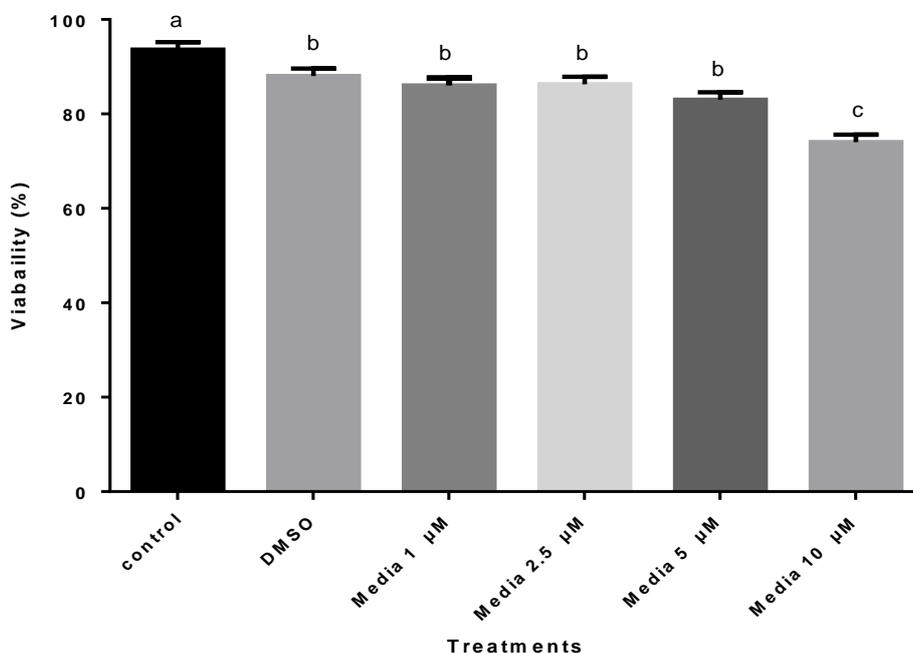


Figure 3. Viability of *in vitro* cultured granulosa cells treated with different concentrations of curcumin (1, 2.5, 5 and 10 μ M) after 24h.

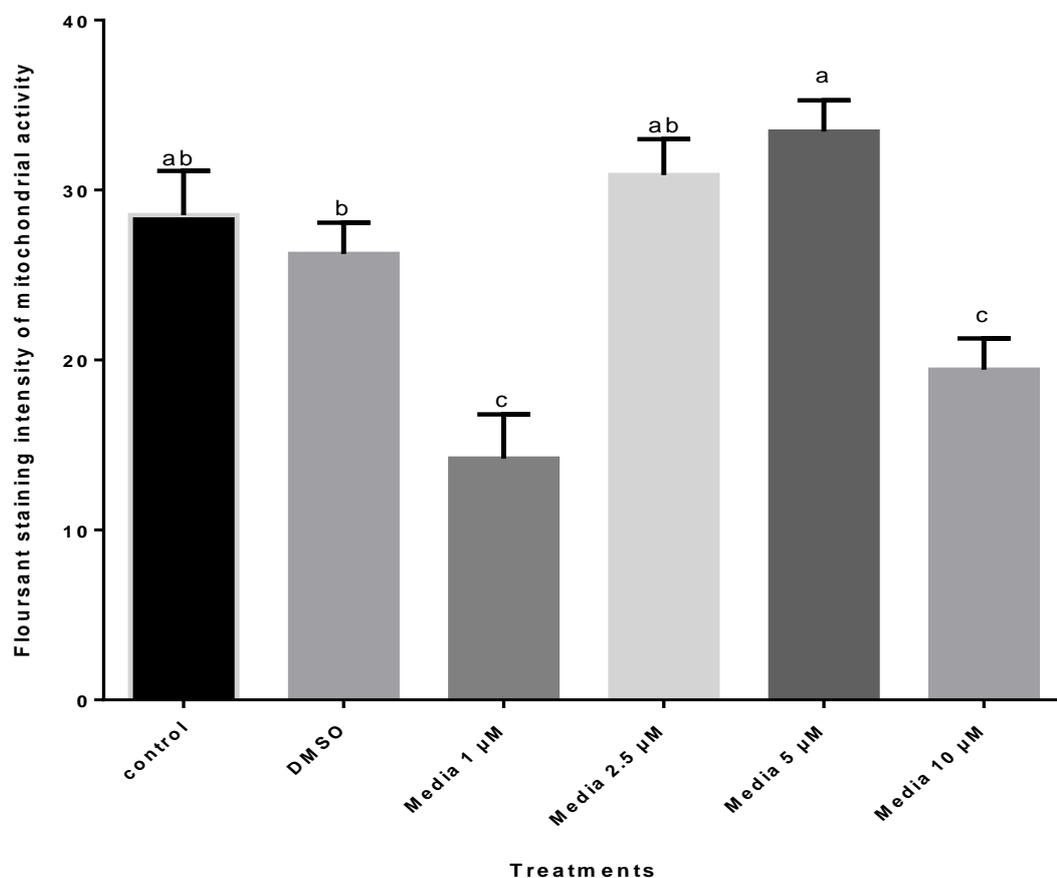


Figure 4. Mitochondrial activity of *in vitro* cultured granulosa cells treated with different concentrations of curcumin (1, 2.5, 5 and 10 µM) after 24h.

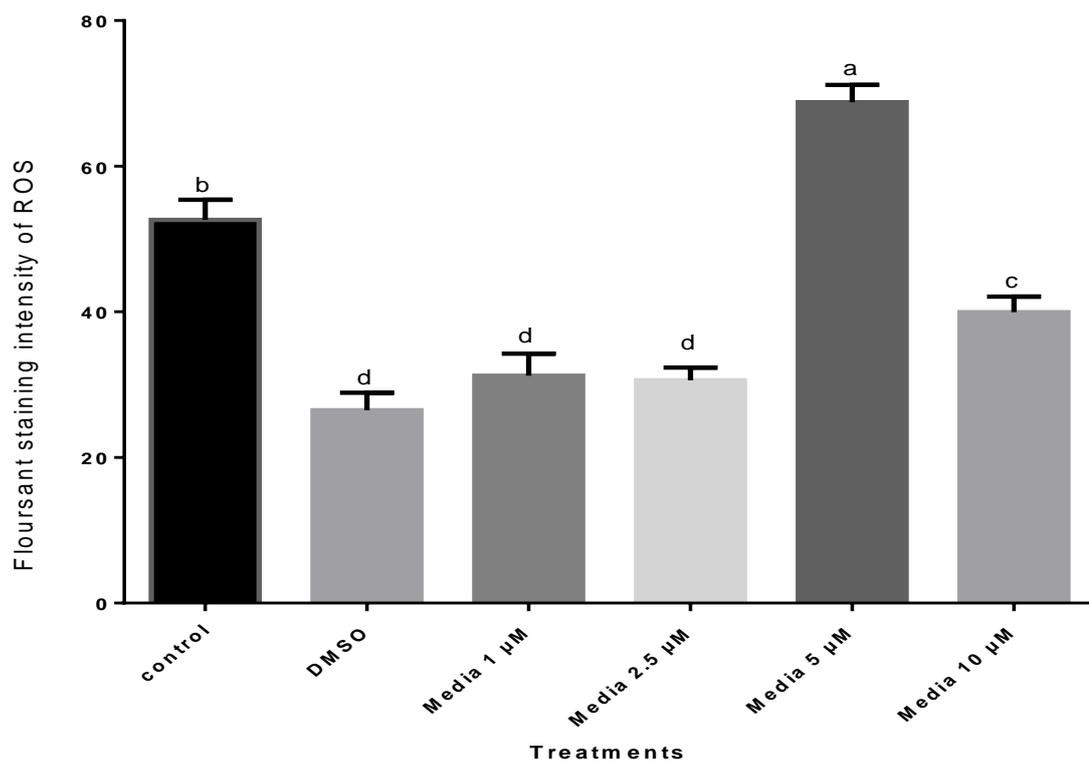


Figure 5. Intracellular reactive oxygen species level of *in vitro* cultured granulosa cells treated with different concentrations of curcumin (1, 2.5, 5 and 10 µM) after 24h.

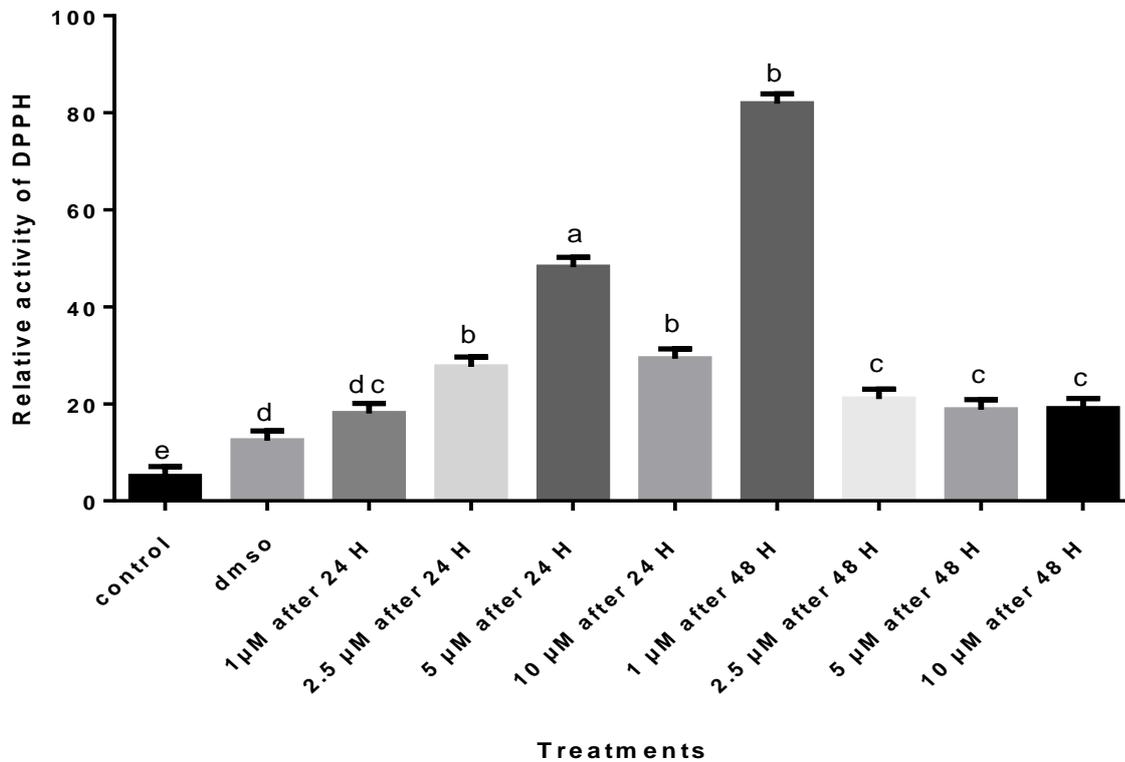


Figure 6. Enzymatic activity of 1,1-diphenyl-2-picrylhydrazyl (DPPH) in *in vitro* cultured granulosa cells treated with different concentrations of curcumin (1, 2.5, 5 and 10 μM) after 24h and 48h.

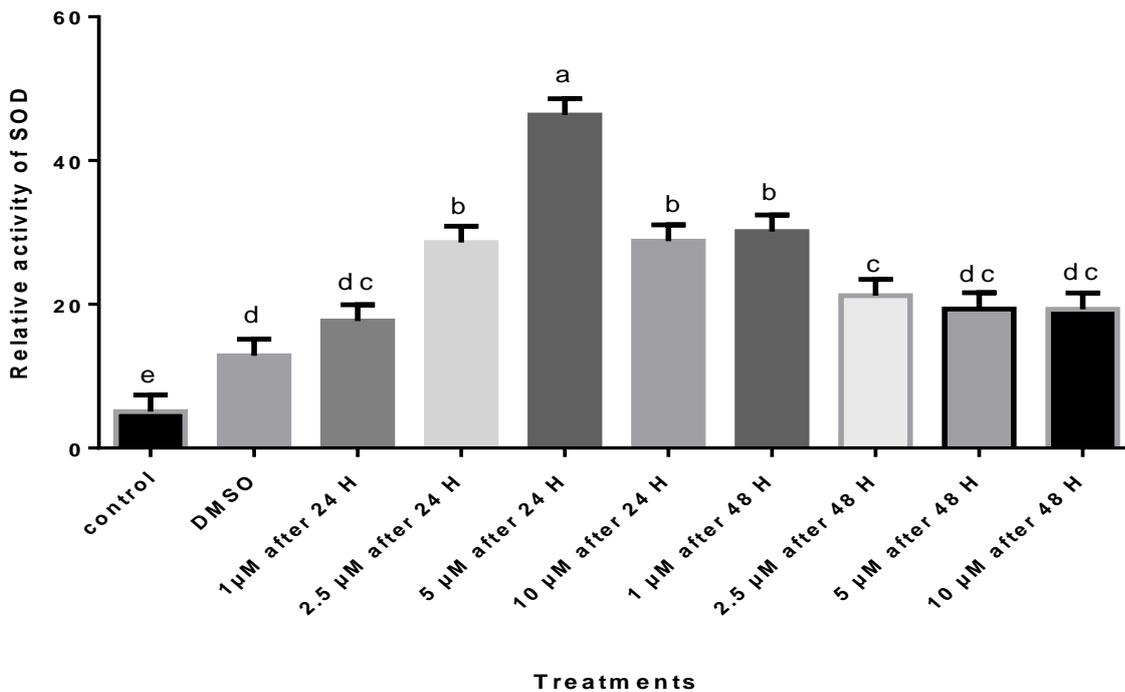


Figure 7. Enzymatic activity of superoxide dismutase (SOD) in *in vitro* cultured granulosa cells treated with different concentrations of curcumin (1, 2.5, 5 and 10 μM) after 24h and 48h.

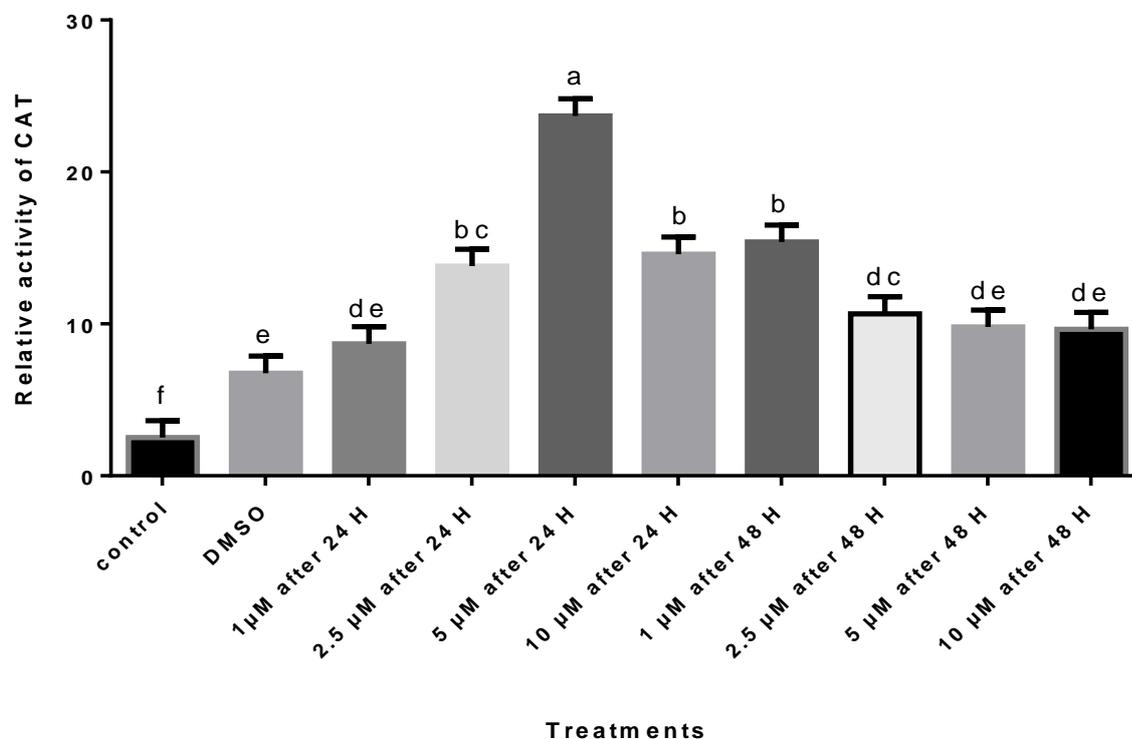


Figure 8. Enzymatic activity of catalase (CAT) in *in vitro* cultured granulosa cells treated with different concentrations of curcumin (1, 2.5, 5 and 10 μ M) after 24h and 48h.

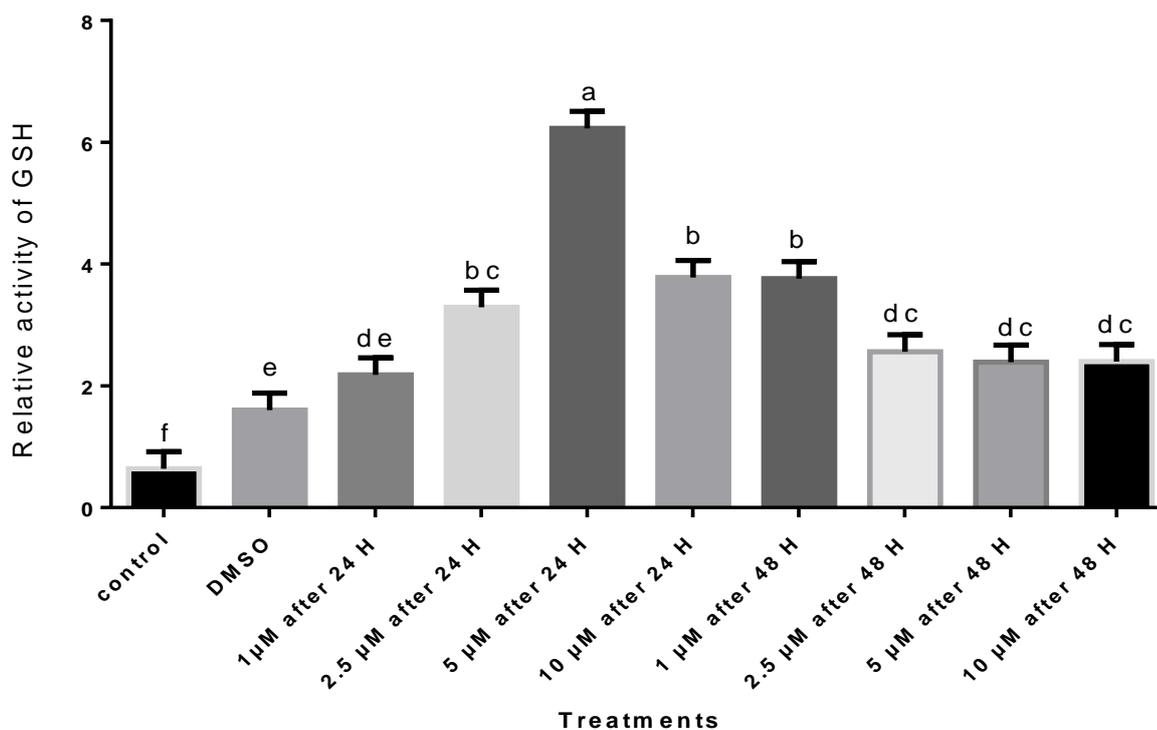


Figure 9. Enzymatic activity of glutathione (GSH) in *in vitro* cultured granulosa cells treated with different concentrations of curcumin (1, 2.5, 5 and 10 μ M) after 24h and 48h.

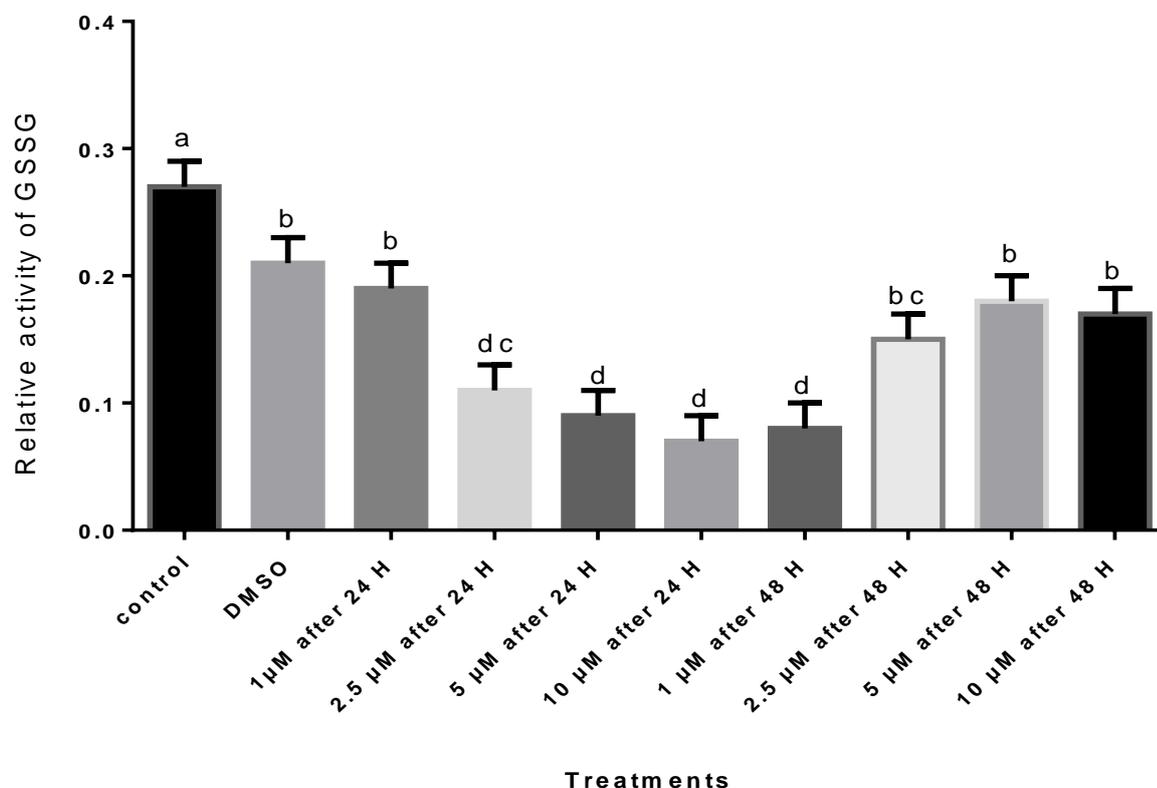


Figure 10. Enzymatic activity of oxidized glutathione (GSSG) in *in vitro* cultured granulosa cells treated with different concentrations of curcumin (1, 2.5, 5 and 10 μM) after 24h and 48h.

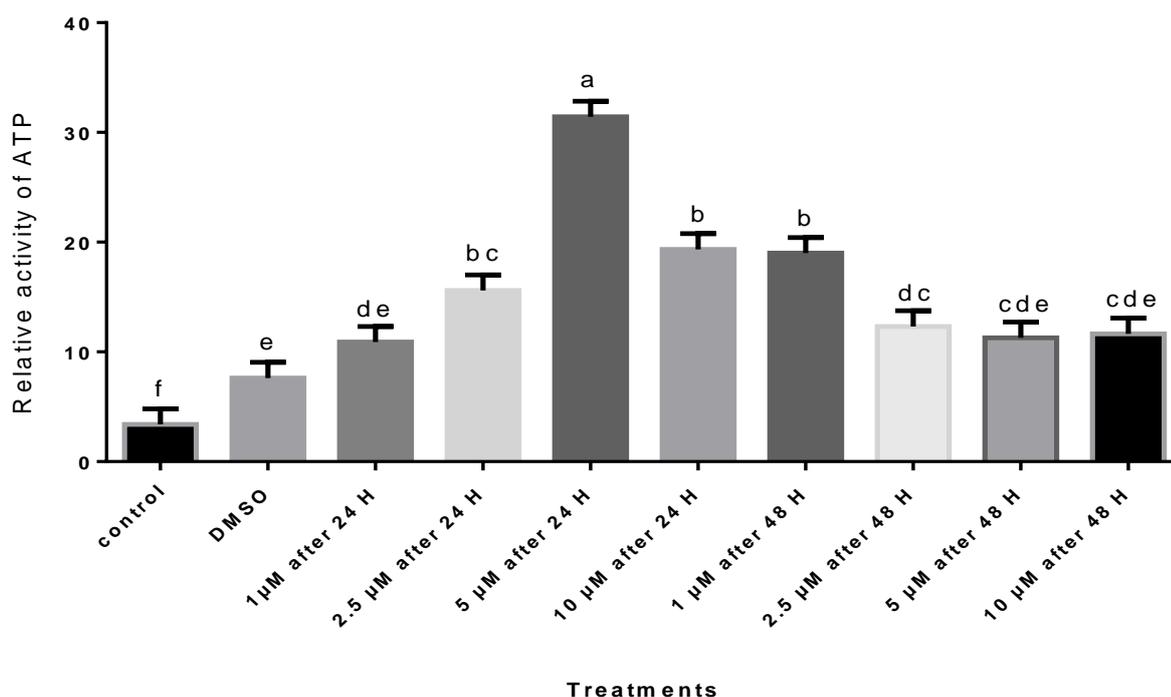


Figure 11. Intracellular adenosine triphosphate (ATP) content in *in vitro* cultured granulosa cells treated with different concentrations of curcumin (1, 2.5, 5 and 10 μM) for 24h and 48h.

DISCUSSION

Granulosa cells represent ovarian somatic cells that are in direct contact with oocytes. The GCs supports the oocyte via secretory activity, protective and nutritive effects. Therefore, GCs play a major role in acquisition of oocyte development potential and ovulation process (Buccione et al., 1990; Joyce et al., 2001; Su et al., 2009). However, these cells are also affected by oxidative stress that could be induced by ROS produced either by normal metabolic activity or as a result of *in vitro* culture conditions (Aggarwal et al., 2005). The induction of oxidative stress could subsequently lead to apoptosis of living cells (Al Dhaheri et al., 2014).

In the current study, the culture medium was supplemented with different concentrations of curcumin to alleviate the excessive ROS accumulated in GCs under *in vitro* culture condition. Results of the present study indicated reduction of granulosa cells viability cultured on groups treated with DMSO (88.0%), 1 μM curcumin (86.0%), 2.5 μM curcumin (86.26%), 5 μM curcumin (83.0%) and 10 μM curcumin (74.0%) compared to control group (93.60 %). In accordance with our results, Kádasi et al. (2012 and 2017) reported reduction in growth of *in vitro* culture swine granulosa cells after curcumin supplementation with 10 and 100 $\mu\text{g.mL}^{-1}$ compared to control and 1 $\mu\text{g.mL}^{-1}$. In addition, it was reported that curcumin down-regulated proliferation of colon cancer cells (Hanif et al., 1997). This negative effect of curcumin in cultured cells is exerted through apoptosis induction (Bhaumik et al., 1999; Liduan et al., 2004; Voznesens' ka et al., 2010). On contrast, Aktas et al. (2012) have shown a positive proliferative effect of curcumin on mice ovarian follicular cells by preventing apoptosis. Indeed, the variation in curcumin effect observed in our study and other investigations could be explained by type of cells under investigations, conditions of culture, dose and duration of treatment (Kádasi et al., 2012 and 2017).

The reduced viability of granulosa cells was coupled with increased level of ROS in groups treated with 5 μM of curcumin compared to other experimental groups in the current study. Although, curcumin is a well-known antioxidant (Mantzorou et al., 2018) that is used for reducing incidence of oxidative stress (Santos-Parker et al., 2017). However, high concentration of curcumin could induce cell death (Raza et al., 2008). Nevertheless, when the production of ROS overcomes the cellular antioxidant capability, this may lead to a problem referred to as oxidative stress (Agarwal et al., 2005). The ROS level could be elevated endogenously during many physiological procedures including ovulation (Agarwal et al., 2005). However, during *in vitro* cell culture, the ROS could be elevated to the level that cause oxidative stress (Rizzo et al., 2012; Castro et al., 2014; Hatami et al., 2014). Interestingly, our results indicated that the level of ROS and mitochondrial activity were elevated on granulosa cells cultured with 5 μM of curcumin which was linked with reduced viability of this group, confirming harmful side effects of increasing level of this compound during cell culture. Oxidative stress that occurred due to supplementation of exogenous oxidants has induced apoptosis in different types of mammalian cells, including hepatocytes (Haidara et al., 2002), epithelial cells (Jungas et al., 2002), and fibroblasts (Ran et al., 2004). On the other side, curcumin has maintained the mitochondrial respiratory function as well as redox status of PC12 cell line without influencing ROS and viability of cells (Raza et al., 2008). This in accordance with our results that demonstrated increased mitochondrial activity and ATP content in GCs supplemented with curcumin for 24 h during *in vitro* culture however, that was coupled with reduced cellular viability.

Several intracellular enzymes comprise the defense systems of mammalian cells. For example, SOD, GPX1 and CAT, GSSG and DPPH are contributing to scavenging capacity of cells to reduce the harmful effects of oxidative stress induced by ROS (Qin et al., 2015). In the current study, the enzyme activity of CAT, SOD, GSH and DPPH was increased after treating cultured granulosa cells with 5 μM of curcumin however all these enzymes were declined significantly reduced after 48 h. A recent study done by Qin et al., (2015) demonstrated a protective effect of curcumin on alleviating oxidative stress of porcine granulosa cells by rescuing the activity of antioxidant enzymes. However, the present study indicated that although curcumin increase the level of different antioxidant enzymes after 24 h of *in vitro* cell culture but it could not sustain this biological action after 48 h and cell viability was reduced due to increased ROS level.

CONCLUSION

The present findings indicated negative effect of *in vitro* culture on granulosa cell viability and redox status. Antioxidant compound namely curcumin increased the negative effect of *in vitro* culture when added at higher concentration (10 μM). However, low concentration (2.5 μM) of curcumin could maintain metabolic activity as well as defense system by up-regulation of antioxidant enzymes for short duration.

DECLARATIONS

Author's contributions

All authors have contributed to Lab work, the experimental design, writing and revision of the manuscript.

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Competing interests

All authors declare no competing interests that might interfere with the data provided in the current manuscript.

Consent to publish

All the authors approved and agreed to publish the manuscript.

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Efficacy of Ivermectin-Based Drugs against Ectoparasites in Broiler Chickens

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ABSTRACT

This research aimed to study the efficacy of two different ivermectin-based drugs against ectoparasites of chickens. In total 1200 Highsex brown chickens aged 1-1.5 years were examined to determine the prevalence of ectoparasites among chickens. The diagnosis of ectoparasites in chickens was established using clinical and entomological methods. For studying drug efficacy, 20 chickens were selected and divided into two groups (experimental and control) of 10 birds each according to the principle of analogs. A prepared ivermectin-based drug consisting of active substance ivermectin and the auxiliary substances including jojoba Resplanta, diethylene glycol monoethyl ether, Tween-80, benzyl alcohol, and purified water, was administered to the experimental group at a dose of 0.4 ml/L of drinking water (400 µg ivermectin per 1 kg of body weight) twice with a 24-hour interval. The treatment was repeated after 14 days. The control group was administered another drug based on ivermectin in the same dose and manner as the drug given in the experimental group. The efficacy of the drugs was determined by counting the number of ectoparasites per chicken before and after treatment. The clinical condition of the birds was monitored from day 1 to day 28 of the experiment. To evaluate the physiological state of chickens, blood and biochemical tests were performed on day 28 of the experiment. The results revealed that the prevalence of infection with *Menacanthus stramineus*, *Menopon gallinae*, and *Dermanyssus gallinae* in chickens was 34.5%, 21.5%, and 12%, respectively. The number of parasites/chicken after treatment between the experimental and the control group was significantly different. The efficacy of the drugs against ectoparasites in the experimental and control group was 95.6-99.0% and 85.1-91.1%, respectively. The blood tests showed that hematological and biochemical parameters were within physiological norms for both groups. Also, a pharmacokinetic study was performed on 18 ISA cross, 40-day-old chickens administered orally with the test drug at the same dose. The results revealed that ivermectin reached maximum concentration at 30-60 minutes after administration to the bird. After 1 hour, the concentration of the active substance of the drug in the blood serum of chickens decreased sharply and reached the limit of quantification by 12-24 hours. In conclusion, this drug can be recommended for use in poultry as an effective and safe drug for the treatment of arachnoentomosis in birds.

Key words: Chickens, Ectoparasites, Ivermectin.

INTRODUCTION

Prevention measures of infectious diseases are carried out in clearly prescribed terms and a certain sequence as a rule in industrial poultry farms (Limarenko et al., 2005). Unfortunately, little attention is paid to the treatment of infectious diseases. Most often it is necessary to deal with the consequences of these diseases, which leads to additional economic losses (Arisov et al., 2014).

Generally, adult chickens are kept on the floor, not in the cage, in modern poultry farms in Russia. This fact can lead to infection with ectoparasites including ticks, fleas, and biting lice, which can be found in poultry farms with different production technologies (Limarenko et al., 2005). Usually, chickens are infected with *Dermanyssus gallinae*, *Argas persicus*, *Epidermoptes bilobatus*, *Knemidocoptes* species, *Citodites* ticks, *Menopon gallinae*, and fleas (Nagornaya, 2015; Safiullin et al., 2015; Sigognault Flochlay et al., 2017). A large number of these parasites in birds lead to a decrease in egg production, loss of body weight and, often, death. Also, ticks are carriers of borreliosis, ornithosis, cholera, and plague (Safiullin et al., 2012). The broad-spectrum antiparasitic, including ivermectin-based drugs, are used to treat ectoparasites in chickens (Melnis, 2016; Nagornaya, 2016; Zubarev et al., 2016). Ivermectin (22,23-dihydroavermectin B1), consisting of the main component of 22,23-dihydroavermectin B1a and the minor component of 22,23-dihydroavermectin B1b, is one of the most active compounds of the avermectin series (Chhaiya et al., 2012). Most of the well-known drugs based on ivermectin are toxic and have a long elimination period and can accumulate in organs and tissues of animals, thus, are difficult to be applied widely in production (Semeryak, 2009).

Many manufacturers of ivermectin-containing drugs use excipients in a certain ratio and concentration, thereby contributing to the formation of a stable complex with the active substance, which enters the target organs and bypasses the undesired organs (Abramov et al., 2014). For example, an antiparasitic drug for farm animals includes ivermectin,

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vitamin E and organic solvents, which provide the formation of a stable complex of ivermectin with a prolonged duration of action. It also increases the resistance of the host organism, prevents local reactions and toxicity (Nepoklonova and Prokhorova, 2013).

The aim of the present study was to study the prevalence of ectoparasites in a poultry farm and the efficacy of an ivermectin-based drug with a new component composition of excipients against ectoparasites in broiler chickens.

MATERIALS AND METHODS

Ethical approval

This study used the most humane and operational methods and manipulations to prevent pain and distress in birds. All painful manipulations were carried out in compliance with regulatory standards of the European Convention for the Protection of Vertebrate Animals Used for Experimental and other Scientific Purposes (ETS 123), Directive 2010/63/EU on the protection of animals used for scientific purposes.

The test drugs

The work included a test drug that consists of active substance ivermectin and the following auxiliary substances: diethylene glycol monoethyl ether (DEME), Tween-80, benzyl alcohol, jojoba Resplanta, and purified water. Jojoba Resplanta (Res Pharma Industriale, Italy) is a composition of jojoba oil, glycereth-8 ether, and water, representing a non-ionizable water-soluble colloidal complex. This drug is a solution for oral administration.

Study of the pharmacokinetics of ivermectin

A total of 18 ISA cross chickens at the age of 40 days, weighing 2.0-2.2 kg, were selected for the study of the pharmacokinetics of ivermectin in the body of birds. Generally, three experimental groups of six animals each were formed from chickens. All chickens were weighed before drug administration to accurately dose the chickens.

The birds were kept in the poultry farm of Pavlovskaya Poultry Factory LLC, Nizhny Novgorod Region, Russia. The birds were monitored by veterinary specialists before and during the experiment. The birds were clinically healthy and no abnormal physiological state was noted. The test drug was administered to the birds once individually orally with water at a dose of 0.04 ml of the drug per 1 kg of body weight, which corresponds to 400 µg of ivermectin per 1 kg of body weight. Blood sampling was carried out before administration of the drug, as well as in the different times after single oral administration (first group: after 0.75, 3, 24; the second group: 0.25, 2, 6, 12; the third group: 0.5, 1, 9 hours post-drug administration). Blood was collected in polyethylene tubes without anticoagulant and serum was separated by centrifugation. Samples were delivered in a thermocontainer to the laboratory of MNITs OZOS LLC and placed in a freezer (-25 °C) until the analysis.

Determination of ivermectin in serum

The technique is based on the determination of ivermectin by high-performance liquid chromatography with pre-column modification with N-methylimidazole and trifluoroacetic anhydride and subsequent detection by fluorescence (MUK, 2009). Quantitative determination was carried out using the internal standard method. To calculate the concentrations of ivermectin in the blood serum samples, the equations obtained for the trend line of the calibration graphs for extracts of model samples of biomatrixes were used:

$$CIVE = (SIVE/SIS - b)/k$$

Where: CIVE is the desired concentration of ivermectin in serum, ng/ml; SIS is peak area of doramectin (internal standard), mV * sec; SIVE is peak area of ivermectin in the sample extract, mV * sec; k and b are coefficient of the calibration dependence.

Experimental groups

The experiment was conducted on 1,200 Highsex Brown chickens (1 to 1.5-year-old) to determine the prevalence of ectoparasites. Twenty chickens were selected and divided into two groups (experimental and control) of 10 birds each according to the principle of analogs. The experimental group was administered the prepared ivermectin-based drug at a dose of 0.4 ml/L of drinking water (400 µg ivermectin per 1 kg of bird weight), twice with a 24-hour interval. The treatment was repeated one more time after 14 days. To prepare a treatment solution of the test drug in a single dose, calculated on the treated number of birds, it was diluted ¼ in daily consumed drinking water. The control group was administered another drug-based on ivermectin in the same dose and manner as the drug tested in the experimental group.

Diagnosis of ectoparasites

The diagnosis of ectoparasites in chickens was established based on examination of the bird's body (ticks are visible to the naked eye) before and after treatment. Ticks were collected by the sweeping method using a brush on a

white sheet of paper. The collected material was preserved in 70% alcohol. Further, the species of insects were determined using a light microscope (eyepiece 10, field lens $\times 40$), and according to the practicum on the diagnosis of infectious animal diseases (Akbaev et al., 2006) and the determinant of Mallophaga in domestic animals (Blagoveshchensky, 1940). The treatment efficacy was evaluated by the number of parasites in the experimental and control group ratio before and after treatment, expressed as a percentage.

Clinical observation and blood sampling

The birds were observed from the 1st to 28th day of the experiment and changes in the bird's clinical condition were recorded. Blood sampling was performed before the start of the experiment and immediately on day 28 of the experiment. On the night before blood sampling, the bird was not fed to prevent the results distortion. Access to the water was not limited. Blood was collected in tubes coated with K3-EDTA anticoagulant for the general analysis. Blood smears were stained according to the Romanowsky-Giemsa method. Stained smears were examined under immersion magnification on a trinocular digital microscope. For the analysis of biochemical parameters, blood was collected in test tubes without an anticoagulant, centrifuged after coagulation at 3,000 rpm for 3-5 minutes to obtain serum (Voronin et al., 2006).

Statistical analysis

Statistical analysis was performed using SAS/Stat software, version 9 (SAS Institute Inc., USA). Differences were considered to be significant at p -value < 0.05 .

RESULTS AND DISCUSSION

Ectoparasites were found on the body of birds, litter, and crevices. The results of entomological studies of all chickens before drug administration showed that birds were infected with the following ectoparasites: *D. gallinae* (12.0%), *M. stramineus* (34.5%), and *M. gallinae* (21.5%). Biting louse *M. stramineus* was found on the chest, thighs and around the cloaca. The skin of birds was pale, with areas of irritation and the presence of scabs. Ruffled plumage, anxiety, bites on affected areas, and a decrease in appetite and body weight were observed in chickens infested with biting louse *M. gallinae*. In cases of infestation with *D. gallinae* an untidy and dull plumage, as well as weight loss, were observed. The results of the evaluation of the efficacy of the ivermectin-based drugs are presented in Table 1.

The number of parasites/chicken after treatment compared to before treatment decreased significantly ($p < 0.05$) in both the experimental and control groups. The efficacy of the test drug against ectoparasites in the experimental group was 95.6-99.0%. The effectiveness of another drug-based on ivermectin was 85.1-91.1%. Also, the results showed that the number of parasites/chicken after treatment between the experimental and the control group was significantly different ($p < 0.05$).

During the study, no negative effect of the drugs on the physiological state of birds was detected. No side effects and complications were noted. Hematological and biochemical parameters of blood were within physiological norms (Table 2). Hematological parameters in the experimental and the control group were slightly different ($p > 0.05$).

Dermanissiosis and malophagosis cause significant economic damage to poultry farms (Safiullin et al., 2012). There is a narrow spectrum of insecticides containing ivermectin to treat these parasitosis at the pharmaceutical market of Russian manufacturers. One of these drugs is an experimentally tested medicinal product (Neomek, "Ecoprom", Russia), which is a solution for oral use. This form is a convenient and less costly way to mass-treat birds at poultry farms and private farmsteads. The significant feature of the drug composition is the presence of an emulsifier, a jojoba Resplanta with a high content of vitamin E and a complex mixture of unsaturated essential fatty acids, which is chemically resistant (Arya and Khan, 2016). Fatty acids with ivermectin form a complex that enters the skin depot. Ivermectin is secreted gradually through the pores on the skin surface and has a prolonged antiparasitic effect. Vitamin E is a natural antioxidant, which can reduce the toxic effect, eliminate immunosuppression and unwanted side effects of the drug. The use of jojoba Resplanta in the drug composition as an auxiliary component helps to increase the bioavailability of ivermectin. According to the pharmacokinetic study of the drug, ivermectin was detected in all chickens 15 minutes after a single oral administration, reaching its maximum after 30-60 minutes. The maximum concentrations of ivermectin in the blood of chickens ranged from 145.5 to 182.7 ng/ml. The concentration of ivermectin decreased by more than 2 times by 2-3 hours. After 1 hour, the concentration of the active substance of the drug in the blood sera of chickens decreased sharply and reached the limit of quantification by 12-24 hours. Ivermectin is rapidly absorbed after oral administration and is also rapidly excreted from the systemic circulation, as can be seen from the data presented. Ivermectin can also accumulate in organs and tissues due to its lipophilic properties, this fact leads to a prolonged antiparasitic effect (Moreno et al., 2015).

The drugs used in the experimental and control groups had the same active substance (ivermectin), but different auxiliary components. The prepared ivermectin-based drug administered to the experimental group contained jojoba Resplanta. The drugs were administered through drinking water at a dose of 400 μg ivermectin per 1 kg of body weight, twice with a 24-hour interval. The treatment was repeated one more time after 14 days. Data are expressed as mean \pm

standard deviation. Means in the same row are slightly different ($p > 0.05$). AST: Aspartate aminotransferase, ALT: Alanine aminotransferase (Lineva, 2003; Nasonov et al., 2014).

The results of the present study in the treatment of ectoparasites are consistent with data from other researchers (Abramov et al., 2014; Melnis, 2016; Xu et al., 2018). The efficacy of the tested drug was slightly higher compared to other drugs based on ivermectin. Abramov et al. (2014) revealed that the drug Ivermek (active substance: ivermectin) for oral administration was highly effective against *M. stramineus*, *M. gallinae*, *Dermatoryktes mutans*, and *Aphaniptera* spp. Melnis (2016) investigated the efficacy of another ivermectin-based drug (Iversan) at the therapeutic dose of 1 ml/10 L of water once and at the same dose twice (with 24-hour interval) and reported 76% efficacy against *D. gallinae*. Xu et al. (2018) reported that the effectiveness of an oral dose of ivermectin at 5.0 mg/kg body weight of chickens against *D. gallinae* was 71.32%. Therefore, the pharmaceutical interaction of the certain ratio of components of the test drug increased the efficacy of the drug on the treatment of arachnoentomosis in birds and minimized adverse reactions and complications.

Table 1. The results of treatment of broiler chickens with ivermectin-based drugs against ectoparasites

Species	Number of parasites/chicken				Treatment efficacy (%)		Reliability of treatment ¹
	Before treatment		14 days after the last treatment		Experimental group (n=10)	Control group (n=10)	
	Experimental group (n=10)	Control group (n=10)	Experimental group (n=10)	Control group (n=10)			
<i>Dermanyssus gallinae</i>	20.30 ± 5.40	20.10 ± 4.20	0.90 ± 0.74	3.00 ± 0.67	95.6	85.1	p < 0.05
<i>Menacanthus stramineus</i>	62.40 ± 9.30	63.20 ± 8.10	0.60 ± 0.51	5.70 ± 0.67	99.0	91.1	p < 0.05
<i>Menopon gallinae</i>	51.60 ± 4.80	52.40 ± 3.80	1.20 ± 1.10	6.60 ± 1.30	97.7	87.4	p < 0.05

The drugs used in experimental and control groups had the same active substance (ivermectin), but different auxiliary components. The prepared ivermectin-based drug administered to the experimental group contained jojoba Resplanta. The drugs were administered through drinking water at a dose of 400 µg ivermectin per 1 kg of body weight, twice with a 24-hour interval. The treatment was repeated one more time after 14 days. Data are expressed as mean ± standard deviation. ¹Reliability of treatment for experimental and control groups (before and after treatment)

Table 2. Hematological and biochemical blood parameters of broiler chickens infected with ectoparasites at 14 days after the last treatment with ivermectin-based drugs

Parameters	Chicken group		Normal range
	Experimental group (n=10)	Control group (n=10)	
Hematocrit (%)	40.89 ± 1.48	41.86 ± 1.93	38-42
Red blood cells (10 ¹² /L)	2.96 ± 0.13	3.03 ± 0.10	3.0-4.0
Hemoglobin (g/L)	108.40 ± 2.66	107.30 ± 3.93	80-120
Platelets (10 ⁹ /L)	58.40 ± 2.70	56.40 ± 4.15	32-100
White blood cells (10 ⁹ /L)	20.13 ± 1.85	21.41 ± 2.39	20-40
Total protein (g/%)	31.50 ± 1.55	30.60 ± 1.59	30-60
Uric acid (mmol/L)	255.30 ± 8.46	252.20 ± 10.89	119-892
Alkaline phosphatase (u/L)	443.00 ± 42.83	400.70 ± 25.40	300-500
AST (u/L)	232.40 ± 10.80	235.90 ± 5.97	107-481
ALT (u/L)	16.30 ± 2.13	15.20 ± 2.26	13-23

CONCLUSIONS

The study demonstrated that the used ivermectin-based drug had a high efficacy (95.6-99%) in the treatment of arachnoentomosis and no side effects and drug complications in chickens were seen. Thus, this drug can be recommended for use in poultry farms as an effective and safe drug for the treatment of arachnoentomosis in birds.

DECLARATIONS

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Competing interests

No conflict of interest exists.

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Sensitivity of Lateral Flow technique for Evaluation of Inactivated Rift Valley Fever Virus Vaccine in Comparison with Serum Neutralization Test

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ABSTRACT

Rift Valley Fever (RVF) is a zoonotic mosquito-borne bunyaviral disease associated with high abortion rate, neonatal death, fetal malformations in ruminants, and mild to severe disease in human. The vaccination has significantly reduced the abortion of ewes and mortality of newborn lambs during an outbreak, and induced immunity in cattle. The evaluation of inactivated RVF vaccine required in vivo and in vitro techniques. The present research aimed to evaluate the sensitivity of the Lateral Flow Device (LFD) in comparison with Serum Neutralization Test (SNT) by reference sera to determine the humoral immune response of the sheep vaccinated with an inactivated RVF vaccine. Three batches of inactivated RVF vaccine were inoculated in three sheep groups. Then samples of their sera were collected weekly, and tested by SNT and LFD. It was found that the sensitivity of LFD at a serum dilution of 1:128 was 95%, while SNT carried out at the fourth week after the vaccination showed that antibody titers was 32,64 and 32. On the other hand, LFD had positive results at dilutions of 1:32, 1:128 and 1:64 for the vaccine batches 1, 2 and 3 respectively. These findings suggest the possibility of using LFD for detection of the immune response of vaccinated sheep to the inactivated Rift Valley Fever Virus vaccine, and it could be improved to be more quantitative in future.

Key words: Lateral flow device, Rift valley fever virus, RVFV inactivated vaccine, Vaccine evaluation

INTRODUCTION

Rift Valley Fever (RVF) is a zoonotic arboviral disease accompanied high abortion rate, neonatal death, and fetal malformations in ruminants, and mild to severe clinical symptoms in human (Baptiste et al., 2018). RVFV has tri-segmented single-stranded RNA genome, which is composed of Large (L), Medium (M), and Small (S) segments (Ikegami and Makino, 2011). RVFV caused recurrent outbreaks in African among ruminants and humans, and has caused additionally outbreaks in the Arabian Peninsula (Pepin et al., 2010).

Over the past forty years, RVFV has been detected in African countries outside its traditional enzootic regions, like Egypt in 1977 (El Akkad, 1978). In 1990, a RVF outbreak outside of Africa was confirmed on the Indian Ocean island of Madagascar for the first time. In 2000, Saudi Arabia and Yemen also reported RVFV infection (Morvan et al., 1991), and until 2007, its geographical coverage included the French island of Mayotte in the Comoros Archipelago (Sissoko et al., 2009). Rift Valley Fever Virus was classified as a Category A priority agent by NIAID/NIH, and was selected as an overlap agent by the US Department of Health and Human Services (HHS, 2005) and US Department of Agriculture (USDA, 2005). Vaccination was the most effective countermeasure against RVFV. An ideal vaccine for livestock should be safe, rapid, long-lasting potent with a single dose, and it should sufficiently prevent viremia to be transmitted by competent vectors (Kortekaas et al., 2011).

For the batch release of inactivated vaccines, practically indirect tests have been developed to minimize the use of laboratory animals, which indicated validity of the correlation with a degree of protection percentage of the susceptible animals. Indirect potency tests often includes serological tests for post-vaccination of suitable species. Alternative methods such as antigen mass, could be used if it was suitably validated (OIE, 2018). Therefore the present study was conducted to detect the protective antibody titer in vaccinated sheep with inactivated RVFV vaccine as alternative indirect potency test in comparison with Serum Neutralization Test (SNT).

MATERIALS AND METHODS

Ethical approval

The institutional Animal Care and Use Committee of the Central Laboratory for Evaluation of Veterinary Biologics, Cairo, Egypt hereby acknowledges the research manuscript and it has been reviewed by research authority and is considered to be compliant with bioethical standards in good faith.

Lateral flow device

According to [Sayed et al. \(2019\)](#), Lateral Flow Device(LFD) was developed and prepared in the Central Laboratory for Evaluation of Veterinary Biologics (CLEVB) for detection of IgG against RVFV as a part of an international scientific project funded by U.S. Civilian Research and Development Foundation (CRDF Global), USA.

Cell line

Baby Hamster Kidney (BHK-21) cell line was supplied by the Department of Rift Valley Fever Vaccine Research Department (DRVFVRD), Veterinary Serum and Vaccine Research Institute (VSVRI), Abbasia, Cairo. The cells were grown and maintained according to [Macpherson and Stocker \(1962\)](#), and used in the SNT.

Vaccine

Three batches of locally manufactured gel adjuvant inactivated RVFV vaccine which were prepared from a local isolate serotype were supplied by VSVRI which were selected for the present study. The existing vaccines were previously evaluated by the CLEVB with satisfactory safety and sterility results.

Live virus

Baby Hamster Kidney (BHK-21) cell culture which adapted strain of RVF at a titer of 10^7 TCID₅₀/ml ([Elian et al., 1996](#)) was supplied by the DRVFVR, and used in SNT to follow up the levels of induced antibodies in vaccinated sheep.

Sheep and experimental design

Fifteen native sheep aged three to six months old were allotted randomly into four groups (four animals in three vaccinated groups and three animals in the control group), and kept in separate insect-proofed stables at animal facility house of CLEVB, Cairo, Egypt. Serum samples of these sheep were previously screened by SNT, and found to be free of specific RVF antibodies (Seronegative). Groups 1, 2 and 3 were vaccinated with RVFV vaccine batches number 1, 2 and 3 respectively, while group 4 was kept without any vaccination as a control group.

Serum neutralization test

Serum samples were collected from all sheep groups at 7, 14, 21 and 28 Days-Post Vaccination (DPV) and three samples of each animal were tested to determine the RVF antibody levels by using the micro titer technique as described by [Ferreira \(1976\)](#). According to [Singh et al. \(1967\)](#), the antibody titer was calculated as the reciprocal of the final serum dilution which was neutralized and inhibited the cytopathic effect in 100 tissue cultures with infective dose 50 (TCID₅₀) of RVFV.

Sensitivity test

The test was carried out according to [Thrusfield \(2007\)](#) on Ovine RVFV antiserum obtained from Viral Large and Pet Animal Vaccines Evaluation department in CLEVB (Validated Serum). The serum samples were diluted by using two-fold dilutions (1, 1/2, 1/4, 1/8, 1/16, 1/32, 1/64, 1/128, 1/256, 1/512, and 1/1024) to determine the minimal concentration of antibodies, which indicated the positive results with the prepared LFD in comparison with antibodies which were detected by SNT. Each serum dilution was tested by 20 strips of prepared LFD for detection of sensitivity percentage.

$$\text{Sensitivity} = \frac{\text{T} +}{(\text{T}+) + (\text{F}-)} = \frac{\text{T} +}{(\text{T}+) + (\text{F}-)} \times 100 \text{ (Stated as \%)}$$

T+= true positive; F- = False Negative

Potency testing of inactivated Rift valley fever virus vaccine utilizing Lateral Flow Device

All blood samples of the sheep groups were taken at 0, 7, 14, 21 and twenty eighth Days Post-Vaccination (DPV); and the sera were tested by SNT and LFD using two folds of serial dilutions, then the results were recorded and analyzed for interpretation.

RESULTS

Detection of the prepared LFD sensitivity by using standard positive ovine RVFV antiserum in comparison with SNT showed that the minimal concentration of antibodies which showed the positive results with prepared LFD was at dilution $7 \log_2$ (1/128) with sensitivity percentage of 95 as indicated in [table 1](#). The humoral immune response in vaccinated sheep (groups 1 and 2) indicated that the protective RVFV serum neutralizing antibody titer ($1.5 \log_{10}$) started from the third week of post-vaccination using SNT and red test line of LFD (positive result) at the same dilution, while these findings in vaccinated sheep (group 3) started from fourth week of post-vaccination as showed in [table 2](#) and [figure 1](#).

Table 1. Sensitivity of prepared lateral flow device using standard positive ovine Rift valley fever virus antiserum in comparison with serum neutralizing test in CLEVB¹, Cairo, Egypt in 2019

Variable	Tested dilutions of standard positive Ovine Rift Valley Fever Virus antiserum											
	1	1/2	1/4	1/8	1/16	1/32	1/64	1/128	1/256	1/512	1/1024	
*Serum Neutralizing Test	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	-ve	-ve	-ve
**Lateral Flow Device (20 strips)	T+	20	20	20	20	20	20	20	19	0	0	0
	T-	0	0	0	0	0	0	0	0	15	0	0
	F-	0	0	0	0	0	0	0	1	0	0	0
	F+	0	0	0	0	0	0	0	0	5	0	0
Sensitivity % of LFD***	100	100	100	100	100	100	100	95	25	0	0	0

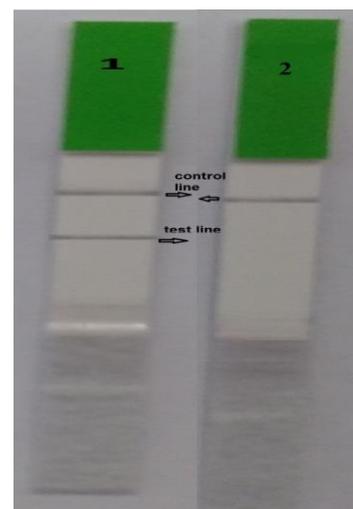
*-ve = result of SNT refer to CPE while +ve = refer to neutralization of virus by serum Antibody. **T+ = true positive, T- = true negative, F+ = false positive, F- = False negative; *** LFD: Lateral Flow Device; ¹Central Laboratory for Evaluation of Veterinary Biologics.

Table 2. Evaluation of the humoral immune response of vaccinated sheep with inactivated Rift valley fever virus vaccine using lateral flow device and serum neutralizing test in CLEVB¹, Cairo, Egypt in 2019

Serum dilutions	*Serum Neutralizing Test (SNT)				**Lateral Flow Device (LFD)			
	Group 1							
	1wpv***	2wpv	3wpv	4wpv	1wpv	2wpv	3 wpv	4 wpv
1/2	*+ve	+ve	+ve	+ve	**+ve	+ve	+ve	+ve
1/4	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve
1/8	*-ve	+ve	+ve	+ve	**+ve	+ve	+ve	+ve
1/16	-ve	-ve	+ve	+ve	-ve	-ve	+ve	+ve
1/32	-ve	-ve	+ve	+ve	-ve	-ve	+ve	+ve
1/64	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
1/128	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
Group 2								
	1wpv	2wpv	3wpv	4 wpv	1wpv	2wpv	3 wpv	4 wpv
1/2	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve
1/4	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve
1/8	-ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve
1/16	-ve	+ve	+ve	+ve	-ve	+ve	+ve	+ve
1/32	-ve	-ve	+ve	+ve	-ve	+ve	+ve	+ve
1/64	-ve	-ve	-ve	+ve	-ve	-ve	+ve	+ve
1/128	-ve	-ve	-ve	-ve	-ve	-ve	-ve	+ve
Group 3								
	1wpv	2wpv	3wpv	4 wpv	1wpv	2wpv	3 wpv	4 wpv
1/2	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve
1/4	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve
1/8	-ve	+ve	+ve	+ve	-ve	+ve	+ve	+ve
1/16	-ve	-ve	+ve	+ve	-ve	-ve	+ve	+ve
1/32	-ve	-ve	-ve	+ve	-ve	-ve	+ve	+ve
1/64	-ve	-ve	-ve	-ve	-ve	-ve	-ve	+ve
1/128	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve

*-ve = result of SNT refer to cytopathic effect while +ve refer to neutralization of virus by serum antibody. **+ve = result of LFD refer to the presence of antibody in serum while -ve means absence of antibody. ***wpv = week post-vaccination; ¹Central Laboratory for Evaluation of Veterinary Biologics.

Figure 1. Interpretation of the prepared lateral flow device for detection of IgG antibodies against Rift valley fever virus. 1: Positive result (Test and control lines are seen); 2: Negative result (Control line is only seen)



DISCUSSION

The sporadic reports of Rift Valley Fever (RVF) outbreaks in neighbor countries in addition to Inter-epizootic periods of RVFV in Egypt illustrated that there was a continuous risk of infection in Egypt. Control of RVF disease in Egypt depended mainly on the vaccination of cattle, sheep and goats. Two types of inactivated RVF vaccines were produced in Egypt (gel and oil) limited a great extent the possibilities of RVFV outbreaks in Egypt (Ahmed, 2011; GOVS, 2008).

The evaluation of inactivated RVFV vaccine in sheep was performed to assess the vaccine's safety and efficacy, while sheep are very sensitive domestic animals, and to demonstrate the specific antibody titer in the sera of vaccinated animals using SNT, indicated $5 \log_2$ (32) or a neutralizing index which is not less than 1.5 after 28th days post-vaccination, confirm the identity of the vaccine virus, and the protection margin (Heba et al., 2020).

The present study was aimed to evaluate the efficacy of existing local commercial inactivated RVFV vaccine batches in sheep using SNT and LFD for the development of an alternative indirect potency test. The sensitivity of the LFD was assessed by comparison to the technique considered as a reference in the present study. The new prepared LFD indicated a diagnostic sensitivity of 95% at the dilution of $7 \log_2$ (1/128) for detection of RVFV antibodies compared with SNT. Thus, the sensitivity test for LFD compared to SNT was agreed with Sastre et al. (2016) who compared the sensitivity of SNT and the Enzyme-Linked Immunosorbent Assay (ELISA) for simultaneous detection of antibodies against African and classical swine fever viruses using developed duplex lateral flow assay.

The humoral immune response in vaccinated sheep (group1, 2 and 3) with local commercial inactivated RVFV vaccine batches (1,2 and 3) respectively indicated a protective neutralizing antibody titer ($1.5 \log_{10}$) $5 \log_2$ at third Week Post-Vaccination (WPV) for groups 1 and 2, but at fourth WPV for group 3, while the LFD showed positive results at $5 \log_2$ at third WPV for groups 1 and 3, and at second WPV for group 2. Regarding the use of LFD for this purpose, Anouk et al. (2016) applied quantitative user Lateral Flow Assays (LFAs) for four immune markers in the whole blood samples from a longitudinal *Bacillus Calmette–Guérin* (BCG) vaccination. On the other hand, Ibrahim et al. (2017) developed Lateral flow immunochromatographic test to detect *Salmonella enteritidis* by specific antibodies in the chicken sera.

CONCLUSION

This study has shown that the LFD was appropriate for semi-quantitative evaluation of serum antibodies induced by RVFV vaccine, and was urgently needed to instantly assess the sera efficacy in the dubious batches of the vaccine. It is possible to use the LFD to detect the immune response of vaccinated sheep to the inactivated RVFV vaccine, and it could be improved in the future to be more quantitative.

DECLARATIONS

Authors' contribution

Dr. Mohamed Abousenna and Dr. Rafik Sayed performed in vitro tests (LFD sensitivity, SNT, and potency using LFD) and interpretation of results. Dr. Darwish Mahmoud conducted in vivo trial (inoculation, sampling and monitoring) while Professor Dr. Mohamed Saad supervised all the research processes, experimental design, and revision. All authors approved the final manuscript.

Competing interests

The authors declare that they have no conflict of interests.

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Molecular Evidence of *Spirometra erinacei* in Asian Wild Frogs (*Rana rugulosa*) from Banyuwangi City, Indonesia

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ABSTRACT

The tapeworm *Spirometra erinacei* is the most frequently species which found in wild frog and causing a serious parasitic zoonosis known as sparganosis. This study aimed to provide molecular evidences of spargana collected from wild frogs which used as food and contribute to provide important implication for prevention and control of sparganosis. A total of 185 Asian wild frog (*Rana rugulosa*) samples were selected from food markets in Banyuwangi City, Indonesia. Molecular identification based on spargana that were collected and coding gene of mitochondrial cytochrome c oxidase 1 (*cox1*) using Polymerase Chain Reaction (PCR) method. Spargana were found in 9.1% (17/185) of the frogs and PCR analysis results identified all specimens belonging to the species *S. erinacei*, therefore indicated that *S. erinacei* is the major causative agent of sparganosis from frogs which sold as food in markets. These findings can be useful to the molecular diagnosis and control of *Spirometra* infections in humans and animals.

Keywords: Asian wild frog, *Rana rugulosa*, Sparganosis, *Spirometra erinacei*.

INTRODUCTION

The species of the genus *Spirometra*, including *S. erinacei* (*S. erinacei*), *S. decipiens*, *S. mansoni*, *S. ranarum*, and *S. mansonoides* are all intestinal parasites of canine and feline hosts (Kavana et al., 2015). The main characters in differentiation of species are the spirally coiled uterus of *S. erinacei* and *S. decipiens* (Jeon et al., 2015). The life cycle of these parasites require 2 different intermediate hosts; the fresh water cyclops as the first intermediate host, and vertebrates such as amphibians and reptiles as the second intermediate hosts. The proceroids develop in cyclops, and the plerocercoids (spargana) develop in frogs or snakes and cause sparganosis in humans (Wiwanitkit, 2005). The tapeworm *Spirometra erinacei* is the most important species of the genus *Spirometra* tapeworms (Nakao et al., 2000). Its plerocercoid larvae (spargana) can lodge in the subcutaneous tissues and sometimes invade the abdominal cavity, eye, and central nervous system of humans causing a serious parasitic zoonosis known as sparganosis (Nithiuthai et al., 2004; Cui et al., 2011).

Sparganosis is a parasitic zoonosis endemic in Asia, Europe, and North America (Kondzior et al., 2018; Sahoo et al., 2018; Scholz et al., 2019). Humans can be infected through the consumption of contaminated water or meat from intermediate hosts or through topical application of raw, contaminated poultices to eyes and open wounds (Jeon et al., 2015; Hong et al., 2016). After entry into humans, the plerocercoid larvae (spargana) migrate to different anatomic locations, where they cause space-occupying lesions as they develop into adults. The sites spargana migrate to include skin and soft tissues, muscles, visceral organs, and the central nervous system. Clinical symptoms range from asymptomatic/mild (e.g., subcutaneous swelling) to severe (e.g., seizure and hemiparesis) depending on the site and size of lesions (Liu et al., 2015).

Sparganosis is an emerging zoonotic disease and public health challenge in Asia, potentially because of the practice of consuming wild frog meat, which is a delicacy in Indonesia (Prasetyo and Safitri, 2019). Although human sparganosis is sporadically distributed around the world, it is most frequently reported in East and Southeast Asia (Qiu and Qiu, 2009). According to a 2009 survey, more than 25% of the local wild frogs in China were infected with spargana (Li et al., 2009). Another recent report showed 104 cases of human sparganosis in China from 2000 to 2006, and more than half, or 53.9%, of cases were caused by eating snakes or frogs (Wu et al., 2007). In Thailand, over 60 sparganosis cases

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have been reported since 1943, and the patients were almost exclusively infected with *S. erinaceiropaei*, with only few cases of *S. proliferum* infections (Anantaphruti et al., 2011; Boonyasiri et al., 2014).

In Indonesia, few cases of *Spirometra* infection have been reported from snakes in Sidoarjo and Mojokerto with total prevalence 68% and 50.85% respectively (Pranashinta et al., 2017; Yudhana et al., 2019). There have been several reports of molecular evidences for the detection of spargana infection in frogs (Jongthawin et al., 2014; Zhang et al., 2015). However, there is no molecular evidence of the parasite species in frogs in Indonesia. The aim of the present study is to provide molecular evidences of spargana collected from wild animals which used as food and consumption in Banyuwangi, East Java Province, Indonesia. These results may contribute to identify the sources of infection, which provide important implication for prevention and control of sparganosis in these areas. Moreover, we designed our analysis based on coding gene of mitochondrial cytochrome c oxidase 1 gene (*cox1*) of spargana isolates from Asian wild frogs, *Rana rugulosa* from Banyuwangi, East Java Province, Indonesia.

MATERIALS AND METHODS

Ethical approval

This study was conducted with permission from the local agriculture department in East Java Province, Indonesia. This study was reviewed and approved by the Animal Care and Use Committee of Faculty of Veterinary Medicine, Universitas Airlangga, Indonesia No.1.KE.190.11.2019.

Parasite samples

A total of 185 living frog specimens (*Rana rugulosa*), commonly known as Chinese edible frog or East Asian wild frog were obtained from five food markets located around the Banyuwangi City (Central, West, East, North, and South parts) East Java Province, Indonesia (114.369227 Longitude and -8.219233 Latitude).

Sparganum collection

The presence of spargana in frogs was examined according to the methods of Ooi et al. (2000). The frogs were euthanized using ethyl-ether anesthesia, weighed, and skinned. The muscles and subcutaneous tissues were carefully observed for the presence of spargana by eyes. Then, the spargana were removed from the muscles or subcutaneous tissues and put in a Petri dish containing physiological saline to observe their movement. The number of spargana collected from each infected frog were counted to estimate the intensity of sparganum infection.

DNA extraction and amplification

Total genomic DNA was extracted from individual plerocercoid sample using the extraction kit (NucleoSpin® Tissue, Macherey-Nagel, Germany) following the manufacturer's protocol. A partial sequence of *cox1* was amplified using the primers Se658-F (5'-TTT GAT CCT TTG GGT GGT GG-3') and Se1124-R (5'-ACC ACA AAC CAC GTG TCA TG-3'), which were designed from the *cox1* gene of *S. erinaceiropaei* (GenBank accession no. AB369250) (Boonyasiri et al., 2013). PCR was performed in a 25 µl of reaction volume containing 10 ng of DNA, 2.5 µl of 10X FastStart High Fidelity Reaction buffer (Roche, Mannheim, Germany), 18 mM MgCl₂, 200 µM dNTPs, 0.2 µM each primer (Invitrogen, Carlsbad, CA), and 0.625 U FastStart High Fidelity Enzyme Blend (Roche). Thermocycling conditions (conducted in GeneAmp PCR System 9700, Applied Biosystems, Singapore) were as follows: 94°C for 5 minutes; 35 cycles of 95°C for 30 seconds, 59°C for 30 seconds, and 72°C for 45 seconds; with a final step at 72°C for 10 minutes. For each PCR experiment, a negative (no DNA) and an amplicons were separated by 1% agarose gel-electrophoresis.

RESULTS

Spirometra spargana were identified in 9.1% (17/185) of the frogs (Table 1). These spargana were 1–20 cm in long and 1–1.5 mm in wide. The prevalence of sparganum infection ranged from 3.0% to 13.6%. Most host samples were infected by several spargana, and 1-3 sparganum was selected from each host sample (Table 1). Moreover, a total of 12 parasite samples were used for molecular identification. Partial sequences of the *cox1* gene were successfully amplified for each sample which shows positive bands at 467 bp (Figure 1).

PCR analysis results identified all 12 individual spargana specimens belonging to the species *S. erinaceiropaei*. This study therefore indicated that *S. erinaceiropaei* is the major causative agent of sparganosis from frogs which sold as street food, particularly in Banyuwangi City, Indonesia.

Table 1. *Spirometra* and host samples from food markets in Banyuwangi City, Indonesia in 2019.

Host species	Source of samples	Number of samples	Infected host samples	Prevalence (%)
<i>Rana rugulosa</i>	Central Banyuwangi	35	3	8.5
<i>Rana rugulosa</i>	East Banyuwangi	44	6	13.6
<i>Rana rugulosa</i>	West Banyuwangi	39	5	5.1
<i>Rana rugulosa</i>	North Banyuwangi	34	2	5.8
<i>Rana rugulosa</i>	South Banyuwangi	33	1	3.0
Total		185	17	7.2

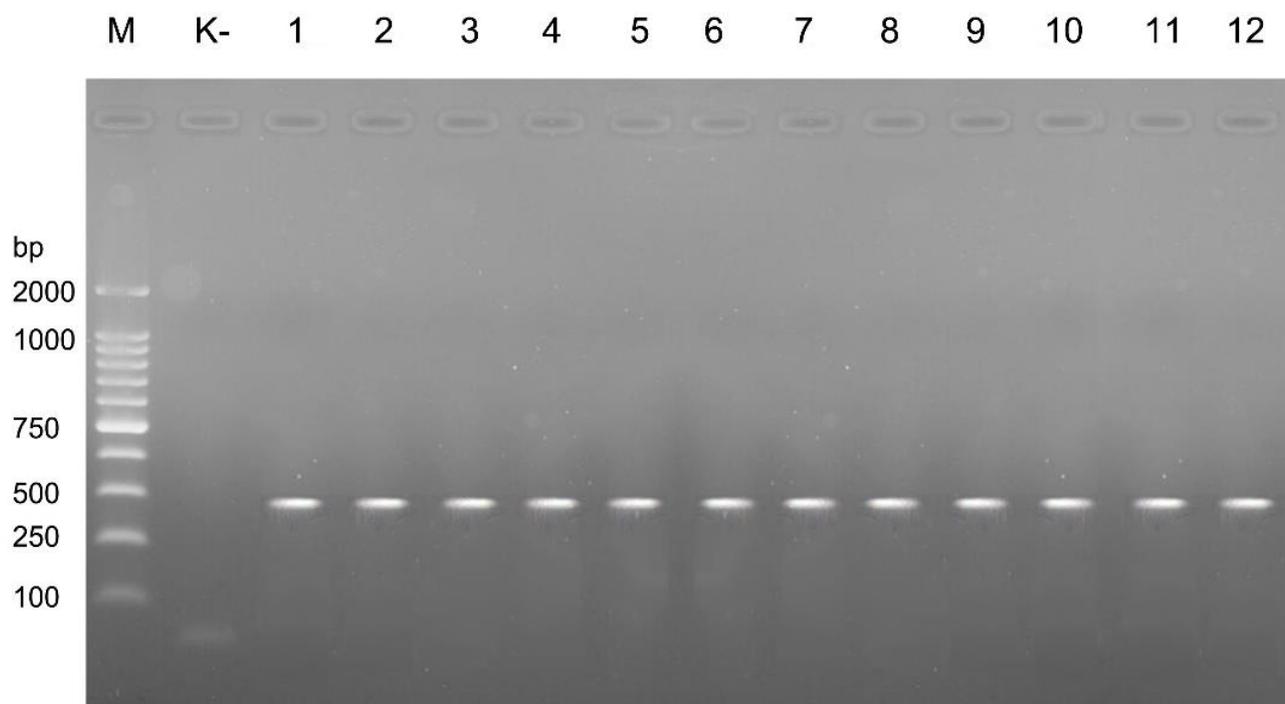


Figure 1. DNA visualization of *cox1* gene of *Spirometra erinaceieuropaei* in polymerase chain reaction products. Lane M=1:100 bp molecular weight standard, K-=Control negative.

DISCUSSION

Sparganosis is a food-borne infection caused by the migrating plerocercoid larvae of the tapeworm *Spirometra* spp. Frogs and snakes are the natural intermediate hosts for these parasites (Cui et al., 2011; Wang et al., 2011). In Chinese cuisine, which also sold in Indonesia, the bushmeat (meat of wild animals used as food) of frog and snake have an important place. The general public like this delicious bushmeat, but it poses as the potential risk of acquiring sparganosis. Spargana of *S. erinaceieuropaei* have also been found in many species of wild frogs, including *Rana nigromaculata*, *R. limnocharis*, *R. temporaria* and *Bufo gargarizans* (Liu et al, 2010; Wei et al., 2014). In a survey of Australian amphibians, *S. erinaceieuropaei* also was found in a variety of tree frogs, including *Litoria caerulea*, *L. aurea*, *L. gracilentata*, and *L. peronii* (Berger et al., 2009). There are several species in the genus *Spirometra* that can cause the disease, and the spargana of these species are often found in frogs, also there have been more than 1,700 global reports of sparganosis published (Liu et al., 2015).

Previous studies in East Java Province, Indonesia the prevalence of spargana was surveyed, but accurate species identification work was not done (Pranashinta et al., 2017; Yudhana et al., 2019).

It seems to the present study is the first report of *S. erinaceieuropaei* infection in frogs (*R. rugulosa*) from Banyuwangi City, Indonesia. Genetic diversity among *S. erinaceieuropaei* specimens from neighboring countries was discovered, and such studies should be extended in order to obtain a more complete understanding of the molecular identification of this parasite in the Asian regions. Molecular taxonomy methods based on suitable markers are well documented for identification of a group of morphologically similar parasites. These markers, such as *cox1* have been used previously for the identification of *S. erinaceieuropaei* (Zhu et al., 2002; Okamoto et al., 2007; Liu et al., 2010; Wang et al., 2011; Liu et al., 2012; Wei et al., 2014).

In the present study, phylogenetic relationship of tapeworms within the Diphyllbothriidae family, based on partial *cox1* sequences clearly distinguished the genus *Spirometra* from *Diphyllbothrium* because of identified the spargana samples as *S. erinaceieuropaei*. Additionally, molecular identification studies from Japan (Okamoto et al., 2007) and

India (Sahoo et al., 2018) of *S. erinaceiropaei* obtained in dogs found similar variations in *cox1* sequences. Future study with a larger number of samples from various localities is needed in order to clarify the genetic diversity of *S. erinaceiropaei* in Indonesia.

CONCLUSION

In present study indicated all spargana species of frogs (*Rana rugulosa*) which sold in food markets around Banyuwangi City, East Java Province, Indonesia were confirmed as *S. erinaceiropaei* with 9.1% (17/185) prevalence rate. The results of this study can help to control of *Spirometra* infections in humans and animals. Additionally, we also propose the control measures for Sparganosis such as periodic inspection of *S. erinaceiropaei* infection in frogs in markets and farms are necessary and scientific propaganda should be carried out by the local governments to inform the food restriction of wild caught frogs generally.

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DECLARATIONS

Authors' contributions

AY is a supervised and project leader. RNP carried out the collection of frog samples, and MNY carried out dissection of frog samples. DKW is a data analysis and help with collected samples. All authors contributed to the drafting and revision of the manuscript. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

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Influence of *Panicum maximum* Replacement of Clover Hay on the Performance of Growing Rabbits

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ABSTRACT

Two experiments were performed to evaluate *Panicum maximum* (Pm) and its effect on rabbits' growth performance. In the first experiment, six adult V-line male rabbits were used to determine the digestible energy in Pm by continuously feeding these 120 gram (g) Pm and 120 g clover hay for 3 days, and then the digestible energy was recorded 1959 kcal/kg. In second experiment, a total of 64 rabbits of V-line, 6 weeks old with average weight of 702 g, were divided into 4 groups, each in 4 replicates (4 rabbits/replicate), the first fed basic diet; control (T1), the 3 groups fed on the diet contained Pm to replace clover hay as a percentage of 15%, 30% and 45%, which corresponds to 4.5%, 9% and 13.5% of the total diet; which represent T2, T3, and T4, respectively. Rabbits were fed *ad libitum* with pellet feed until the end of growth attempt (14 weeks). The results indicated that the proximate analysis of Pm was 11.65% crude protein, 2.67% crude fat, and 30.66% crude fiber. Rabbits in T4 group significantly had the best final weight, daily weight gain, and Feed Conversion Ratio FCR. All groups had high crude protein digestibility except the group fed T3 diet. The total number of cecum bacterial count was improved in all tested groups. In conclusion, feeding growing rabbits with Pm up to 45% instead of clover hay achieved higher growth performance and lower cecum coliform bacteria.

Key words: Cecum bacteria, Growth performance, *Panicum maximum*, Rabbits.

INTRODUCTION

Clover hay is a conventional feed ingredient in rabbit nutrition in Egypt. However, the price is rising sharply, so it is necessary to find alternatives which are included in diets to produce balanced pellet feeds by using local raw materials that are available at a low price. Thus, the inclusion of new ingredients in diets that maintain performance and keep costs down was welcomed by rabbit producers.

Many countries in West and central of Africa and others in the tropical regions of South America and Asia used *Panicum maximum* (Pm) as forage for animals. That plant is related to the Poaceae family. It has many common names like Guinea grass, buffalo grass, and zacate Guinea. This grass was traditionally used in these aeries as a fiber source in the diet of growing rabbits (Liu et al., 2018). It is also a promising feed resource because of its various advantages such as high quality, which contains 10.5% Crude Protein (CP), 2.5% Ether Extract (EE), 30.4% Crude Fiber (CF), and 7.5% ash as reported by Ironkwe and Ukanwoko (2016). Also, it has a fast-growing rate, easy adaptation to the environment. However, only few researches have been done on the application as feed for livestock. In this respect, Udeh et al. (2007) observed that rabbits fed Pm recorded the highest feed intake compared to other forages as *Centrosema pubescens* and *Sidaacuta*. In addition, Amata and Okorodudu (2016), found that rabbits fed concentrate diet plus Pm (1:2) had higher weight gain values than rabbits fed diets concentrate diet with *Myrianthus arboreus* or concentrate with *Gmelina arborea*. Moreover, Ezea et al. (2014) concluded that pregnant rabbit fed concentrate plus a mix of forage (containing Pm) recorded better weight gain of pregnant rabbits and their litters compared to the control group (fed commercial diet). Uzegbu et al. (2010) found that pigs that fed 5% fresh green Pm had better final body weight than the control group and other pigs that fed 10, 15, or 20 % fresh green Pm.

The current study aimed to evaluate the effect of inclusion Pm instead of clover hay to the diet on the growth performance of growing rabbits.

MATERIALS AND METHODS

Location of the study

The experimental work was carried out at the El-Serw Research Farm, in the Dametta governorate in Northern Egypt, Animal Production Research Institute, Ministry of Agriculture.

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Ethical approval

The protocol for the present study was carried out at the meeting of the Animal Production Research Institute Scientific and Ethics Committee (Protocol No. 02-03-03-429).

Preparation of *Panicum maximum*

The grass was chopped to a length of 90 centimeter (cm) (about 1 month old) and air-dried inside an empty room on the farm, turned over in the morning and evening then packed with Pm hay until the start of the experiment.

Experimental design

Two experiments were conducted in the present study. In the first experiment, six adult V-line male rabbits were used in the farm where we did the experimental work to determine Digestible Energy (DE) by feeding each rabbit with 240 gram (g) (120 g clover hay + 120 g Pm) for seven days was divided into 4 days for adjustment, followed by further 3 days for data collection, where the feed intake was calculated and the feces were collected. Then feces dried and analyzed for gross energy according to the official methods (AOAC, 2000). In the second experiment, a total number of 64 V-line rabbits at the age of 6 weeks, with an average weight of 702 g \pm 6 were divided equally into 4 groups in 4 replicates, each with 4 rabbits (8 male and 8 female) existed for each group. Control group was fed a basal diet (T1), while the others three groups were fed graded Pm levels of 15%, 30%, and 45% to replace the percentage of clover hay to 4.5%, 9%, and 13.5% of the whole diet, which represents T2, T3, and T4, respectively. Experimental diets in pellet form and water were offered *ad-libitum* during the 8-weeks growth period. The composition and calculated analysis of the tested diets were listed in table 1 and covered the requirement of growing rabbit (Agriculture Ministry Decree, 1996). All diets contained almost 17% CP with 2500 kcal DE; DE/kg. Rabbits were housed in wire batteries with an open and good ventilation system. Meanwhile, live weight and feed intake were recorded, body weight gain and feed conversion ratio were calculated.

Table 1. Feed ingredients and calculated analysis of the experimental diets

Ingredients (Percentage)	T1	T2	T3	T4
Clover hay (12%)	30	25.50	21.00	16.50
<i>Panicum maximum</i>	---	4.50	9.00	13.5
Yellow corn	22.03	22.03	22.03	22.03
Wheat bran	22.17	22.17	22.17	22.17
Soybean meal (44%)	19.30	19.30	19.30	19.30
Molasses	3.00	3.00	3.00	3.00
Di calcium phosphate	2.27	2.27	2.27	2.27
Salt	0.50	0.50	0.50	0.50
Vitamin & mineral mix*	0.30	0.30	0.30	0.30
Limestone	0.24	0.24	0.24	0.24
D.L. Methionine	0.14	0.14	0.14	0.14
Anticoccidia (Diclazuril)	0.05	0.05	0.05	0.05
Total	100	100	100	100
Calculate analysis				
Crude protein (%)	17.19	17.18	17.16	17.14
Digestible energy (Kcal/kg)	2530	2538	2546	2554
Crude fiber (%)	13.35	13.38	13.41	13.44
Crude fat (%)	2.64	2.67	2.70	2.70
Calcium (%)	1.14	1.10	1.05	1.01
Total phosphorus (%)	0.86	0.86	0.85	0.85
Lysine (%)	0.92	0.92	0.92	0.92
Methionine +cysteine (%)	0.70	0.69	0.67	0.66

*Each 3 kg contain: 6000000 IU Vitamine A; 900000 IU Vitamin D3; 40000 mg Vitamin E; 2000 mg Vitamin K3; 2000 mg Vitamin B1; 4000 mg Vitamin B2; 2000 mg Vitamin B6; 10 mg Vitamin B12; 50 mg Biotin; 10000 mg Pantothenic acid; 50000 Niacin; 3000 mg Folic acid; 250000 mg Choline; 8500 mg Mn; 50000 mg Zn; 50000 mg Fe; 200 mg I; 100 mg Se, 5000 mg Cu, and 100 mg Co. T1: control diet, T2: 15% *Panicum maximum*, T3: 30% *Panicum maximum*, T4: 45% *Panicum maximum*

Digestible trail

At the end of the experimental period, a digestibility trial was conducted by using 5 rabbits / treatment to determine the digestibility coefficient of the nutrients (Dry Matter (DM), Organic Matter (OM), CP, CF, EE, and Nitrogen Free Extract (NFE)) and nutritive value of DE, Total Digestible Nutrients (TDN), and Digestible Crude Protein (DCP) according to Fekete (1985). Feed and dried feces were analyzed according to AOAC (2000).

Carcass characteristics and blood sampling

At the end of the experimental period, five animals randomly were removed from each group and slaughtered to study carcass characteristics. Head, foreparts, hind parts, trunk, cecum, total edible parts (heart, kidneys, and liver), abdominal fat and Gastrointestinal Tract (GIT) were weighed and carcass percentage was calculated according to [Cheeke \(1987\)](#). Blood samples were taken from the previous animals during slaughter in heparinized test tubes to determine Hemoglobin (Hb, g/dl), Hematocrit (Ht %), and Red Blood Cells (RBCs, $10^6 / \text{mm}^3$). The parameters which calculated are Mean Corpuscular Volume (MCV) = $\text{Ht} \times 10 / \text{RBC's} (\mu\text{m}^3)$, Mean Corpuscular Hemoglobin (MCH) = $\text{Hb} \times 10 / \text{RBC's} (\text{Pg})$, and Mean Corpuscular Hemoglobin Concentration (MCHC) = $\text{Hb} \times 100 / \text{Ht} (\text{g/dl})$. White Blood Cells ($\text{WBCs} \times 10^3 / \mu\text{l}$) and its differentiation (neutrophil, eosinophil, lymphocyte, and monocyte). All measurements performed according to [Clark et al. \(2009\)](#).

Bacterial count

To determine the total bacterial count and coliform bacteria were collected in digesta from 12 rabbits (3 rabbits per treatment) and used for microbial assays using the spread plate technique described by [Quinn et al. \(1994\)](#). The microbial level was expressed as a Colony-Forming Unit (CFU) per gram of sample. The different isolated bacterial colonies were further identified according to [Holt et al. \(1994\)](#).

Tissues Specimens

Tissues specimens were taken during the dressing process, with the proper tissue samples obtained from the liver for histo-pathological examination. The tissues were immediately fixed in a 10 % formalin-saline solution. Dried samples were cleaned in 70% absolute alcohol. All samples were then dehydrated in ascending strengths of 70% absolute alcohol. After clearing in xylol, infiltration, and embedding in paraffin wax, the tissue block was sectioned and then stained by hematoxyl in and eosin stains, according to [Mescher \(2016\)](#). The histological sections (4 to 5 microns) were examined with a light microscope supplied with an electric lamp, and the magnification power was 400X.

Statistical analysis

The data collected was analyzed statistically by the analysis of variance using the SAS Institute's General Linear Model (GLM) ([SAS, 2004](#)). Significant differences between treatments were performed using the Duncan's multiple range test ([Duncan, 1955](#)). The model used in the current experiment was $Y_{ij} = \mu + T_i + e_{ij}$. μ = overall mean of Y_{ij} , T_i = effect of treatment, $i = (1, 2, 3 \text{ and } 4)$, and e_{ij} = experimental error.

RESULTS

The proximate composition analysis, the amino acid profile and the tannin content of Pm are given in [table 2](#). Pm contains nearby similar CP to clover hay (11.65% and 12%), CF (30.66% and 30%), crude fat (2.76% and 2.1%) and DE (1959 and 1780) which indicate that Pm is suitable to replace clover hay in rabbit nutrition.

First experiment

According to the analysis by [AOAC \(2000\)](#), the DE of Pm was 1959 kcal/kg, and knowing its DE, all diets were formulated and then used in the second experiment.

Second experiment

The effect of different Pm levels on the growth performance of growing rabbits is presented in [table 3](#). The rabbits fed higher Pm level recorded significantly the best final weight compared to the control and T3 groups, but without a significant difference from T2. The same trend was observed with daily weight gain. Although feed intake was not significantly influenced by the treatments examined, it is noticeable that all groups that received different Pm levels recorded higher Feed Intake (FI) value rather than control, which indicates that the increase the inclusion of Pm in the feed the palatability of the food increased.

In terms of feed conversion ratio, a group of T4 recorded the best FCR without significant differences from the T2 group, but with significant differences from the control and a group of T3. As shown in [table 4](#), the digestibility of CP was significantly enhanced in control group, T2 and T4, while the group of T3 recorded the lowest value. However, control groups and T4 achieved a significantly higher CF digestibility than T2 and T3 groups. The rest of nutrient digestibility (DM, OM, EE and NFE) did not significantly affected via different groups. In respect of nutritional values, only the DCP was significantly affected, which followed the same trend of CP digestibility. On the other hand, TDN and DE had no significant effects from different treatments. The carcass characteristics of rabbits fed graded Pm inclusion levels ([table 5](#)) did not affect by the treatments, with the exception of the carcass and foreparts percentage, where the T2 and T3 groups recorded significantly higher carcass percentage values than the control group, while foreparts percentage was significantly improved in T3 compared to control and T4 groups, but insignificantly in T2.

The effect of different levels of Pm inclusion on the serum hematological parameters is listed in table 6. Rabbits fed T3 diet recorded a significantly higher RBC's count of 13.1% compared to the control group, 16.4%, and 19.8% compared to T2 and T4, respectively. On the other hand, Ht%, Hb, MCV, MCH and MCHC values had no significant influence on the treatments studied. White blood cells and their fractions were almost significantly affected by the inclusion of Pm in growing rabbit's diet. It is clear that rabbits fed T4 diet recorded fewer WBC count and neutrophil cells compared to other treatments. In addition, both groups of T2 and T4 recorded significantly higher lymphocyte counts of 17.6% and 31.8%, respectively, compared to the control group. The group of T2 recorded significantly the worst value of eosinophil. However, none of the treatments indicated an improvement in the monocyte count.

As shown in table 7, all of the groups tested had a higher total bacterial count in the cecum than the control group. Increasing the dietary Pm level, was significantly decreased the total bacterial count. Rabbits fed T3 recorded a significantly higher total coliform bacteria count, followed by the control group, then the T4 group and finally the lowest count was recorded for the T2 group.

According to the histological assessment, plates 1 to 4 showed liver histological sections of different treatments. The liver of control group showed degeneration in hepatocytes, focal area of hepatocellular necrosis, and edema in the sinusoids (Figure 1a). In the second group (T2), the central vein showing dilatation, dilated sinusoids and degeneration in hepatocytes (Figure 1b). In the third group (T3), the portal area showed dilated portal blood vessel, newly formed bile ductules, the focal area of hepatocellular necrosis and the hepatocyte, which revealed degeneration (Figure 1c). While in the fourth group (T4), there was granular degeneration of the hepatocytes, focal area of the hepatocellular necrosis and dilated hepatic sinusoids (Figure 1d).

Table 2. Chemical composition and amino acids profile of *Panicum maximum*.

Chemical composition		Amino acids profile			
Crude protein (%)	11.65	Methionine	0.16%	Therionine	0.34%
Moisture (%)	81.7	Lysine	0.49%	Aspartic	0.80%
Calcium (%)	0.415	Valine	0.48%	Isoleucine	0.36%
Phosphorus (%)	0.16	Alanine	0.61%	Leucine	0.67%
Ash (%)	13.1	Glycine	0.39%	Tyrosine	0.32%
Crude fat (%)	2.76	Proline	0.39%	Phenylalanine	0.45%
Crude fiber (%)	30.66	Glutamic	0.98%	Histidine	0.18%
Digestible energy (kcal/kg)	1959	Serine	0.32%	Argenine	0.45%
Anti-nutritional factors		Cysteine	0.21%		
Tannins (%)	1.734				

Table 3. Effect of inclusion of *Panicum maximum* on growth performance of growing rabbits.

Characteristics	T1	T2	T3	T4	SEM
Initial weight (g)	709	712	713	702	6.25
Final weight (g)	1736 ^b	1848 ^{ab}	1744 ^b	1988 ^a	50.8
Daily weight gain (g)	18.3 ^b	20.3 ^{ab}	18.4 ^b	23.0 ^a	0.91
Total feed intake (g)	5017	5175	5293	5131	75.4
FCR (g feed/g gain)	4.99 ^a	4.59 ^{ab}	4.96 ^a	3.82 ^b	0.22

^{a, b}: Means in the same row with different superscripts are significantly different ($p \leq 0.05$). T1: control diet, T2: 15% *Panicum maximum*, T3: 30% *Panicum maximum*, T4: 45% *Panicum maximum*

Table 4. Effect of inclusion of *Panicum Maximum* on digestion coefficients and nutritive values of growing rabbits.

Nutrients	T1	T2	T3	T4	SEM
Digestion coefficient (%)					
Dry matter	69.24	65.48	66.61	66.84	2.53
Organic matter	71.19	67.88	69.23	65.73	2.51
Crude protein	74.23 ^a	73.18 ^a	68.46 ^b	72.40 ^a	1.01
Crude fiber	42.70 ^A	31.30 ^B	34.22 ^B	42.26 ^A	1.54
Ether extract	79.15	76.20	77.08	76.84	1.69
Nitrogen free extract	76.39	74.26	70.21	72.83	4.18
Nutritive values					
Total digestible nutrients (TDN)	66.52	63.53	64.43	61.90	2.69
Digestible crude protein (DCP)	12.62 ^a	12.44 ^a	11.64 ^b	12.31 ^a	0.172
Digestible energy; kcal/kg (DE)*	2946	2814	2854	2742	119.47

^{a, b}: Means in the same row with different superscripts are significantly different ($p \leq 0.05$). ^{A, B}: Means in the same row with different superscripts are significantly different ($p \leq 0.01$). *DE (kcal/ kg) = TDN \times 44.3 according to Schneider and Flat, (1975). T1: control diet, T2: 15% *Panicum maximum*, T3: 30% *Panicum maximum*, T4: 45% *Panicum maximum*

Table 5. Effect of inclusion of *Panicum maximum* on carcass characteristics of growing rabbits.

Items (%)	T1	T2	T3	T4	SEM
Carcass	61.88 ^c	65.4 ^a	65.2 ^{ab}	62.6 ^{bc}	0.685
Head	4.98	5.23	5.74	5.19	0.236
Fore parts	19.04 ^{bc}	19.46 ^{ab}	20.17 ^a	18.32 ^c	0.267
Hind parts	20.64	21.73	22.61	23.42	1.423
Trunk	13.22	13.46	13.31	13.54	0.369
Cecum	5.09	5.01	4.70	5.21	0.549
Total edible parts	3.84	3.74	3.55	3.69	0.316
Abdominal fat	0.77	0.77	0.67	0.68	0.042
GIT*	15.07	12.27	14.84	14.72	0.72

^{a, b, c}: Means in the same row with different superscripts are significantly different. ($p \leq 0.05$). * GIT: gastrointestinal tract, T1: control diet, T2: 15% *Panicum maximum*, T3: 30% *Panicum maximum*, T4: 45% *Panicum maximum*

Table 6. Effect of inclusion *Panicum maximum* on serum hematological parameters of growing rabbits.

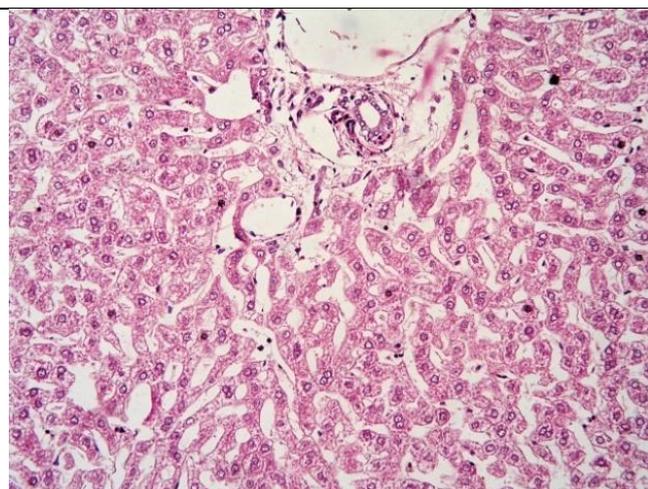
Parameters	T1	T2	T3	T4	SEM
RBC($10^6 / \text{mm}^3$)	5.34 ^B	5.19 ^B	6.04 ^A	5.04 ^B	0.154
Ht (%)	32.95	31.3	34.55	32.05	1.263
Hb (g/dl)	11.1	10.85	11.7	10.55	0.545
MCV (μm^3)	61.7	60.3	57.35	63.5	1.503
MCH (Pg)	20.75	20.9	19.5	20.8	0.744
MCHC(g/dl)	33.65	34.75	34.0	32.7	0.514
WBC ($\times 10^3 / \mu\text{l}$)	9.3 ^a	10.4 ^a	10.1 ^a	6.65 ^b	0.737
Neutrophile (%)	49.5 ^A	43.5 ^A	44.0 ^A	34.0 ^B	1.968
Lymphocyte (%)	42.5 ^C	50.0 ^{AB}	48.0 ^{BC}	56.0 ^A	1.854
Monocyte (%)	6.0	5.5	6.0	8.0	1.25
Esinophile (%)	2.0 ^A	1.0 ^B	2.0 ^A	2.0 ^A	0.001

^{A, B, C}: Mean in the same row with different superscripts are significantly different. ($p \leq 0.05$). ^A and ^B Means in the same row with different superscripts are significantly different ($p \leq 0.01$). Mean Corpuscular Volume (MCV) = $\text{Ht} \times 10 / \text{RBC's} (\mu\text{m}^3)$, Mean Corpuscular Hemoglobin (MCH) = $\text{Hb} \times 10 / \text{RBC's} (\text{Pg})$ Mean Corpuscular Hemoglobin Concentration (MCHC) = $\text{Hb} \times 100 / \text{Ht} (\text{g/dl})$. T1: control diet, T2: 15% *Panicum maximum*, T3: 30% *Panicum maximum*, T4: 45% *Panicum maximum*

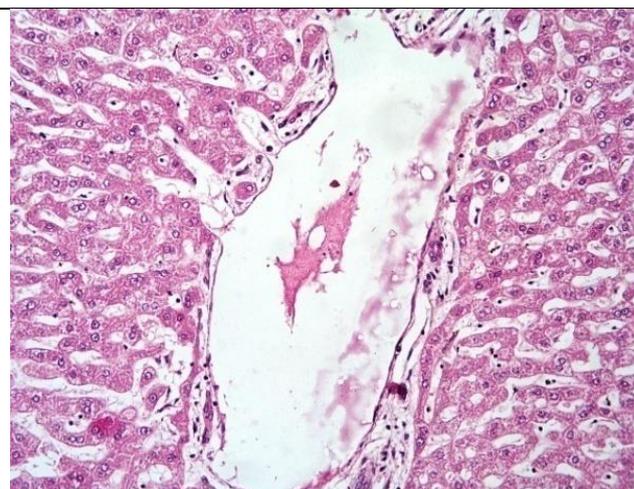
Table 7. Effect of inclusion *Panicum Maximum* on cecum bacterial count in growing rabbits.

Bacterial count	T1	T2	T3	T4	SEM
Total bacterial count	$7.11 \times 10^{10\text{C}}$	$5.3 \times 10^{12\text{A}}$	$3.0 \times 10^{12\text{B}}$	$8.86 \times 10^{10\text{C}}$	0.626
Coli-form count	$2.99 \times 10^{8\text{B}}$	$1.26 \times 10^{6\text{D}}$	$4.99 \times 10^{8\text{A}}$	$7.47 \times 10^{6\text{C}}$	1.139

^{A, B, C, D}: Means in the same row with different superscripts are significantly different ($p \leq 0.01$). T1: control diet, T2: 15% *Panicum maximum*, T3: 30% *Panicum maximum*, T4: 45% *Panicum maximum*



a. Degeneration in the rabbits' hepatocytes, focal area of hepatocellular necrosis, and edema in the sinusoids in the Control group.



b. The rabbits' central vein showing dilatation, dilated sinusoids and degeneration in hepatocytes in T2 (fed 15% *Panicum maximum*)

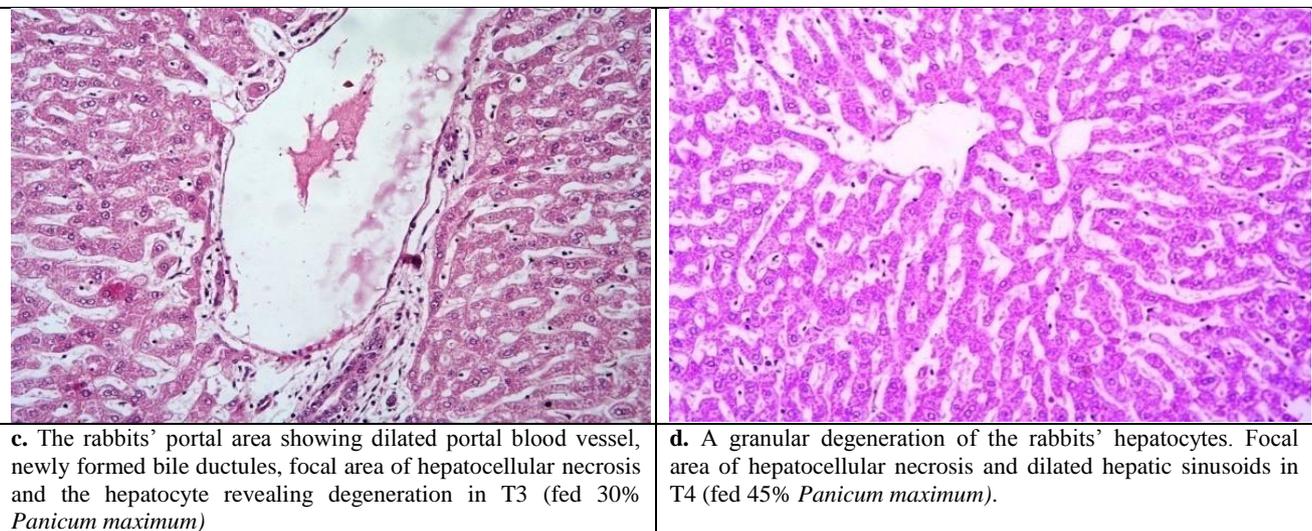


Figure 1. Liver histology of growing rabbits fed different inclusion levels of *Panicum maximum*

DISCUSSION

Rabbits fed T4 recorded 14.5% significantly higher final weight compared to the control group. This enhancement may be due to better quality of mixing Pm hay (grass hay) with legume hay (clover hay) than only the clover hay (control diet) as documented by [Ezenwa and Aken'Ova \(1998\)](#). In this respect, [Uzegbu et al. \(2010\)](#) concluded that the inclusion of pig diet by 5% fresh green guinea grass (Pm) increased the final weight and weight gain. An earlier study on feeding lambs with a diet contained guinea grass with coconut or fishmeal, reported a better growth performance ([Hammond and Wildeus, 1991](#)). Also, [Ajayi et al. \(2008\)](#) documented that feeding goats with Pm with *Lablab purpureus* gave the highest performance as presented in weight gain. Recently, [Salama and Abo El-Azayem \(2018\)](#) found that replacing clover hay with either 25% or 50% of the biologically treated discarded palm fronds resulted in 13.8% and 10.1% improvements in final weight compared to control group. Moreover, [Abd- El Ghany et al. \(2016\)](#) documented that rabbits fed diets containing conocarpus treated with bacteria and fungi had significantly ($p \leq 0.05$) higher live body and daily weight gain than those fed with the other experimental diets. On the contrary, [Ifeanyichukwu et al. \(2007\)](#) studied the effects of inclusion in different forages (Pm, *Centrosema pubescens* and *Sida acuta*) on the growth performance of rabbit. They concluded that there was no significant variation in weight gain between all groups. It is known that fiber content affects the performance of growing rabbits, depending on the fiber source (lignification, cell wall complexity, and different hemicellulose), as reported by [García et al. \(2002\)](#).

In the present study, higher feed intake of rabbits in all groups fed with Pm may be due to the higher palatability of the diets. This finding was confirmed by [Ifeanyichukwu et al. \(2007\)](#), who reported that Pm-fed rabbits indicated the highest feed intake, followed by those fed with *C. pubescens*, and *S. acuta* compared to the control group. [Phimphachanhvongsod and Ledin \(2002\)](#) also had found that feeding growing goats with guinea grass recorded a significantly higher feed intake than others fed with *Gliricidia sepium* leaves. In addition, the improvement in FCR in the 45% Pm T4 group was 23.4% compared to the control group. This decreased in FCR indicated professional feed consumers, which confirmed the findings of [Bamikole and Ezenwa \(1999\)](#), who reported that feeding rabbits with forage (Pm) with concentrate achieved better FCR than others were fed only by forage. Moreover, [Uzegbu et al. \(2010\)](#) documented that feeding pig with 5% fresh Pm achieved lower feed intake with better FCR by 20.2% compared to the untreated group. The enhancement in growth performance may be due to insignificant differences in CP and CF between T4 and the control group. These results were in agreement with [Ajayi et al. \(2008\)](#), who concluded that goats fed Pm with *Lablab purpureus* had higher values of nitrogen digestibility and utilization than goats fed either *Stylosanthes guianensis*, *Lablab purpureus*, *Aeschynomene histrix*, or *Centrosema pubescens*. On the other hand, [Bamikole and Babayemi \(2004\)](#) demonstrated that the CP digestibility did not affect goats fed guinea grass compared to the untreated group. In addition, [Salama et al. \(2016\)](#) concluded that there were no significant differences in dry matter, OM, CP, and CF between rabbit feed with olive cake (as a substitute for clover hay) and others fed with the control diet. In the current study, the percentages of carcass and foreparts in groups of T2 and T3 indicated a significantly higher percentages than in control group, which agrees with [Salama et al. \(2016\)](#), who reported that replacing clover hay in rabbit diets by diets containing 30% olive cake with 1% bentonite resulted in insignificant variations in the carcass. [Abd-El Ghany et al. \(2016\)](#) concluded that rabbits fed 15% and 30% conocarpus and treated with fungi to replace clover hay achieved the highest carcass weight. In the present study, although the RBC's values were in the normal range, it was observed that the RBC values were significantly decreased in the control, T2 and T4 groups in comparison to T3 group and this may be due to the presence of tannins in Pm, which is considered as an anti-nutritional factor. The same

conclusion was made by Yusuf et al. (2012), who found that RBC's were lower in goats fed 25% Pm/ 75% NL compared to the control group. It is known that hematological parameters (RBC's and WBC's) are usually related to health status and are of diagnostic importance for the clinical evaluation of the health state. Moreover, any change in the hematological status of the animals explains the impacts of the nutrients and ration ingredients used, while, these parameters were within the normal range and indicate that the animals have a good physiological, pathological and nutritional status. Therefore, any changes in hematological parameters could be used to clarify the influence of nutritional factors and ingredients in the diet on living creature (Ganong, 1999). Due to the increased WBC's count and their fractions in the control group, may the poor growth performance in this group could be explained, which the most of nutrients were forwarded to the synthesize and development of immune organs, hence reduced the number of nutrients available for growth as reported previously by Tajodini et al. (2014). White Blood Cells are known to be the body's first lines of defense (Ganong, 1999). The highest number of cecum harmful bacteria in the group of T3 could explain the lowest growth performance in the present study, although T2 and T4 achieved the lowest number of coliform bacteria. The results confirmed the previous findings of Abd- El Ghany et al. (2016), who reported that the total bacteria count in rabbits fed either 15% or 30% conocarpus treated with *Trichoderma reesi* (partial replacement of clover hay), was significantly higher than control rabbits. Lelkes (1987) concluded that increasing cellulose in food increased the number of cellulomonace bacteria, which improved cellulose digestion.

CONCLUSION

Panicum maximum can replace up to 45% of the clover hay in the growing rabbit's diet, which caused higher growth performance than the control group by improving the digestibility of CP, CF and decreasing the coliform bacteria count.

DECLARATIONS

Authors' contribution

Dr. Amira M Refaie collaborated with the idea of this research and wrote the manuscript. Dr. Walaa A Salama worked on the main idea and design of the experiment. Dr. Ahmed E. Shams Eldeen performed the experimental chemical and statistical analyses. Dr. Malak M. Boshra and Dr. Ahmed Alazab carried out the practical part of the experiment, and Dr. Fouad S Khalil tabulated the experimental data and offered the grass used in this research.

Competing interests

The authors have declared that there is no competing interest.

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Detecting Intestinal Parasitic Infections in Laboratory Mice

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ABSTRACT

A total of 150 Laboratory mice divided into four age groups consisted of 4, 6, 8 and 10 weeks old were used in this study by placing each animal individually in a special cage within the period between October 2019 to the end of February 2020 at the Research and Graduate Studies Laboratory University of Mosul, Iraq. This study aimed to investigate intestinal parasitic infections in laboratory mice, stool samples were collected for 150 laboratory mice and periodically to perform laboratory tests that included direct slide examination and using the concentration method to detect eggs of worms and cysts of protozoa parasites, the culture of parasites also was used by prepared manufactured culture media to develop parasites. The infection was diagnosed in 136 (90.66%) mice while the rest 14 (9.33%) mice did not record any parasitic infection (clean). The higher rate of infection 58% was reported for *Trichomonas muris* followed by *Entamoeba muris* and *Giardia muris* which found in 22%, 15.3% respectively. In the other hand the infection with *Hymenolepis diminuta* was recorded in 16% from infected cases by identifying the eggs of this worm in stool samples. This study shows the high rate of parasites infection in laboratory mice which might have negative effects on the result of previous scientific researches, in addition to wasting effort, time, and materials.

Keywords: *Entamoeba muris*, *Giardia muris*, Laboratory mice, *Trichomonas muris*

INTRODUCTION

Laboratory animals throughout the previous years contributed significantly to the progress of scientific research at the level of composition and vital function (Clark et al., 1997) and it play`s an essential role in medical experiments, it has been widely used in assessing the safety of many medicines, food and chemicals , In addition to laboratory experiments to diagnose the pathogens of many diseases and to produce vaccines (Tsegaye and Shiferaw, 1999; Velev et al., 2018), however, in some cases, laboratory animals themselves may develop various laboratory causes, especially parasitic agents, which overlap and affect negatively the result of scientific research to be conducted in addition to wasting effort Scientific, time, and material losses (Baker, 2007; Tanideh et al., 2010), These severe parasitic infections may be internal or external and the parasite is one of the organisms that live on or in other types of organisms and that often causes harmful diseases if they are not beneficial to the host and the parasites live parallel to the livelihood these parasites organisms always try to identify and reduce the host's immunity for the purpose of preserving themselves and surviving (Behenke et al., 2003; Evgenya and Oleg, 2020)

The influence of the parasites may extend to the host's behavior (McNair and Timmons, 1977; Webster, 1994) and even alter its immune status (Bashir et al., 2002; Kamal et al., 2002) and its growth (Mullink, 1970) although mice are tolerant to tolerance and reception (Rahemo et al., 2012) large numbers of parasites have reported cases of parasitic infections in them that have reached out of control limits, as in the case of a pinworm, especially if they hit laboratory mice and the arthropod may play an important role in this process by being an intermediate host or a mechanical carrier of these parasites or their larval roles, therefore, the process of diagnosing infection in laboratory mice is a major challenge (Behnke et al., 2003; Baker, 2007; Parkinson et al., 2011) and the parasite's influence may extend to the host's behavior (McNair and Timmons, 1977; Webster, 1994)

The parasites or their eggs confuse the laboratory diagnosis process, which made relying on one laboratory diagnostic method that can give wrong results that negatively affect the overall laboratory work and therefore it is better to use more than one method because the diagnostic process is not easy It is very important to keep in mind that in many cases of parasitic infections like *Entamoeba histolytica* were most probably confused with *Entamoeba dispar* because they did not differentiate morphologically between identical species in microscopic examinations (Mehmet and William, 2003).

The ability of the immune system of wild mice to tolerate and resist parasitic infections is much more than the ability of the immune system of laboratory mice when placed under the same controlled laboratory conditions (Stephen et al., 2017). Therefore, there is a great diversity in the types of parasites that can infect laboratory mice compared to the

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wild (Baker, 2007). So, through the current research, the focus was on most of the intestinal parasitic species that can cause infections in laboratory mice through laboratory tests, since most studies and researchers rely on observing the general morphological health status of laboratory mice when conducting experiments without attention to the microbiological state of the Laboratory mice, which will adversely affect the overall results of scientific research under study.

MATERIALS AND METHODS

Study samples

The present study was conducted within the period between October 2019 to the end of March 2020 at the Research and Graduate Studies Laboratory of the Department of Life Sciences, College of Education for Girls, University of Mosul, Iraq to investigate intestinal parasitic infections in laboratory mice of the Swiss type (Swiss albino Balb / C). The study sample included a laboratory examination of 150 laboratory mice (4-10 months of age) that kept in isolated cages in room temperature and fed on the commercial diet (Erbil feed Co., Iraq) and sterile water was prepared for drinking. The all mice were divided into four age groups considered of 4th, 6th, 8th and 10th weeks by placing each animal individually in a separate cage within the period between the October 2019 to the end of March 2020.

Sample collection and examination

Freshly excreted stool samples were collected for 150 laboratory mice and periodically to perform laboratory tests that included direct examination by preparing smear stools stained with iodine and giemsa stain and using the concentration method (highly saturated sodium salt solution) by mixing one gram of freshly excreted stool with 100 ml of distilled water and the sample was filtered through sterile medical gauze and by three layers to remove suspended matter, eject the suspension by centrifuging at speed of 2300 rpm for one minute.

The filtrate was poured and the centrifugation process was repeated at the same speed after adding 2 ml of water. this process was repeated until clear leachate is reached, then add 2 ml of zinc sulfate to the end of the test tube after disposal of the filtrate with the glass slide cover fixed to the test tube and place the sample In a centrifuge at the same speed and time as before, the cover of the slide was raised and fixed to a glass slide and the sample was stained with an iodine dye to detect eggs of worms and cysts of protozoa parasites. A dissection of infected mice was also performed to obtain whole intestinal contents where the animal was anaesthetized with chloroform by placing the lab mouse in a sealed glass chamber containing a piece of cotton wet with the drug (Padmanabhan et al., 1981; Cicero et al., 2018) and the intestine removed and then emptying its contents in a sterile Petri dish with normal saline solution and then prepare the stained slides that examined later by using microscopy (Nicon Co., Japan) at the power of 40X and 100X to diagnose parasitic pathogens, according to method of Parkinson et al. (2011).

The cultivation of parasites

The process of growing parasites in the laboratory and using prepared and manufactured culture media is one of the important research methods in the process of diagnosing parasites. Therefore, through current research, culture media was used to develop parasites.

Diamond's Medium Trypton, Yeast, Maltose

This medium, suggested by Shaio et al. (1981) was used to grow the Trichomonas parasite, which is prepared for every 100 ml of distilled water and the following substances: The materials were dissolved in distilled water and mixed using an electric magnetic stirrer for one hour and then filtered using Whatman NO.2 filter paper to get rid of insoluble materials (Table 1). The acid function was set at 6.2 and the sterility of the medium using the sterilizer at 121 C at a pressure of 15 Ibs for 15 minutes (Sobel et al., 1999).

The culture media of the Entamoeba parasite

Culture media manufactured and imported from the Indian company (HIMEDIA Laboratories) According to the first two mediums, it consists of the following components as mentioned in table 2. The second culture medium, HiVeg Media, which is also imported from the Indian company HIMEDIA Laboratories, consists of the following materials as mentioned in table 3.

These media were prepared *in vivo* by dissolving 33 grm of medium powder in 1000 ml of distilled water and mixed using the device of the magnetic electric stirrer and after the powder was completely thawed it was distributed on

test tubes with a tight cover and the sterility of the medium using the sterilizer at 121C and pressing 15 lbs for 15 minutes, after completing the sterilization.

The tubes were placed diagonally to harden, and about half of the inclined surface was covered with sterile and diluted horse serum in a ratio of 1: 6 with a solution of physiological salt added to the middle (5) carrying a loop of sterile rice flour in the oven for one hour at a degree of 161 ° C.

These media were inoculated by adding 0.5 ml of the emulsion containing the parasite to the parasite's tubes and incubated for 24 hours at a temperature of 37 ° C. The samples were examined to ensure the growth of the parasite every 24 hours by preparing the stained smears and recording the results (Al-Idrissi et al., 2008).

Ethical approval

The present experimental research was conducted in compliance with the health protection guidelines of experimental animals regulated by the ethical committee in the Department of Biology of Mosul University, Iraq and in according to Helsinki declaration on Ethical Principles.

Table 1. Ingredients for Diamond's medium

Ingredients	g/l	Company
Tryptone soya broth	2.0	Thermo Scientific, USA
Yeast extract	1.0	Thermo Scientific, USA
D-Maltose	0.5	Sigma-Aldrich, USA
L-Ascorbic acid	0.02	Sigma-Aldrich, USA
Agar	0.05	Sigma-Aldrich, USA
Inhibited human plasma	10.0 ml	
Stryptomycin sulphate	1.0	Sigma-Aldrich, USA
Nystatine	500000 IU	Awamedica Pharmaceutical, Iraq

Table 2. Ingredients for culture media

Ingredients	g/l
Liver infusion	272.0
Protease pepton	5.5
Sodium Beta – glycerophosphate	3.0
Sodium chloride	2.7
Agar	11.0

Table 3. The culture medium

Ingredients	g/l
HiVeg infusion	10.0
HiVeg peptone	5.5
Sodium Beta – glycerophosphate	3.0
Sodium chloride	2.7
Agar	11.0

RESULTS AND DISCUSSION

The infection was diagnosed in 136 mice (90.66%) while the rest 14 mice (9.34%) mice did not record any infection. As presented in table 4, the higher rate of infection, 58% was reported for *Trichomonas muris*. Figure 1 is exactly in agreement with the finding of Bicalho et al. (2007) and Rahemo et al. (2012). As well as the result was shown that the rate of infection of *Entamoeba muris* (figure 2) and *Giardia muris* (figure 3) was 22%, 15.3% respectively infection with these parasites was lower than the result that noticed by Rahemo et al. (2012), but it's disagreement with the result of Bicalho et al. (2007). In other hand, the infection with *Hymenolepis diminuta* was recorded in 16% of infected cases by identification of the eggs of this worm (figure 4). The high rate of infection may be due to the living of mice in the same plastic cage, dirty wooden chips and contamination of water and food, but the lower rate, 11.3% was observed for

Balantidium coli, (figure 5) it has been considered as the reservoir. When performing a diagnostic laboratory examination of stool samples overlap, leading to a misdiagnosis of the sample due to the presence of parasitic arthropods as shown in (figure 6) (Reedha and Aseel, 2019).

In table 5, the results indicated that the overall rate of infection with different types of intestinal parasites was 90.66% most of the infections were single parasites (70.67%). The results showed there is equals bilateral and multiple infections (10%). The higher rate of bilateral infection was with *Trichomonas muris* and *Hymenolepis diminuta*. In multiple infections the higher rate was for *Trichomonas muris*, *Entamoeba muris*, and *Hymenolepis diminuta* (4.67%). The *Trichomonas muris* and *Entamoeba muris* are classified commensal agents as they are not associated with animal health alterations with probable involvements in experimental outcomes. Sharp and La Regina (1998) declared *Giardia muris* as a pathogenic agent. These results have converged with the results of Rahemo et al. (2012) which indicated that the highest rate of infection by *Trichomonas muris* and this study showed infection by *Giardia muris*. The infection with *Hymenolepis diminuta* was observed in this study which had reported by Garedaghi and Khaki (2014) that found some rodents with the same infection.

Table 4. Diagnosed parasites in fresh fecal smears of mice

Smear No	Parasite	+ve	Percent
1-	<i>Entamoeba muris</i>	33	22
2-	<i>Trichomonas muris</i>	87	58
3-	<i>Giardia muris</i>	23	15.3
4-	<i>Balantidium coli</i>	17	11.3
5-	<i>Hymenolepis diminuta</i>	24	16

No: number; *150 mice were studied

Table 5. Type of infections in the stool of examined mice.

Parasites	Number of infections	Percent
Single parasitic infection		
<i>Entamoeba muris</i>	13	8.67
<i>Trichomonas muris</i>	65	43.33
<i>Giardia muris</i>	12	8
<i>Balantidium coli</i>	5	3.33
<i>Hymenolepis diminuta</i>	11	7.33
Total	106	70.67
Co-parasitic infection		
<i>Entamoeba muris</i> & <i>Giardia muris</i>	3	2
<i>Trichomonas muris</i> & <i>Balantidium coli</i>	4	2.67
<i>Trichomonas muris</i> & <i>Hymenolepis diminuta</i>	6	4
<i>Trichomonas muris</i> & <i>Entamoeba muris</i>	2	1.33
Total	15	10
Multi parasitic infection		
<i>Trichomonas muris</i> , <i>Giardia muris</i> , <i>Entamoeba muris</i> & <i>Balantidium coli</i>	3	2
<i>Trichomonas muris</i> , <i>Entamoeba muris</i> & <i>Hymenolepis diminuta</i>	7	4.67
<i>Giardia muris</i> , <i>Entamoeba muris</i> & <i>Balantidium coli</i>	5	3.33
Total	15	10

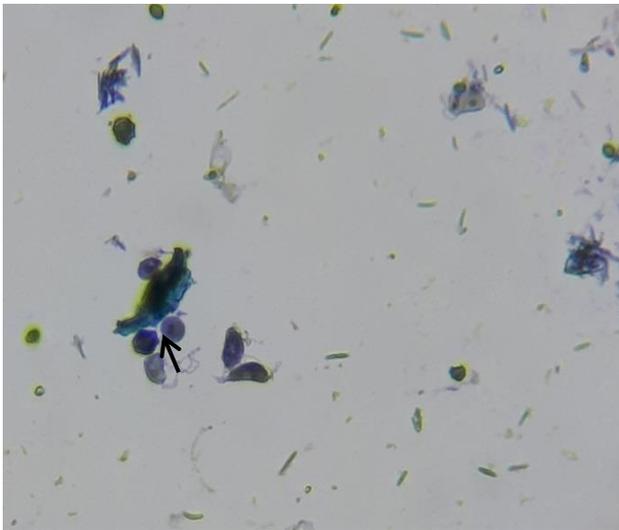


Figure 1. Microscopic view of *Trichomonas muris* trophozoite (arrow) from mice fecal sample stained by giemsa stain ($\times 40$ magnification)

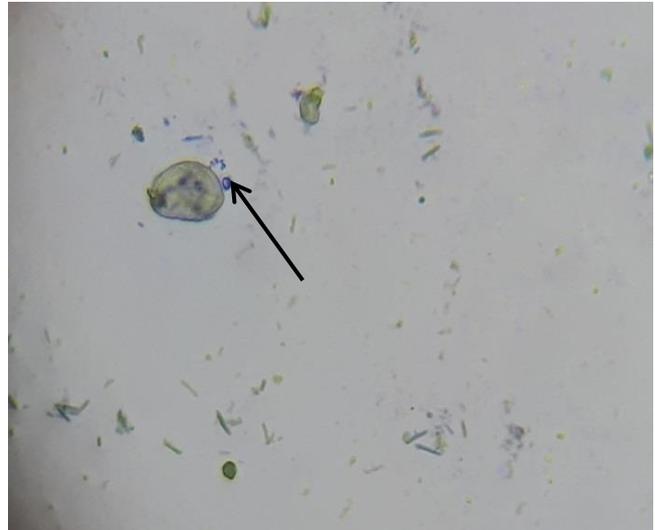


Figure 2. Microscopic view of *Entamoeba muris* cysts (arrow) from mice fecal sample stained by iodine stain ($\times 40$ magnification)

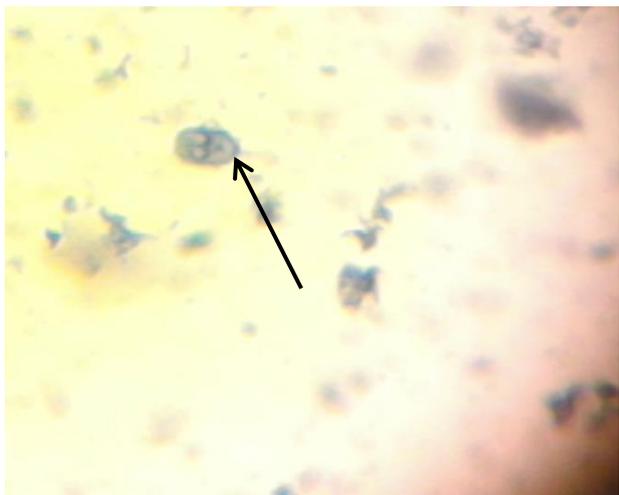


Figure 3. Microscopic view of *Giardia muris* trophozoite (arrow) from mice fecal sample stained by iodine stain ($\times 40$ magnification)



Figure 4. Microscopic view of *Hymenolepis diminuta* egg (arrow) from mice fecal sample ($\times 100$ magnification)

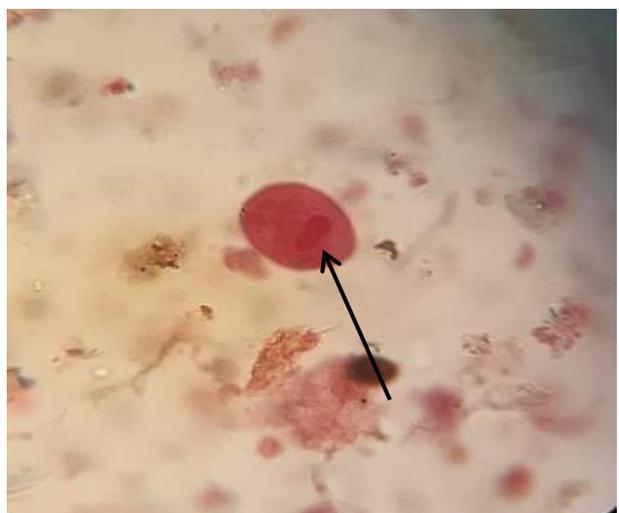


Figure 5. Microscopic view of *Balantidium coli* trophozoite (arrow) from mice fecal sample stained by giemsa stain ($\times 40$ magnification)

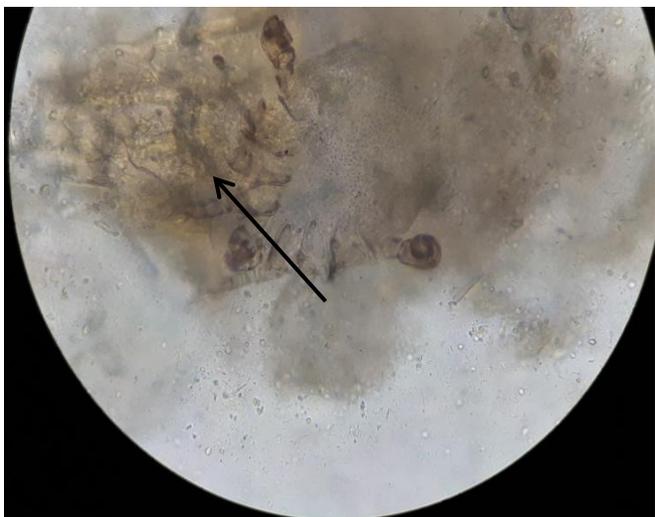


Figure 6. Microscopic view of adult *Tick* (arrow) from mice fecal sample ($\times 40$ magnification)

DECLARATIONS

Authors' contribution

Firas M. B. Alkhashab contributed to data analysis and manuscript writing and performed the experimental works. Aseel Isam Jamal aldeen Alnuri and Rana Suhail Abdallah Al_Juwari were involved in the development of the methodology and experimental works. Finally, after careful consideration, the final revised manuscript was approved by all authors.

Competing interests

The authors have declared no conflict of interest.

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Toxicity, Anthelmintic Efficacy and Proteolytic Activity of Chitosan-Encapsulated Bromelain within the Gastrointestinal Tract of Small East African Goats

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ABSTRACT

The development of resistance to anthelmintic drugs has prompted researches into alternative methods for controlling intestinal nematodes in ruminants. This study aimed to evaluate the anthelmintic efficacy, proteolytic activity, and toxicity of bromelain encapsulated in chitosan within the gastrointestinal tract (GIT) of Small East African goats in Kenya. Twelve healthy indigenous male goats were divided into four groups contained three goats in each group. Treatment groups included: G1, chitosan-encapsulated bromelain (90 mg/kg); G2, chitosan-encapsulated bromelain (270 mg/kg); G3, positive control (albendazole 7.5 mg/kg); and G4, negative control. The animals were orally treated with the drugs in a single dose. The hematological and serum biochemical parameters were determined using standard methods. The strongyle fecal egg count was evaluated weekly using a modified McMaster technique. To determine the proteolytic activity of nanoencapsulated bromelain within the GIT, another set of twelve goats was used and administered 270 mg/kg of encapsulated bromelain. Every four hours, three goats were sacrificed and the proteolytic activity of the drug was determined in the different organs of the GIT. Significant differences were observed between the mean PCV of goats treated with 270 mg/kg encapsulated bromelain and non-treated goats on days 21 and 28 post-treatment. The mean aspartate aminotransferase, urea, and creatinine levels of treated and control goats did not significantly differ during the experiment period. Also, no significant difference was observed between the mean alanine aminotransferase level of treated and untreated goats 28 days post-treatment. The administration of encapsulated bromelain was not associated with any clinical sign and mortality. The reduction in fecal egg count in G1 and G2 at 28 days post-treatment was 9.5% and 22.6%, respectively. The encapsulated bromelain remained proteolytically active along the goat GIT but its protease activity varied according to the type of GIT organ and time elapsed since administration. In conclusion, chitosan-encapsulated bromelain is safe, but have low efficacy against GIT strongyle nematodes when given as a single dose. Future studies should evaluate higher and repeated doses of encapsulated bromelain for controlling GIT nematodes.

Key words: Bromelain, Chitosan, Efficacy, Goats, Nanoencapsulation, Proteolytic activity.

INTRODUCTION

Livestock parasites are associated with major economic losses worldwide and have a considerable impact on farm profitability (Sackett et al., 2006; Roeber et al., 2013; Rashid et al., 2019). For instance, in Kenya, economic losses associated with haemonchosis alone in sheep and goats are estimated at US\$26 million, while returns could be enhanced as much as 470% by controlling haemonchosis (Mukhebi et al., 1985; Kareru et al., 2008). A 15-year retrospective study in central Kenya revealed that 32% of sheep mortalities were due to parasitic diseases, of which 63% were associated with helminthiasis (Kagira and Kanyari, 2001). In addition to mortality, helminthiasis reduces growth and reproductive performance in goats (Waller, 2006; Lashari and Tasawar, 2011).

For control of helminthiasis, farmers mainly rely on anthelmintic treatments (Vercruysse et al., 2018). Unfortunately, the currently available commercial anthelmintics are associated with problems such as loss of efficacy as a result of the emergence of resistance (Wanyangu et al., 1996; Waruiru et al., 1998; Gatongi et al., 2003). Despite the high rate of resistance development in gastrointestinal tract (GIT) nematodes (Waruiru et al., 2003; Wanyangu et al., 1996; Nalule et al., 2011; Woodgate et al., 2017), the discovery of new anthelmintics is very slow, the main reason being the lack of investment and lengthy process in drug discovery (Behnke et al., 2008). Given the resistance to the anthelmintics, high cost of drugs, and lack of newer anthelmintics, there is a need to find alternatives or complementary methods for nematode control. The use of plant extracts has been considered a possible efficacious and environmentally acceptable method to control GIT nematodes (Newman et al., 2012; Ribeiro et al., 2014).

Bromelain, a cysteine proteinase, is one of the plant-derived products which possess anthelmintic properties (Hunduza, 2018). Its use as anthelmintic has faced some constraints including the requirement of multiple dosing and

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rapid movements in the ruminant GIT; it requires only 20 minutes to pass through the small intestine (Stepek et al., 2005; Buttle et al., 2011). Shiew et al. (2010) also found that the activity of bromelain is lowered by the low pH found in the abomasum of ruminants; the drug is further degraded by rumen microbiota, resulting in an ineffective contact between parasite and drug. A study by Hunduza (2018) indicated that the encapsulation of bromelain in chitosan enhanced the *in vitro* activity against all the stages of *Haemonchus contortus* isolated from goats. Moreover, it revealed that encapsulated bromelain compared to pure bromelain had a higher inhibitory activity on egg hatch. It is, therefore, necessary to conduct *in vivo* studies on the efficacy and safety of this compound.

To the authors' knowledge, the proteolytic activity of cysteine proteinases has not yet been examined in different parts of the GIT of goats but attempts have been made in several *in vitro* studies to treat the abomasal nematode, *H. contortus*, with these enzymes (Hunduza, 2018). Therefore, there is a need to assess the stability of the proteolytic activity of bromelain in various parts of the GIT of goats where a number of nematodes reside. Hence, this study was designed to evaluate toxicity, anthelmintic efficacy, and proteolytic activity of chitosan-encapsulated bromelain within the GIT of Small East African goats.

MATERIALS AND METHODS

Ethical approval

Approval for animal experiments was obtained from the Animal Ethics Committee of Jomo Kenyatta University of Agriculture and Technology (JKUAT, REF: JKU/2/4/896B). The protocols were approved by the Institutional Animal Care and Use Committee of JKUAT.

Animals

In total, 24 Small East African healthy male goats, aged between 8 and 30 months and weighing between 13-21 kg, were used in this study. Twelve of them were used for the assessment of the toxicity and anthelmintic efficacy of chitosan-encapsulated bromelain and another set of twelve goats was used to determine the proteolytic activity of nanoencapsulated bromelain within the GIT. They were ear-tagged and kept in a goat house where they were acclimatized to the diet within 14 days before the start of the study. Animals were group-housed in pens (3 goats in each) located within the JKUAT, in Kiambu County, Kenya. Prior to the start of the experiment, each animal was screened for the presence of strongyle eggs by the Fecal Egg Count (FEC) examination. The goats were fed on 1.5 kg of concentrate feed and 1 kg of wheat hay twice per day (9 a.m. and 3 p.m.). The concentrate feed comprised beet liquid molasses, maize germ, and soybean meal (Aroma Feed Suppliers, Kenya). Feed blocks (Aroma Feed Suppliers, Kenya) were used to supplement essential minerals.

Bromelain extraction and encapsulation in chitosan nanoparticles

Bromelain extraction was performed as described by Kahiro et al. (2018). The extracted bromelain was purified using a 10 kDa dialysis membrane. Thereafter the ionic gelation method was used to encapsulate bromelain into chitosan (Fan et al., 2012; Hunduza, 2018). Briefly, after mixing equal volume (30 ml) of extracted bromelain (4 mg/ml) with 1% sodium tripolyphosphate (STPP), 12 ml of the bromelain-STPP mixture was added to 20 ml of 1% chitosan under vigorous and continuous stirring. Following the centrifugation at 15000 rpm for 45 minutes, the obtained pellet was washed with distilled water prior to freeze-drying at -60 °C using a freeze dryer (MRC Ltd., Israel). The Fourier transform infrared spectrophotometer analysis was used to confirm the successful conjugation of bromelain to the chitosan nanoparticles.

Treatments

Treatment groups were formed after randomization based on the number of eggs per gram (EPG) of feces, such that the mean EPG of the animals in each group was more than 500 (Coles et al., 2006). Each group had three animals. The treatment was done in a single oral dose. Group 1 received 90 mg/kg of encapsulated bromelain and group 2 received 270 mg/kg of encapsulated bromelain. Group 3 was the negative control (infected, non-treated). Group 4 (albendazole 7.5 mg/kg body weight) only served as a positive control in the anthelmintic efficacy assessment. Goats were fasted overnight prior to dosing. Following the period of fasting, the animals were weighed and then the encapsulated bromelain (90 and 270 mg/kg) was administered orally using drenching guns. The goats were monitored for 28 days.

Clinical observations

Observations were made and recorded systematically and continuously as per the guidelines (OECD, 2002). Each animal was observed during the first 30 minutes following the drug administration. Special attention was given during the first 4 hours and daily thereafter, for a total of 14 days to observe any changes in behavior and any clinical signs associated with toxicity. Temperature and body weight of goats were measured at 09:00 am using a digital thermometer

(Kruise, Denmark) and a 100 kg spring balance scale (Salter Model, Capital Scales, South Africa), respectively. This was done prior to treatment and weekly during the experiment period. Changes in the weight of individual goats were calculated and compared with that of the control animals. Changes were considered as adverse effects of drugs if the body weight loss observed was more than 10% of the initial recorded body weight (Nurul et al., 2018).

Blood Sample collection

Blood samples (3 ml) from each goat were taken from the jugular vein in ethylene-diamine-tetraacetic acid (EDTA) test tubes. This was done at 09:00 am weekly.

Packed cell volume

Packed Cell Volume (PCV) was determined using the microhematocrit method (Hansen and Perry, 1994; Githiori et al., 2004). Briefly, an aliquot of blood with anticoagulant from each goat was put in microcapillary tubes and then centrifuged at 14,000 rpm for 10 minutes. After centrifugation, samples were analyzed using a microcapillary reader (Hawksley, England).

Determination of serum biochemical parameters

The serum was prepared by allowing the whole blood to clot. Thereafter the clot was removed by centrifuging at 2,000 x g for 10 minutes. The resulting supernatant was used for biochemical tests. Aspartate aminotransferases (AST), alanine aminotransferases (ALT), urea, and creatinine were analyzed using standard diagnostic test kits on automated clinical biochemistry analyzer (Reflotron Plus System®, model: Cobas 4800 Detection Analyzer; India).

Assessment of the anthelmintic efficacy

FEC examination was performed before treatment and weekly during the experiment period. Fecal samples were weekly collected from the rectum of the goats using flesh gloves. Aliquots of the fecal sample from each goat were placed in a plastic bottle (Indosurgicals Pvt. Ltd., India). The fecal samples were analyzed using a modified McMaster technique (Zajac and Conboy, 2012) with a precision of 100 EPG using an Olympus B 201 microscope (Optical Element Corporation, Melville, USA) at 10× magnification. The percentage reduction in FEC was calculated using the formula described by Kochapakdee et al. (1995).

Assessment of proteolytic activity in gastrointestinal tract

Twelve goats were orally administered 270 mg/kg of encapsulated bromelain as a single dose. Every 4 hours, a group of 3 goats was sacrificed and the entire GIT was removed from each goat, from the rumen to the large intestine, then washed in a Petri dish of Phosphate-Buffered Saline (PBS), and split into six sections: the rumen, reticulum, abomasum, omasum, small intestine, and large intestine. These sections were opened longitudinally and the ingesta contents (100 grams) placed in a beaker containing PBS. Thereafter the contents were filtered using a sieve (250 µm size) into a beaker. The amount of enzyme present in each section over the 16 hours was measured by performing the casein enzymatic assay as described by Hunduza (2018).

Statistical analysis

All statistical analyses and graphical presentations were carried out using R (version 3.6.0) and GraphPad (Version 7.02), respectively. The Students t-test was used to compare the mean weight, temperature, AST, ALT, urea and creatinine levels of treated with those of non-treated goats. Differences in the proteolytic activity in the different organs of the GIT were tested using ANOVA. The significance was based on a p-value <0.05.

RESULTS

Toxicity assessment of encapsulated bromelain

Clinical observations

There were no mortality and clinical signs of toxicity observed in the goats after a single oral dose of 90 and 270 mg/kg of encapsulated bromelain. The mean body temperature of pre-treated goats ranged from 38.5 to 39.4 °C. Following treatment, no significant differences were observed between the mean body temperature of goats treated with 90 mg/kg encapsulated bromelain and that of non-treated goats ($p>0.05$). The same observation was made between goats treated with 270 mg/kg encapsulated bromelain and non-treated goats. Prior to treatment, the mean body weight of treated goats was 16.8 kg and ranged from 16.1 to 17.3 kg while that for goats of the negative control group was 17.6 kg. Following treatment, there were no significant differences between the mean body weight of the non-treated goats and that of goats treated with 90 mg/kg encapsulated bromelain ($p>0.05$). Likewise, no significant differences were observed

between the mean body weight of goats treated with 270 mg/kg encapsulated bromelain and that of non-treated goats ($p>0.05$).

Effect of bromelain on the packed cell volume and serum biochemical parameters

The mean PCV of the untreated goats was 28.5% and 28.9% on day 21 and day 28 of the experiment, respectively. The pre-treatment mean PCV of goats treated with 270 mg/kg encapsulated bromelain was 33% (range: 32-34%). Significant differences were observed between the mean PCV of goats treated with 270 mg/kg encapsulated bromelain and that of non-treated goats on day 21 ($p=0.0460$) and day 28 post-treatment ($p=0.027$). However, no significant differences were observed between the mean PCV of goats treated with 90 mg/kg of encapsulated bromelain ($p>0.05$) and that of non-treated goats from day 7 to day 28 post-treatment (Figure 1). The mean ALT level of untreated goats was 15.74 U/L on day 0 of the experiment. Prior to treatment, the mean ALT level of goats treated with 90 mg/kg encapsulated bromelain was 15U/L while that of goats treated with 270 mg/kg encapsulated bromelain was 14.8 U/L. The mean ALT level of untreated goats on day 7 (15.7 U/L) was significantly higher ($p<0.05$) than that of goats treated with 90 mg/kg (14.8 U/L) and 270 mg/kg encapsulated bromelain (14.9 U/L) on day 7 post-treatment. On day 0, day 14, and day 28 post-treatment, no significant differences were observed between both groups treated with encapsulated bromelain and the non-treated goats. The mean AST level of untreated goats was 114.8 U/L (range: 112-117 U/L). Before treatment, the mean AST level of goats treated with 90 mg/kg encapsulated bromelain was 115.8 U/L while that of goats treated with 270 mg/kg encapsulated bromelain was 116.3 U/L. Following treatment, no significant differences ($p>0.05$) were observed between the mean AST level of treated goats (both 90 and 270 mg/kg encapsulated bromelain) and that of untreated goats (Figure 3). The mean urea level of untreated goats was 6.1 mmol/L (range: 5.5-6.5 mmol/L) on the day 0 experiment. The mean pre-treatment urea level of goats treated with 90 mg/kg encapsulated bromelain was 5.8 mmol/L while that of goats treated with 270 mg/kg was 6.2 mmol/L. Following treatment, no significant differences ($p>0.05$) were observed between the mean urea level of both treated groups and that of untreated group (Figure 4). The mean serum creatinine level of untreated goats was 56.9 μ mol/L and ranged from 54 to 61.2 μ mol/L. The mean pre-treatment creatinine level of goats treated with 90 mg/kg and 270 mg/kg were 57.5 and 60 U/L, respectively. After treatment, no significant differences ($p>0.05$) were observed between the creatinine levels of encapsulated bromelain treated goats (both 90 and 270 mg/kg) and the non-treated goats (Figure 5).

Figure 1. Mean packed cell volume of untreated and treated goats with single-dose of encapsulated bromelain. PCV: Packed Cell Volume; d0: day 0; d7: day 7 post-treatment; d14: day 14 post-treatment; d21: day 21 post-treatment; d28: day 28 post-treatment. * Columns with an asterisk symbol are significantly different ($p<0.05$) from the control group on the same day. Each treatment (90 and 270 mg/Kg) was compared separately to the control group using the Students t-test.

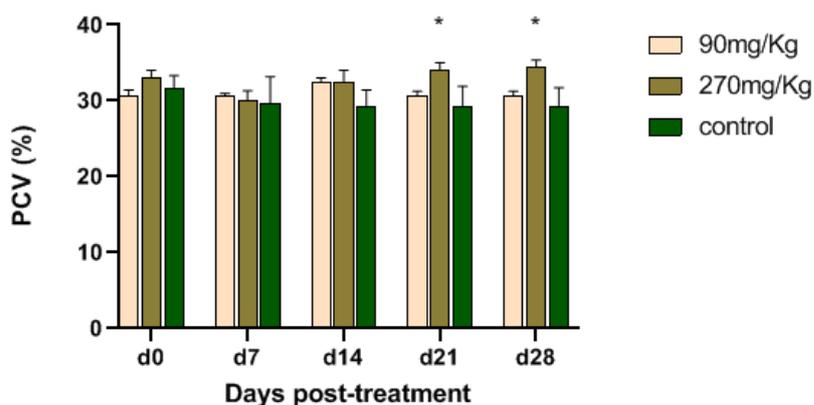


Figure 2. Mean concentration of alanine aminotransferase in untreated and treated goats with single-dose of encapsulated bromelain. ALT: Alanine aminotransferase; d0: day 0; d7: day 7 post-treatment; d14: day 14 post-treatment; d21: day 21 post-treatment; d28: day 28 post-treatment. * Columns with an asterisk symbol are significantly different ($p<0.05$) from the control group on the same day. Each treatment (90 and 270 mg/Kg) was compared separately to the control group using the Students t-test.

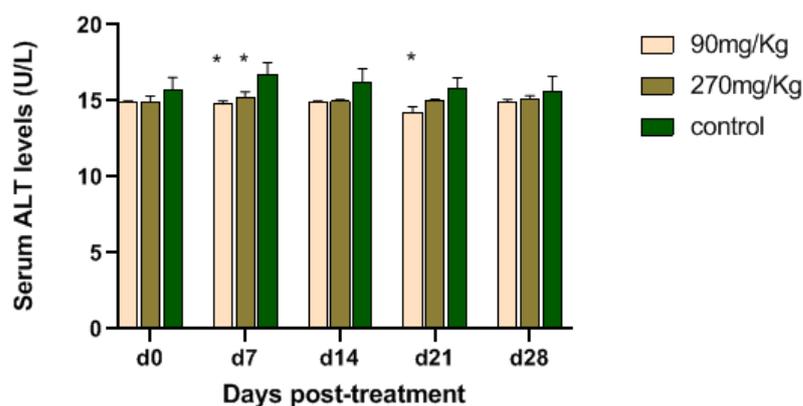


Figure 3. Mean concentration of aspartate aminotransferase in untreated and treated goats with single-dose of encapsulated bromelain. AST: Aspartate aminotransferase; d0: day 0; d7: day 7 post-treatment; d14: day 14 post-treatment; d21: day 21 post-treatment; d28: day 28 post-treatment.

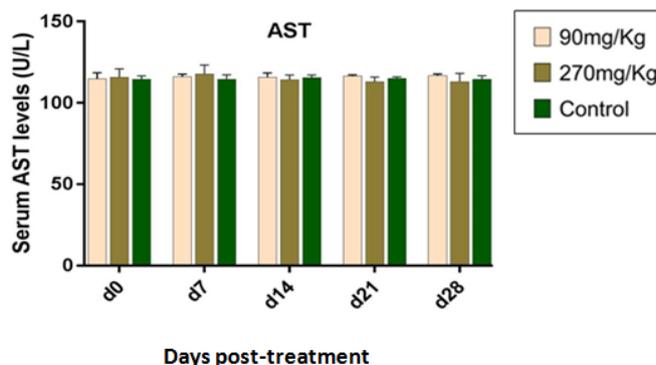


Figure 4. Mean urea level of untreated and treated goats with single-dose of encapsulated bromelain. d0: day 0; d7: day 7 post-treatment; d14: day 14 post-treatment; d 21: day 21 post-treatment; d28: day 28 post-treatment.

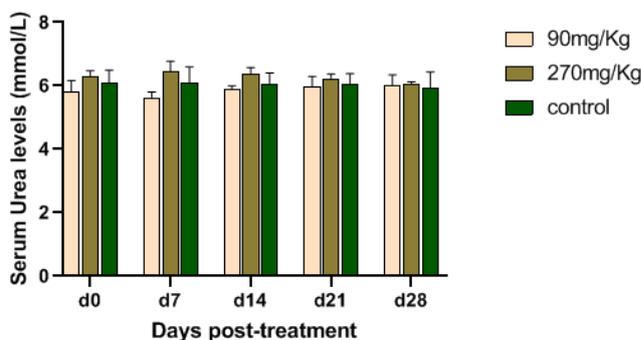
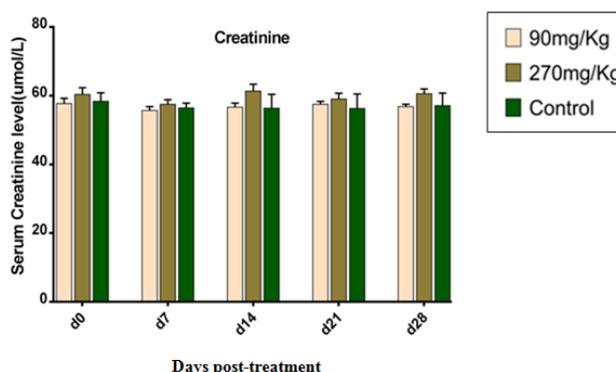


Figure 5. Mean serum creatinine level of untreated and treated goats with single-dose of encapsulated bromelain. d0: day 0; d7: day 7 post-treatment; d14: day 14 post-treatment; d21: day 21 post-treatment; d28: day 28 post-treatment



***In vivo* assessment of anthelmintic efficacy of encapsulated bromelain**

Before treatment, the mean strongyle FECs for treated goats with a single dose of 90 and 270 mg/kg of encapsulated bromelain were 1,050 and 1,550 EPG, respectively. Following treatment (on day 28 post-treatment), the percentage reduction in FEC was 9.5% and 22.6% for treated goats with 90 and 270 mg/kg of encapsulated bromelain, respectively. The percentage reduction in strongyle eggs of goats treated with 270 mg/kg was significantly higher ($p < 0.05$) than that of the group treated with 90 mg/kg encapsulated bromelain on all treatment days (day 7, 14, 21 and 28 post-treatment). In comparison, the mean percentage of strongyle FECs for the untreated group increased by 22% while a reduction of 100% was recorded for the goats treated with albendazole (7.5 mg/kg) on day 28 post-treatment (Figure 6).

Protease activity of encapsulated bromelain in the different organs of the gastrointestinal tract

The protease activity of chitosan-encapsulated bromelain in the different digestive organs varied with time as the encapsulated bromelain was passing through the GIT of goats. Four hours after the drug administration, the encapsulated bromelain activity was lowest in the reticulum (0.124 U/ml, range: 0.04-0.18 U/ml) while 8 hours after the drug administration, the activity was highest in the rumen (3.11 U/ml, range: 2.83-3.3 U/ml) and lowest still in the reticulum (0.117 U/ml, range: 0.02-0.18 U/ml). The overall encapsulated bromelain activity (sum of activities in the different organs) gradually increased over 12 hours and then decreased ($p=0.001$). The highest enzyme activity (3.142 U/ml, range: 2.83-3.3 U/ml) was recorded in the large intestine at 12 hours after drug administration (Table 1).

Figure 6. Mean percentage reduction in fecal egg count of goats treated with a single dose (90 or 270 mg/kg) of chitosan-encapsulated bromelain. EB: Encapsulated bromelain; d0: day 0; d7: day 7 post-treatment; d14: day 14 post-treatment; d21: day 21 post-treatment; d28: day 28 post-treatment. * The asterisk symbol indicates a significant difference ($p < 0.05$) between treatment groups on the same day.

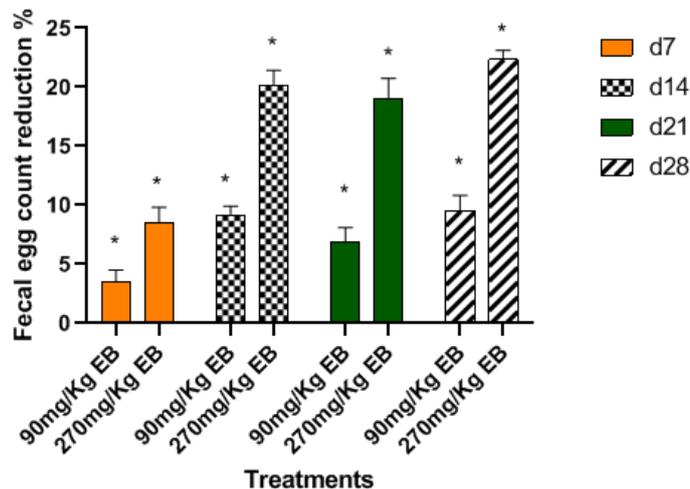


Table 1. Mean enzyme activity of encapsulated bromelain in digestive organs of goat at different time points

Organ	Enzyme activity after drug administration (Units/ml)				p-value
	4 hr	8 hr	12 hr	16 hr	
Reticulum	0.124±0.062	0.117±0.073	0.106±0.058	0.034±0.016	0.408
Rumen	0.665±0.361	3.11±0.217	1.146±0.288	0.251±0.117	<0.0001
Omasum	0.603±0.141	2.128±0.257	1.808±0.451	0.397±0.186	0.001
Abomasum	0.191±0.018	0.398±0.151	1.446±0.294	0.345±0.161	<0.0001
Small intestine	0.002±0.0001	0.398±0.124	0.339±0.087	0.074±0.034	0.099
Large intestine	0.001±0	0.018±0.004	3.142±0.218	0.402±0.18	<0.0001
Overall activity	1.586±0.582	6.169±0.826	7.987±1.396	1.503±0.69	0.001
p-value	0.007	<0.0001	<0.0001	0.064	

Data are expressed as mean ± standard deviation

DISCUSSION

The current study evaluated the anthelmintic efficacy, toxicity, and proteolytic activity of bromelain encapsulated in chitosan in the GIT of Small East African goats. In this study, no mortality and clinical signs were observed when encapsulated bromelain as a single dose was administered to goats. Therefore, the median lethal dose (LD_{50}) of encapsulated bromelain may be considered to be greater than 270 mg/kg in goats. This finding is in agreement with the results of Dutta and Bhattacharyya (2013) who similarly did not observe any toxicity after oral administration of acute and sub-acute doses of the aqueous extract of *Ananascomosus* (pineapple) crown leaf to rats. According to Taussig et al. (1975), bromelain has very low toxicity and its LD_{50} is greater than 10 g/kg in mice, rats, and rabbits. Similarly to this study, Pavan et al. (2012) also did not report any toxic effect associated with daily administration of bromelain to dogs in increasing levels up to 750 mg/kg after six months. The PCV observed in this study for healthy goats was lower compared to the values (32.5-43.7%) reported by Al-Bulushiet al. (2017) for Sahrawi goats. Earlier reports for Jabali goats showed PCV values in the same range (37.4-43.7%) (Al-Bulushi et al., 2017). Further, the obtained PCV values were within the normal range of the species whose values vary among breeds of goats (Radostitis et al., 2000). The observation that there were no significant differences between the PCV of treated and untreated goats indicates that the administration of encapsulated bromelain does not affect erythrocyte production and physiology and thus is safe for use in animals.

Following treatment, the serum creatinine, urea, ALT and AST levels of encapsulated bromelain treated goats were within the normal range of the species and did not show any significant difference across the groups. This indicates no liver and kidney damage up to 270 mg/kg of encapsulated bromelain administered as a single dose. The range of ALT level observed in this study is comparable with the finding by Tibbo et al. (2008) who reported the ALT level of indigenous Arsi-Bale, Central Highland, and Long-eared Somali goat breeds ranged from 14.0-20.2 U/L. The obtained values for urea were within the range reported for normal healthy goats described by other studies (Kaneko et al. 2008; Chikwanda and Muchenje, 2017). This study is consistent with previous results obtained by Buttle et al. (2011) who did not observe any sign of toxicity or gross lesions in the sheep administered single and repeated doses of papaya latex cysteine proteinase during the post-mortem examination.

In comparison to the anthelmintic efficacy obtained in this study (22.6%) when encapsulated bromelain (270 mg/kg) was administered to goats as a single dose, Domingues et al. (2013) reported a lower efficacy for plain bromelain

(180 mg) in sheep (3.7%). This disparity can be attributed to the differences in the animal species and the administered dosages. Another possible explanation of the differences in anthelmintic efficacy can be the beneficial effects of nanoformulation as a drug delivery system (Bhatnagar et al., 2014). According to Dimitrov (2012), nanoparticles including chitosan improve the efficacy of drugs by preventing enzymatic degradation and enhancing the absorption of the intestinal epithelium. Similarly, George and Abraham (2006) reported that incorporation of proteins into a chitosan matrix protected these biomolecules. In the same trend, Mahlangu (2018) declared that the encapsulation of bromelain into chitosan enhanced its activity against bacteria isolates from mastitis infected goats. A critical fact to take into consideration regarding the use of proteins as anthelmintic is their possible degradation by proteases found in the GIT, including the rumen. Thus, the nanoformulation is a very important step in the development of protein drugs for oral delivery since it protects and promotes their availability in nematode habitat organs (Dos Santos Soares et al., 2019).

The anthelmintic efficacy reported in this study is consistent with the findings of Buttle et al. (2011) who found sheep receiving repeated treatment of papaya latex supernatant (100 µmol active cysteine proteinase), daily for 4 days, had fewer *H. contortus* worms compared to those that received the single treatment. The lower anthelmintic efficacy of a single dose treatment as compared to repeated doses can be explained by the fact that repeated administration of the drug extends contact period between cysteine proteinases and the parasite resulting in an increased efficacy (Buttle et al., 2011). Another possible reason is the rapid movement of cysteine proteinase in the GIT of ruminants (Steppek et al., 2006), which make a drug administered as a single dose to have little chances and short time of getting in contact with the worms. The lower anthelmintic efficacy of a single dose treatment compared with repeated doses suggests that, following dilution in the GIT, the enzymes require prolonged contact time with the worms in order to demonstrate effectiveness (Buttle et al., 2011). Thus, experiments with repeated treatment doses for 90 and 270 mg/kg of encapsulated bromelain are needed in order to see the possibilities of achieving effectiveness greater than 22.6%.

Similar to the findings of this study, Stepek et al. (2007) reported a variation in the enzyme activity of papaya latex with time in the different organs as the drug was passing through the GIT of mice. In the current study, it was noted that four hours after the drug administration the encapsulated bromelain had already passed the rumen thus very low activity was recorded in that GIT site. This confirms the report by Stepek et al. (2005) who indicated the limitation of bromelain in ruminants was their rapid movement in the GIT. Twelve hours after the drug administration, a build-up of activity in the large intestine was observed and this may be due to an increase in enzyme concentration following absorption of water in the small intestine (Steppek et al., 2007).

Apart from *H. contortus*, there are many other parasite species infecting the stomach and intestines of sheep and goats. These include *Teladorsagia (Ostertagia) spp.*, *Trichostrongylus spp.*, *Trichostrongylus*, *Nematodirus spp.*, *Bunostomum spp.*, *Oesophagostomum spp.*, *Cooperia spp.*, and *Strongyloides spp.* (Urquhart et al. 1996; Hutchinson, 2009). The observed build-up of encapsulated bromelain activity in the large intestines 12 hours after the drug administration shows that the drug will be effective against the intestinal nematodes residing in the large intestine of the host animal (Hutchinson, 2009). The omasum and abomasum showed the second and third highest encapsulated bromelain proteolytic activity 12 hours after the drug administration, respectively, indicating that the drug will be effective against nematodes such as *Haemonchus spp.* and *Ostertagia spp.*, which are located in the abomasum of ruminants (Hutchinson, 2009). The present study demonstrated that encapsulated bromelain remained intact and proteolytically active within the GIT, which is consistent with the findings of Hale (2004). Therefore, encapsulated bromelain can act as an effective anthelmintic treatment for different strongyle residing along the GIT of small ruminants.

CONCLUSION

In conclusion, chitosan-encapsulated bromelain administered orally in a single dose up to 270 mg/kg is not associated with any adverse clinical signs of toxicity and mortality in goats. This compound was not very effective in reducing the burden of strongyle eggs in goats. Additionally, the encapsulated bromelain remained intact and proteolytically active along the gastrointestinal tract of goats, although the protease activity of it varied with time in the different organs as the drug was passing through the gastrointestinal tract. With regards to the low efficacy of single-dose bromelain observed in this study, further studies with repeated doses of encapsulated bromelain are needed.

DECLARATIONS

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Authors' contribution

All authors conceived and designed the experiment. Wasso Shukuru and John Kagira performed the experiment. Naomi Maina performed the biochemical assays. All authors analyzed data and wrote the manuscript. All authors read and approved this manuscript.

Competing interests

All authors declare that they have no conflict of interest.

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Effect of Combined Plant Essential Oils on *Dermanyssus gallinae*: *In vitro* and *in vivo* study

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ABSTRACT

The present study was carried out to evaluate the effect of plant essential oils on *Dermanyssus gallinae* (*D. gallinae*). *In vitro* six groups of red mites, 20 mites in each group were exposed to direct spray of combined plant essential oils (Alisal) in rate of 0.25% on mites. activity and changes under stereomicroscope showed that sprayed mites completely stopped movements at both 1- and 2- h after treatment with completely stretched legs and white bead-like spots of oils accumulation on legs and bodies at 1- and 2- h. while, the non-treated mites were active with pale light brown colour. *In vivo* effect of Allisal to control red mite infestation in laying hens was investigated. In case of drinking water method mite count reduction rate on the bird was 60 %, 10%, and 0% as well as 0%, 0% and 10% in their traps at 4,7, and 12 days respectively, from the start of treatment in drinking water. While, in spray method mite reduction was 40%, 20%, and 10% on birds and 0%, 0% and 30% in the traps. On the other hand, water intake, feed intake, general health condition, skin health, and feather condition scores were improved at 4th day post treatment (DPT). Total lesion score at 12 DPT was improved. The present study concluded that *in vitro* combined plant essential oils have rapid and strong acaricide effect in contact sprays. *In vivo*, there was obvious improvement in groups treated with plant oils than non-treated group. Drinking water treated birds showed good results than spray treated group. Therefore, it is recommended to use combined plant essential oils in *D. gallinae* control strategies in poultry.

Key words: Acaricides, Chickens mite, *Dermanyssus gallinae*, Plant essential oils, Red mite.

INTRODUCTION

Dermanyssus gallinae (*D. gallinae*) infestation has significant impacts on poultry industry and productivity. Significant economic losses are due to reduction in weight gain and egg production, moreover their role as a disease vector (Flochlay et al., 2017). Also, Blood feeding mite can cause losses due to anemia where layers lose more than 3% of its blood volume every night. In severe cases hens may die from severe anemia (Van Emous, 2005). The veterinary and human medical impact needs adequate therapeutic measures to control parasites in poultry (Meyer-Kuhling et al., 2007; Roy et al., 2009; Sparagano et al., 2009). Where, *D. gallinae* is involved in transmission of many pathogenic agents responsible for severe outbreaks in both animals and humans (Chirico and Tauson, 2002; Moro et al., 2009). In a study it's concluded that northern fowl mite infestation (NFM) has negative impact on interior egg quality and hen integument (Vezzoli et al., 2016). *D. gallinae* life cycle possessed five stages (egg, larva, protonymph, deutonymph, adult) Infestation can triple its numbers in only 10 days where nymphs need a blood meal for metamorphosis and adult females need blood meals for egg maturation. The mites are small and grey in color but may appear red if they have filled with blood after feeding on the bird (Pritchard et al., 2015). Various synthetic contact acaricides such as permethrin, carbaryl, diazinon, dichlorvos are the most used for control *D. gallinae* infestation lead to drug chemical pollution and the development of resistance (Flochlay et al., 2017). In addition, drug and chemical residues in eggs and meat are an important problem for human health (Cernea, 2006; Kim et al., 2007). So that, there is an urgent need for using of herbal organic pharmaceuticals products as an alternative control measures to avoid drawbacks and maintain a good animal health (Lee et al., 1997; Abbas et al., 2014).

Plant essential oils are rich sources of bioactive components to control of mite infestations (Kim et al., 2004). The main chemical constituent of intact garlic is the amino acid allicin, an alkyl derivative of cysteine alkyl sulfoxide, which may vary from 0.2 to 2.0% fresh weight (Michahelles, 1974; Lutomski, 1987). Garlic contains at least 100 sulfur-containing compounds basic to medicinal uses. Allicin represents 70-80 % of the total thiosulfinates (Lawson and Hughes, 1992; Lawson, 1993; Srivastava et al., 1995). Valuable phytochemicals such as phenolic compounds (2554 µg/g), carotenoids (2.92 µg/g), and ascorbic acid (1798 µg/g) are contained in the rosehip seed. Also, it was rich in polyunsaturated fatty acids, linoleic acid (54.05%), linolenic acid (19.37%), and phytosterols, mainly β-sitosterol (82.1%) (Ilyasoglu, 2014). Rapeseed oil is plant-based oil extracted from the seeds of the rapeseed plants contained

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higher levels of total tocopherols and carotenoids. So that, it widely used in animal nutrition (Marčić et al., 2009).

Acaricide/insecticide activity of some plant extracts, oils and active components were examined and used as alternative to the chemical acaricides to control chicken's mite infestation (Kim et al., 2004; Magdaş et al., 2010; George et al. 2010 and 2014). The activity of numerous essential oils were evaluated against adult *D. gallinae* which collected from poultry using direct contact and fumigation methods, the results showed great effect due to action in the volatile oils (vapour phase) (Kim et al., 2004). *In vitro* the effect of eleven essential oils using direct contact method was evaluated and the results revealed that the most effective oils were sweet basil, coriander, peppermint and summer savory (Kim et al., 2004; Magdaş et al., 2010). Acaricidal activity of plant bioactive components was evaluated against *D. gallinae* by contact toxicity to carvacrol and thymol, the author concluded that two components were found to be toxic to *D. gallinae* with LD50 values of 1 and 3.15 µg/cm, respectively (Tabari et al., 2015). Carvacrol-thymol combination in ratio 4:1 at 2 % concentration displayed good residual toxicity and was effective against *D. gallinae* till 14 days post spraying (Masoumi et al., 2016). Garlic essential oil was toxic to *T. molitor* larva, followed by pupa and adult. Diallyl disulfide was the most toxic induced symptoms of intoxication and necrosis in larva, pupa, and adult of *T. molitor* between 20–40 h after exposure, therefore, garlic essential oil and their compounds have the potential for pest control (Plata-Rueda et al., 2017). The rosehip-seed oil plant oils were efficient natural phytochemicals against the treated larvae of cotton leaf-worm and combination of oils has synergistic action against the 4th larval instar of *S. littoralis* (Mesbah et al., 2006). Rapeseed oil used in spray treatment against spider mites, green peach aphid, pear psylla, summer population of *P. ulmi* and *T. urticae* resulted in efficacy rate 97.4% -84.1 % at 7-11 DPT (Marčić et al., 2009). Antimicrobial activity of the synergistic action of essential oils mixture was also reported (Mesbah et al., 2006; Bassolé and Juliani, 2012; Hyldgaard et al., 2012; Masoumi et al., 2016). Susceptibility of *D. gallinae* to combined essential oil was carried out by direct contact fumigation or spray to fulfill contact toxicity (Kim et al., 2004; Magdaş et al., 2010; Faghihzadeh Gorji et al., 2014; Rahimian and Sparagano, 2017).

The present study was aimed to evaluate the acaricidal potential of combined plant essential oils (includes garlic, rosehip, rapeseed and polysorbate) *in vitro* using direct contact method and *in vivo* through drinking water and direct spray on *D. gallinae* (red mite) of chickens.

MATERIAL AND METHODS

Red mite collection

Five hundred and twenty red mites (*D. gallinae*) of different stages were collected from naturally infested 5 layer poultry farms. The mites were collected with the aid of a brush in plastic jars and were used for tests within 2 days of collection. Until experiment duration, the mites were kept at 24 C° under a photoperiod of 16:8 h light/dark (Magdas et al, 2010). The collected mites were used for testing of efficacy of mixture of vegetable oil extracts (Allisal®) *in vitro* and *in vivo* (Faghihzadeh Gorji et al., 2014; Rahimian and Sparagano, 2017).

Essential oils

Allisal® is a liquid supplement product of ENVISAL EUROPE BV Vlambloem 85B 3068 JG Rotterdam, Netherlands. It was an aqua suspension of natural plant oils contained garlic oil, rosehip oil, rapeseed oil and polysorbate at concentration of 2.5%, 4.2%, 4.8% and 14.0% respectively. The product was diluted to 0.25% in water and used for *in vivo* and *in vitro* study.

In vitro study

Grouping and treatment for using direct contact with the mixture of plant oils. Six groups, 20 mites each were transferred to separate glass petri dishes (3 replicates) and exposed to the following treatments: Group 1 kept as non-treated control (sprayed with only water). Group 2 was sprayed with 0.25% plant oils mixture in water. All treated mite groups were observed under stereomicroscope at 1 and 2 h after direct spray, non-moving mites considered dead, the time for parasite death was recorded and photos taken for comparative evaluation of the acaricidal effect.

In vivo evaluation of plant oil mixture effect on red mite

Sixty mites infested 45 weeks-old layer hens having marked signs were selected and transferred to our laboratory. Birds were randomly divided into 3 equal groups (1 to 3); 20 hens each. Two hundred mites for each treated group were released for 4 days before start of treatment the average mite count from the first date will be set as 100% and the following counts will be compared to the first count. Each group was kept in separate cage on cross straw litter, feed on commercial layer ration under natural day light dark time. Hen groups were treated as group 1 kept as control non-treated. Groups 2 and 3 were treated with 0.25% plant oils mixture for 4 days, followed by 3 days stop of treatment and

return other 3 days treatment via drinking water and spray, respectively. Four traps were laid in each cage corner and were examined with counting of mite for calculation of counts % at 4,7 and 12 days from start of treatment and release it again. All groups were subjected to daily observation with recording of comb color, skin color, scape in skin and feather condition for calculation of lesion score. Also, water intake, feed intake and laid egg were considered in observation.

Scoring

The feather cover on six different body areas (head/neck, breast, vent, wings, back, and tail) was scored using a 0 to 2 scale, with 0 indicating that all body parts were completely feathered, 1 indicating that one or more body areas had damaged feathers or featherless spots < 5 cm in diameter, and 2 indicating that there were one or more body areas with featherless spots \geq 5 cm (Nicol et al., 2009; Welfare Quality, 2009; Vezzoli et al., 2016). The normal layer hens have no lesion (Score 0). The layers with lesions known as one lesion (score 1), two lesions (score 2), three lesions (score 3), four lesions (score 4). The all counted lesions were observed on feather, skin color, scabs and comb color.

Statistical analysis

The groups were compared at each time point by one-way analysis of variance (ANOVA) in SPSS (Statistics software, 2017) (Allen, 2017). The level of significance of the formal tests was set at 5%.

RESULTS

In vitro results of anti-red mites

Mite's description

Mites were directly subjected to treatment and examined under stereomicroscope until complete stop movements and their features were recorded at 1 and 2 hours after treatments (Figure 1). The non-treated mites showed active movement under stereomicroscope with pale light brown color at 1 and 2 hours after treatment (Figures 1A and 1B). The Allisal essential oil sprayed mites with 0.25% solution were completely stopped movements which considered dead at 1 and 2 hours after treatment with completely stretched legs and white bead like spots of oils accumulation on legs and bodies (Figures 1 C and 1 D). Results of essential oils contact on *D. gallinae* at different dose were listed in table (1). For the 0.25% Allisalesential oils after 1 and 2 h of contact a strong acaricide effect of 100% death of red mites at 1 and 2 h was seen.

In vivo results of anti-red mites

In vivo effect of Allisal oil to control red mite infestation in laying hens (Table 2). First treatment showed that at the 3rd day post- administration in drinking water mite was reduced to 60% on bird and 0% in traps, while in sprays; the mite population was reduced to 40% on bird and 0% in trap. At the 7th day (1st day of starting 2nd treatment) mite count was reduced to 10% and 0% on bird and in trap; respectively, while in spray it was reduced to 20% and 0% on birds and in trap; respectively. By the 12th day (2nd day after full treatment course) birds treated via drinking water showed no mite on hens and 10% in traps, while those spray treated showed 10% on birds and 30% in traps. Infested chickens showed dirty dull feather, red spots in pale skin and area free from feather at 0 time, these signs started to improve in treated via drinking water and spray at the 4th day (figure 2) while feathers and skin returned to normal at the 12th day as compared with non-treated. Lesion score (table 3) drinking water treated group 2 at the 4th day of treatment showed lower feather, skin color, scabs, comb color and total score (0.70, 0.6, 0.70, 0.50 and 2.50) than those of infected non-treated group 1(0.75, 0.7, 0.85, 0.65and 2.90), respectively. The spray treatment (group 3) showed slight improvement (0.70, 0.5, 0.70, 0.50 and 2.40). The improvement in feather, skin color, scabs, comb color and total score were the highest at 12 days post-treatment in water (0.30, 0.4, 0.30, 0.30and 1.30 followed by spray (0.70±, 0.6, 0.60,0.50and 2.30) compared with non-treated (0.80, 0.8, 1.00, 0.80 and 3.45), respectively. At 4th day there is marked improvement in water and feed intake, bird's activity and egg production (quantity and quality) were started to improve in water treated groups than spray and non-treated groups from the 4th day till the 12th. In general drinking water treated birds showed suitable general health condition and lower lesion scores than spray treated as compared with non-treated control group.

Table 1. Acaricidal effect of Allisal essential oils against *D. gallinae* collected from layers farms

Treatment	Dose	Mites Mortality (%)	
		1 h.	2hs
Non treated	Water	0	0
Allisal®Oils	0.25%	100	100

Table 2. Effect of Allisal 0.25% in drinking water and direct spray on red mites count in infested treated hens

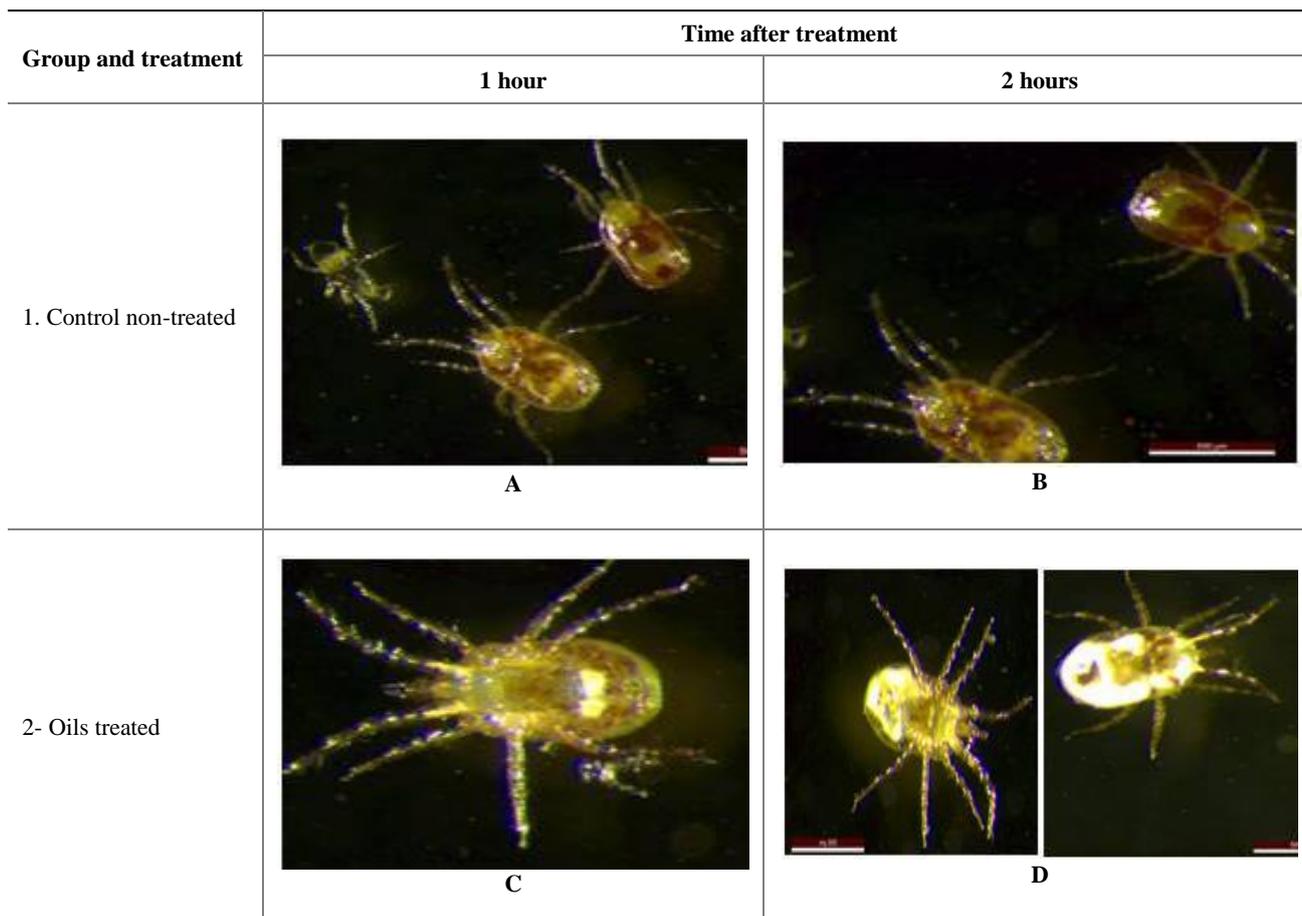
Parameter	Observation/ days							
	Drinking water				Spray			
	0	4D	7	12	0	4	7	12
Red mites count on birds (%)	100	60*	10	0	100	40	20	10
Red mites count in traps (%)	100	0	0	10	100	0	0	30

*All numbers are rounded to the nearest ten.

Table 3. Lesion score of Allisal 0.25% treatment in drinking water and direct spray on red mites in infested laying hens

Treatment	Time/ days	Lesion				Total
		Feather	Skin colour	Scabs	Comb colour	
		Mean* ± SD**	Mean ± SD	Mean ± SD	Mean ± SD	
Non-treated	0	0.65±0.49	0.6±0.53	0.75±0.44	0.55±0.51	2.50±0.69
	4	0.75±0.45	0.7±0.49	0.85±0.37	0.65±0.49	2.90±0.79
	7	0.80±0.42	0.75±0.45	0.90±0.31	0.80±0.41	3.25±0.64
	12	0.80±0.41	0.8±0.419	1.00±0.0	0.80±0.41	3.45±0.51
Drinking water	4	0.70±0.48	0.6±0.52	0.70±0.48	0.50±0.53	2.50±0.53
	7	0.60±0.52	0.5±0.523	0.80±0.42	0.4±0.52	2.30±0.82
	12	0.30±0.48	0.4±0.51	0.30±0.48	0.30±0.48	1.30±1.16
Spray	4	0.70±0.48	0.5±0.53	0.70±0.48	0.50±0.53	2.40±0.69
	7	0.60±0.52	0.5±0.53	0.60±0.52	0.60±0.52	2.40±0.84
	12	0.70±0.48	0.6±0.52	0.60±0.51	0.50±0.53	2.30±1.05

*Means: Mean number of birds with lesion/ total birds per group. ** SD: Standard deviation.

**Figure 1.** Acaricidal effect of essential oils against *D. gallinae* collected from layers farms

DPT	Control non-treated	Drinking water	Direct spray
0			
4			
7			
12			

Figure 2. Effect of Allisal essential oil at 0.25% concentration in drinking water and water direct spray in red mites natural infested laying hens. DPT: Day post treatment. 0: Time before start treatment. 4-, 7- and 12- Days post- treatment

DISCUSSION

Recently, the susceptibility of field collected *D. gallinae* from naturally infested commercial chicken houses to essential oil mixture and/or prepared nanoparticles as acaricide was evaluated. The susceptibility of collected *D. gallinae* to essential oil as acaricide was carried out by direct spray to fulfill contact toxicity (Faghihzadeh Gorji et al., 2014; Rahimian and Sparagano, 2017).

In the present study, we used essential oils mixture (Allisal) which contains garlic oil, rosehip oil and rapeseed oil to evaluate their effect on the chicken's mites in vitro and vivo. As shown in (figure 1 and table 1) results of in vitro essential oils contact on *D. gallinae* at different doses of the 0.25% Allisal after 1 and 2 h of contact were revealed that the non-treated mites showed active movement under stereomicroscope with at 1 and 2 h after treatment. The Allisal essential oil sprayed mites with 0.25% solution were completely stopped movements which considered dead at 1 and 2 h after treatment with completely stretched legs and white bead like spots of oils accumulation on legs and bodies A strong acaricide effect of 100% death of red mites at 1 and 2 h was seen. This in accordance with Kim et al. (2004 and 2007)

and George et al. (2010a, b, 2014) the authors concluded that plant-derived essential oils are shown to have a lethal characteristics, where garlic and thyme oils were the most effective. In the other hand, the synergistic action of essential oil for cuticular penetration was also reported (Tong and Bloomquist, 2013; Tak and Isman, 2015). Where the outer part of the mite exoskeleton, known as the epicuticle, consists of a layer of wax, which further limits water loss, and a cement layer, which protects the cuticle from external abrasion (Pritchard et al., 2015; Flochlay et al., 2017). The hydrophobic nature of the oils can cause mechanical effects on the parasite by disrupting the cuticular waxes and blocking the spiracles, leading to death by water stress or suffocation (Burgess, 2009). On the other hand, some of essential oils can be toxic effect on the insect nervous system (Mills et al., 2004; Lopez and Pascual-Villalobos, 2010) and maybe they have similar effects in other parasites.

The synergistic acaricidal action of essential oil was recorded, and the treated mites showed stop movement and detached legs and abnormal keratin surface of dark brown color at 2 h (Amagase et al., 2001). High concentration extract of Garlic (*Allium sativum*) juice and Chrysanthemum (*Chrysanthemum cinerariae folium*) were found to be effective, against *D. gallinae* (Maurer et al., 2009).

Strong acaricide effect with 100% deaths at 1.2 and 3 hours was previously reported by many researchers. Carvacrol (essential oil of *Origanum vulgare*), garlic extract, cinnamon, eucalyptus and mint extract reduction in mite control with 92%, 96%, 66.97, 80.85 and 90.19%; respectively (Rahimian and Sparagano, 2017). The acaricidal activity of essential oils was tested on movable *D. gallinae*; regardless of the stage of their development, garlic extract was 96% effective after two successive sprays (Faghihzadeh Gorji et al., 2014).

Results of in vivo effect of Allisal oil to control red mite infestation in laying hens showed in (table 2 and figure 2) and revealed that in drinking water treated group mites reduction rate on the bird was 60 %, 10%, and 0% as well as 0%, 0% and 10% in their traps at 4,7, and 12 days respectively, from the start of treatment in drinking water. While, in spray method mite reduction rate was 40%, 20%, and 10% on birds and 0%, 0% and 30% in the traps. On the other hand, water intake, feed intake, general health condition, skin health, and feather condition scores were improved at 4th (DPT). Total lesion score at 12 (DPT) was improved. Where feathers and skin returned to normal at the 12th day as compared with non-treated group (Table 3). Drinking water treated group showed suitable general health condition and lower lesion scores as well as egg production improvement more than spray treated group.

The improvement in all parameters proved safety of used essential oil mixture in red mite control in poultry. These results may be attributed to efficacy of used oil as antihistaminic, anti-inflammatory, high vitamin E and antioxidant activity. These findings are consistent with the result recorded by (Rabinkov et al., 1998; Miron et al., 2000; Xu et al., 2011; Pillaiyar et al., 2017; Qing, 2017). Moreover, essential oil had been approved as food additives and fall in the category of generally recognized as safe by the US FDA (Bilsland and Strong, 1990). For example, Rapeseed oil is plant-based oil contained higher levels of total tocopherols and carotenoids. So that, it widely used in animal nutrition (Marčić et al., 2009).

CONCLUSION

From the obtained results its concluded that the treatment with special and formulated essential oil mixture is highly effective for *D. gallinae* when used in drinking water than direct spray (0.25ml/liter) as acaricidal. It can be considered as safe recommended alternative to chemical compound used in control strategies of *D. gallinae* (red mites) in poultry industry.

DECLARATIONS

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Competing interests

The authors declare that they have no competing interests.

Author's contributions

Aziza M. Amer and Mohamed M. Amer designed, planned the study, Hoda M. Mekky and Hanaa S. Fedawy collecting samples. All authors shared performed experimental work, manuscript writing, drafted, revised the manuscript, and approved the final manuscript.

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Toxicity Assessment of a Multicomponent Antiparasitic Drug in Animals

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ABSTRACT

The important aspect of the high quality new pharmaceuticals is safety assessment in animals in practical conditions. Toxicity assessment of the new antiparasitic multicomponent drug (Inspector Quadro Tabs) composed of lufenuron, praziquantel and moxidectin in the form of tablets for cats and dogs was carried out. The parameters of acute oral toxicity were determined on white mice and white rats and subchronic toxicity was observed after repeated oral administration to rats. Embryotoxicity and teratogenicity of the drug were also evaluated. As a result of toxicological studies, median lethal doses (LD₅₀) of the drug during oral administration to the 60 white mice were established which were equal to the following: LD₅₀ = 14800 mg/kg (Karber's method), 13800 mg/kg (Miller and Tatener's method); to the white rats LD₅₀ > 16912 mg/kg; according to the generally accepted classification, the drug belongs to the fourth class of hazard (low hazard substances). It was established that doses of 1691 mg/kg, 846 mg/kg and 338 mg/kg were threshold in a subchronic experiment on the rats. Moreover, it was found that the drug did not possess embryotoxic and teratogenic properties in pregnant female rats. Experimental results have confirmed the low toxicity of a new antiparasitic multicomponent drug.

Key words: Acute toxicity, Antiparasitic Drug, Embryotoxicity, Mice, Rats, Subchronic Toxicity

INTRODUCTION

Nowadays, the urgent task of the modern veterinary science is a development of safe and effective antiparasitic drugs with a new combination of actual substances. The combined parasitic diseases of animals can be often found in veterinary practice so antiparasitic drugs should have several substances, which are active against different kinds of parasites (Arisov and Smirnova, 2016). However, besides the high efficiency, drugs should also be safe and have low toxicity level. Before carrying out clinical studies of the drug efficiency, preclinical trial is usually made, which cover studying of the toxicity of the drug in laboratory animals. Preclinical trials use standard methods of studying of the acute and subchronic toxicity and embryotoxicity of the drug in laboratory animals, according to guidelines for conducting preclinical studies of drugs (Mironov, 2012). For example, Nazarova (2014) and Polishuk et al. (2015) have used standard methods of studying toxicity for the determination of safety of the nanocrystal powders of metals which have been used as biologically active supplements for feeding animals.

Lutfullin et al. (2017) and Lutfullin et al. (2018) carried out studies on the toxicological parameters of the antiparasitic NB compound (in these authors' article it is presented only such a name of the drug as "NB compound", concealed data) on white mice and white rats, which included monitoring of hematological and biochemical blood parameters, pathoanatomical studies of animal corpses, fetuses, etc. One of the most dangerous effects of drugs is embryotoxic and teratogenic ones; therefore, studying the effects of drugs on the reproductive function of animals is an important task in veterinary medicine. Direct impact on the mother's body, the natural physiological barrier (placental barrier), and the direct effect on the fetus is possible when using the drugs. Embryotoxicity and fetotoxicity can occur in increased levels of embryonic mortality, changes in body weight, craniocaudal size of the fetus, delayed skeletal ossification, and an increase in perinatal pathology (Burkov, 2013).

MATERIALS AND METHODS

Ethical approval

This study used the following methods to reduce the pain and suffering of the experimental animals. The most humane and sparing methods and procedures were used towards animals; the means to prevent pain and suffering at animals were also used. Any painful procedures with animals were carried out according to the guidelines and standards:

European Convention for the Protection of Vertebrate Animals Used for Experimental and other Scientific Purposes (ETS 123). Strasbourg, 1986. Directive of the European Parliament and the Council of the EU 2010/63/EU of September 22, 2010 on the protection of animals used for scientific research. When carrying out the operations with the animals, we also used the guide for the care and use of laboratory animals National Academy press (Washington, USA).

Experimental groups

Sixty white male mice, 84 white male rats, 42 white female rats were held in the vivarium of All-Russian Research Institute of Fundamental and Applied Parasitology of Animals and Plants, a branch of the Federal State Budgetary Scientific Institution, Federal Research Center - All-Russian Scientific Research Institute of Experimental Veterinary Medicine. The animals were randomized into groups and did not take part in any studies before, 6 groups of 10 mice and 4 groups of 6 rats were formed to study the parameters of acute oral toxicity of the drug, 4 groups of 10 animals were formed to study subchronic toxicity in rats, 62 rats were used to study the embryotoxicity of the drug which were divided into 7 experimental groups of 6 female rats and 20 male rats in each group. Feeding was carried out *ad libitum* with complete extruded feed for laboratory animals and access to water was free. The temperature and humidity in the vivarium were constantly monitored (Air temperature: 18–22 °C; relative humidity: 50–65%). To study the parameters of acute oral toxicity of the drug in mice, five experimental and one control groups of white male mice weighing 19–22 g were formed which each group contained 10 individuals. To study the acute oral toxicity of the drug in rats, 3 experimental and 1 control groups of white outbred male rats weighing 185–210 g were formed, in which group there were 6 individuals. Subchronic toxicity was studied in 40 male rats with an initial mass of 165–200 g; they were divided into 3 experimental and 1 control groups with 10 animals in each group. The embryotoxicity of the test drug with repeated administration was studied on 42 white female rats weighing 200–220 g. For fertilization of the females, 20 males weighing 250–335 g (contained separately) were used. All females were divided into 7 experimental groups, with 6 individuals in each group. Males were placed for a night to females in the estrus and proestrus stages in a 4:1 ratio. The day of sperm detection in the vagina of females after placing males was counted as the first day of pregnancy (Figure 1).

Determination of toxicological parameters of the drug in laboratory animals

For this purpose, the drug was tested in mice in dose of 20000, 16800, 13600, 10400 and 6400 mg/kg. For ease of administration, the drug in the amount of 20.0 g was crushed, diluted in 15 ml of water, thoroughly mixed in a porcelain bowl with a pestle and the volume of the resulting suspension became 25.0 ml. Then this suspension was administered into fast mice once through intragastric probe with dose of 0.25; 0.21; 0.17; 0.13 and 0.08 ml per 10 g of animal mass, respectively. The control animals were given only drinking water in the amount of 0.25 ml per 10 g body mass. Dose of 10147, 14882 and 16912 mg/kg of the drug was tested in rats. For ease of administration, the drug in the amount of 23 g was crushed, diluted in 20 ml of water, thoroughly mixed in a porcelain bowl with a pestle and the volume of the resulting suspension became 34.0 ml. Then this suspension was administered into fast rats once with guide of an intragastric probe in doses of 1.5; 2.2 and 2.5 ml per 100 g of animal weight, respectively. The control animals were given only drinking water in the amount of 2.5 ml per 100 g body mass. The general condition and behavior of the animals, the occurrence of intoxication symptoms, and the possible deaths on days 1, 3, 7, 9, and 14 were monitored within 14 days.

Determination of subchronic toxicity in laboratory animals

The tested drug was administered intragastrically on daily basis in 14 days. The doses of 1691, 846 and 338 mg/kg (1/10, 1/20, and 1/50 of maximum possible for the administration into a stomach) were tested in rats. For ease of administration, 8.0 g of the drug was diluted in 30 ml of water, mixed thoroughly, and 34 ml of suspension was obtained. Then this suspension was administered using an intragastric probe in doses of 0.72; 0.36 and 0.14 ml per 100 g of animal weight, which corresponds to dose of 1691, 846 and 338 mg/kg. The control animals were given only drinking water in the amount of 0.72 ml per 100 g. During the entire period of use of the drug, the general condition and behavior of the animals, reactions to stimuli (sound, light), symptoms of intoxication, and possible death were observed. On the first day after the last administration of the drug (15th day of the experiment), half of the animals from each group were euthanized and blood samples were taken to determine hematological and biochemical parameters. Ten days after the last administration (24th day of the experiment) the second half of the animals were euthanized and blood samples were taken to assess the degree of reversibility of possible toxic processes after repeated use of the drug. Blood samples were taken, organ samples were taken from all rats in each group, the mass of the organs was determined, and the mass coefficients were calculated. The functional state of the central nervous system was assessed by visual observations of motor activity and reactions to external stimuli.

Equipments

The main indicators of rat peripheral blood were determined on a PCE 90-vet hematology analyzer (ERMA INC, Japan). When calculating leukocyte formula, blood smears were stained according to the Romanovsky-Giemsa method using a set for fixing and staining for hematological and cytological studies Haemocrafix (Russia). Stained smears were examined under a light microscope (Levenhuk D670T, USA). Blood biochemical parameters were determined on a Biosystems A-15 analyzer (Spain). Macroscopic examination of organs (liver, lungs, kidneys, heart, spleen, stomach and intestines) were carried out.

Determination of drug embryotoxicity

The tested drug was administered to pregnant female rats in 1, 2 and 3 experimental groups in a form of suspension in 1% starch gel in therapeutic dose, and also female rats in 4, 5 and 6 groups received drug and doubled therapeutic dose according to Mironov (2012), (20 mg of lufenuron, 10 mg of praziquantel, and 0.6 of moxidectin per 1 kg of the animal's weight) orally during the critical period of embryogenesis (1-6, 6-16 and 16-19 days of pregnancy). The therapeutic dose of the drug divided by the actual substances which were 10 mg of lufenuron, 5 mg of praziquantel, and 0.3 mg of moxidectin per 1 kg of the animal's weight. To prepare the suspension, 120 mg of the drug were crushed in a porcelain bowl with a pestle and mixed with 10.0 ml of 1% starch gel. The resulting suspension was administered to rats of the experimental groups in doses of 0.5 ml/100 g (First, second and third groups) and 1.0 ml/100 g (Fourth, fifth and sixth groups). The control group of pregnant females received a solution of 1% starch paste without the drug. On the 20th day of embryogenesis, all pregnant animals were killed by the method of decapitation. At necropsy the uterus and ovaries of female rats were removed (Figure 2).

In the ovaries, the number of corpus luteums (CLs) of pregnancy was counted. The implantation sites, live, dead, and resorbed fetuses were also counted in the uterus. Fetuses were examined under a binocular magnifying glass to detect external anomalies, then they were weighed and the size of the embryo was measured, along with the mass and the diameter of the placenta. The ratio of the number of CLs, implantation sites and live fetuses was used to calculate indicators of total embryonic mortality, pre-implantation and post-implantation death. After weighing, the fetuses in the litter from each female were divided into two equal parts. To study the skeletal system according to the Dawson method, half of the fetuses were left in 95% ethanol for 7-10 days, the alcohol was changed periodically. Then the embryo was immersed in a 1% KOH solution for clearing soft tissue. After 2-3 days (when the bone marks were visible), the fetuses were taken out of alkali, washed with water and transferred to a solution consisting of 150 ml of glycerol, 800 ml of distilled water and 10 g of KOH, as well as a few drops of 1% alizarin red solution. After 3-5 days, the ossified areas of the skeleton were painted in intense red-violet color. For bleaching of soft tissues and final enlightenment, the fetuses were transferred to a solution of glycerin, distilled water and KOH in the same ratios, but without the addition of a 1% solution of alizarin red. Then the fetuses were dehydrated by slow processing them through a mixture of alcohol, glycerin and water in different proportions (1: 2: 7; 2: 2: 6; 4: 4: 2), equal parts of alcohol and glycerin, pure glycerin with 1-2 drops of formalin. The length of the centers of ossification of the skeleton of the stained embryo (scapular, brachial, ulnar, radial, femoral, large and small tibialis) was measured under a stereoscopic binocular microscope magnifier using an ocular micrometer (Figure 3). The remaining fetuses were put into Bowen's fluid for 14 days for microanatomical analysis for the presence of abnormalities of internal organs according to the Wilson method modified by Andrey Pavlovich Dyban (Dyban, 1986). According to the scheme, 8 transverse sections were made with the razor blade (Figure 4); all sections were analyzed under a MBS-2 stereomicroscope for the presence of internal anomalies in the fetuses. First, the head was removed from the body with the parallel cut to the lower jaw bone; then the series of cuts were performed: perpendicular to the lower jaw directly behind the vibrissae (condition of the lower jaw, anterior part of the hard palate and nasal cavity); through the middle of the eyeballs and olfactory bulbs; through the large lateral cranial diameter (the state of the brain: the cerebral cortex, lateral, third and fourth ventricles); parallel to the third cut (for the study of the cerebellum and the medulla oblongata). In front of the front legs - the neck is cut off from the body (esophagus, trachea, spinal cord, large vessels); directly behind the forelimbs through the organs of the chest (heart, lungs, bronchi, esophagus, spinal cord); midway between the seventh incision and the umbilical ring (the study of the liver and the state of the diaphragm); below the umbilical ring (intestine, pancreas, kidneys, ureters, bladder, rectum, genitals).

Embryo death at the pre and post-implantation stages of development (embryonic effect), the presence of abnormal development of the internal organs and skeleton (teratogenic effect), fertility, mass and length of fetuses, diameter and mass of the placenta, anogenital distance and the anogenital index, the length and number of bone ossification points in the fetuses of rats were assessment criteria of embryotoxic and teratogenic effects of the drug.



Figure 1. Sperm cells detected in a vaginal smear of the female rat of the second experimental group during embryotoxic studies of the drug after her fertilization. Thin filaments are circled in red. The microscope Levenhuk D670T (USA), the magnification x100.



Figure 2. Dissection of the female rat with the extraction of the uterus (the female rat of the third experimental group during embryotoxic studies of the drug)



Figure 3. Fetuses of rats colored according to the Dawson's method. Ossified areas of the skeleton are painted red-violet, soft tissues are discolored.



Figure 4. Transverse cuts of fetuses of rats (Wilson's method, modified by A.P. Dyban)

RESULTS

Study of the acute oral toxicity in laboratory animals

During the observation of the laboratory animals, the dependence of the ratio of dead animals to the volume of the administered dose was observed. After the administration of the dose of 20000, 16800, and 13600 mg/kg, animals become despondent and anorexic and took lateral position before the death. During the autopsy, hyperemia of the gastric and intestinal mucous membrane and also swelling were revealed. The generalized results of the experiment to determine acute toxicity in mice are presented in table 1.

The most toxic dose was 20000 mg/kg in the 1st experimental group in which all the animals of the group was died. The dose of 16800 mg/kg was found to be less toxic in the 2nd experimental group in which the mortality of 60% of animals was noted. The indicators of the percentage of the increase in live weight in mice of the 2nd and 3rd experimental groups were statistically significantly lower than in the control group: $10.39 \pm 8.95\%$ versus $28.23 \pm 4.92\%$ and $7.40 \pm 9.82\%$ versus $28, 23 \pm 4.92\%$, respectively. Indicators of the percentage of live weight gain of mice in the 4th and 5th experimental groups did not undergo significant changes compared to the same indicator in the control group.

Mice of the fifth experimental group which received the drug at a dose of 6400 mg/kg showed no statistically significant difference in live weight, the behavior and general condition did not differ from control animals. Based on the obtained data, the LD₅₀ was calculated by the Karber's method as well as by the Miller- Tainter methods (Mironov, 2012).

$$LD_{50} = LD_{100} - \frac{\sum(zxd)}{n}$$

LD₁₀₀: Dose caused death of all animals;

z : The arithmetic average of the number of animals which death was observed under the influence of two adjacent doses;

d : Interval between two adjacent doses;

n : Number of animals in a group.

LD₅₀ of the drug (Inspector Quadro Tabs) in oral administration to mice, count according to the Karber's method, was equal to 14,800 mg/kg of the weight of the animal. In order to have a complete understanding of the LD₅₀, which can vary depending on the individual characteristics of animals of a given species and age, it is necessary to know the amplitude of its variation. To calculate the confidence interval of the general average LD₅₀ used the following formula:

$$\text{Confidence interval of the general average} = LD_{50} \pm t * S LD_{50}$$

LD₅₀: Lethal dose, counted according to the diagram; t: Test of validity; S LD₅₀: Error index LD₅₀; Confidence interval of the general average = $13,800 \pm 2,340.9$ mg/kg

Thus, LD₅₀ counted according to the Miller and Tatener's method is in the range between 11459.1 mg/kg and 16140.9 mg/kg (LD₅₀ = $13800 (11459.1 \div 16140.9)$ mg/kg). Therefore, taking into consideration LD₅₀ counted by two methods and according to the common hygienic classification (GOST 12.1.007), drug (Inspector Quadro Tabs) belongs to the fourth class of hazard (low hazard substances). During the study of acute oral toxicity in rats, the general condition of the animals of all experimental groups that received doses of 10147, 14882, and 16912 mg/kg was satisfactory throughout the experiment, there were no changes in the behavior of the rats, and their levels of appetite and thirst were normal. No disturbance of coordination of movements and convulsions were noted, the tone of skeletal muscles corresponded to the norm; response to tactile, pain, sound and light stimuli was adequate. The hair of the experimental animals was smooth, shiny, without alopecia; the integrity of the skin was not broken, there was no hyperemia; the color of the visible mucous membranes corresponded to the norm; the frequency and depth of respiratory movements and the

rhythm of heart contractions were not changed. It should be noted that a dose of 16912 mg/kg is the maximum possible for a single oral administration to animals of this species and this dose did not lead to the death of animals. Thus, the LD₅₀ of the (Inspector Quadro Tabs) drug exceeds the dose of 16912 mg/kg, which means that this drug (Inspector Quadro Tabs) belongs to the 4th hazard class (low hazardous substances).

Study of the subchronic toxicity

As a result of clinical examination of animals during the experiment, no signs of intoxication were observed in rats of the experimental groups. The general condition of the animals remained satisfactory, changes in behavior were not observed, appetite and thirst were not changed, and physiological parameters corresponded to the norm. The weight of the rats of the experimental groups in all weighing periods was not statistically significantly different from those of control animals. The relative mass of organs is a simple, but very clear indicator of the toxic effects of drugs with their long-term use. The calculation of the relative mass of each organ was carried out according to the formula:

$$S = (m / M) * 100$$

S: The relative mass of the body, m: The mass of the organ (g), M: The body weight of the animal (g).

The results of calculations of mass coefficients indicate that at doses of 1691, 846 and 338 mg/kg, the mass coefficients of all organs of animals on the first day after the last injection of the drug did not differ statistically significantly from those of the control group. Also, no significant differences were found in the rates of the mass coefficients of the organs of the experimental animals compared with the control group in 10 days after the last administration of the tested drug (Table 2).

According to the results of the macroscopic study of organs, differences between groups were not established, and therefore the data of rat necropsy is presented as average for all groups. During external examination of rats, no secretions from natural openings were found, the hair was shiny, there were no alopecia lesions, the teeth were preserved, the visible mucous membranes were pale pink, shiny, deformation or swelling of the extremities were not detected, the development of the external genitalia was in accordance with the physiological norm. Thoracic and abdominal cavities did not contain effusion, the position of the internal organs of the thoracic and abdominal cavities were anatomically correct, the parietal and visceral pleura and peritoneum were thin, shiny, and smooth. A macroscopic examination of the liver, lungs, kidneys, spleen, and heart after administration of the drug to rats at the three doses indicated that there were no changes in the structure, shape and size of the internal organs, the surfaces were smooth, shiny, of a consistent consistency and color without indurations. In all animals of all experimental groups on the 15th day of the experiment, the structure of the stomach corresponded to the norm, the thickness of the stomach wall was not changed, and there was no hyperemia. The mesenteric membranes and glands of rats were not changed, the vessels were not filled with blood, the mesenteric lymph nodes are not enlarged, the intestines contain a small amount of yellowish pasty mass, and the mucous membrane was not changed. During an autopsy performed on the 24th day of the experiment, there were no differences in the macroscopic structure of the organs of rats of the experimental groups in comparison with the control analogues.

An objective determination of toxic properties in the experiments also included the determination of hematological parameters of rats. At first and tenth day after the last administration of the drug to rats of the experimental groups, no statistically significant changes in blood parameters were detected compared with the control group. According to the results of determination of the biochemical parameters of blood serum after prolonged use of the drug (Inspector Quadro Tabs), the following was noted: on the 15th day of the experiment in rats of the first experimental group, an increase in the content of direct bilirubin was observed: $0.72 \pm 0.30 \mu\text{mol/l}$ versus $0.34 \pm 0.14 \mu\text{mol/l}$ in the control group (Table 3), which may indicate the hepatotoxic effect of the tested dose. In the rats of the other groups, there were no statistically significant differences in the biochemical indicators compared with the control. Summarizing all the results of the experiment, we can conclude that the doses of the drug (Inspector Quadro Tabs) of 1691 mg/kg, 846 mg/kg and 338 mg/kg when administered orally for 14 days are threshold.

Study of the embryotoxicity of the drug

As a result of the study, it was found that the drug for veterinary use (Inspector Quadro Tabs) in therapeutic and double therapeutic doses when administered orally to pregnant rats does not affect the morphometric parameters of the fetuses of rats when administered during pregnancy and does not cause developmental abnormalities. The study of the status of pregnant rats in the dynamics did not reveal the symptoms of the general toxic effect of the drug, the behavior; the consumption of water and food by the animals of the experimental and control groups did not differ. The drug in the tested doses did not affect the number of corpus luteum in the ovaries, did not have an embryo-lethal action, and did not cause the preimplantation death of fetuses. In groups of rats treated with the drug, the number of fetuses did not differ from number in the control group. It was established that the drug does not change the parameters of growth and development of the fetuses; during the study of the fetuses of rats of the control and experimental groups, developmental

anomalies were not observed. When studying the process of ossification of different parts of the skeleton of the fetuses of rats it was observed that the drug does not affect the length and number of points of ossification of large bones.

Table 1. The results of the experiment to determine the acute oral toxicity of the multicomponent antiparasitic drug in mice

Group	Number of mice	Dose (mg/kg)	Dose (ml/10g)	Died	Survived
1 experimental	10	20000	0.25	10	0
2 experimental	10	16800	0.21	6	4
3 experimental	10	13600	0.17	3	7
4 experimental	10	10400	0.13	2	8
5 experimental	10	6400	0.1	0	10
Control	10	H ₂ O (fresh)	0.25	0	10

Table 2. Mass coefficient of rat organs at 10 days after the last administration of the multicomponent antiparasitic drug (n = 5)

Organs	Control	Dose		
		1691 (mg/kg)	846 (mg/kg)	338 (mg/kg)
Liver	4.42 ± 0.96	4.33 ± 0.52	4.04 ± 0.38	4.28 ± 0.35
Kidney	0.69 ± 0.14	0.65 ± 0.05	0.63 ± 0.04	0.68 ± 0.02
Spleen	0.25 ± 0.06	0.26 ± 0.1	0.25 ± 0.06	0.29 ± 0.06
Lungs	0.61 ± 0.03	0.54 ± 0.12	0.57 ± 0.08	0.58 ± 0.15
Heart	0.37 ± 0.1	0.33 ± 0.03	0.32 ± 0.02	0.37 ± 0.07

Note: *P value > 0.05

Table 3. Biochemical parameters of blood serum of rats after 14 day of administration of the multicomponent antiparasitic drug (n = 5)

Parameters	Unit	Control	Dose		
			1691 (mg/kg)	846 (mg/kg)	338 (mg/kg)
Total bilirubin	µmol/l	2.74 ± 1.10	2.10 ± 0.69	2.54 ± 1.32	3.44 ± 0.48
Direct bilirubin	µmol/l	0.34 ± 0.14	0.72 ± 0.30*	0.62 ± 0.57	0.68 ± 0.56
Aspartate aminotransferase	U/l	155.20 ± 32.34	127.00 ± 39.53	135.00 ± 37.87	150.00 ± 21.52
Alanine aminotransferase	U/l	52.60 ± 3.12	47.80 ± 11.29	46.20 ± 14	48.00 ± 7.18
Urea	µmol/l	5.04 ± 1.18	4.54 ± 0.84	4.64 ± 0.69	4.26 ± 0.69
Creatinine	µmol/l	39.80 ± 2.83	39.60 ± 4.78	42.60 ± 5.59	41.60 ± 7.32
Total protein	g/l	63.40 ± 2.08	65.40 ± 5.38	64.40 ± 2.86	62.40 ± 1.88
Alkaline phosphatase	U/l	242.6 ± 75.78	258 ± 84.55	298.6 ± 47.24	266.8 ± 76.28
Alpha Amylase, total	U/l	644 ± 58.96	691.6 ± 41.04	675.80 ± 49.39	621.2 ± 152.71
Glucose	µmol/l	6.46 ± 0.91	5.80 ± 0.69	6.92 ± 0.83	6.18 ± 0.51
Lactate dehydrogenase	U/l	1394.6 ± 516.15	1176.8 ± 302.46	1252.2 ± 555.39	961 ± 264.49

Note: *P value > 0.05

DISCUSSION

It is well known that some insectoacaricides may have undesirable side effects when administered to specific species, for example the administration of fipronil to the rabbits during treatment of the psoroptic scab (Elhawary et al., 2018). In turn, present toxicological studies of the multicomponent drug “Inspector Quadro Tabs” containing 3 antiparasitic components found the absence of a side effect as embryotoxicity in pregnant animal. During the process of studying the toxicological parameters of the drug (Lufenuron, moxidectin, praziquantel), experiments on the study of allergenic properties were carried out (Stepanova and Koshkarev, 2019). As a result of research, it was found that the drug, after a 20-day sensitization of guinea pigs with epicutaneous, nasal and conjunctival samples, does not cause a specific response of the body and does not possess allergenic properties. During the study of acute oral toxicity of the drug containing moxidectin and praziquantel, it was found that the drug belongs to the 4th hazard class, and its dose of 142 mg/kg is inactive (Arisov and Smirnova, 2016). The studied multicomponent antiparasitic drug containing moxidectin, praziquantel and lufenuron is also a low-hazard substance. In the veterinary practice, there was a registered case in which 3 dogs were orally administered a topical preparation containing imidacloprid and moxidectin as active substances (See et al., 2009). All toxic effects were reversible and after 48 hours, all dogs were clinically healthy. Tamparon et al. (2015) proved that abamectin, belonging to the group of avermectins, has a high toxicity for aquatic invertebrates, which is observed with lytic activity of sea urchin spermatozoa. Insecticide interferes with fertilization,

preventing gamete fusion. But it is known that the main difference between avermectins and milbemycins (moxidectin) is the presence of a disaccharide in the avermectins molecule, which is absent in milbemycins (Safiullin, 2006), which may explain their lower toxicity to animals. Numerous studies have also proved the low toxicity of praziquantel, which allows it to be used for therapeutic purposes (Zuskova et al., 2018; Sun et al., 2016), which is confirmed by our studies of a multi-component preparation with praziquantel content of 8 - 11%. When studying the toxicity and efficacy of the drug with the content of lufenuron for the treatment of fish ectoparasites, it was found that there were no histological signs of acute and chronic toxicosis, as well as mortality of fish (Tang et al., 2019), which confirms the low toxicity of lufenuron when used in drug (Inspector Quadro Tabs) for animals. In the present study examining of the drug embryotoxicity at a therapeutic and double therapeutic dose (20 mg of lufenuron, 10 mg of praziquantel, 0.6 mg of moxidectin per 1 kg of body mass weight) by oral administration to the pregnant rats, showed that the drug has no effect on the morphometric parameters of the rat fetuses while administration in pregnancy period did not cause any abnormalities. The drug did not affect the number of CL in the ovaries in the tested doses, did not have an embryo-lethal effect and did not cause pre-implantation death of the embryos. The study of the toxicological parameters of the combination of actual substances lufenuron, moxidectin and praziquantel in the tablet form was carried out for the first time and the results showed that the drug belongs to the group of low-hazard substances, the drug is safe for use in clinical studies of therapeutic efficacy in parasitic diseases of the target animal species.

CONCLUSION

As a result of the study, acute oral toxicity of the drug (Inspector Quadro Tabs) was evaluated in white outbred rats and white outbred mice. It was determined that LD₅₀ of the drug during single oral administration to the rats is more than 16912 mg/kg. During a single oral administration of the drug to white outbred mice, LD₅₀ calculated according to the Karber's method was 14800 mg/kg of animal weight; The LD₅₀ calculated by the Miller and Tainter's method was 13800 (11459.1 ÷ 16140.9) mg/kg. According to the generally accepted hygienic classification, the drug belongs to the fourth hazard class - low hazardous substances (GOST 12.1.007). In a subchronic experiment on rats, it was established that doses of 1691 mg/kg, 846 mg/kg, and 338 mg/kg are threshold. The Inspector Quadro Tabs does not possess embryotoxic and teratogenic properties, does not cause changes in linear measurements and fetal weight, the number of fetuses in the litter, or anatomical disorders and skeletal ossification.

DECLARATIONS

Authors' Contribution

Irina A. Stepanova planned this investigation, took part in its development and controlled the experimentation, drafted the manuscript. Gulnara B. Arisova carried out all the preparations for the experiment, made manipulation with laboratory animals, Mikhail V. Arisov observed and monitored the state of animals, did an autopsy of fallen animals, calculated and interpreted the results. Irina A. Stepanova examined the acute and subchronic toxicity of the drug; Mikhail V. Arisov and Gulnara B. Arisova carried out the investigation of embryotoxic properties. All authors read and approved the final manuscript.

Competing interests

Authors state no conflict of interest.

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Consent to publish

All the authors approved and agreed to publish the manuscript

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Immunoreactivities to α -SMA and S-100 Proteins in the Testis of the African Four-toed Hedgehog (*Atelerix albiventris*)

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ABSTRACT

The African four-toed hedgehog is a small nocturnal mammal, characterized by a short-grooved brown or grey spine covering the dorsum of the body with a band of whitish fur running across their forehead, little is known about the reproductive biology of this animal. The present study aimed to evaluating the validity of immunohistochemistry in the differential labelling of the different cellular components of the testis of the African four-toed hedgehog. Paraffin-embedded testicular sections were stained by conventional histological technique using ten male African four-toed hedgehogs captured from the wild animals in Ibadan, Nigeria. Primary antibodies against alpha smooth muscle actin (α -SMA) and S-100 were applied on paraffin sections. The peritubular myoid cells, the testicular capsule and vascular endothelium expressed strong immunostaining for α -SMA. The spermatogenic cells, Sertoli and Leydig cells, peritubular myoid cells, the testicular capsule, straight tubules as well as rete testis and vascular endothelium all expressed positive immunostaining for S-100. α -SMA and S-100 proteins play active roles in cytoskeletal physiology of testis of the African four-toed hedgehog while S-100 protein plays additional role in the structural formation and maintenance of the blood-testis barrier during the process of spermatogenesis in the animal. It is concluded that α -SMA and S-100 proteins has active roles in the cytoskeletal structure of testis and physiology of the African four-toed hedgehogs.

Keywords: African four-toed hedgehog, Immunoreactivities, Spermatogenic cells, Sertoli cells, Testis.

INTRODUCTION

The order insectivore comprises several families that include shrews, moles, hedgehogs, solenodons, tenrecs and golden moles (Bedford et al., 2000). The African four-toed hedgehog (*Atelerix albiventris*), family *Erinaceidae*, widespread in West Africa, is an inhabitant of plains, savannas, and grassland (Kingdon, 1997). It has been described as a small nocturnal mammal, characterized by a short-grooved brown or grey spine covering the dorsum of the body with a band of whitish fur running across their forehead. The fifth toe on the hind feet, which is present in other species of Hedgehogs is reduced to vestigial hallux in this animal, hence the name “four-toed” and there is no sexual dimorphism (Reeve, 1994; Coker et al., 2018).

Despite its exploitation as a popular pet in the United States (Mori and O'Brien, 1997) as well as its use in biomedical research, the animal is listed as least concern in the IUCN red list category in view of its wide distribution and high tolerance to a degree of habitat modification (Cassola, 2016). It has been reported that *Atelerix albiventris* reaches sexual maturity in the wild at about 1-year of age but sexual maturity may be attained at 2-4 months (Smith, 1992; Symonds, 1999). Nevertheless, there is no record of its successful captive - breeding thus far (Santana et al., 2010).

Previous reports on the African four-toed hedgehog have focused on reproductive characteristics (Smith, 1992; Symonds, 1999; Bedford et al., 2000); skull morphometry (Couture et al., 2015; Girgiri et al., 2015); disease occurrence in captive animals (Gardhouse and Eshar, 2015) and appendicular skeleton morphology (Girgiri et al., 2016). Thus, there is paucity of research information on the reproductive biology of the African four-toed hedgehog (Olukole et al., 2020). In particular, nothing, to our knowledge, has been reported on the presence, localisation and activities of proteins in the testis of this animal.

This study was therefore aimed to investigate the immunohistochemical localization of alpha smooth muscle actin (α -SMA) and S-100 proteins in the testis of the African four-toed hedgehog as contribution to existing literature and possible improvement in the understanding of the testicular architecture and function and the captive-breeding programmes of this animal.

MATERIALS AND METHODS

Animals and tissues

Ten male African four-toed hedgehog, captured from the wild in Ibadan, Oyo State, Nigeria, between the months of May and October (wet season), 2016 were used for the study. The male hedgehogs had an externally visible penis, located in the mid-abdomen, but the testicles were recessed into pouches close to the anus ([World Heritage Encyclopedia, 2017](#)). Hedgehogs were anaesthetised with a subcutaneous injection of 20 mg/kg ketamine in the area of the back ([Henke et al., 2007](#)), sacrificed and the testis was retrieved immediately and fixed in buffered neutral formalin.

Ethical approval

Experiments were carried out according to the guidelines and approval of the institutional ethical committee of the University of Ibadan, Nigeria (UIACUREC: 12/17/05).

Histology and immunohistochemistry

Samples of the testes were fixed in Bouin's fluid and embedded in paraffin blocks. Sections 2-4 μm thick were stained with Haematoxylin and Eosin ([Alkafafy et al., 2012](#)). The slides were then studied under a light microscope (Olympus BX63, Germany, with DP72 camera). Immunohistochemistry was carried out as reported by [Aire and Ozegebe \(2007\)](#). Briefly, paraffin-embedded tissues of testis were cut and mounted on slides pre-coated with polylysine. They were deparaffinised, rehydrated and heat-treated for antigen retrieval. To reduce endogenous peroxidase activity, sections were incubated for 5 min in hydrogen peroxide (3% in distilled water). In order to block non-specific binding sites, the slides were rinsed in a 0.01 M phosphate buffered saline solution (PBS, pH: 7.4), containing bovine serum albumen, for 5 minutes. Prior to immunostaining, validation trials for each of S-100 and α -smooth muscle actin antibodies in this species were carried out using four different dilutions (1: 50, 1: 100, 1:200 and 1:400). Immunostaining of slides were carried out for one hour at room temperature, using the LSAB-plus kit (Dakocytomation, Glostrup Co, Denmark) monoclonal antibodies against S-100 and α -smooth muscle actin at dilutions of 1:100 and 1:400, respectively. The slides were then rinsed in PBS followed by incubation for 15 minutes in a link antibody (Biotinylated secondary antibody, LSAB-plus kit; Dakocytomation) and then in peroxidase-labelled streptavidin. This was followed by addition of 3,3'-diaminobenzidine tetrachloride solution (DAB) from the LSAB+[®] kit to visualise antigen localization. Negative controls involved the primary antibody replaced by bovine serum albumen. Smooth muscle was used as a positive control for α -smooth muscle actin, while tonsillar tissue was used as positive controls for S-100. The testis sections were counterstained with hematoxylin for 30 seconds, washed in water, dehydrated through graded ethanol, cleared in xylene and mounted with DPX permanent mounting media (Sigma-Aldrich Co, MO, USA). Sections were then examined under light microscope. Immunoreactivities to S-100 and α -smooth muscle actin were designated as absent (-), weak (+), moderate (2+) and strong (3+) based on visual examination.

RESULTS

Histology

The testis was ensheathed by a capsule with seminiferous tubules of various sizes and shapes, mostly spherical to oval (Figure 1A). Wedged among adjacent seminiferous tubules was the interstitium containing interstitial Leydig cells and blood vessels. Highly-convoluted seminiferous tubules formed about 80% of testis tissue of the animal with each having a basement membrane lined with Sertoli cells as well as germ cells arranged in successive layers representing different stages of spermatogenesis and spermiogenesis (Figure 1B).

Immunohistochemistry

The main immunohistochemical findings are summarized in table 1.

Alpha smooth muscle actin (α -SMA)

Strong immunoreactivities to α -SMA were observed for the peritubular myoid cells, the testicular capsule and vascular endothelium (Figures 2A and B). The testicular capsule showed more intense reactivity in the inner than in the outer portions (Figure 2B). Moderate immunoreactivities to α -SMA were observed for the straight tubules as well as rete testis (Figures 2C and D). However, the spermatogenic cells, Sertoli cells and Leydig cells were immunonegative to α -SMA.

S-100

Strong immunoreactivities to S-100 were observed for the peritubular myoid cells and testicular capsule (Figures 3B and D). The spermatogenic cells, Sertoli cells, Leydig cells, straight tubules as well as rete testis and vascular endothelium all expressed moderate immunoreactivities to S-100 (Figures 3A and C).

DISCUSSION

The histological features of the testis of the African four-toed hedgehog are similar to those reported for the African greater cane rat, *Thryonomys swinderianus*, (Olukole et al., 2009; Olukole and Obayemi, 2010), the African giant rat, *Cricetomys gambianus*, (Oke, 1982; Oke, 1988) and rodents generally (Bacha and Bacha, 2000; Massanyi et al., 2003; Young et al., 2006). Similar to the case in the African greater cane rat, contractile myoid cells were found lining the peritubular tissue of the seminiferous tubules of the African four-toed hedgehog (Olukole and Obayemi, 2010). However, unlike the case of the African greater cane rat, majority of the seminiferous tubules of the African four-toed hedgehog were highly convoluted (Olukole et al., 2009).

The present study is a new report on the localization of alpha smooth muscle actin (α -SMA) and S-100 in the testis of the African four-toed hedgehog. The more intense immunoreactivity to α SMA observed in the inner portion of the testicular capsule of the African four-toed hedgehog, suggests that contractile activity of the testicular capsule is greater in the inner than in the outer portions of the testicular capsule of the animal. Similar observation has been documented in the rat (Banks et al., 2006). Conversely, Abd-Elmaksoud (2009) reported α SMA-positive reaction to be more in the outer portion of the testicular capsule of the rabbit. α SMA has been reported to be widely distributed throughout the tunica albuginea of the testicular capsule in the avian species such as Sudani duck and pigeon (Abd-Elmaksoud, 2009). It can be inferred that the contractile smooth muscle arrangement observed in the testicular capsule of the African four-toed hedgehog may be essential in the movement of spermatozoa out of the testis of the animal.

The α SMA-positive reaction observed in the testicular peritubular tissue and vasculature in this study has been observed in several mammals and birds (Sekido and Lovell-Badge, 2013; Valdez et al., 2014; Domke et al., 2019). One of the functions of peritubular myoid cells has been shown to be the regulation of Sertoli cell functions in animals (Anthony, 1991). Sertoli cell's stimulation of total protein by peritubular cells have linked to increases in androgen-binding protein production (Hadley et al., 1985).

Hence, peritubular myoid cells of the African hedgehog, due to its strong reactivity to α SMA are expected to collaborate with Sertoli cells in the structural formation and maintenance of the blood-testis barrier in the animal. Collectively, the positive immunoreactivities to α SMA observed for the testicular capsule, the peritubular myoid cells, straight tubules as well as the rete testis of the African four-toed hedgehog is suggestive of a collaborative contractile activity in the movement of spermatozoa produced in the seminiferous tubules into the excurrent ducts of the testis of the animal.

The strong immunoreactivities to S-100 protein observed for the peritubular tissue and Sertoli cells further shows the possibility of a collaborative function by the duo in the maintenance of the blood-testis barrier in the African hedgehog. Similarly, S-100 protein has been localized in the Sertoli cells of various animals includes bull, dog, boar, rat, rabbit, ram, stallion and boar (Riuzzi et al., 2011; Abd-Elmaksoud et al., 2014) as well as in the monkey, buffalo (Cruzana et al., 2003) and humans (Li et al., 2010; West and Watson, 2010). S-100 protein has been shown to regulate cell division and cell morphology in a calcium-dependent manner (Donato, 2001; Abd-Elmaksoud et al., 2014) Also, S-100 protein in the Sertoli cells is assumed to be involved in the microtubule assembly-disassembly as well as in the secretory, protective and absorptive functions of the Sertoli cells of mammals (Cruzana et al., 2003).

The S-100 positive reaction with spermatogenic and Leydig cells of the African four-toed hedgehog suggests that the protein is involved in the processes of spermatogenesis as well as steroidogenesis. S-100 positive reaction has been reported for the Leydig cells of turtle and mammals (Young et al., 2006; Olukole et al., 2018). However, the testicular germ cells as well as the Leydig cells of the rabbit have been shown to be immunonegative to S-100 (Abd-Elmaksoud et al., 2014).

The strong immunoreactivity to S-100 exhibited by the testicular vasculature in this study tallies with other studies that have shown that the endothelial cells of capillaries, veins, and lymphatic vessels are regularly S-100 protein immunoreactive in rat, rabbit, boar, ruminants, European bison and man (Michetti et al., 1985; Amselgruber et al., 1994; Czykier, 1999; Abd-Elmaksoud et al., 2014). Testicular S-100 protein has been reported serve in the prevention of cytoplasmic calcium overload which has been demonstrated to cause cellular apoptosis as well as the induction of mitochondria calcium overload thereby leading to testicular mitochondrial malfunctioning (Orrenius et al., 2003).

CONCLUSION

In conclusion, α SMA and S-100 proteins have active roles in the cytoskeletal structure of testis and physiology of the African four-toed hedgehogs while S-100 protein plays additional and complementary roles in the structural formation and maintenance of the blood-testis barrier as well as in steroidogenesis and spermatogenesis in the animal.

Table 1. Distribution of α SMA and S-100 proteins in the testis of the African four-toed hedgehogs.

ATB	SPC	SC	LC	PMC	TC	VE	ST	RT
α SMA	-	-	-	+++	+++	+++	++	++
S-100	++	++	++	+++	+++	++	++	++

Antibody (ATB); spermatogenic cells (SPC); Sertoli cell (SC); Leydig cell (LC); peritubular myoid cell (PMC); testicular capsule (TC); vascular endothelium (VE); straight tubule (ST); rete testis (RT). Negative reaction (-); moderately positive reaction (++); strongly positive reaction (+++).

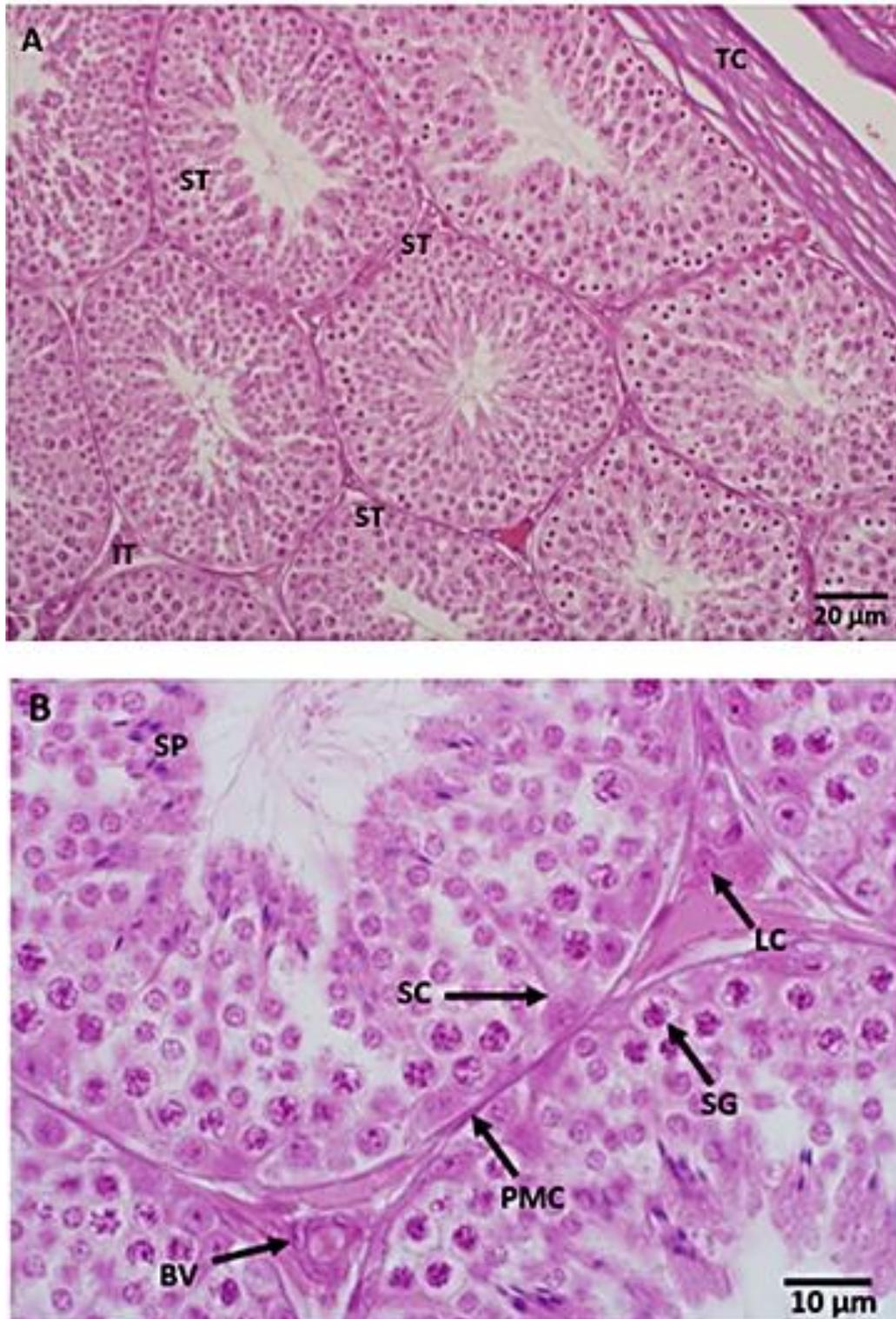


Figure 1. Photomicrographs of the testis of the African four-toed hedgehog (H&E). **A:** testicular capsule (TC), seminiferous tubule (ST) and interstitial tissue (IT). **B:** spermatocyte (SP), Sertoli cell (SC), blood vessel (BV), peritubular myoid cell (PMC), spermatogonium (SG), Leydig cell (LC).

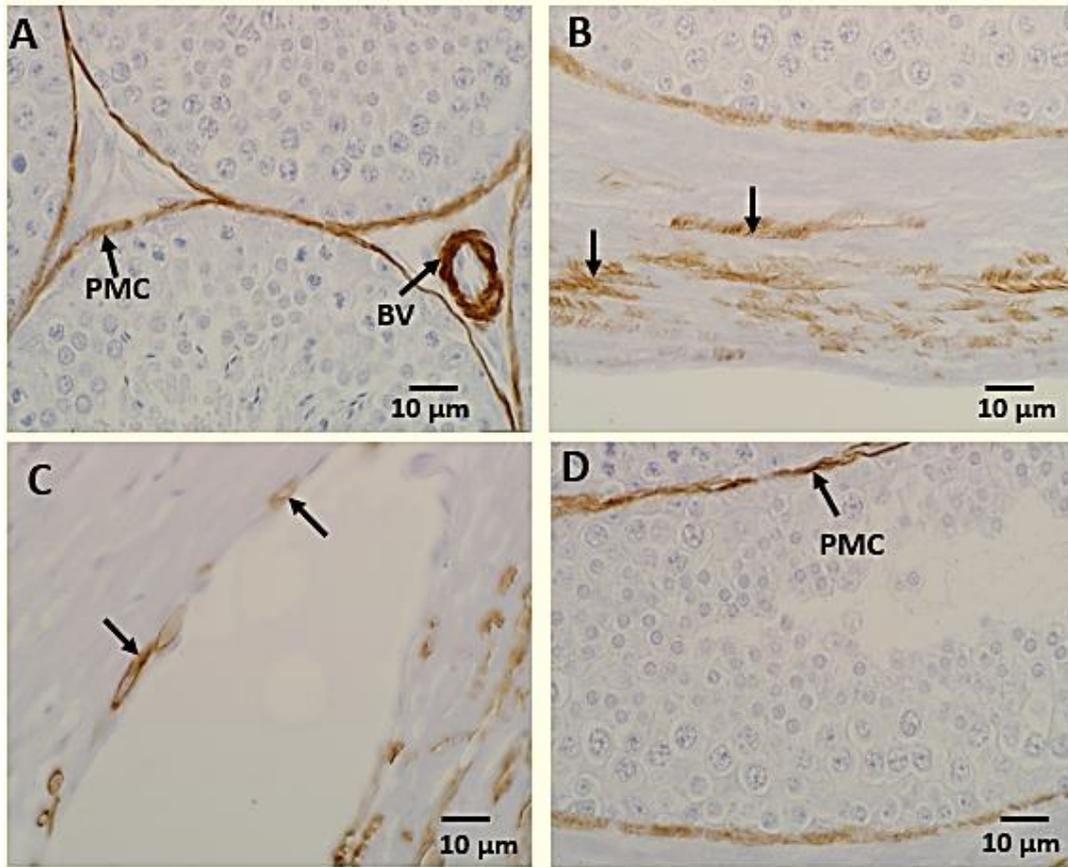


Figure 2. **A:** Localization of alpha smooth muscle actin (α SMA) in the testis of the African four-toed hedgehog. Blood vessel (BV) and peritubular myoid cell (PMC). **B:** Positive reactions (arrows) in testicular capsule. **C:** Positive reactions (arrows) in the rete testis. **D:** Positive reactions (arrows) in the peritubular tissue of the straight tubules (α SMA).

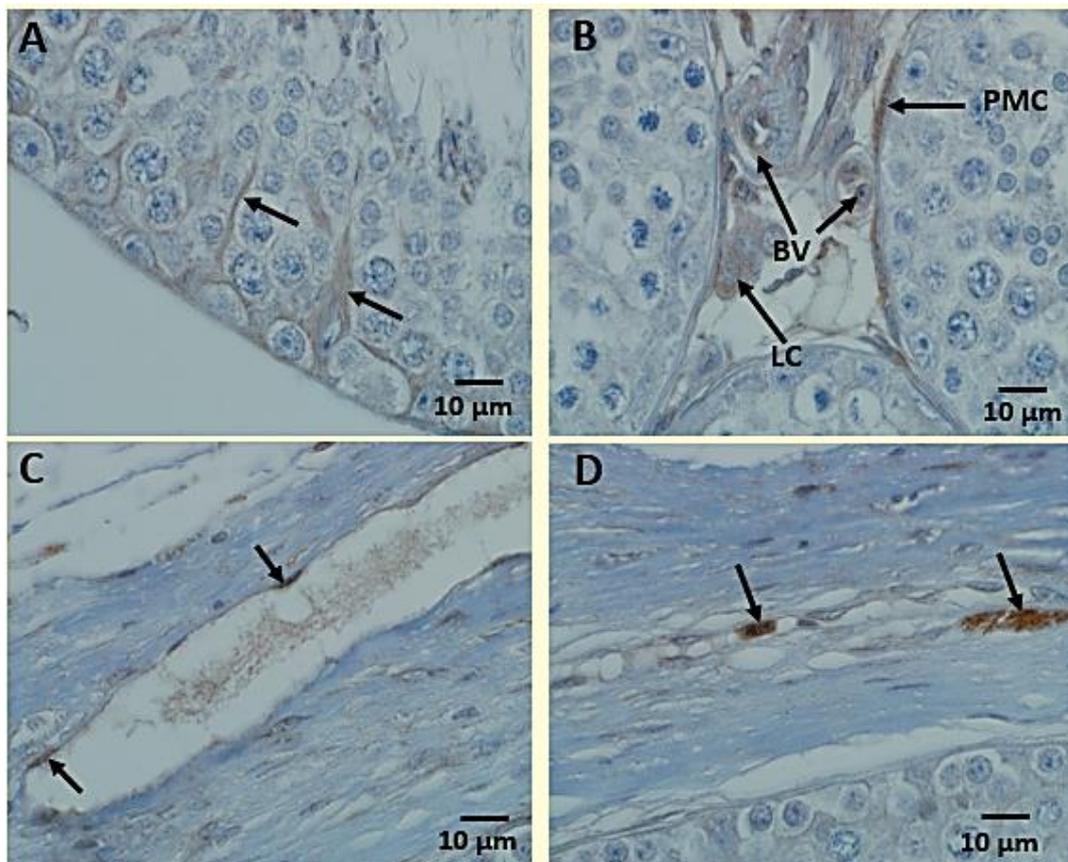


Figure 3. Localization of S-100 protein in the testis of the African four-toed hedgehog. **A:** Positive reaction with the Sertoli cells (arrows). **B:** Positive reactions with the blood vessels (BV), peritubular myoid cell (PMC) and Leydig cell (LC). **C:** Positive reactions (arrows) in the rete testis. **D:** Positive reactions (arrows) in testicular capsule.

DECLARATIONS

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Competing interests

The authors declare that they have no competing interests.

Authors' contributions

Samuel Gbadebo Olukole conceived the study and participated in its design and coordination and drafted the manuscript. Oluwakayode Michael Coker participated in its design and coordination and helped to draft the manuscript. Bankole Olsiji Oke participated in its design and coordination. All authors read and approved the final manuscript.

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The Labial and Zygomatic Salivary Glands in Mixed Breed Dogs in Trinidad: Anatomical Location, Histological Features and Histochemical Characteristics

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ABSTRACT

The objective of this investigation was to give detailed descriptions of the anatomical location, histological features and histochemical characteristics of the labial and zygomatic salivary glands in mixed breed dogs. This study was performed on five heads of adult mixed breed dogs of both sexes. The heads were dissected to detect in situ position of the labial and zygomatic salivary glands. The glands were dissected and examined grossly. Samples of the glands were taken, processed and stained using hematoxylin and eosin and Masson's Trichrome for histological examination as well as Periodic Acid-Schiff, Alcian Blue (pH 2.5 and 1.0) and a combination of Periodic Acid-Schiff and Alcian Blue (pH 2.5 and 1.0) techniques for histochemical examination. The labial and zygomatic salivary glands were located in the lower lip and in the orbit respectively and they were surrounded by fibrous capsules containing collagen fibers. They were minor, compound, mixed tubuloalveolar glands. They composed of mucous acini, mucous acini with serous demilunes and isolated serous acini. The secretion of the glands (chiefly mucous) consisted of neutral mucins, acid carboxylated mucins and acid sulphated mucins. The duct system of the glands was intralobular (intercalated and striated ducts) and interlobular ducts. The anatomical location as well histological and histochemical structures of the labial and zygomatic salivary glands were important to classify the glands and their secretion as well as to give veterinarians knowledge during clinical examination of the oral and orbital regions, and to recognize normal and pathological conditions.

Keywords: Anatomy, Dog, Labial, Salivary glands, Zygomatic

INTRODUCTION

The salivary glands are the accessory structures of the gastrointestinal tract and their exocrine secretion (saliva) prepare ingesta for digestion as it contains mucopolysaccharides, water, enzymes and lubricating glycoprotein (Adnyane et al., 2010; Al-Abbad, 2011). They are developed at various locations with different structures and secretions which are either serous, mucous or seromucous (Jaskoll et al., 2002). The major salivary glands include parotid, mandibular and sublingual, while the minor salivary glands include the lingual, buccal and labial salivary glands, which are found in the lingual, buccal and labial mucosa respectively (Kimura et al., 1998; Popovici et al., 2003; Barone, 2009; Miclăuş, 2012). The diversity of structures and secretions of the salivary glands depends on the animal's feeding habits (Tandler and Phillips, 1998). Zygomatic and molar glands are major salivary glands which are present in carnivores and cat respectively (Dyce et al., 2004; Mohammadpour, 2010). However, in other animals the zygomatic glands are classified either as minor (Dellman and Eurell, 1998) or major (Evans and Christensen, 1979). Tumors of salivary glands have been reported in the horse, cattle, sheep, goat, dog and cat (Head, 1976; Carberry et al., 1988). Pleomorphic labial gland adenoma was recorded in a dog (Izawa et al., 2017). Zygomatic gland mucocele associated with exophthalmos is less common in dogs (McGill et al., 2009 and Cannon et al., 2011); however, it was reported in a cat and ferret (Miller and Pickett, 1989; Speakman et al., 1997). Zygomatic salivary glands tumors in the orbit of the dog were recorded previously (Buyukmihiç et al., 1975; Attali-Soussay et al., 2001). There is no available study documenting the histomorphological and histochemical features of the labial and zygomatic salivary glands in mixed breed dogs. It is important for veterinarians to recognize normal and pathological conditions during examination and investigation of the oral and orbital cavities of dogs. Moreover, the labial gland is used in autologous transplant to stimulate the lubrication of the ocular surface and the zygomatic salivary glands problems can cause exophthalmos.

So the present study aimed to indicate the anatomical location, histological aspect and histochemical features of the labial and zygomatic salivary glands of mixed breed dogs.

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MATERIALS AND METHODS

A total of five apparently healthy adult mixed breed dogs (3 males and 2 females) were used for the current study. Fresh euthanized dogs with pentobarbital (1ml/4.5 kg, IV) were obtained from the Trinidad and Tobago Society for the Prevention of Cruelty to Animals (TTSPCA) and transferred to the anatomy laboratory, School of Veterinary Medicine, The University of the West Indies, Trinidad and Tobago. The head of each dog was dissected by removing the bony structures such as the zygomatic arch and mandible, followed by the removal of the masseter, temporalis and pterygoid muscles. The labial and zygomatic glands were dissected and excised from the adhering tissues. The position of the glands and their related structures were described and photographed using a Sony 12 megapixel digital camera (Sony Coporation, Japan). Tissue specimens were taken from the labial and zygomatic glands and fixed in 10% neutral buffered formalin for 24-48 hours, then dehydrated in ascending grades of ethanol, cleared in xylene and then embedded in paraffin blocks. Sections of 5µm thickness were prepared using a microtome and stained by hematoxylin eosin for general histological examination and Masson's trichrome stain for detecting collagen fibers (Culling et al., 1985). Periodic Acid-Schiff (PAS), Alcian Blue (AB pH 2.5 and 1.0) and AB-PAS (pH 2.5 and 1.0) techniques were used to detect and differentiate the types of the secretion of the labial and zygomatic salivary glands (Culling et al, 1985; Cunha et al., 2016). The stained sections were observed under a light microscope (Olympus BX40 with an Olympus DP 15 megapixel digital camera, Japan) at different magnifications and photomicrographs were taken.

Ethical approval

The ethical approval from the University of the West Indies, St. Augustine, Trinidad and Tobago ethical committee (CEC906/02/19) was obtained.

RESULTS

Anatomical location

The labial salivary gland of the mixed breed dogs was a lobulated gland located in the lower lip. It extended from the lower second premolar tooth to the distal edge of the lower molar tooth. The gland was located within the connective tissue, deeply embedded in the mucosal lining of the vestibular cavity (Figure 1). The zygomatic salivary gland was present as a pyramidal, lobulated organ in each side of the head. It was located ventrolaterally in the orbit, under the rostral end of the zygomatic arch and dorsolaterally to the pterygoideus muscles. It was covered by adipose tissue and encapsulated. The medial surface of the gland was slightly grooved by the maxillary vessels and nerve (Figure 1).

Histological features

The zygomatic and labial salivary glands of the mixed breed dogs were enclosed in a well-developed capsule containing collagen fibers. Interlobular connective tissue septae were originated from the tunica albuginea dividing the gland into various sized lobules (Figure 2). The labial and zygomatic salivary glands were compound, exocrine, tubuloacinar mixed glands containing mucous acini, mucous acini with serous demilunes (mixed seromucous units) and sporadic serous acini. The mucous secretory units, which secrete only mucous, were predominant. The mucous cells had basally located, flattened nuclei with foamy cytoplasm. The serous demilunes with cells had rounded central nuclei and were capped as a half-moon on the mucous cells. The sporadic serous acini showed rounded nuclei with dark cytoplasm. Myoepithelial cells and were present as spindle-shaped cells incorporated between the basement membrane and epithelium of the secretory cells. The ducts of the labial and zygomatic salivary glands were intralobular and interlobular. The intralobular ducts consisted of intercalated and striated ducts which were lined by cuboidal epithelium and tall columnar cells with basal striation respectively. The interlobular ducts were the main excretory ducts in the labial and zygomatic salivary glands. They were lined by stratified columnar epithelium and they were situated in the connective tissue septum between the gland's lobules (Figure 2).

Histochemical characteristics

The histochemical evaluation of the labial and zygomatic salivary glands showed that the mucous secretory units and serous demilunes in the seromucous acini and sporadic serous clusters as well as the striated ducts were positive to the PAS stain and looked magenta red due to the presence of the neutral mucins (Table 1 and figures 3 and 4). The mucous secreting cells were positive to AB (pH 2.5) due to the presence of the acid mucins with strongly staining of acid carboxylated mucins (Figures 3 and 4) and to the AB (pH1.0) due to the presence of the acid sulphated mucins and looked blue (Figures 3 and 4). The combination of AB-PAS showed that the mucous cells stained reddish purple at pH 2.5 (Figures 3, 4 and 5 and bluish purple at pH 1.0 (Figures 3, 4 and 5). On the other hand, the reaction of the serous demilunes (Figures 3 and 5) were negative to AB (pH 2.5 and 1.0).

Table 1. Histochemical features of the mucous secretory units and serous demilunes of the labial and zygomatic salivary glands in the mixed breed dogs in Trinidad

Technique / Gland	Labial salivary gland		Zygomatic salivary gland	
	Mucous secreting cells	Serous demilunes	Mucous secreting cells	Serous demilunes
PAS	+	+	+	+
Alcian Blue (pH 2.5)	+	-	+	-
Alcian Blue (pH 1.0)	+	-	+	-
AB-PAS (pH 2.5)	+	-	+	-
AB-PAS (1.0)	+	-	+	-

PAS: Periodic Acid-Schiff; AB: Alcian Blue; +: positive staining; -: negative staining

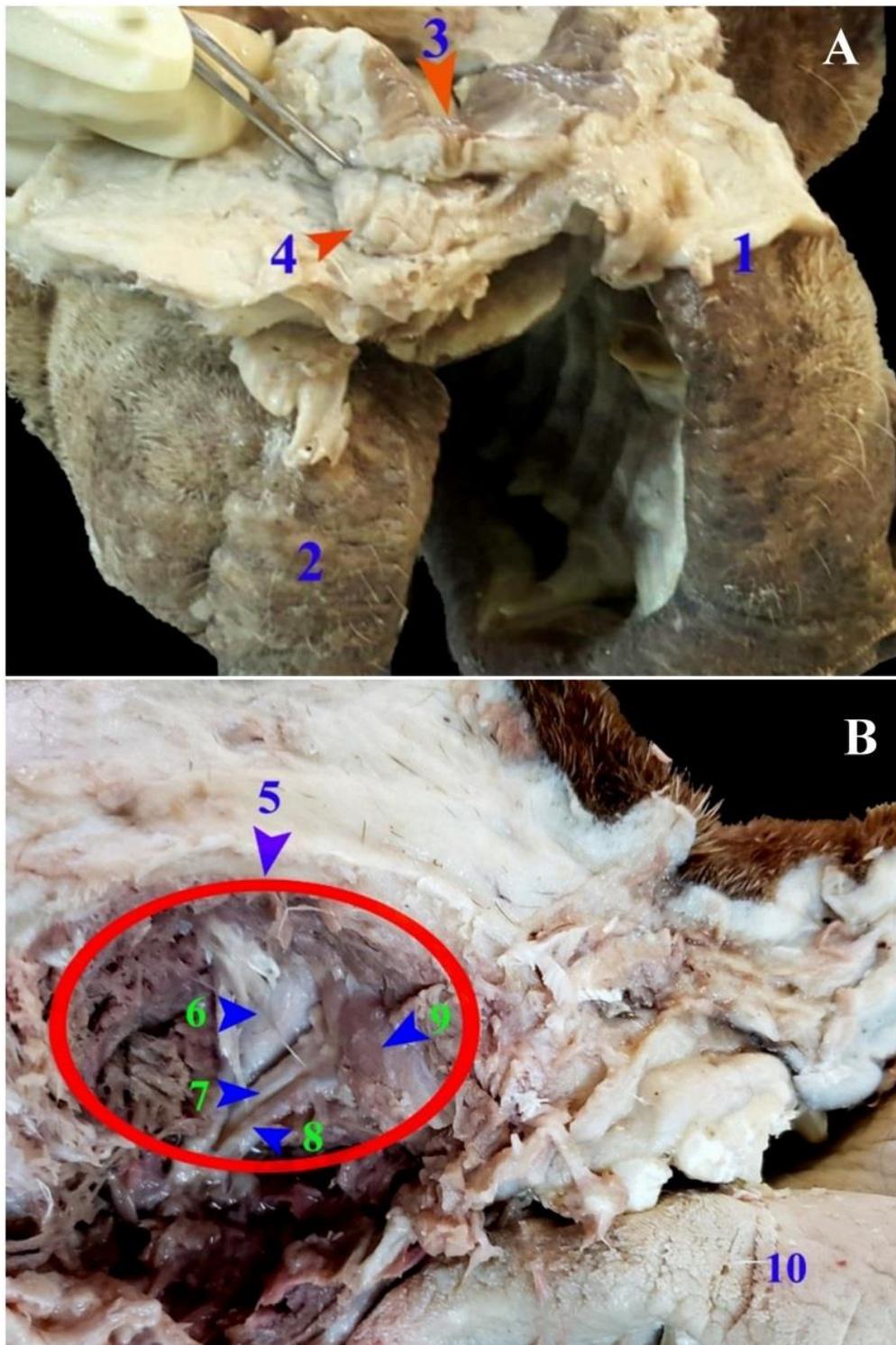


Figure 1. Photographs showing the location of the labial (A) and zygomatic (B) salivary glands in the mixed breed dog. 1: Upper lip; 2: Lower lip; 3: Labial commissure; 4: Labial salivary gland; 5: Orbital cavity; 6: Eye ball; 7: Maxillary nerve; 8: Maxillary artery; 9: Zygomatic salivary gland; 10: Tongue.

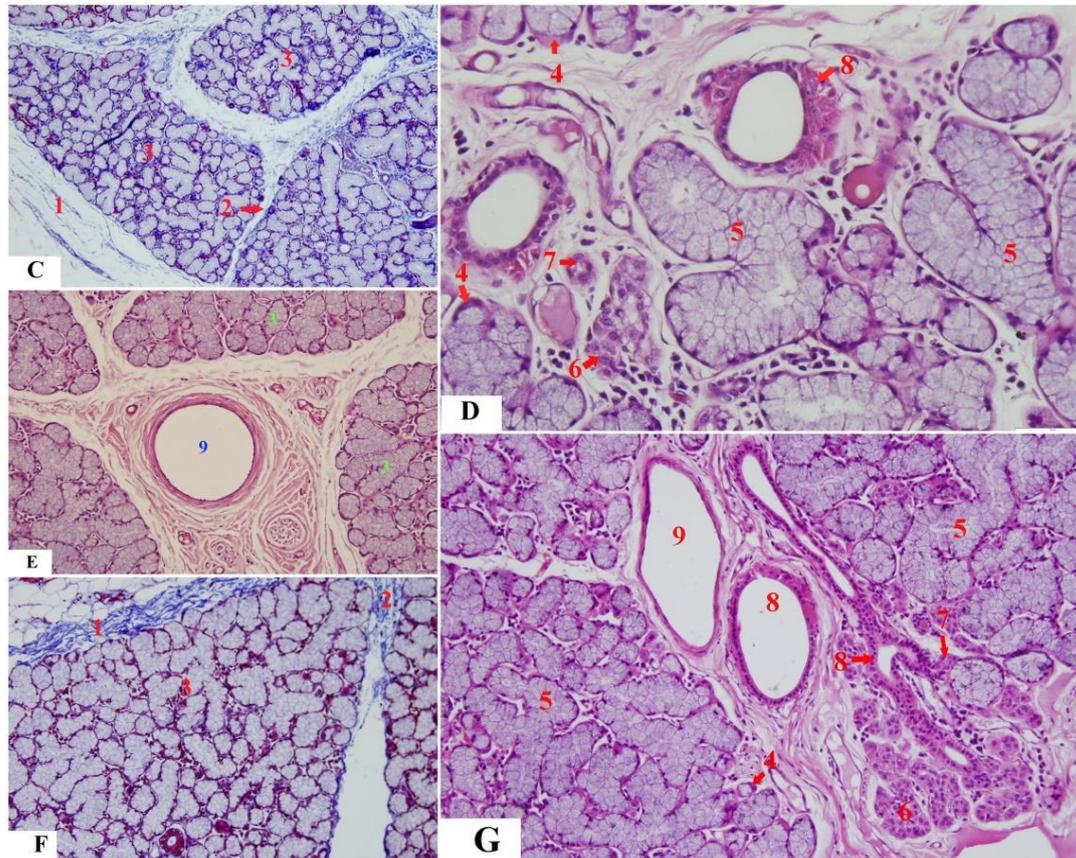


Figure 2. Photomicrographs showing the general histology of the labial (C, E and D) and zygomatic (F and G) salivary glands of the mixed breed dog stained with H & E (DX40; E x 20 and GX20) and Masson's Trichrome stain (CX10 and FX20). 1: Connective tissue capsule containing collagen fibers; 2: Interlobular septum; 3: Gland lobules; 4: Serous demilunes; 5: Mucous secretory units; 6: Serous acini clusters; 7: Intercalated duct; 8: Striated duct; 9: Interlobular duct.

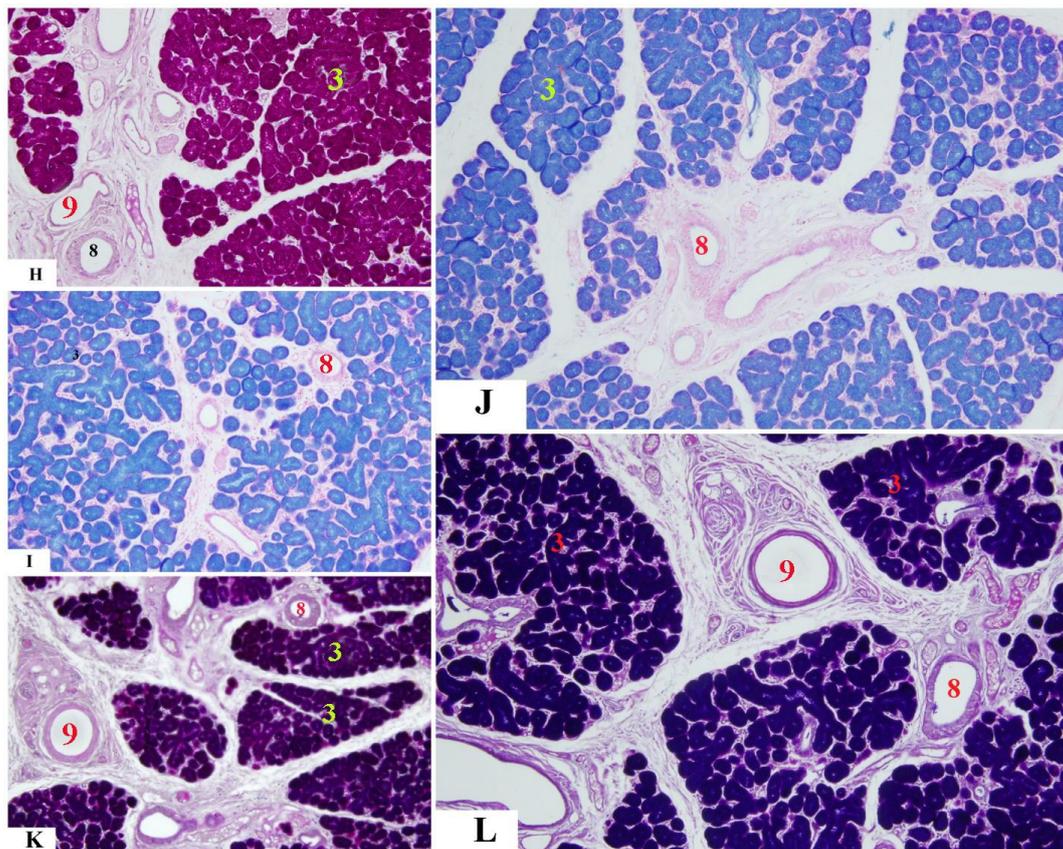


Figure 3. Photomicrographs showing the histochemical characteristics of the mucous secreting cells of the labial salivary gland of the mixed breed dog stained with PAS (HX10), AB (pH 2.5, I X10 and pH 1.0, JX10) and AB-PAS (pH 2.5, KX10 and pH 1.0, LX10). 3: Gland lobules; 7: Intercalated duct; 8: Striated duct; 9: Interlobular duct.

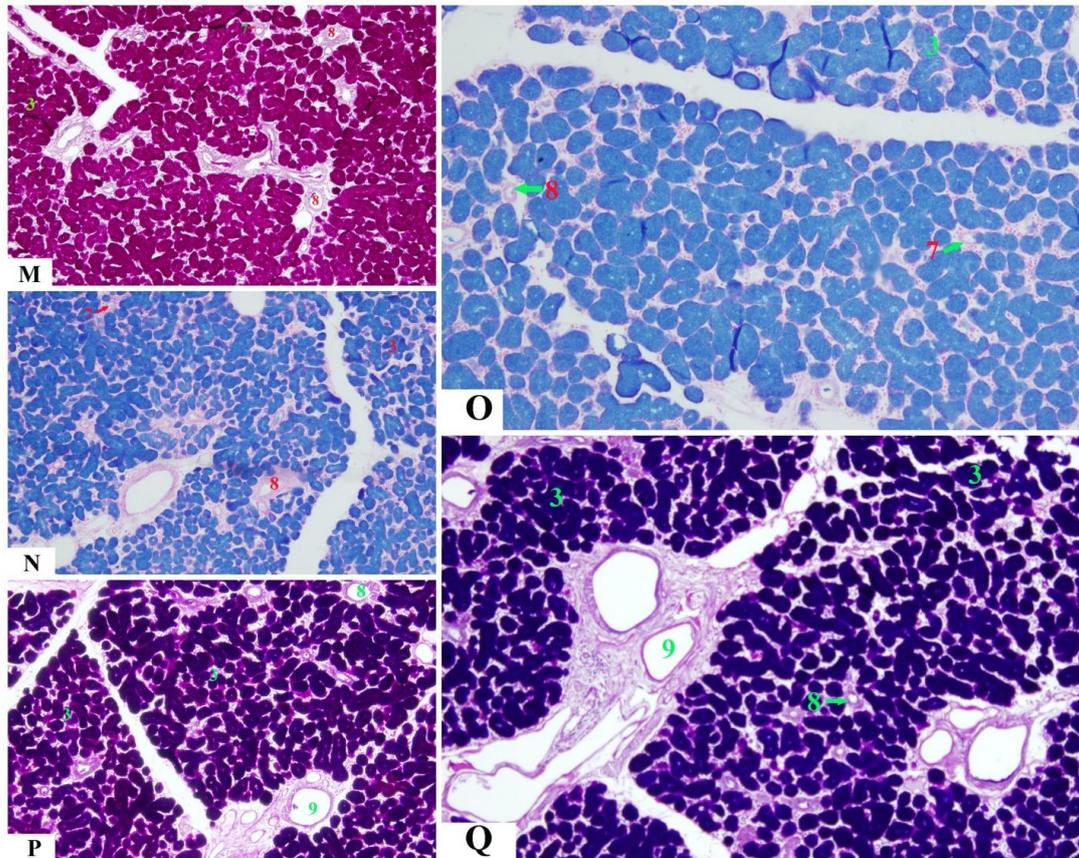


Figure 4. Photomicrographs showing the histochemical characteristics of the mucous secreting cells of the zygomatic salivary gland of the mixed breed dog stained with PAS (MX10), AB (pH 2.5, N X10 and pH 1.0, OX10) and AB-PAS (pH 2.5, PX10 and pH 1.0, QX10). 3: Gland lobules; 8: Striated duct; 9: Interlobular duct.

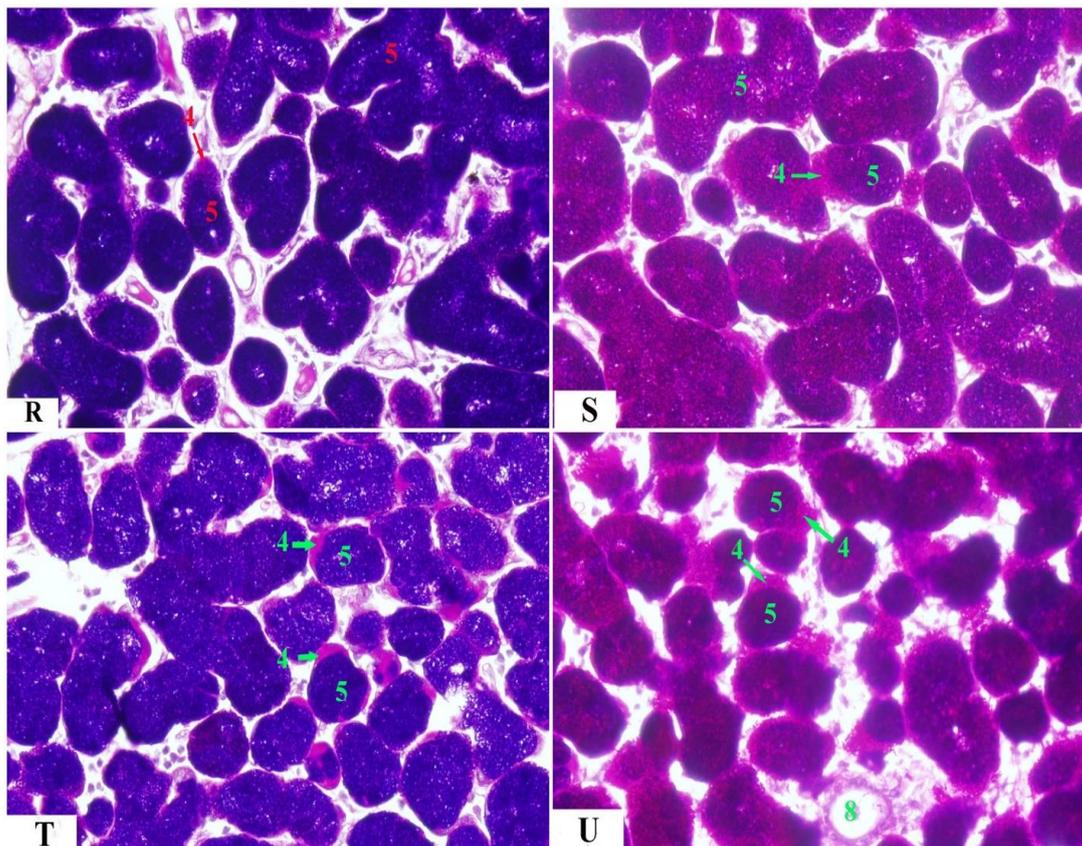


Figure 5. Photomicrographs showing the histochemical characteristics of the mucous secreting cells and serous demilunes of the labial salivary gland (R and S) and zygomatic salivary gland (T and U) of the mixed breed dog stained with AB-PAS (pH 1.0, R and TX40 and pH 2.5, S and UX40). 4: Serous demilunes; 5: Mucous secretory units; 8: Striated duct.

DISCUSSION

There was not much available literature reporting on the labial and zygomatic salivary glands of mixed breed dog in Trinidad. However, the zygomatic salivary gland of the Philippine non-descript dog and the labial salivary gland of the dog (Maala et al., 2008; Cunha et al., 2016) respectively were described.

The present results as well as that in dog (Angélico et al., 2011; Rocha, 2012 and Cunha et al., 2016) stated that the labial salivary gland was easily palpable in the lower lip between the lower second premolar and the second molar teeth. Moreover, the zygomatic salivary gland of the mixed breed dog was a pyramidal shaped gland located inside the orbit. Similar result was observed in mesaticephalic dog (Diesem, 1975). However, Maala et al. (2008) reported the same location but the gland was irregular in shape in the Philippine non-descript dog.

The labial and zygomatic salivary glands were surrounded by a connective tissue capsule with collagen fibers; a similar result is noticed in the labial salivary gland of the dog (Rocha, 2012; Cunha et al., 2016), zygomatic salivary gland of the dog (Maala et al., 2008), labial gland of the buffalo (Jabbar, 2010) and molar salivary gland in domestic cat (Mohammadpour, 2010). On the other hand, the minor salivary glands are lack of connective tissue capsule (Bacha and Bacha, 2003). Moreover, the labial and zygomatic salivary glands were divided into lobules by the connective tissue which surrounded and intermingled the gland as mentioned in the rat (Hand et al., 1999), labial salivary gland of the dog (Cunha et al., 2016), zygomatic salivary gland of the dog (Maala et al., 2008), labial salivary gland of the buffalo (Jabbar, 2010) and molar salivary gland in domestic cat (Mohammadpour, 2010).

Similar to the observations in the labial salivary gland in the dog (Rocha, 2012; Cunha et al., 2016), zygomatic salivary gland in the dog (Dellmann and Eurell, 1998; Maala et al., 2008; Gomi et al., 2017), zygomatic salivary gland in the Leopard and cat (Sadeghinezhad et al., 2016), molar salivary gland in the domestic cat (Mohammadpour, 2010) and Weber's salivary gland of the rabbit (Haddao and Yasear, 2018), the secretion of the labial and zygomatic salivary glands of the mixed breed dog was mixed and primarily mucous. However, the secretion is classified as mucous in the dog (Banks, 1991). However, the labial salivary gland is serous glands in the buffalo (Jabbar, 2010) and in the Giant rat (Asojo and Aire, 1983). On the other hand, the labial gland has mucoserous acini capped by serous demilunes in the camel (Taib and Jarrar, 1987). On the other hand, the secretion of the zygomatic gland of the Japanese serow is chiefly serous (Tsuchimoto et al., 1984).

The current study as well as Cunha et al. (2016) and Gomi et al. (2017) in the dog and Jabbar (2010) in the buffalo stated that the secretions of the labial and zygomatic salivary glands pass via the intralobular (intercalated and striated) and interlobular ducts. However, Frappier (2006) mentioned that the minor salivary glands are lacking in striated ducts. However, the intercalated duct was absent in the zygomatic salivary gland, while the intralobular and interlobular ducts were present; the former ones appeared as oval ducts, lined by cuboidal epithelium, without basal striations in dog (Dellmann and Eurell, 1998; Maala et al., 2008) and in the labial gland of the camel (Taib and Jarrar, 1987). On the other hand, there are no intercalated or striated ducts in molar salivary gland in domestic cat (Mohammadpour, 2010). The striated duct is absent in the Weber's salivary gland of the rabbit (Haddao and Yasear, 2018).

In the samples of the current study of the labial and zygomatic salivary glands of the mixed breed dog showed that the mucous secretory units and serous demilunes secrete neutral mucins as they were positive to PAS stain. All acid mucous of the mucous secretory units was positive to AB (pH 2.5) with strongly staining of acid mucins, while acid, sulphated mucins were stained by AB (pH 1.0). Similar results were observed in the labial salivary gland in the dog (Giudice et al., 2005; Cunha et al., 2016), zygomatic salivary gland in the dog (Sozmen et al., 1999; Maala et al., 2008) and labial salivary gland of the buffalo (Jabbar, 2010) and camel (Taib and Jarrar, 1987). However, the mucous acini of the weber's salivary gland of the rabbit were negative to PAS and AB (1.0) while the mucous acini and serous cells were positive to PAS (Haddao and Yasear, 2018).

The current study showed that the combination of PAS-AB stain showed that the mucous cells of the labial and zygomatic salivary glands stained bluish purple at pH 1.0 and reddish purple at pH 2.5 which indicated that mucous cells secrete neutral mucins more than acidic mucins. Moreover, the mucous cells secrete large amounts of acidic sulphated mucins; similar results were reported in the zygomatic salivary of the dog (Sozmen et al., 1999). However, Maala et al. (2008) stated that the zygomatic salivary gland secretes small amount of acidic sulphated mucins. On the other hand, the mixed mucins of the mucous cells of the zygomatic salivary glands are more acidic in leopard and cat (Sadeghinezhad et al., 2016). However, the weber's salivary gland secretes acidic mucins from the mucous cells and neutral mucins from the serous cells (Haddao and Yasear, 2018).

The mucin of the labial salivary glands was characterized by its high viscosity, low solubility and stickiness which made them able to lubricate the mucosa of the food passage and prevent its drying. Furthermore, it facilitates the passage of the food from the mouth to the esophagus by coating food boluses and keeping the integrity while protecting the mucosa of the pharynx and esophagus from mechanical injury. Similar findings were mentioned by Samar et al. (1995), Maala et al. (2008), Munyala et al. (2009) and Cunha et al. (2016).

CONCLUSION

The labial and zygomatic salivary glands of mixed breed dogs were tubuloacinar with mixed seromucous secretions. The duct system consists of intercalated, striated and interlobular ducts in the labial salivary and zygomatic salivary glands. The secretions of the glands were composed of neutral, acid carboxylated and acid sulphated mucins.

DECLARATIONS

Competing interests

The author declared that there is no conflict of interests.

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Pharmacokinetic Characteristics of the Drug Based on Moxidectin for Young Stock and Small Breed of Domestic Animals

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ABSTRACT

The pharmacokinetic characteristics of moxidectin in the blood serum of dogs and cats after a single cutaneous (spot-on) application of drug for veterinary use “Inspector Mini” to prevent and treat arachnoses, entomoses and intestinal nematodes in kittens and puppies as well as in small breed dogs and cats were investigated. Twelve outbred dogs and cats of different ages and weights were involved in present study. All the animals were weighed to determine the exact dosage of the drug. The determination of moxidectin in blood serum was carried out by high performance liquid chromatography with pre-column modification of N-methylimidazole and trifluoroacetic anhydride followed by fluorescence detection. According to the results of the study, it was found that moxidectin was well absorbed into the systemic circulation and reached to maximum concentration in the blood serum of dogs and cats after 4-10 days. After treatment with the drug, moxidectin was determined in the blood serum of animals after 12 hours at concentration of 2 ng/ml. Significant concentrations of moxidectin in the blood serum of animals remained for 28 days after topical application (spot-on). Moxidectin was detected in the blood serum of animals at the end of the experiment (after 30 days) which indicates its therapeutic effect for at least one month after the application of the drug.

Key words: Blood Serum, Cats, Dogs, Moxidectin, Pharmacokinetics

INTRODUCTION

The main methods against parasites of carnivores are preventive treatment of animals for which more and more new drug are constantly being developed (Arisov et al., 2018; Arisova et al., 2019). However, it should be noted that most of these drugs have limitations for especial ages and weights. In this regard, small breed dogs and cats and as well puppies and kittens with a body weight of less than 1 kg remain unprotected from parasites. Taking into account the urgency of this problem, the drug names “Inspector Mini” was developed for veterinary use to treat small breed dogs, cats, puppies and kittens against ectoparasites, nematodes and dirofilariasis as well. The drug combination contains 5 mg/ml of moxidectin as an active substance. Moxidectin is a semisynthetic compound of the milbemycin group (macrocyclic lactones) has a profound systemic and contact effect against the larvae and adults of ectoparasites and nematodes, has a stimulating effect on gamma-aminobutyric acid release, increases the permeability of membranes for chlorine ions which inhibits the activity of parasitic nervous cells causing a disorder of muscle innervation, paralysis and death of ectoparasites and nematodes. Pharmacokinetics and metabolism of moxidectin was studied in rats, sheep and cattle. According to the data of scientific research, moxidectin has a higher intrinsic potency against some parasites, especially filarial nematodes, than the avermectins. It has also high distribution in lipid tissues, poor metabolism and a long half-life. So that effective concentrations of moxidectin persist for longer time in target hosts, which makes it possible to have a low resistance to parasites. Moreover, moxidectin has a high safety index (Prichard and Geary, 2019). The compound Moxidectin is mostly released intactly from the body. The therapeutic efficacy of macrocyclic lactones in arachnoses and entomoses is high (Nolan and Lok, 2012; Balandina, 2017; Bepalova et al., 2018). The drug is used for animals by the drip (spot-on) application on a dry intact skin in places out of reach of licking (between shoulder blades at the base of the neck at the following doses (Table 1).

The drug is used in a dose of 0.2 ml per 1 kg of animal weight during treatment. Safety of the drug for use in animals at recommended dosage regimen was confirmed during studying its tolerability. According to the extent of exposure, “Inspector Mini” belongs to low-hazard substances (class 4 hazard according to GOST 12.1.007-76), so it does not have a local irritation, resorptive-toxic and sensitizing effect at the recommended doses (Simaeva et al., 2017).

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Table 1. Dose of Inspector Mini according to body weight of dogs and cats

Animal species	Animal mass (kg)	Dose of the drug (ml)	AI concentration(mg/kg)
Dogs, puppies	0.5 – 2.0	0.4	1 – 4
Cats, kittens	0.5 – 2.0	0.4	1 – 4

MATERIALS AND METHODS

Six outbred dogs weighted 2.6-4.5 kg and aged 8-14 months along with six outbred cats weighted 1.0-1.9 kg and aged 6-9 months were used in the experiment. These animals were kept in nurseries with no history of chemotherapy and health problems before the investigation (30 days). For accurate dosing of the drug all animals were weighed. The drug was applied to the animals once by the spot-on method at the rate of 1 mg moxidectin per 1 kg of body weight of dog or cat. Blood sampling was performed prior to the study (0 h) and 12, 24, 72, 120, 168, 240, 360, 480, 600 and 720 hours after application of the drug. The blood samples were collected in polymer tubes without an anticoagulant and blood serums were separated and frozen up to the beginning of the study. Moxidectin in the blood serums of animals was determined by HPLC on Shimadzu Prominence LC20 chromatograph with RF-20Axs fluorimetric detector. Quantitative determination was carried out with the external standard method. The procedure of calibrating chromatographic data was used to carry out a qualitative and quantitative analysis of the obtained blood serum extracts (liquid extraction with hexane). The calibration procedure has two goals: determination of the retention time of the analyzed component for its following identification (qualitative analysis of samples) and determination of the analyte concentration with a calibration curve (external standard method). The example of the chromatogram of blood serum extract of moxidectin standard solution obtained by HPLC is presented in figure 1.

The calibrating procedure on moxidectin in the eluent solution was repeated twice in different days. For calibration procedure, solutions with concentrations of 100, 50, 10, 5, 1 ng/ml were used. For calibration procedure on serum samples, solutions of moxidectin with concentrations of 1, 5, 10, 50 and 100 ng/ml were used. The average extraction rate of moxidectin from blood serum was 79%. Metrological approval of test procedure on the content of moxidectin in the blood serum was carried out in accordance with Hartmann et al. (1998), Rockville (2000), Chiap et al. (2003), Epstein (2004), Senyuva and Gilbert (2011) and Prichard and Barwick (2012). Several blood serum solutions (samples for calibration) with concentrations of 1, 5, 10, 50, and 100 ng/ml were used for the experiment. The obtained data are presented in tables 2 and 3.

Thus, the proposed method was linear in the range of 1-100 ng/ml ($R > 0.99$) and showed good precision and accuracy. The method makes it possible to identify moxidectin in the model and blood serum samples. The pharmacokinetic parameters were calculated with PK Solver program (Zhang et al., 2010).

Ethical approval

The most humane and operational methods and manipulations were used in the study; immediate measures to prevent pain and distress in dogs and cats. All painful manipulations with the bird were carried out in accordance with regulatory standards: European Convention for the Protection of Vertebrate Animals Used for Experimental and other Scientific Purposes (ETS No. 123; Strasbourg, 18 March 1986), Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes (Strasbourg, 22 September, 2010).

Table 2. Linearity and parameters of the test method for blood serum

Limit of detection (LOD), (ng/m)	Limit of quantification (LOQ), (ng/m)	Calibration curve	Linearity range, (ng/ml)
0.29	0.97	$y = 11165 * R > 0.999$	1 – 100

LOQ: limit of quantification, LOD: limit of detection.

Table 3. Parameters of precision and accuracy of the method

Matrix	Measuring range, (ng/ml)	RSD (%)	Δ (%)
Blood serum	1.0 – 5.0	7.7	37.4
	5.0 – 100.0	6.9	12.4

RSD: Relative Standard Deviation, Δ : accuracy factor, LOD: Limit of detection

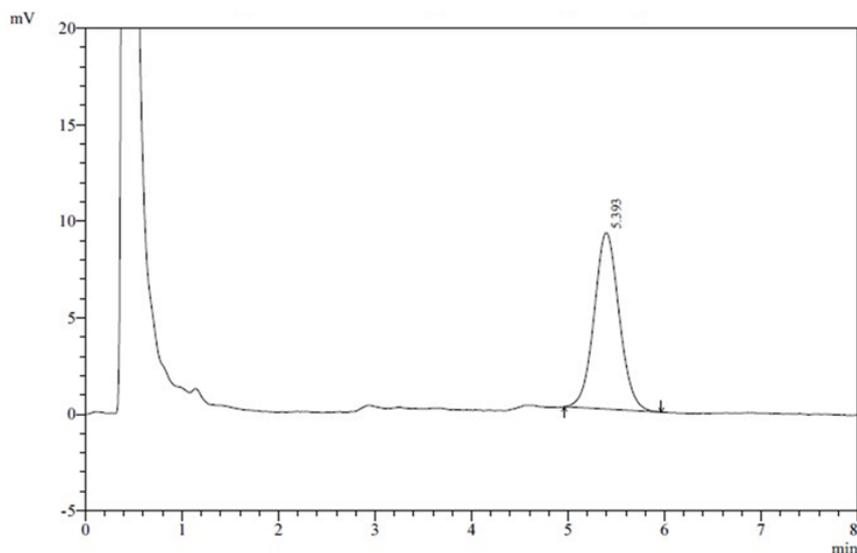


Figure 1. Chromatogram of the blood serum extract of moxidectin standard solution (10 ng/ml). Derivative of moxidectin in 5.4 minute is presented in figure

RESULTS

As a result of studying the pharmacokinetic characteristics of the drug after spot-on application to dogs and cats, moxidectin was determined in the blood serums of animals after 12 hours at a concentration about 2 ng/ml. The maximum concentration of moxidectin in the blood serum of dogs reached 3.8 ng/ml on the third day of the study and remained at roughly the same level (3.6 ng/ml) up to the seventh day. Then there was a gradual decrease in the concentration of the active substance to 1.3-1.7 ng/ml in the blood serums by 30 days after a single dose of the drug “Inspector Mini” which is used in veterinary (Figure 2).

The studies of the pharmacokinetics of moxidectin in cats showed that the maximum concentration of active ingredient also reached 5.3 ng/ml on the third day of the study and remained at the level of 5.3-5.1 ng/ml up to 7 days after treatment. Then the moxidectin concentration gradually decreased to 1.5-3.5 ng/ml by 30 days of the study (Figure 3). The calculation of pharmacokinetic parameters of moxidectin in the blood of dogs and cats are presented in table 4. The obtained data showed that moxidectin is absorbed into the blood in both species used in present study. The active ingredient was found in the blood serum of dogs and cats at the end of the experiment (30th day) which indicated its therapeutic effect for at least 1 month after application.

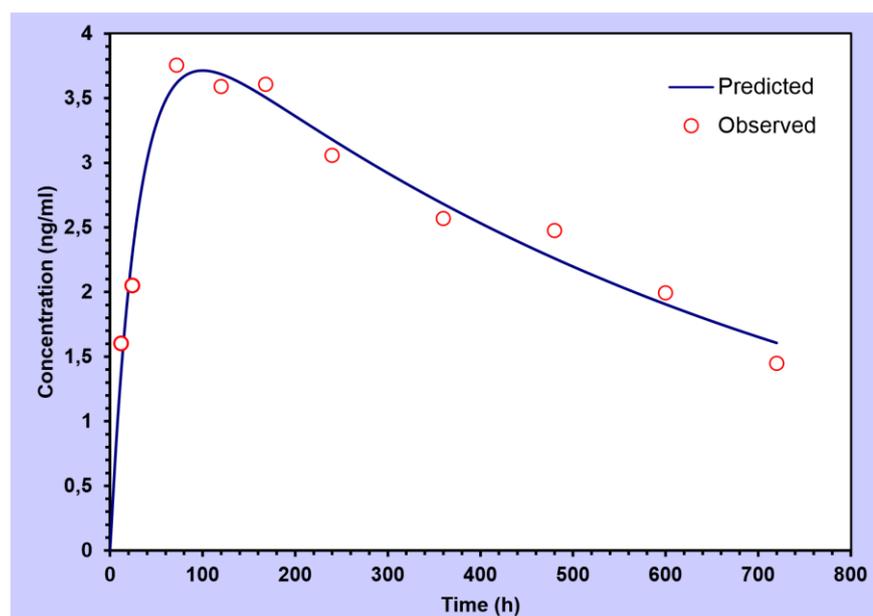


Figure 2. Concentration-time pattern of moxidectin in the blood serum of dogs

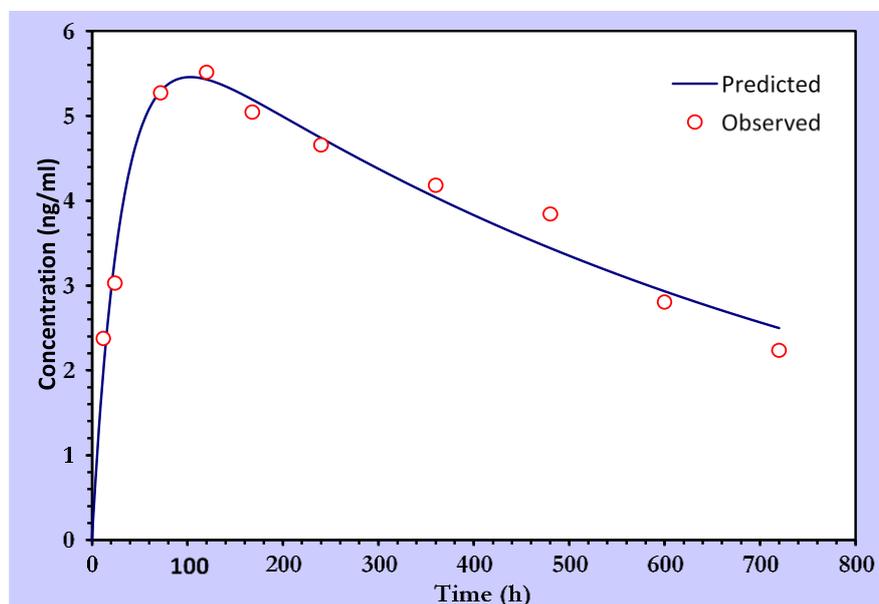


Figure 3. Concentration-time pattern of moxidectin in the blood serum of cats

Table 4. Pharmacokinetic parameters of moxidectin in the blood of dogs and cats

Parameter	Pharmacokinetic parameters of moxidectin in the blood of dogs		Pharmacokinetic parameters of moxidectin in the blood of cats	
	Average	RSD (%)	Average	RSD (%)
K_1, h^{-1}	3.83×10^{-2}	54.7	3.82×10^{-2}	53.8
K_{el}, h^{-1}	1.60×10^{-3}	44.1	1.29×10^{-3}	46.4
T_{half}, h	504	39.6	617	36.7
$V_d, ml/kg$ body weight	246805	41.4	170868	39.7
Cl, ml/kg body weight/h	337	4.7	196.2	24.3
T_{max}, h	104	40.8	112.4	44.6
$C_{max}, ng/ml$	3.8	31.2	5.5	27.8
$AUC_{(0-Inf)}, ng/ml \cdot h$	1884	24.2	2936	24.7
$AUC_{(0-T)}, ng/ml \cdot h$	2970	4.7	5357	24.4
$AUMC_{(0-Inf)}, ng/ml \cdot h^2$	2262839	33.2	5126399	52.3
MRT, h	766	34.7	929	32.9

RSD: Relative Standard Deviation, K_1 : Langmuir constant (absorption rate of AI in injection site), K_{el} : elimination constant (elimination rate), T_{half} : elimination half-life of drug substance, V_d : apparent volume of distribution, Cl: Clearance (drug clearance from the system), T_{max} : time-to-peak concentration of drug substance, C_{max} : value of maximum concentration of the drug substance, AUC: Area Under Curve (area under curve of the drug substance in blood), AUMC: Area Under Multiplication Curve (area under the first moment versus time curve), MRT: Mean Residence Time.

DISCUSSION

The present study showed that the dynamics of distribution and elimination of moxidectin in the body of these two species of carnivores is approximately the same although the serum concentrations of the studied compound were higher in cats. High values of the area under the curve and the volume of distribution indicate the accumulation of moxidectin in tissues, particularly in the subcutaneous fat layer, which provides drug activity against ectoparasites. It should be noted that the long-term prolongation of moxidectin (high values of AUC) in the blood of dogs and cats confirms the preventive effect of “Inspector Mini” against cardiac dirofilariasis. The calculated elimination half-life and MRT values prove that the moxidectin circulates in the animal body for a long time. Comparison of the pharmacokinetics of the combination of Inspector Quadro (fipronil, pyriproxifen, praziquantel and moxidectin) revealed the presence of moxidectin in the blood serum of cats within 1 month and in dogs - within 40 days after a single dose application (spot-on) in identified concentrations (Arisov et al., 2018; Arisova et al., 2019). This drug is intended for the treatment and prevention of endo- and ectoparasites in dogs and cats from 1 kg and at least 7 weeks old according to the instruction for use. In the pharmacokinetic studies of the complex antiparasitic drug «Gelminthal» (syrup), moxidectin remained in the cats and dogs’ blood for a long time (up to 30 days) which indicates its therapeutic effect for at least 1 month after use (Arisov et al., 2016a). These findings suggest the use of the drug based on moxidectin to prevent dirofilariasis in

domestic animals. The relevant studies of moxidectin of Gelminthal tablets found that the pharmacokinetics of this substance are almost the same in dogs and cats which had good distribution in tissues and preservation in serum samples up to 25 days (Arisov et al., 2016b). The Contraindication of Gelminthal tablets is for animals with body weight less than 0.5 kg. The results of the pharmacokinetics study of moxidectin by other researchers showed that it is rapidly absorbed through the skin and is slowly removed (Xiao et al., 2019). The skin half-life of moxidectin is longer, potentially covering the entire mite life cycle (Bernigaud et al., 2016), which is important for the effective treatment against ectoparasites.

When comparing the comparative plasma dispositions of ivermectin (IVM), doramectin (DRM) and moxidectin (MXD) following subcutaneous administration in rabbits, it was found that moxidectin was absorbed faster from the injection site and reached to the peak in plasma concentration (C_{max}) significantly earlier than IVM and DRM. The mean plasma residence time and terminal half-life ($t_{1/2\lambda z}$) were longer for DRM and MXD compared with IVM (Gokbulut 2010). This finding indicated that the persistence of moxidectin are significantly long and it has a positive impact on its efficacy against parasites or utility relating to interdosing interval. There is a scientific evidence that the complex antiparasitic drug “Inspector Total” (fipronil and moxidectin), which is used for skin administration in dogs and cats, protects animals from 14 types of parasites and prevents from reinfestation for up to 2 months (Gavrilova 2013). But this drug is not used for puppies and kittens under 7 weeks old and the treatment of dogs and cats under one kg is carried out carefully under veterinarian supervision. It should be noted that complex drugs might not always be suitable for young animals due to the toxicity of any active component. Thus, the developed new drug based on moxidectin “Inspector Mini” for dermal administration is an alternative to protect young stock and small breed domestic animals from helminths and ectoparasites.

CONCLUSION

As a result of pharmacokinetic studies of moxidectin in the blood serum of dogs and cats, it was found that the active ingredient of the drug for veterinary use the Inspector Mini was absorbed into the blood of carnivores after spot-on application and was determined within 30 days, thereby providing the necessary therapeutic and preventative effect against nematodes and ectoparasites.

DECLARATIONS

Acknowledgements

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Authors' contribution

Irina P. Belykh designed the study, interpreted the data, and drafted the manuscript, carried out the research work, compared, analyzed data and critical checking of this manuscript and also final acknowledgement of the version to be published.

Competing interests

Author state no conflict of interest.

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Quality Evaluation of Nile Tilapia Fish (*Oreochromis niloticus*) Fillets by Using Chitosan and Nanochitosan Coating during Refrigerated Storage

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ABSTRACT

Using natural preservatives has a probability to improve the quality and integrity of fish products. Such research investigated the antimicrobial and antioxidant effects of chitosan and chitosan nanoparticles casing on the quality of tilapia (*Oreochromis niloticus*) fish fillets through refrigerated storage. In the present investigation solutions of chitosan (1 and 2%) and nanochitosan (1 and 2%) were applied for the casing of tilapia fish slices thereafter stored at 4°C for 15 days. Uncoated (control) and coated fish fillets pieces were examined intermittently for bacteriological parameters (Total bacterial count, Proteolytic bacterial count, Lipolytic bacterial count, and *Staphylococcus aureus* count), quality parameters (pH, total volatile basic nitrogen (TVB-N), and thiobarbituric acid reactive substances, TBARS) and sensory features. Results showed that 2% chitosan and 2% chitosan nanoparticle solutions were the optimal concentrations for improving the quality of tilapia fish fillets until 10 days of refrigerated storage period compared to the control group. However, using 2% chitosan nanoparticles showed higher antimicrobial activity, strong ability in preventing protein degradation, retarding lipid oxidation, accepted pH values and delay in declining of sensory score more than 2% chitosan solution during the storage period. Therefore, 2% chitosan nanoparticles as a natural preservative can be utilized for the conservation of quality properties and expanding the shelf life of tilapia fish slices through chilled storage.

Key words: Bacteriological and quality parameters, Chitosan, Nanochitosan, Tilapia fish fillets

INTRODUCTION

Fish products are highly susceptible to quality deterioration, probably due to lipid oxidative reactions, in particular PUFAs. Such reactions are stimulated (catalyzed) by the presence of high heme and nonheme protein concentrations. These proteins are known to contain iron and other metal ions in their structures (Decker and Haultin, 1992). In addition, the quality of seafood is strongly affected by autolysis, bacterial contamination and loss of protein functionality (Jeon et al., 2002). Tilapia (*Oreochromis niloticus*) is a freshwater fish species that has been commonly cultured worldwide and sold in general stores and food market chains, but its preservation has been a problem for a long time due to its brief shelf life. Hence, effective methods to extend the shelf-life of tilapia need to be created. In order to improve the microbial quality and increase the shelf-life of seafood products, food preservation methods like freezing, chemical preservation, salting, and modified atmosphere packaging were utilized. In spite of the simple and widespread use of preservatives, both food processors and consumers have wanted to reduce the use of synthetic chemicals to preserve foods. As a result, interest in the application of natural agents as bio-preservatives has been growing, while most natural agents have low antimicrobial activity spectrums and only effect in very high concentrations. Chitosan shows antimicrobial action against a wide range of foodborne microorganisms, thereby gaining attention as a possible natural preservative for food (Raafat and Sahl, 2009; Friedman and Juneja, 2010). Chitosan, a linear polysaccharide of randomly distributed β -(1-4)-linked D-glucosamine and N-acetyl-D-glucosamine is a biocompatible polysaccharide gotten from chitin deacetylation that is commonly found in nature, such as in shrimps, crabs and fungi. Chitosan coatings have been widely used in the food industry due to certain benefits such as edibility, biodegradability, cosmetic appearance and barrier properties, being non-toxic and non-polluting, as well as being a carrier of food additives (i.e., antioxidants, antimicrobials). So, by preventing bacterial growth and delaying lipid oxidation, these coatings can maintain the quality of raw, frozen, and processed foods including fish products. Chitosan antimicrobial action has been illustrated against many bacteria, fungi and yeasts, possessing a high killing rate against Gram-positive and Gram-negative microbes but low poisonous towards mammalian cells (Kong et al., 2010). The antimicrobial action mechanism of chitosan has not yet been completely elucidated but several theories have been suggested. Due to interactions between the positively charged chitosan molecules and bacterial cell membrane charged negative, the most plausible explanation is a shift in cell

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permeability. This interaction results in the leakage of protein and other intracellular components (Papineau et al., 1991; Fang et al., 1994). Other techniques are the interaction of diffused hydrolysis products with microbial DNA, resulting in mRNA and protein synthesis inhibition (Sudarshan et al., 1992) and the chelation of metal, spore components and fundamental nutrients (Cuero et al., 1991). Nanoparticles are prepared from natural or artificial polymers with the order of 100 nanometers (nm) or less in one or more dimensions (Sinha and Okamoto, 2003). Nanoparticles have demonstrated unusual physical and chemical characteristics due to special effects such as the quantum size, small size, surface, and macro quantum tunnel effects. Due to the advantages of chitosan nanoparticle over other conventional materials, their use as food packaging materials has increased recently (Ramezani et al., 2015). In addition, chitosan nanoparticles have inhibited bacterial growth in food because of the antimicrobial properties (Du et al., 2009). Furthermore, using nanoparticles of chitosan-tripolyphosphates retained antioxidant activity in vitro using free radical scavenging and reducing power tests (Zhang et al., 2008). Therefore, it is beneficial to produce natural preservative coatings or films with antioxidant and antibacterial activities that increase the shelf life of fish and fish products. Hence, the object of this research was to investigate the antimicrobial and antioxidant effects of chitosan and chitosan nanoparticles coatings on the quality of chilled (4 ± 1 °C) Tilapia fish fillets.

MATERIALS AND METHODS

Ethical approval

The current study was approved by the Ethical Committee for life fish sampling at the Animal Health Research Institute, Agriculture Research Center (ARC), Egypt (License No. AHRI, 184429).

Preparation of chitosan and chitosan nanoparticles

Chitosan solution was made by dissolving 1% (w/v) chitosan (Meron Chemical Co., low molecular weight, moisture 10% max., Marine Chemicals, India) in 1% (v/v) acetic acid. To realize the total scattering of chitosan, the solution was blended using a magnetic stirrer at room temperature (25 °C) for melting totally. Glycerol was added up to 0.75 mL/g as a plasticizer and blended for 10 min. 2% chitosan was also prepared in the same way. Nanoparticles were attended by cross-linking of chitosan-sodium tripolyphosphate solution (Ch-TPP). Chitosan (1%) was melted in 1% acetic acid. Sodium tripolyphosphate solution (1%, w/v) was melted in distilled water. By magnetic stirring at room temperature (25-30 °C), 4 mL of sodium tripolyphosphate solution was included in 100 mL of chitosan solution. The blend was mixed for 60 min, at that point, treated with sonication (Model 300VT, 115 V, 60Hz, Manassas, VA, USA) at 1.5 kW for 10 min, sometime recently being utilized for further examination and also, 2% nanochitosan was prepared by the same way (Du et al., 2009). Figure 1 showed that the average particle size (nm) of the Ch-TPP nanoparticle was measured using a Transmission electron microscope (TEM) of 2000 kV (Jem-100SX model, Japan) in the Faculty of Medicine, Tanta University. The average particle size (nm) of Ch-TPP nanoparticle was 100 nm.

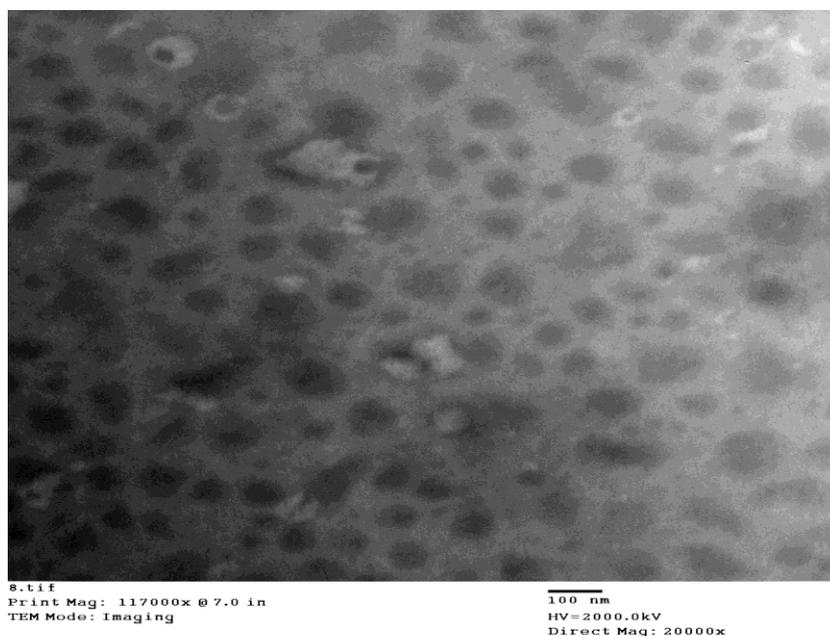


Figure 1. Transmission electron microscope of high voltage (2000 KV) exposed to the average particle size (100 nm) of the Ch-TPP nanoparticle with direct magnification (20000x) and the image of 8.tif (tagged image file format) with print magnification of 117000x@7.0.

Sample preparation

Thirty tilapia fish (*Oreochromis niloticus*) with mean weight of 450-500 g were bought from a regional fish shop in Kafrelsheikh town. The fish were freshly hunted and were kept in ice with a fish/ice proportion of 1:2 (w/w) and transported to the laboratory inside 1 h. Upon entry, the fish were washed in cool faucet water then every fish was accurately filleted by hand. Two skins on fillets were gained from every fish after taking off the head and bone. 60 slices of fish (fillet: 5 cm × 10 cm) were partitioned randomly into five treating sets (12 fillets in each set) and were given a dip treatment in 1% acetic acid (control), 1% chitosan, 2% chitosan, 1% nanochitosan, and 2% nanochitosan solution up to 20 min. At that point, the fillets were taken out and permitted to deplete for 2 h at 20°C on a pre-sterilized metal net to make the edible casing, at that point stocked at 4° C for the following quality evaluation (Alboghbeish and Khodanazary, 2018). Sensorial, Physicochemical, and microbiological examinations were carried out at 5-days interims up to 15 days to confirm the total goodness of fish.

Sensory analysis

The total acceptability of tilapia fish slices was confirmed by a five-point measure taking into consideration texture, color, and smell. Specialists (6-member trained panel) were recorded for sensory features, such as color discoloration (score 5 means no discoloration; till score 1: which means extraordinary discoloration); smell (score 5: means amazingly desirable; till score 1: refers to greatly unacceptable/off-odors), and texture (score 5: means firm; and score 1: refers to extremely smooth). The mediums of these scores were described as total acceptability (Score 5: greatly desirable; 4: good; 3: average; 2: questionable and finally score 1: greatly inadmissible). Shelf life standards supposed that repudiation would happen when the sensory traits declined underneath 4.0 (Ojagh et al., 2010).

Physicochemical examination

Measurement of pH

The pH rate was measured by utilizing an electrical pH meter (Bye model 6020, USA) according to Pearson (2006).

Measurement of total volatile basic nitrogen

TVB-N of *Oreochromis niloticus* fish fillets was measured as stated in ES: 63-9/ (2006).

Measurement of thiobarbituric acid reactive substances

This test depends on determination of malonaldehyde (MDA) as an end product of lipid peroxidation and was done according to ES: 63-10/ (2006).

Microbiological analysis

10 grams of fish meat was carried aseptically to a stomacher bag including 90 mL of 0.1% peptone water. Fish flesh was homogenized for 60 s using a stomacher beneath sterilized conditions, to obtain 1/10 dilution. Serial dilutions were getting ready to be utilized for enumeration of total bacterial count (TBC), proteolytic (PBC), Lipolytic (LBC), and *Staphylococcus aureus* count. TBC was cultivated on standard plate count agar, PBC (on skim milk agar), LBC (on butterfat agar) and *Staphylococcus aureus* (on Baird Parker agar). Fish fillets bacterial counts were confirmed as stated in APHA (2002). The TBC was incubated at 37°C / 48hr; PBC, and LBC at 30°C for 48 hr and 37°C for 48 hr for *Staphylococcus aureus* count. The bacterial colonies were counted as CFU/g.

Statistical analysis

All estimations were reproduced three times to every set and average values ± standard errors were registered for each case. Analysis of variance (ANOVA) was done and average comparisons were achieved by Duncan's multiple range tests utilizing SPSS (Statistical Package for the Social Sciences) to evaluate the importance of differences among average values. P values lower than 0.05 were deemed statistically significant.

RESULTS

Sensory analysis

The findings of the sensory assessment of fish fillets are shown in table 1. During the first days of storing period, no significant differences have been identified between the control sensory scores and other treatments where all the score values were 4.97 ± 0.03 ($P < 0.05$). On the fifth day, the control samples displayed an observed decrease in the freshness score (2.83 ± 0.17) which became unacceptable, while at fifth day, 1% chitosan and 1% nanochitosan freshness score were 4.17 ± 0.17 ; however, 2% chitosan and 2% nanochitosan were 4.67 ± 0.17 . Until day tenth, it observed that 2% chitosan and 2% nanochitosan treated samples had significantly ($P < 0.05$) higher scores (4.1 ± 0.21 and 4.33 ± 0.17) in the overall acceptability than the other treatment groups (1.5 ± 0.29 , 2.83 ± 0.4 and 3 ± 0.29 in control, 1% chitosan and 1% nanochitosan, respectively).

Table 1. Statistical analysis results of overall acceptability values of fish fillet samples

Groups	Control	Chitosan (1g Ch/100ml DW)	Chitosan (2g Ch/100ml DW)	Nanochitosan (1g NCh/100ml DW)	Nanochitosan (2g NCh/100ml DW)
First day	4.97±0.03 ^a	4.97±0.03 ^a	4.97±0.03 ^a	4.97±0.03 ^a	4.97±0.03 ^a
5 th day	2.83±0.17 ^b	4.17±0.17 ^a	4.67±0.17 ^a	4.17±0.17 ^a	4.67±0.17 ^a
10 th day	1.5±0.29 ^c	2.83±0.4 ^b	4.1±0.21 ^a	3.0±0.29 ^b	4.33±0.17 ^a
15 th day	1.17±0.17 ^c	2.17±0.17 ^b	3.17±0.3 ^a	2.33±0.17 ^b	3.67±0.17 ^a

Means of different superscript letters within the same row differ significantly in $P < 0.05$. Ch: Chitosan, NCh: Nanochitosan, DW: Distilled water

Physicochemical examination

pH

During refrigerated storing, changes in pH values were seen in table 2. The first pH values of all fish specimens was 6.18±0.15, 6.18±0.16, 6.09±0.12, 6.13±0.01, and 5.96±0.07 in control, 1% chitosan, 2% chitosan, 1% nanochitosan, and 2% nanochitosan, respectively, and increased to 6.72±0.26, 6.58±0.02, 6.29±0.01, 6.39±0.02, and 6.21±0.01 at fifth day, respectively. At tenth day, pH values were 8.22±0.05, 6.87±0.05, 6.30±0.02, 6.46±0.02 and 6.24±0.03, respectively until reached to 8.63±0.06, 6.88±0.04, 6.48±0.02, 6.62±0.02, and 6.30±0.02, respectively at fifteenth day of refrigerated storage.

Total volatile basic nitrogen

Table 3 showed the changes in Total volatile basic nitrogen (TVB-N) values of fish samples during refrigerated storing. The values of TVB-N initially were 6.18±1.24, 6.03±1.18, 5.04±0.83, 6.04±1.22, and 4.89±0.73 mg/100 g of fish in control, 1% chitosan, 2% chitosan, 1% nanochitosan, and 2% nanochitosan respectively. TVB-N rates of Tilapia fillets gradually increased in all treatments with storing time. At fifth day TVB-N values were 36.73±0.26, 22.03±0.1, 16.73±0.34, 19.15±0.13, and 14.01±1.1 mg/100 g of fish, respectively. TVB-N values recorded 47.88±0.79, 29.99±0.63, 20.68±0.9, 27.04±1.32, and 17.95±0.53 mg/100 g of fish at tenth day, respectively. Finally, at the fifteenth day of storing period, samples coated with chitosan and nanochitosan had markedly lower TVB-N values ($P < 0.05$) than control samples (54.62±0.27 mg/100 g of fish). Mean of TVB-N values of 2% chitosan and 2% nanochitosan were 22.93±0.55 and 19.75±0.93 mg/100 g of fish, respectively on day fifteenth of storing.

Thiobarbituric acid reactive substances

The initial values of Thiobarbituric acid (TBA) reactive substances in table 4 was 1.26±0.45, 1.12±0.40, 0.67±0.19, 0.90±0.04 and 0.56±0.15 mg MDA/kg of fish in untreated (control), 1% chitosan, 2% chitosan, 1% nanochitosan and 2% nanochitosan group, respectively. Values of TBA in the control, chitosan, and nanochitosan coating samples increased with storing time. At fifth day, TBA values reached 4.83±0.06, 3.89±0.07, 2.24±0.04, 3.23±0.29, and 1.18±0.24 mg MDA/kg of fish, respectively. On day 10, samples coated with 1% and 2% chitosan (4.69±0.05, 3.55±0.20) and 1% and 2% nanochitosan (4.24±0.06, 3.21±0.04) had markedly lower TBA rates other than the control value (5.60±0.04 mg MDA/kg of fish) ($P < 0.05$). TBA mean values of 2% chitosan and 1 and 2% nanochitosan were 4.16±0.09, 4.31±0.04, and 3.95±0.05 mg MDA/kg of fish, on day fifteenth of storing time, respectively. While, control and 1% chitosan were 6.87±0.03 and 4.78±0.06 mg MDA/kg of fish.

Microbiological examination

During refrigerated storing, the variation in total bacterial count (TBC) was shown in table 5. The first TBC values were 5.15±4.23, 5.0±4.64, 4.18±3.54, 5.04±4.58 and 3.97±3.36 log₁₀ cfu/g in control, 1% chitosan, 2% chitosan, 1% nanochitosan and 2% nanochitosan, respectively. At fifth day TBC reached 5.92±5.41, 5.11±4.36, 4.88±4.45, 5.08±4.36 and 4.95±3.46 log₁₀ cfu/g, respectively. Among all treatments, sample treated with 2% chitosan and 2% nanochitosan (5.29±4.82 and 4.99±4.51 log₁₀ cfu/g) had lower TBC at tenth day than control, 1% chitosan and 1% nanochitosan (6.26±5.41, 6.11±5.97, and 6.08±5.62 log₁₀ cfu/g, respectively) and within the acceptable limit of Egyptian Organization for Standardization and Quality Control (EOS) (2005).

The variations in proteolytic (PBC) and lipolytic bacterial counts (LBC), respectively through the storage periods are presented in tables 6 and 7. The first values of PBC in the fish slices were 4.57±2.95, 4.41±2.95, 4.08±3.18, 4.11±4.41, and 4.04±3.98 and for LBC were 3.98±3.89, 3.71±3.71, 3.28±3.60, 3.68±4.28, and zero log₁₀ cfu/g, in control, 1% chitosan, 2% chitosan, 1% nanochitosan and 2% nanochitosan group, respectively. During the storage period PBC and LBC values increased gradually within each treatment. At fifth day, PBC values were 5.23±3.76, 5.11±4.18, 5.04±4.08, 5.08±3.94 and 4.72±4.32 log₁₀ cfu/g, while LBC values were 5.46±4.26, 5.36±4.58, 4.71±4.53, 5.3±5.32 and 4.51±4.04 log₁₀ cfu/g in control, 1% chitosan, 2% chitosan, 1% nanochitosan, and 2% nanochitosan samples, respectively. At tenth day of the storing time, values of the treated sets with 2% chitosan and 2% nanochitosan (5.85±5.28 and 5.71±5.41 log₁₀ cfu/g) had markedly lower ($P < 0.05$) PBC other than the other treated groups (untreated (control) 7.08±6.34, 1% chitosan 6.99±6.08, and 1% nanochitosan 6.98±5.88 log₁₀ cfu/g) and also markedly lower LBC ($P < 0.05$) with 2% chitosan and 2% nanochitosan treatment groups (5.88±5.23 and 5.58±5.23 log₁₀ cfu/g) than the other

treated groups (control: 6.79 ± 6.18 , 1% chitosan: 6.70 ± 6.43 , and 1% nanochitosan: 6.65 ± 6.04 log₁₀ cfu/g). Finally, at fifteenth day PBC mean values recorded 8.52 ± 8.11 , 7.36 ± 6.89 , 6.34 ± 6.04 , 7.32 ± 7.11 , and 6.23 ± 5.81 while LBC were 7.69 ± 6.72 , 7.15 ± 7.45 , 6.51 ± 6.26 , 7.1 ± 7.34 , and 6.39 ± 5.92 log₁₀ cfu/g in control, 1% chitosan, 2% chitosan, 1% nanochitosan and 2% nanochitosan group, respectively.

Table 8 revealed that *Staphylococcus aureus* count in fish samples immersed in 2% nanochitosan coating was negative in all storage period. Initial *Staphylococcus aureus* count was 3.64 ± 3.30 , 3.5 ± 3.0 , 3.0 ± 2.85 , 3.34 ± 3.49 log₁₀ cfu/g, and zero in control, 1% chitosan, 2% chitosan, 1% nanochitosan and 2% nanochitosan group, respectively. At fifth day *Staphylococcus aureus* count reached 5.77 ± 5.11 , 5.45 ± 4.58 , 4.72 ± 4.26 , 5.32 ± 4.72 log₁₀ cfu/g, and zero; Then increased to reach 6.91 ± 5.92 , 6.84 ± 6.18 , 5.38 ± 5.11 , and 6.8 ± 6.15 in control, 1% chitosan, 2% chitosan, and 1% nanochitosan group, respectively at fifteenth day.

Table 2. Statistical analysis results of pH values of fish fillet samples

Groups	Control	Chitosan (1g Ch/100ml DW)	Chitosan (2g Ch/100ml DW)	Nanochitosan (1g NCh/100ml DW)	Nanochitosan (2g NCh/100ml DW)
First day	6.18 ± 0.15^a	6.18 ± 0.16^a	6.09 ± 0.12^a	6.13 ± 0.01^a	5.96 ± 0.07^a
5 th day	6.72 ± 0.26^a	6.58 ± 0.02^{ab}	6.29 ± 0.01^b	6.39 ± 0.02^{ab}	6.21 ± 0.01^b
10 th day	8.22 ± 0.05^a	6.87 ± 0.05^b	6.30 ± 0.02^{cd}	6.46 ± 0.02^c	6.24 ± 0.03^d
15 th day	8.63 ± 0.06^a	6.88 ± 0.04^b	6.48 ± 0.02^d	6.62 ± 0.02^c	6.30 ± 0.02^e

Means of different superscript letters within the same row differ significantly at $P < 0.05$. Ch: Chitosan, NCh: Nanochitosan, DW: Distilled water

Table 3. Statistical analysis results of TVB-N values of fish fillet samples

Groups	Control	Chitosan (1g Ch/100ml DW)	Chitosan (2g Ch/100ml DW)	Nanochitosan (1g NCh/100ml DW)	Nanochitosan (2g NCh/100ml DW)
First day	6.18 ± 1.24^a	6.03 ± 1.18^a	5.04 ± 0.83^a	6.04 ± 1.22^a	4.89 ± 0.73^a
5 th day	36.73 ± 0.26^a	22.03 ± 0.1^b	16.73 ± 0.34^d	19.15 ± 0.13^c	14.01 ± 1.1^e
10 th day	47.88 ± 0.79^a	29.99 ± 0.63^b	20.68 ± 0.9^c	27.04 ± 1.32^b	17.95 ± 0.53^c
15 th day	54.62 ± 0.27^a	35.92 ± 0.20^b	22.93 ± 0.55^c	34.64 ± 0.9^b	19.75 ± 0.93^d

Means of different superscript letters within the same row differ significantly at $P < 0.05$. Ch: Chitosan, NCh: Nanochitosan, DW: Distilled water

Table 4. Statistical analysis results of TBA values of fish fillet samples

Groups	Control	Chitosan (1g Ch/100ml DW)	Chitosan (2g Ch/100ml DW)	Nanochitosan (1g NCh/100ml DW)	Nanochitosan (2g NCh/100ml DW)
First day	1.26 ± 0.45^a	1.12 ± 0.40^a	0.67 ± 0.19^a	0.90 ± 0.04^a	0.56 ± 0.15^a
5 th day	4.83 ± 0.06^a	3.89 ± 0.07^b	2.24 ± 0.04^c	3.23 ± 0.29^b	1.18 ± 0.24^c
10 th day	5.60 ± 0.04^a	4.69 ± 0.05^b	3.55 ± 0.20^d	4.24 ± 0.06^c	3.21 ± 0.04^e
15 th day	6.87 ± 0.03^a	4.78 ± 0.06^b	4.16 ± 0.09^{cd}	4.31 ± 0.04^c	3.95 ± 0.05^d

Means of different superscript letters within the same row differ significantly at $P < 0.05$. Ch: Chitosan, NCh: Nanochitosan, DW: Distilled water

Table 5. Statistical analysis results of Total bacterial count (TBC) of fish fillet samples

Groups	Control	Chitosan (1g Ch/100ml DW)	Chitosan (2g Ch/100ml DW)	Nanochitosan (1g NCh/100ml DW)	Nanochitosan (2g NCh/100ml DW)
First day	5.15 ± 4.23^a	5.0 ± 4.64^a	4.18 ± 3.54^b	5.04 ± 4.58^a	3.97 ± 3.36^b
5 th day	5.92 ± 5.41^a	5.11 ± 4.36^b	4.88 ± 4.45^b	5.08 ± 4.36^b	4.95 ± 3.46^b
10 th day	6.26 ± 5.41^a	6.11 ± 5.97^{ab}	5.29 ± 4.82^b	6.08 ± 5.62^{ab}	4.99 ± 4.51^b
15 th day	7.89 ± 7.72^a	7.71 ± 7.38^a	6.78 ± 6.65^a	7.69 ± 7.34^a	6.28 ± 6.08^a

Means of different superscript letters within the same row differ significantly at $P < 0.05$. Ch: Chitosan, NCh: Nanochitosan, DW: Distilled water

Table 6. Statistical analysis results of proteolytic bacterial counts of fish fillet samples

Groups	Control	Chitosan (1g Ch/100ml DW)	Chitosan (2g Ch/100ml DW)	Nanochitosan (1g NCh/100ml DW)	Nanochitosan (2g NCh/100ml DW)
First day	4.57± 2.95 ^a	4.41±2.95 ^a	4.08±3.18 ^a	4.11±4.41 ^a	4.04±3.98 ^a
5 th day	5.23±3.76 ^a	5.11±4.18 ^{ab}	5.04±4.08 ^b	5.08±3.94 ^b	4.72±4.32 ^c
10 th day	7.08±6.34 ^a	6.99±6.08 ^a	5.85±5.28 ^b	6.98±5.88 ^a	5.71±5.41 ^b
15 th day	8.52±8.11 ^a	7.36±6.89 ^b	6.34±6.04 ^b	7.32±7.11 ^b	6.23±5.81 ^b

Means of different superscript letters within the same row differ significantly at $P < 0.05$. Ch: Chitosan, NCh: Nanochitosan, DW: Distilled water

Table 7. Statistical analysis results of lipolytic bacterial counts of fish fillet samples

Groups	Control	Chitosan (1g Ch/100ml DW)	Chitosan (2g Ch/100ml DW)	Nanochitosan (1g NCh/100ml DW)	Nanochitosan (2g NCh/100ml DW)
First day	3.98± 3.89 ^a	3.71±3.71 ^a	3.28±3.60 ^a	3.68±4.28 ^a	-
5 th day	5.46±4.26 ^a	5.36±4.58 ^a	4.71±4.53 ^a	5.3±5.32 ^a	4.51±4.04 ^a
10 th day	6.79±6.18 ^a	6.70±6.43 ^a	5.88±5.23 ^b	6.65±6.04 ^a	5.58±5.23 ^b
15 th day	7.69±6.72 ^a	7.15±7.45 ^a	6.51±6.26 ^a	7.1±7.34 ^a	6.39±5.92 ^a

Means of different superscript letters within the same row differ significantly at $P < 0.05$. Ch: Chitosan, NCh: Nanochitosan, DW: Distilled water

Table 8. Statistical analysis results of *Staphylococcus aureus* count of fish fillet samples

Groups	Control	Chitosan (1g Ch/100ml DW)	Chitosan (2g Ch/100ml DW)	Nanochitosan (1g NCh/100ml DW)	Nanochitosan (2g NCh/100ml DW)
First day	3.64± 3.30 ^a	3.5±3.0 ^a	3.0±2.85 ^a	3.34±3.49 ^a	-
5 th day	5.77±5.11 ^a	5.45±4.58 ^{ab}	4.72±4.26 ^b	5.32±4.72 ^b	-
10 th day	6.76±6.32 ^a	6.72±6.75 ^a	5.82±4.83 ^a	6.61±6.15 ^a	-
15 th day	6.91±5.92 ^a	6.84±6.18 ^a	5.38±5.11 ^b	6.8±6.15 ^a	-

Means of different superscript letters within the same row differ significantly at $P < 0.05$. Ch: Chitosan, NCh: Nanochitosan, DW: Distilled water

DISCUSSION

Sensory evaluation

Fish acceptability and its products through storing relied on the variations in their sensory characteristics. Fish fillets were deemed to be satisfactory for human consuming until the sensory grade reached 4 (Ojagh et al., 2010). Among treatments, the most elevated score was gotten for the fish slices coated with 2% nanochitosan.

Physicochemical analysis

pH

2% Chitosan and 2% nanochitosan groups were remarkably lower in pH values than the other sets ($P < 0.05$) due to the suppression in development of bacteria (Shahidi, et al., 1999) and were acceptable according to EOS (2005) where pH of fish meat shouldn't exceed 6.5. The gradual increase of pH rates in refrigerating storing periods, probably due to the collection of fundamental components created from both autolysis handled by endogenous enzymes and microbial enzymatic activities (Nirmal and Benjakul, 2011). Similar observations were made by Alboghbeish and Khodanazary (2018). The pH is a substantial determinant of microbial development and seafood with elevated pH has a high spoilage possibility and a brief shelf life (Newton and Gell, 1981).

Total volatile basic nitrogen

The Total volatile basic nitrogen (TVB-N) value is a pointer of spoilage, which is basically consisted of trimethylamine, dimethylamine, and ammonia resulted from the degeneration of proteins and non-protein nitrogenous components by the action of spoilage microbes and endogenous enzymes. It was noticed that the rate of TVB-N rising was extremely slower in fish slices coated with chitosan and nanochitosan rather than the control samples. Besides, a significant difference ($P < 0.05$) was in TVB-N values between 1% and 2% chitosan, and also, among 1% and 2% nanochitosan treated groups on days 5, 10, and 15. Rates of TVB-N in groups of 1% chitosan and nanochitosan were higher than 2% chitosan and nanochitosan groups, this might be ascribed to the higher antimicrobial action of 2% chitosan and nanochitosan compared to 1% chitosan and nanochitosan. TVB-N mean values of 2% chitosan and 2% nanochitosan at fifteenth day of storage were acceptable according to EOS (2005) rather than the other groups where

TVB-N of fish meat should be 30mg/100g. Ramezani et al. (2015) and Ojagh et al. (2010) revealed that pretreatment of silver carp and rainbow trout with 2% nanochitosan and 2% chitosan respectively, might delay the rising in the TVB-N rates compared to the other treated groups. Also, Fan et al. (2009) explained that chitosan coating decreased TVB-N values obviously and consequently slowed the deterioration of silver carp.

Thiobarbituric acid reactive substances

Thiobarbituric acid reactive substances value has been commonly used to measure the grade of lipid oxidation and the existence of TBA reactive substances is attributable to the second step auto-oxidation through which aldehyde and ketone are produced from peroxides oxidation. During the storage period, samples coated with 2% chitosan and nanochitosan had significantly lower TBA values than samples coated with 1% chitosan and nanochitosan ($P < 0.05$). At day 15 of storing, TBA mean values of 2% chitosan and 1, 2% nanochitosan were acceptable according to EOS (2005) rather than control and 1% chitosan where TBA exceeded 4.5 MDA/kg of examined samples. The raising in TBA levels of samples through storing can be ascribed to the partial dehydration of fish and interaction of lipids with the oxygen of air (Kilincceker et al., 2009). Therefore, the use of chitosan coating possibly will reduce lipid oxidation in fish samples due to the antioxidant activity and its low oxygen permeability characteristic. Solval et al. (2014) confirmed that the coating of chitosan nanoparticles through frozen storage could reduce the TBARS content in the shrimp. Also, Alboghbeish and Khodanazary (2018) reported that 2% chitosan and nanochitosan may minimize lipid oxidation levels in *Carangoides coeruleopinnatus* fillets during refrigerated storage.

Microbiological analysis

It is noteworthy that TBC of fish fillets in the control group raised quickly during the storage period and was significantly higher than the other treated groups ($P < 0.05$), demonstrating the antimicrobial action of chitosan and chitosan nanoparticles and exceeded the maximum acceptability level of EOS (2005, 10^6 cfu/g) on day 10. Therefore, the treatment of fish coated with 2% chitosan and 2% nanochitosan might delay the development of total bacteria more efficiently, compared with 1% chitosan and 1% nanochitosan. The mechanism of action of chitosan seems to be related to the disruption of the lipopolysaccharide layer of outer membrane of Gram-negative bacteria (Pereda et al., 2011), as well as to its role as a buffer against oxygen transfer (Jeon et al., 2002). Seafood spoiled by proteolytic and lipolytic bacterial strains which are capable of producing extracellular protease and lipase enzymes that can break down protein and fat to substances with low molecular weight. Protease enzymes can target the nitrogen molecules that occur naturally in meat, causing severe deteriorating color and odor changes in foods even when preserved in refrigeration or frozen (Ali, 2011). Chitosan and nanochitosan treated samples showed a decrease in PBC and LBC values compared to control samples that suggest the antimicrobial activity of chitosan and chitosan nanoparticles. The groups treated with 2% chitosan and 2% nanochitosan had significantly lower PBC and LBC ($P < 0.05$) than the other treated groups at tenth day of the storage period. Proteolytic and lipolytic bacteria could be responsible for a variety of food odor and flavor problems. Some of the common psychrotrophic bacteria are intensely proteolytic and/or lipolytic and cause severe defects in dairy, meat, poultry and fish products when high counts (10^6 per g or ml or higher) are reached during chilled storage (Vanderzant et al., 1985). The results confirmed the antibacterial properties of nanochitosan as stated by Ramezani et al. (2015). Dipping of samples in this solution prevented oxidation of flesh and water absorption and thus inhibited bacterial growth, as Fan et al. (2009) observed when investigating the effect of chitosan coating on the quality of silver carp and shelf life during frozen storage. In addition, nanochitosan solution is known to degrade bacterial cell walls naturally, rendering them vulnerable to lysis, which has resulted in lethal consequences (Liu et al., 2004). Fish samples immersed in 2% nanochitosan coating were negative for *Staphylococcus aureus* in all storage periods. During the storage period, there is a decrease in *Staphylococcus aureus* count of 2% chitosan group than the other treated groups and control samples ($P < 0.05$). Qi et al. (2004) stated the higher antibacterial activity of chitosan nanoparticles against *Escherichia coli*, *Staphylococcus aureus*, and *Salmonella typhimurium* than chitosan due to the extraordinary nature of the nanoparticles, the nanoparticles are probable to have a larger surface area and a greater affinity with the microbial cells, producing a quantum-sized effect. On the contrary, Sadeghi et al. (2008) mentioned that the impact of chitosan nanoparticles on *Staphylococcus aureus* is less inhibitive than free-soluble polymers, where nanoparticles have less positive binding charges available to link to the negative bacterial cell wall. On the other hand, Du et al. (2009) stated that nanoparticles of chitosan tripolyphosphate loaded with different metal ions display greater antibacterial activity against *Escherichia coli*, *Salmonella choleraesuis*, and *Staphylococcus aureus*.

CONCLUSION

Chitosan is a potential resource that is sustainable, non-toxic and biodegradable and has gained significant attention in the final two decades. Results of this study displayed a shelf-life of fewer than 5 days for untreated tilapia (*Oreochromis niloticus*) slices, while a shelf-life of 10 days was observed for 2% chitosan and 2% nanochitosan treated samples according to various quality and spoilage parameters, where the bacterial and chemical examination was associated with

the sensory assessment. In addition, 2% nanochitosan demonstrated a greater ability to inhibit TVB-N and TBARS content compared to other treated groups, resulting in delaying the deterioration of fresh tilapia slices and prolonged shelf life during chilled storage. So, our work demonstrated the antioxidant and antimicrobial activity of chitosan and nanochitosan as a natural preservative for preserving of tilapia fillets during refrigerated storage.

DECLARATIONS

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Competing interests

The authors declared that they have no competing interests.

Consent to publish

All authors agree on article publication

Author`s contributions

WME and SAY found research idea, planned the study design, performed data, performed the laboratory work, collected of fish samples in the experiment, drafted and preparation the manuscript. MNS shared in the research's idea and preparation of working solutions. The final manuscript was read and accepted by all authors.

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Developmental Competence of Buffalo Oocytes Cultured Under Different Oxygen Tensions after Selection with Brilliant Cresyl Blue

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ABSTRACT

The aim of this investigation was to follow up *in vitro* preimplantation development of buffalo cumulus-oocyte complexes (COCs) after BCB test and followed by *in vitro* maturation under two different levels of oxygen tension. Cumulus-oocyte complexes (n=1045) were selected with BCB staining (oocytes with any degree of blue color in cytoplasm was defined as BCB+, oocytes without any degree of blue color in cytoplasm was defined as BCB-) in addition to a third control group. The previous experimental groups (BCB+, BCB-, control) were matured *in vitro* under low (5%) and high oxygen tension (20%), followed by *in vitro* fertilization and *in vitro* culture of presumptive zygotes. There were no differences ($P \leq 0.05$) in cleavage, morula and transferable embryos rates among BCB+, BCB- and control group. However, blastocyst rate was greater significantly in control group (14.4 ± 2.0) than BCB-COCs (8.4 ± 1.9). According to the oxygen tension effect, the rate of morula and transferable embryos was increased ($P \leq 0.05$) in buffalo COCs developed under low oxygen tension (11.6 ± 1.4 and 23.8 ± 1.9) compared to high oxygen tension group (7.4 ± 1.4 and 17.9 ± 2.1). In addition, cleavage, morula, blastocyst and transferable embryos rates were greater in BCB+ under low (43.6 ± 3.9 , 14.9 ± 2.5 , 14.1 ± 2.9 and 28.4 ± 3.6) than high oxygen tension group (33.5 ± 3.9 , 7.1 ± 2.5 , 11.6 ± 2.9 and 18.8 ± 3.6) which may reflect enhanced biological processes controlling early development. Moreover, blastocyst rate was significantly higher in control group cultured under high (12.0 ± 2.9) and low (16.9 ± 2.8) oxygen level than their counterparts of BCB- group (9.3 ± 2.9 and 7.6 ± 2.6 , respectively). In conclusion, there was no differences in embryo development between BCB+ and BCB-, COCs; therefore, oocyte selection based on BCB staining is not an effective tool to select developmental competent buffalo COCs. Buffalo morula and transferable embryos prefer low oxygen tension for early development, which should be applied during *in vitro* embryo production of this species.

Keywords: Brilliant cresyl-blue staining, Cumulus-oocyte complex, Morula, Preimplantation.

INTRODUCTION

Egyptian buffaloes represent an important livestock resource for producing meat and milk production in agriculture national economy. Reproductive efficiency is a key factor affecting female buffalo productivity; however, buffaloes are characterized by late of puberty and sexual maturity, long postpartum, poor oestrous signs, low pregnancy rate and long calving intervals (Barile, 2005). Noteworthy, several Assisted Reproductive Technologies (ART's) are becoming important tools that applied for accelerating the genetic gain of milk production and enhancing efficacy of dairy animal's reproductive performance (Hafez, 2015).

In this regard, *in vitro* production of buffalo embryos (IVEP) technique has improved during the last decades. However, the quality and rate of transferable embryos produced still low than that required for breeding programs (Gasparrini, 2007; Goszczynsk et al., 2018). It is well established that the development of embryos depends on various factors like culture conditions (*in vivo* versus *in vitro* conditions), constituents of culture media, pH and oxygen tension (Simopoulou et al., 2018).

Suboptimal conditions for *in vitro* culture eventually reduced the development rate and quality of blastocysts because of reactive oxygen species (ROS) accumulated during pre-implantation development due to insufficient oxygen level (Bavister, 1995; Karagenc et al., 2004; Oyamada and Fukui, 2004; Torres-Osorio et al., 2019). The primary gas phases used in IVF laboratories are 5% carbon dioxide (CO₂) in air (20% O₂), and 90% Nitrogen (N₂). The oxygen level of most mammalian species in the oviduct and uterus ranged from 2 to 8 % (Fischer et al., 1992). There is a balanced system in any living cell, which keeps the oxygen species generation and scavenging in balance (He et al., 2017). An imbalance between the Reactive Oxygen Species (ROS) produced and the intracellular antioxidants causes oxidative stress (Fiers et al., 1999).

Various types of ROS cause oxidative stress, which either produced intracellularly or derived from extracellular environment. Indeed, the main sources of ROS are free radicals such as superoxide anion, hydrogen peroxide, hydroxyl

radical, hydroxyl ion, and nitric oxide produced during oxygen reduction reactions (Balaban et al., 2005; Agarwal et al., 2006). The effects of ROS in mammalian reproductive tract could be negative or positive depends on the duration of exposure and level of ROS (Agarwal et al., 2005). Indeed, an adequate amount of ROS is required for ovarian steroid biosynthesis and induction of ovulation events.

Physiological levels of ROS are required during *in vitro* maturation (IVM) to reinitiate meiosis of the oocytes (Shkolnik et al., 2011). However, an elevated level of ROS during *in vitro* maturation can cause chromosomal errors and finally reduced developmental competence of the oocyte (Sasaki et al., 2019). Increased ROS generation has been shown to cause alterations in the organization of microtubules and chromosomal alignment of metaphase II meiotic spindles in mouse oocytes (Cheung et al., 2016; Kala et al., 2016). Moreover, minimal levels of oxidative stress during embryonic growth may be beneficial for early development (Combelles et al., 2009; Shkolnik et al., 2011). In contrast, embryos exposed to high levels of ROS are morphologically poor quality and at risk of early embryonic development blockage. An increase in ROS production has been found to result in embryonic arrest at two-cell stage of development (Du plessis et al., 2008; Ashraf et al., 2016). Therefore, the current study was designed to assess the developmental competence of buffalo oocyte after *in vitro* maturation under two levels of oxygen tension followed by *in vitro* fertilization and early embryonic development up to the blastocyst stage.

MATERIALS AND METHODS

Experimental design

Good quality COCs of grade A and B were used in the current investigation. In this experiment, good quality cumulus-oocyte complexes (COCs) were distributed in three groups (BCB+, BCB- and control) and according to oxygen tension divided into two subgroups as follows: Control 5% n=160, Control 20% n=174, BCB+5% n=160, BCB+20% n=197, and BCB-5% n=169, BCB-20% n=185).

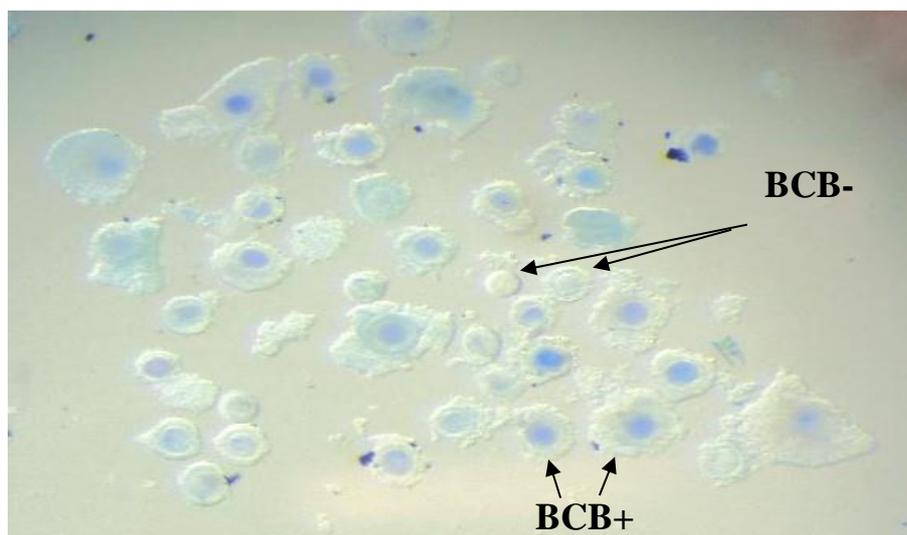
Collection of ovaries and oocyte retrieval

Buffalo ovaries (n=1045) were collected from local slaughterhouses (El-Monieb and El-Warak) during Winter season, from October 2019 to March 2020. Within 3 hrs after slaughtering, ovaries were transported to the laboratory (Embryology Lab, Research Park, Faculty of Agriculture, Cairo University) in physiological saline (0.9% NaCl supplemented with 50 µg / ml gentamycin) and maintained at 34-37°C. Ovaries were washed once in 70% ethanol and three times in warm sterile physiological saline. Cumulus-oocyte complexes (COCs) were collected by aspiration of antral follicles using 18-gauge needle attached to a 10-ml syringe. Oocytes were washed twice in washing medium TCM-199-Hepes medium supplemented with 2% fetal bovine serum (FBS), 0.3 mg/ml glutamine and 50 µg/ml gentamycin. The evaluation of recovered COCs was done under a stereo microscope, according to their cumulus layers and ooplasm homogeneity as stated by (Chauhan et al., 1998). Compact COCs that had a mass of ≥ 5 layers of cumulus cells and homogeneous ooplasm (Grade-A) and those had 3-4 layers of cumulus cells and homogeneous ooplasm (Grade-B) were used in the current study.

Brilliant cresyl blue staining test

Cumulus-oocyte complexes were washed three times in Dulbecco's PBS (DPBS) and adjusted by adding 0.4 % BSA (Fraction V), then COCs were exposed to 26 mM of BCB (B-5388, Sigma) and diluted in mDPBS at 38.5 ° C for 90 min in a humidified air atmosphere without CO₂. Directly after washing twice in mDPBS, COCs were examined under a stereomicroscope and according to their cytoplasm coloration were divided into two classes (Figure 1); oocytes with any degree of blue cytoplasm coloration (BCB+) and oocytes with no blue cytoplasm coloration (BCB-). While control group was incubated immediately without BCB exposure.

Figure 1. Brilliant cresyl blue stained of Egyptian buffalo oocytes. Blue stained oocytes are designated as brilliant cresyl blue (BCB +) and colorless oocytes are designated as brilliant cresyl blue (BCB-).



***In vitro* oocytes maturation**

Before commencing the maturation process, the maturation medium was prepared in advance. The medium was held for equilibrium at 38.5° C and 5 % CO₂ and incubated for 2 hrs. All COC groups were washed twice in the ripening medium consisting of TCM-199 combined with 10 % FBS (F7524, Sigma, Germany), 1 µg / ml estradiol-17β (E2758, Sigma, USA), 0.15 mg / ml glutamine, 22 µg / ml, Na-pyruvate (P-4562;Sigma, USA), 5 µg / ml FSH (F2293, Sigma, USA), and 50 µg / ml gentamycin. A total of 10-15 COCs were seeded from each category (Control, BCB+, BCB-) in drops of 100 µl of maturation medium under mineral oil (M8410, Sigma, USA) in a 3.5 cm Petri dish. The cumulus-oocyte complexes were incubated at 38.5° C for 24 hrs in a humidified atmosphere with 5 % CO₂ under 5% oxygen level or 20% oxygen level.

***In vitro* fertilization**

Frozen straw of Egyptian buffalo bull semen (0.25 ml) was thawed in warmed water at 38°C for 30 seconds and opened after wiped with 70% ethanol alcohol. Spermatozoa were washed twice in the sperm washing medium namely Tyrode Albumin Lactate Pyruvate (TALP) medium (modified Ca²⁺ free TALP medium) by centrifugation at 1800 rpm for 5min and one time in fertilization medium (modified TALP medium) according to [Stinshoff et al. \(2011\)](#). Spermatozoa pellet was re-suspended in the fertilization medium. The concentration of sperm was adjusted to be 2×10⁶ ([Gasparrini, 2002](#)). Matured oocytes (10-15 oocytes per drop) were placed in a small petri dish containing 50 µl droplets of fertilization medium. Aliquots of the sperm suspension were added to each droplet containing mature oocytes. The oocytes and sperm were co-cultured in an incubator at 38.5°C in 5% CO₂ in air, with maximal humidity for 18 hrs.

***In vitro* culture**

The received zygotes were washed four times after 18 hrs of fertilization by repeated pipetting to denude cumulus cells. The presumptive zygotes of all groups (control, BCB+ and BCB-) were placed into 50 µl droplets of *in vitro* culture medium in 4 well petri dish and covered with paraffin oil. The culture medium composed of TCM-199 supplemented with 3 mg/ml BSA, 22 µg/ml Na-pyruvate, 10 µl/ml NEAA (100 X), 20 µl/ml EAA (50 X) and 50 µg/ml gentamycin sulphate. During the culture period, presumptive zygotes were kept at 38.5°C in a humidified atmosphere of 5% CO₂ in air. Half of the medium was changed every 48 hrs, the cleavage rate was checked after 48 hrs post insemination (Figure 2) and blastocyst rate was checked on day 7 (Figure 3).

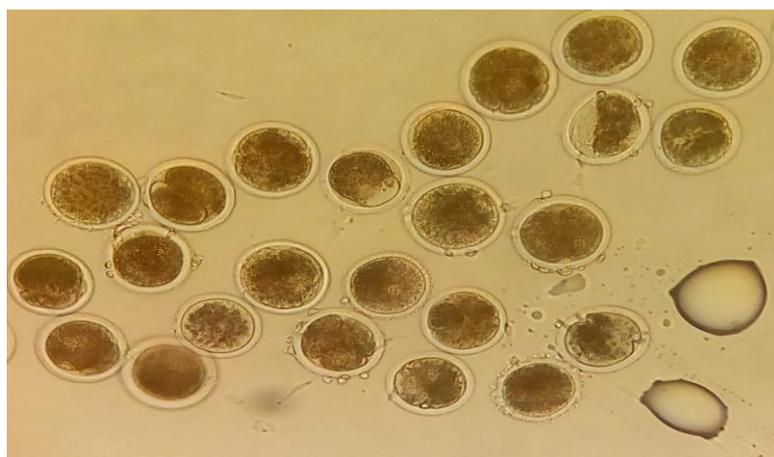


Figure 2. Embryos at 2-Cell, 4-Cell, 8-Cell and degenerated embryos after 48 hours of culture under oxidative stress (20% O₂) in Egyptian buffalo oocytes

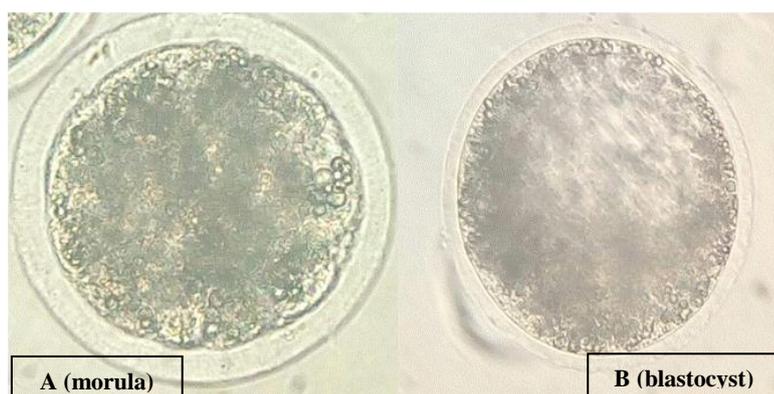


Figure 3. Embryos at morula and blastocyst stages under 5% O₂ level in Egyptian buffalo oocytes

Statistical analysis

Statistical analysis of current data was done using the SPSS program package (SPSS Inc., Chicago, 183 Illinois, USA). The effect of oxygen level (5% versus 20%) and oocyte type (BCB+ versus BCB- versus control) on the previous traits were tested by applying the following model:

$$Y_{ijk1} = \mu + R_i + A_j + (R_i * A_j)_{ijk} + e_{ijk1}$$

Where:

Y_{ijk1} = individual observation; μ = Overall mean; R_i = Effect of BCB staining (BCB+ =1 and BCB- =2); A_j = Effect of oxygen level (5% =1 and 20% =2); (R_i*A_j)_{ijk} = The interaction between BCB and oxygen level; e_{ijk1} = Experimental error supposed to be randomly distributed (0, σ^2). The data were expressed as mean \pm standard error. Probability values up to $\leq 5\%$ was considered significant.

Ethical approval

The present work has been conducted in accordance with the guidelines of the ethical committee of Faculty of Agriculture, Cairo University. The experimental lab work was executed at Cairo University Research Park, Faculty of Agriculture, Cairo University, Egypt.

RESULTS AND DISCUSSION

Although the efficiency of *In vitro* embryo production has improved in Buffalo, the rate of transferable embryos (TE) and the rate of development to term is still very low (Gasparrini, 2002). The outcome and quality of *in vitro* produced embryos are generally still lower than *in vivo* counterparts in other mammalian species (Marsico et al., 2019). Different factors play a key role in assessing the quality of *in vitro* production (IVP). The competence of COCs to reach transferable embryos stages is considered one of the main factors influencing the yield of the pre-implantation embryos (Reader et al., 2017), while the conditions of culture affect the quality of the blastocysts (Rizos et al., 2002). In the current study, we investigated the interaction of oxygen tension levels with the quality of buffalo oocytes (using BCB staining model) after *in vitro* maturation, fertilization and early embryonic development up to the blastocyst stage.

Embryonic development of buffalo oocytes under two levels of oxygen tension

The results indicated that there were no significant differences between experimental groups (BCB+, BCB-, and control) neither in cleavage rate nor in the rates of morula and transferable embryos developed under the two oxygen levels. However, the cleavage, morula, blastocyst and transferable embryos rates were greater in BCB+ under low than high oxygen level (Table 1). Moreover, blastocyst rate was significantly increased in the control group compared to BCB- oocyte group when cultured under low and high oxygen tension (Table 1 and figure 4). The improvement of development rates of preimplantation embryos of BCB+ group under low compared to high oxygen tension may reflect enhanced biological processes controlling early development. Indeed, embryo development rate was enhanced when the intracellular mitochondrial activity was increased under low level of oxygen (Ma et al., 2017; Belli et al., 2019). Noteworthy, High oxygen tension could cause oxidative stress which directly compromises early embryonic development by disturbing mitochondria (Ma et al., 2017).

The increased blastocyst rate of control group compared to BCB- is due to lower total number of embryo cells and mitochondria content of BCB- group (Fakruzzaman et al., 2013). Selection of COCs based on BCB staining during IVP of bovine indicated improvement of blastocyst development rate, quality, activity of mitochondria and increased expression of pregnancy associated candidate genes in BCB+ compared to BCB- group (Fakruzzaman et al., 2013). This indicates that the increased development rate of BCB+ embryos under low oxygen tension is due to its inherent intracellular potential.

Table 1. Cleavage rate, morula rate, blastocyst rate and transferable embryos rate of buffalo COCs either selected with BCB staining or cultured under low and high oxygen tension

Groups	Oxygen level (5%)				Oxygen level (20%)			
	Cleavage* rate (%)	Morula rate (%)	Blastocyst rate (%)	Transferable embryos** (%)	Cleavage* rate (%)	Morula rate (%)	Blastocyst rate (%)	Transferable embryos** (%)
Control	35.5 \pm 3.7 ^{a***}	7.7 \pm 2.3 ^a	16.9 \pm 2.8 ^a	24.3 \pm 3.4 ^a	31.6 \pm 3.9 ^a	6.1 \pm 2.5 ^a	12.0 \pm 2.9 ^a	17.9 \pm 3.6 ^a
BCB+	43.6 \pm 3.9 ^a	14.9 \pm 2.5 ^a	14.1 \pm 2.9 ^{ab}	28.4 \pm 3.6 ^a	33.5 \pm 3.9 ^a	7.1 \pm 2.5 ^a	11.6 \pm 2.9 ^{ab}	18.8 \pm 3.6 ^a
BCB-	30.3 \pm 3.6 ^a	12.1 \pm 2.2 ^a	7.6 \pm 2.6 ^b	18.9 \pm 3.2 ^a	34.5 \pm 3.9 ^a	9.0 \pm 2.5 ^a	9.3 \pm 2.9 ^b	17.1 \pm 3.6 ^a

Values with different superscripts across treatments within the same column indicate significant differences ($P \leq 0.05$). *Cleavage rate= Numbers of oocytes cleaved/Numbers of oocytes inseminated; **Transferable embryos (Day 7): numbers of morula and blastocyst embryos; ***Least Square Means \pm Standard Error; BCB+: oocytes with any degree of blue color in cytoplasm; BCB-: oocytes without any degree of blue color in cytoplasm

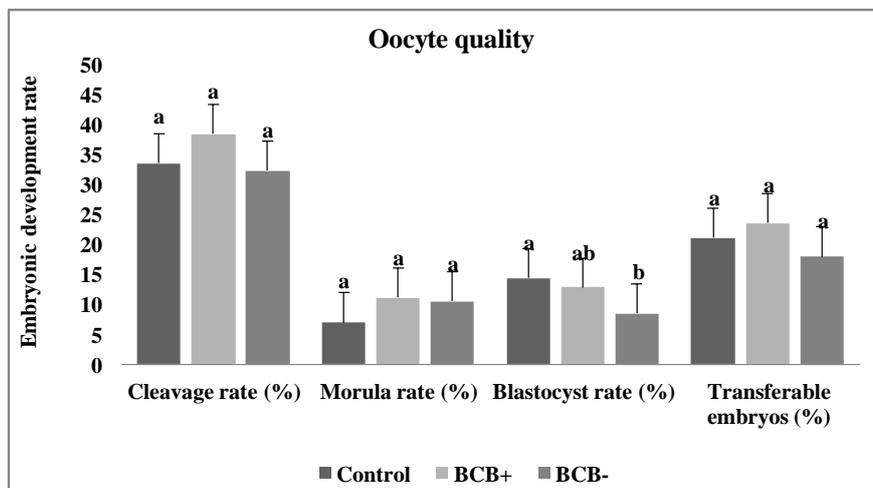


Figure 4. Effect of oocytes brilliant cresyl blue staining on *in vitro* embryonic development of Egyptian buffalo oocytes. Bars with different superscripts (a, b) indicate significant differences ($P \leq 0.05$).

Effect of brilliant cresyl blue staining

The inefficiency of *in vitro* embryo production was associated with oocyte quality at the beginning of maturation (Gasparrini, 2002). Identifying and selecting oocytes with high developmental competence is a critical step towards successful embryo technology (Egerszegi et al., 2010). Many studies used brilliant cresyl blue (BCB) staining tests to monitor and identify oocyte quality for different species such as buffalo and ovine (Manjunatha et al., 2007; Wang et al., 2012).

Previous work has shown that BCB+ oocytes are more competent and yield more blastocysts compared to BCB- oocytes. BCB testing has been shown to enable the selection of larger oocytes with higher levels of *in vitro* maturation and fertilization and an increased percentage of normal fertilized oocytes (Wang et al., 2012). The ability of BCB+ derived oocyte to be fertilized and developed to morula and blastocyst stages was significantly higher than morphologically selected oocytes only (Rodriguez-Gonzalez et al., 2003; Alm et al., 2005; Manjunatha et al., 2007; Silva et al., 2013). Indeed, BCB selects larger oocytes with more mitochondria and activation of the maturation-promoting factor (MPF) (Catalá et al., 2011). On the other side, less competent oocytes (BCB-) displayed fertilization failure or developmental arrest due to delayed mitochondrial DNA (mtDNA) replication attributed to the delayed expression of their nuclear replication factors.

Our results indicated a lower blastocyst rate in BCB- oocytes than control group (Table 2 and figure 4). In agreement with our findings, Wongsrikeao et al. (2006) reported that BCB staining has a negative impact on the cleavage and development of porcine embryos after *in vitro* maturation. Opiela et al. (2008) consider the BCB staining test as questionable because of the lack of significant differences in blastocyst formation between BCB+ and control group. Pawlak et al. (2014) stated that BCB staining test was less successful selection method with high apoptosis incidence and major variations in the diameter of the BCB+ oocyte. In contrast, Manjunatha et al. (2007) stated that before *in vitro* maturation, staining of buffalo oocytes with BCB stain had identified developmentally competent oocytes for IVP. Relative to conventional oocyte selection based solely on morphology, the yield of blastocyst has been significantly improved in BCB+ selected oocytes. Fathi et al. (2017) found that in BCB+ groups, indices of early embryonic development; cleavage, morula and blastocyst stage growth were substantially improved compared to the BCB- and control groups.

Table 2. Effect of oocytes quality on *in vitro* embryonic development of Egyptian buffalo oocytes

Treatments	Cleavage rate* (%)	Morula rate (%)	Blastocyst rate (%)	Transferable embryos** (%)
Control	33.6 ± 2.7 ^{a***}	6.9 ± 1.7 ^a	14.4 ± 2.0 ^a	21.1 ± 2.5 ^a
BCB+	38.5 ± 2.8 ^a	11.1 ± 1.8 ^a	12.8 ± 2.1 ^{ab}	23.6 ± 2.5 ^a
BCB-	32.4 ± 2.7 ^a	10.5 ± 1.7 ^a	8.4 ± 1.9 ^b	18.0 ± 2.4 ^a

Values with different superscripts (a, b) across treatments within one column indicate significant differences ($P \leq 0.05$). *Cleavage rate= Numbers of oocytes cleaved/Numbers of oocytes inseminated; **Transferable embryos (Day 7): numbers of morula and blastocyst embryos; ***Least Square Means ± Standard Error; BCB+: oocytes with any degree of blue color in cytoplasm; BCB-: oocytes without any degree of blue color in cytoplasm

Oxygen concentration

Karagenc et al. (2004) found that high oxygen concentrations (20% in air) decreased the percentage of embryonic development due to increased level of ROS in rodents, swine (Kitagawa et al., 2004; Booth et al., 2005), goats (Batt et al., 1991) and bovine (Takahashi et al., 2000). According to the oxygen tension effect, the rate of morula and transferable

embryos was increased ($P \leq 0.05$) in buffalo COCs developed under low oxygen tension compared to high oxygen tension group (Table 3 and figure 5).

Table 3. Effect of oxygen levels on *in vitro* embryonic development in Egyptian buffalo oocytes

Oxygen level	Cleavage rate* (%)	Morula rate (%)	Blastocyst rate (%)	Transferable embryos** (%)
5%	36.5 ± 2.2 ^{a***}	11.6 ± 1.4 ^a	12.8 ± 1.6 ^a	23.8 ± 1.9 ^a
20%	33.2 ± 2.3 ^a	7.4 ± 1.4 ^b	10.9 ± 1.7 ^a	17.9 ± 2.1 ^b

Values with different superscripts (a, b) across treatments within one column indicate significant differences ($P \leq 0.05$). *Cleavage rate= Numbers of oocytes cleaved/Numbers of oocytes inseminated; **Transferable embryos (Day 7): numbers of morula and blastocyst embryos; ***Least Square Means ± Standard Error; BCB+: oocytes with any degree of blue color in cytoplasm; BCB-: oocytes without any degree of blue color in cytoplasm

However, there were no significant differences in cleavage and blastocyst rates under two oxygen levels. In terms of blastocyst formation, embryo developmental competence was significantly higher in embryos grown under low concentration of oxygen (5% O₂ + 5% CO₂ + 90% N₂) than those developed in atmospheric oxygen (air + 5% CO₂) in bovine (Thompson et al., 1990; Liu and Foote, 1995), mouse (Orsi and Leese, 2001) human (Dumoulin et al., 1999) and porcine (Booth et al., 2005). Similarly, for the development of high-quality ovine blastocysts, high oxygen concentration (20 versus 5% O₂) during IVF was found to be unfavorable (Leoni et al., 2007). Amin et al. (2014) found that there was no significant difference in the cleavage rate between groups. On the other hand, cultivating embryos below 5% oxygen resulted in blastocyst levels significantly higher ($P \leq 0.05$) than those cultivated at 20% oxygen.

The high concentration of O₂ in the culture media can increase certain enzymatic reactions, such as xanthine oxidase and NADPH oxidase activity, leading to increased levels of ROS in embryonic cells (Goto et al., 1993; Lopes et al., 2010). The high level of intracellular ROS increased incidence of DNA damage, lipid peroxidation, and apoptosis, which subsequently reduced embryo development (Takahashi, 2012). The physiological level of oxygen has enhanced embryo development through regulation of development related genes (Rinaudo et al., 2006; Bermejo-Álvarez et al., 2010).

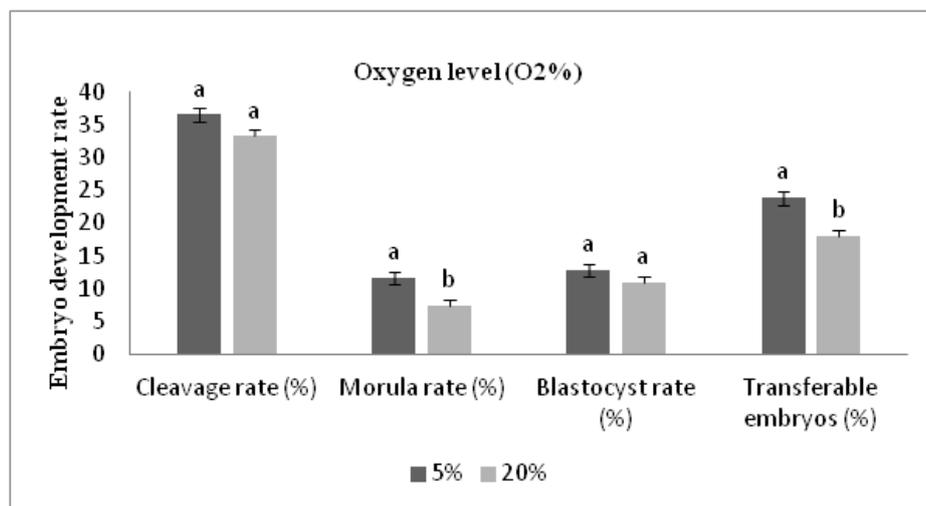


Figure 5. *In vitro* embryonic development of Egyptian buffalo oocytes under two oxygen levels. Bars with different superscripts (a, b) indicate significant differences ($P \leq 0.05$).

CONCLUSION

The results of the present investigation have clearly indicated that preimplantation embryo development is similar in BCB+ and BCB- COCs, which discourages the use of this test in the selection of buffalo COCs. Moreover, transferable buffalo embryos (morula and blastocyst) developed at a higher rate under low oxygen tension (5% O₂). Therefore, it is recommended to culture buffalo pre-implantation embryos at 5% of oxygen during *in vitro* development.

DECLARATIONS

Competing interests

The authors have no competing interests, and we are with respect to this search and in agreement with each other. In addition, we have no conflict with authorship or article publication.

Author`s contribution

All authors have contributed to design of the study, writing and revision of the manuscript. In addition, Dalia Abdel Rahman Ahmed who conducted the laboratory work, analyzed the data wrote the research article. All the authors approved and agreed to publish the manuscript.

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Butanol Fraction of *Kelussia odoratissima* Mozaff Inhibits the Growth of *Leishmania major* Promastigote and Amastigote

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ABSTRACT

Naturally derived compounds have been used to treat several infectious diseases including leishmaniasis. The study aimed to investigate the *in vitro* effects of *Kelussia odoratissima* Mozaff extract on *Leishmania major* promastigote and amastigote. Dried leaves of *K. odoratissima* were fractionated by 3 solvents including aqueous, butanol, and ethyl acetate. The results showed that the butanol fraction of *K. odoratissima* showed the highest anti-*Leishmania* effects against *L. major* promastigotes. Ninety four percent growth inhibition of the promastigote was observed when cells were treated with the 1,280 µg/mL butanol fractions. Moreover, 100% inhibition of amastigotes was detected after treatment with the butanol fraction. Half maximal inhibitory concentration (IC₅₀) of the butanol fraction in promastigotes and amastigotes was 264.1 and 154.1 µg/mL, respectively. The obtained results suggested the potential medicinal benefits of *K. odoratissima* butanol fraction as an alternative treatment for leishmaniasis caused by *L. major* infections.

Key words: Amastigotes, Butanol fraction, *Leishmania major*, *Kelussia odoratissima* Mozaff, Promastigotes

INTRODUCTION

Leishmaniasis is a group of infectious diseases caused by intracellular protozoa parasites of the genus *Leishmania* (Murray et al., 2005). Nowadays, leishmaniasis is prevalent in 98 countries, with up to 350 million people at risk of infection and an estimated 1.5 to 2 million new cases occurring annually (McGwire and Satoskar, 2014). Clinical manifestations of leishmaniasis include mainly cutaneous (CL), mucocutaneous (MCL) and visceral (VL) forms, with symptoms ranging from skin and mucosal to systemic ulceration which, if untreated, can lead to death. Cutaneous leishmaniasis (CL) is caused by *Leishmania major*, *L. tropica*, and *L. aethiopia*. In addition, the infection is usually limited to the skin and lymphatic system (Murray et al., 2005). A recent review focuses on the immune response displayed against cutaneous leishmaniasis, the most common zoonotic infection (Gabriel et al., 2019). *L. major* can infect the immune cells, including macrophages and dendritic cells. The organism has two infective stages of the life cycle, including promastigote and amastigote. Interestingly, the amastigote can survive within the phagosome of macrophages (Murray et al., 2005).

Pentavalent antimony compounds, such as meglumine antimoniate (glucantime®), are being used as a first-line treatment for leishmaniasis (Oliveira et al., 2011). The use of these medications poses a health risk due to their intrinsic toxicity (Sundar and Chakravarty, 2010), especially when systemic therapy should be used for cutaneous leishmaniasis (Palumbo, 2009). A recent study interestingly reported the alarmingly increasing frequency of drug resistance to antimonial compounds in the treatment of *Leishmania* infections (Aït-Oudhia et al., 2011). Therefore, there are ongoing studies to develop and evaluate new medications for the treatment of leishmaniasis (Monge-Maillo and Lopez-Velez, 2013; Serakta et al., 2013).

In an attempt to overcome *Leishmania*-resistant antibiotics, medicinal plants have been used as alternative agents against *Leishmania* infection (Sen and Chatterjee, 2011). *Kelussia odoratissima* Mozaff (wild celery; mountain celery) is a medicinal herb that belongs to the *Umbelliferae* family. This plant grows at high altitudes (more than 2000 meters above sea level). This self-growing plant, growing up to 50-200 cm, is found exclusively in Iran. In spring, the plant is

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harvested from its habitat and presented on the market (Mahmoudi et al., 2014). The essential oil of *K. odoratissima* consists of bioactive components such as coumarins, flavonoids, and phthalides (Ahmadipour et al., 2015). *Kelussia* is one of the most valuable plants used in Iran, and it is traditionally consumed as a medicinal plant to treat analgesic, inflammatory, sedative, cough, and ulcer effects (Ahmadi et al., 2007). This medicinal herb shows the potential to prevent pulmonary hypertension (Ahmadipour et al., 2015). Recent findings confirm that flavonoid and phthalide compounds, as a major part of the plant, have anti-inflammatory, anti-viral, anti-diabetic, anti-cancer, and anti-toxin effects (Salehi et al., 2019). Limited studies have been done to test *K. odoratissima* extracts against *Leishmania* spp.

The aim of this study was therefore to determine *in vitro* anti-*Leishmania* activity of *K. odoratissima* fractions, including aqueous, ethyl acetate, and butanol fractions on promastigote and amastigote of *L. major*.

MATERIALS AND METHODS

Plant sample

The plant sample was purchased in dried form from the local grocery store, Shiraz, Iran in April 2015. It was then dried and stored in the freezer at -18°C. The sample was powdered in a coffee grinder prior to the experiment.

Preparation of crude extracts

To prepare crude extracts of *K. odoratissima*, 1000 g of dried leaves was powdered. The extraction was performed by the percolation method using 95% ethanol with a flow rate of 1 mL/minute for 10 days. After filtration, the solvent was removed using a rotary evaporator. The remaining semi-solid material was then freeze-dried and placed into a sterile glass and stored at -20°C for further use (Dong et al., 2015).

Chlorophyll and oil elimination

The removal of chlorophyll and oil from the total extract was carried out using medium pressure liquid chromatography (MPLC) system, bypassing the sample dissolved in 65% ethanol through a reverse-phase column (RP-18) (Reid and Sarker, 2012). Subsequently, the total extract was concentrated by a rotary evaporator.

Fractionation of the total extract

As a downstream process, the prepared extract was fractionated based on the polarity of its components. Due to high polarity, proteins and carbohydrates were dissolved in the aqueous phase. Moderately polarity flavonoid, glycosides, and saponins dissolved in the butanol phase, and the low polarity terpenoids, phenolic compounds and ketones remained in the ethyl acetate phase. Accordingly, three fractions, namely aqueous, ethyl acetate, and butanol fractions were prepared from the total extract. Fractionation was performed using the liquid-liquid extraction (LLE) method (Tang et al., 2012). Ethyl acetate (100 mL) and distilled water (100 mL) were equally added to 20 g of the total extract. After stirring, the mixture was transferred to a decanter in which two phases formed and the ethyl acetate was separated from the aqueous phase. Once again, 100 mL of ethyl acetate was added to the aqueous phase and then separated. In the next step, 100 mL of butanol was added to the remaining aqueous phase in the decanter. The mixture was well-stirred and the butanol phase separated from the aqueous phase. The last process was repeated as well. Finally, the three extracts were dried and refrigerated until use (Tang et al., 2012).

Parasite and cell culture

The standard strain of *Leishmania major* promastigotes (MRHO/IR/75/ER) was provided by the Department of Parasitology, Isfahan University of Medical Sciences, Isfahan, Iran. The parasite was maintained *in vitro* by repeated subculture in Roswell Park Memorial Institute (RPMI) medium supplemented with 10% fetal bovine serum (FBS), 100 µg/mL streptomycin, 100 IU/mL penicillin at 24°C. Murine macrophage cells (J774-A1) were obtained from Pasteur Institute, Tehran, Iran. Cells were cultured in RPMI-1640 supplemented with 2 mM L-glutamine, 10% FCS, 100 µg/mL streptomycin, and 100 IU/mL penicillin at 37°C under 5% CO₂ (Gupta et al., 2005).

In vitro Anti-*Leishmania* Activity

Anti-Promastigote Assay

Approximately 1×10^6 promastigotes, which were in their early stationary phase, were incubated at 24°C. A separate stock solution was provided for each of the three fractions: aqueous, butanol and ethyl acetate. These extracts were diluted with dimethyl sulfoxide (DMSO) and transferred to Eppendorf tubes to reach final concentrations of 40, 80, 160, 320, 640, 1000 and 1280 µg/mL. Moreover, the following groups were considered for each fraction: a positive control with amphotericin B and the parasite at a concentration of 1 µg/mL, a negative control (culture medium with promastigotes and DMSO), and a blank (culture medium). After 24, 48, 72 hours of incubation, promastigotes detached from each tube and the final number of viable parasites (with 0.4% of viable trypan blue considered) was counted with a hemocytometer. The experiments were repeated three times and performed twice for each experiment.

Anti-Amastigote Assay

For this purpose, at first 2 cm² cover slips were placed in the wells of 6-chamber slides (Lab-Tek, Nalge Nunc International, NY, USA). In the final step, 200 µL of macrophage cells (J774-A1) (10⁵/mL) were added to each well and incubated at 37°C in 5% CO₂ for 2 hours. Then, 200 µL (10⁶/mL) of promastigotes were added to the murine macrophages (10:1) and incubated for 24 hours. Free parasites were removed by washing the wells with RPMI-1640 medium and the infected macrophages treated with 50 µL of various concentrations (40-1280 µg/mL) of aqueous extract at 37°C in 5% CO₂ for 48 hours (Gupta et al. 2005). Finally, Giemsa stained methanol-fixed dried slides were studied under a light microscope (Olympus BX41TF, New York, USA). Moreover, macrophages containing amastigotes without extracts and parasites and macrophages with extracts and parasites were considered as positive and negative controls, respectively. Macrophages were infected with the amastigote cells. The infected macrophages were treated with different concentrations of each fraction and incubated for 48 hours. The inhibitory activity was investigated and compared with the control. Anti-*Leishmania* activity of the three extracts were measured by the amastigote count in every 100 macrophages examined and comparing them with those obtained in the positive control. Each experiment was performed triplicate, with two replicates for each. Moreover, two other extract fractions (ethyl acetate and butanol) of *K. odoratissima* M. were used for this study according to the method mentioned above.

Statistical analysis

The IC₅₀ was calculated and the graphs plotted using SigmaPlot™13 (Systat Software Inc, CA, USA). The percentage of growth inhibition (% GI) used for the calculated IC₅₀, was obtained with respect to growth control as follows (Tunc et al., 2015).

$$\text{Growth inhibition (\%)} = [1 - (\text{Growth}_{\text{treatment}} / \text{Growth}_{\text{control}})] \times 100$$

RESULTS AND DISCUSSION

The anti-*Leishmania* activity of 3 fractions including aqueous, ethyl acetate and butanol of *K. odoratissima* against *L. major* promastigote and amastigote was determined. It was observed that the 3 fractions significantly inhibited the growth of *L. major* in a concentration and time-dependent manner (P<0.001). Growth inhibition of 94% and 69% of promastigotes was inhibited when cells were treated with the fractions for 72 hours, respectively. The butanol fraction of *K. odoratissima* showed the highest anti-*Leishmania* effects against *L. major* promastigotes (figure 1C), compared to the aqueous fraction (figure 1A) and the ethyl acetate fraction (Figure 1B). Growth inhibition of 94% and 69% of promastigotes was found to be inhibited when cells were treated with the 1,280 µg/mL butanol and ethyl acetate fractions for 72 hours, respectively. However, the aqueous fraction slightly suppressed the growth of *L. major*.

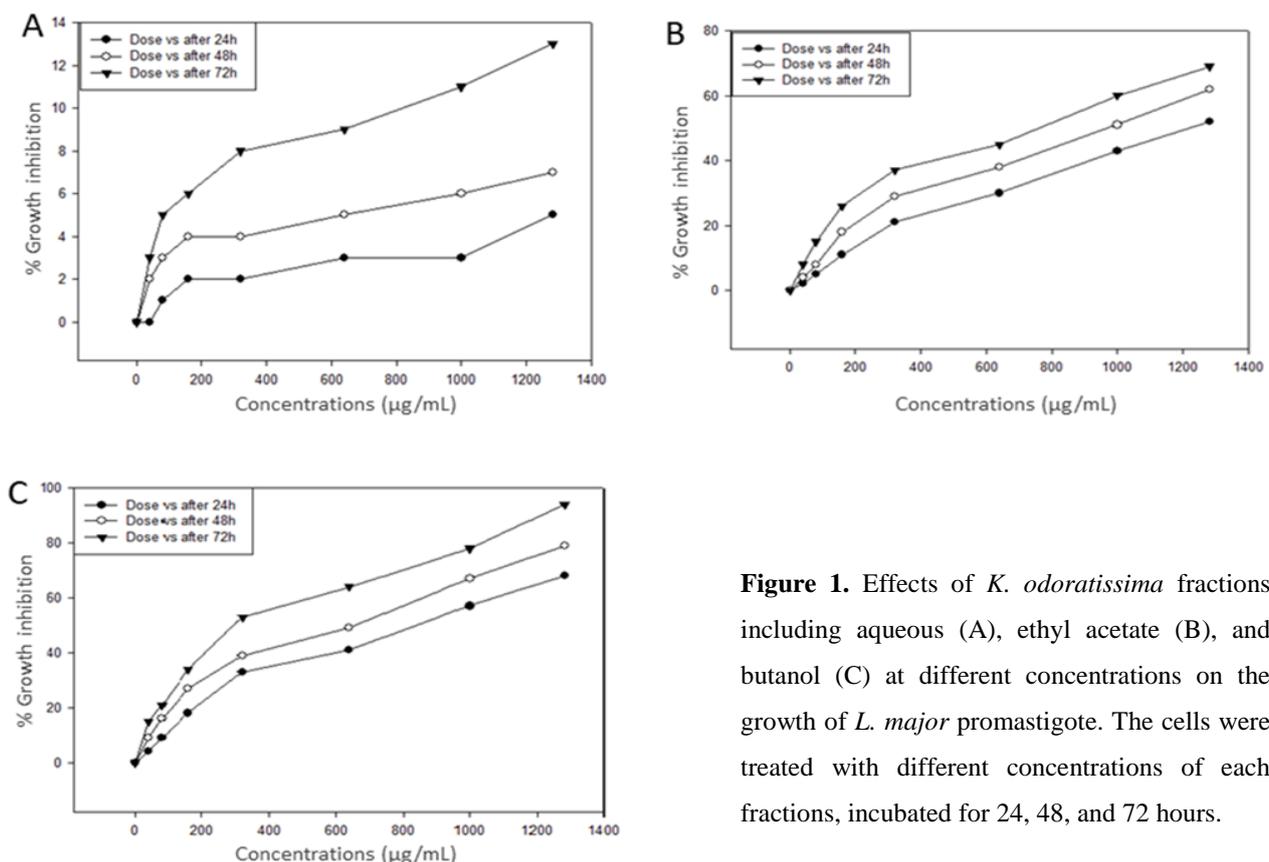


Figure 1. Effects of *K. odoratissima* fractions including aqueous (A), ethyl acetate (B), and butanol (C) at different concentrations on the growth of *L. major* promastigote. The cells were treated with different concentrations of each fractions, incubated for 24, 48, and 72 hours.

At 72 hours, half-maximal inhibitory concentration (IC₅₀) of the ethyl acetate and butanol fractions on the promastigotes was 341.56 and 264.1 µg/mL (table 1), respectively. However, the IC₅₀ of aqueous extract against promastigotes could not be calculated due to the percentage inhibition of growth that was remarkably low for 72 hours incubation.

The butanol fraction of plant species demonstrated the highest anti-*Leishmania* effects against *L. major* amastigotes, followed by ethyl acetate and aqueous fraction. At 48 hours, a 100% inhibition of amastigote cell growth was detected after the treatment with the 1,280 µg/mL butanol compared to the control (Figure 2). In addition, 89% and 51% inhibition of amastigote growth were inhibited when cells were treated with ethyl acetate and aqueous fractions at 1280 µg/mL for 48 hours, respectively. As shown in table 2, the IC₅₀ of the aqueous, ethyl acetate, and the butanol fractions on the amastigotes were 5.5.8, 221.7, and 154.1 µg/mL, respectively.

Table 1. IC₅₀ of different *K. odoratissima* fractions against *L. major* promastigote

Time	IC ₅₀ (µg/mL) with 95% confidence		
	Aqueous	Ethyl acetate	Butanol
After 24 hours	-	530.68	449.75
After 48 hours	-	444.33	418.94
After 72 hours	-	341.56	264.1

Table 2. IC₅₀ of different *K. odoratissima* fractions against *L. major* amastigote

Time	IC ₅₀ (µg/mL) with 95% confidence		
	Aqueous	Ethyl acetate	Butanol
After 48 hours	515.8	221.7	154.1

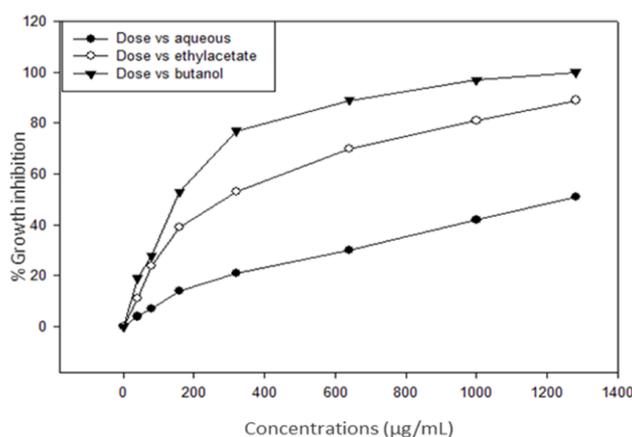


Figure 2. Effects of *K. odoratissima* fractions including aqueous, ethyl acetate, and butanol at different concentrations on the growth of *L. major* amastigote.

Leishmaniasis is one of the most dangerous neglected parasitic diseases, not currently known an effective drug or vaccine to ensure effective immunity against this scourge (Ghorbani et al., 2018). Traditional evidence has been indicated the use of natural plant products may be useful for specific medical conditions (Ghorbani et al., 2018). Historically, many oils and plant extracts have been used for control bacterial, fungal and parasitic diseases (Akthar et al., 2014).

In the present study, the anti-*Leishmania* effect of different fractions of *Kelussia odoratissima* Mozaff on *Leishmania major* was investigated. The butanol fraction was found to have the highest anti-*Leishmania* activity against promastigotes and amastigotes, followed by the ethyl acetate fraction. However, no clear anti-*Leishmania* effects of the aqueous fraction on *L. major* promastigote were observed during the incubation periods. It indicates that the aqueous fraction may not be effective as anti-*Leishmania* compounds. Many studies indicate that several metabolites such as flavonoids, saponins, alkaloids, and some other components in plant extracts have potent anti-*Leishmania* effects (Tasdemir et al., 2006; Shah et al., 2014). Flavonoid compounds have been reported to have antibacterial, antiviral, -larval, -inflammatory, -toxin, -oxidant, and -stress properties. These compounds have also been reported on the treatment properties of diabetes mellitus, ulcerative colitis, cancer, hyperlipidemia, and tranquilizer (Ahmadi et al., 2007). Anti-bacterial and antiviral activities of *K. odoratissima* mediated by flavonoids and phthalides in this medicinal plant were reported (Surai, 2014).

In a study, 105 flavonoid components reported a strong anti-*Leishmania* (Tasdemir et al., 2006). While, another study has shown that Propolis has an inhibitory effect on parasite proliferation (Duran et al., 2008), and this property may be due to the presence of large amounts of flavonoids (Silva et al., 2008). Flavonoid is considered the main chemical compound of the plant (Ahmadi et al., 2007) and its anti-*Leishmania* effect may be related to the flavonoid compound. In the literature review, it can be inferred that many plants have moderate to strong activity against *Leishmania* spp. by different mechanisms of action (Rodrigues et al., 2014). Another study also revealed that plant

extracts can also be used as adjuvants in vaccination against *Leishmania* spp. (Kaur et al., 2014). Recently, secondary metabolites from *Cyathus cf. crassimurus* have been reported anti-*Leishmania* activity against both promastigote and amastigote cells of *L. mexicana* (Mbekeani et al., 2019). Interestingly, *Urtica dioica* extract demonstrated *in vivo* anti-*Leishmania* activity against zoonotic cutaneous leishmaniasis in BALB/c mice. It has been reported that Th1 cells were activated throughout *U. dioica* treatment resulting in cytokine activation (Alireza et al., 2020). Therefore, the anti-*Leishmania* effect of this plant, together with its tissue repair effect, made it a promising candidate for the treatment of cutaneous leishmaniasis caused by *L. major*.

CONCLUSION

The present study shows the potent anti-*Leishmania* effects of *K. odoratissima* on *L. major* promastigote and amastigote. The butanol fraction of *K. odoratissima* showed the highest anti-*Leishmania* effects against both *L. major* promastigotes and amastigotes. The present findings highlight that 100% inhibition of *L. major* amastigotes was detected after treatment with the butanol fraction. Hence, it suggests the potential medicinal benefits of *K. odoratissima* butanol fraction as an alternative treatment of leishmaniasis caused by *L. major* infections. The *in vitro* examinations are in the preliminary step to prove the efficacy and safety of medicinal plants and a comprehensive study involving HPLC, metabolomics, and nanotechnology is recommended to further validate these potent compounds for application in the treatment of some diseases such as leishmaniasis.

DECLARATIONS

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Competing interests

The authors have not declared any conflict of interest.

Author`s contribution

Farzaneh Mirzaei, Mohammadali Nilforoushzadeh, and Amir Maleksabet designed the study. Mohsen Hosseini supervised the laboratory procedures. Farzaneh Mirzaei and Sayed Hossein Hejazi performed the experiments. Manuscript preparation and the statistical analysis were conducted by Roghayeh Norouzi, Abolghasem Siyadatpanah, Watcharapong Mitsuan, Veeranoot Nissapatorn and Maria de Lourdes Pereira. All authors have read and approved the final manuscript.

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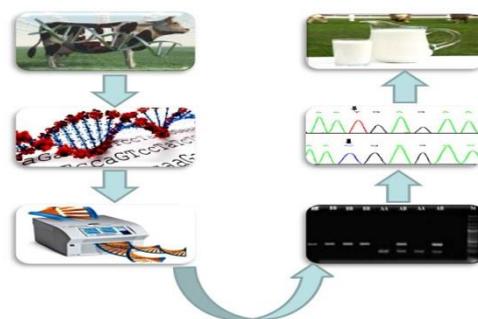
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5. Names of authors and title of journals, published in non-latin alphabets should be transliterated in English.
6. A sample of standard reference is " 1th Author surname A, 2th Author surname B , 3th Author surname C. 2013. Article title should be regular and 7 pt . *World Vet. J.*, Add No. of Volume (Issue No.): 00-00."
7. The color of [references in the text](#) of article is [dark blue](#). Example: ([Preziosi et al., 2002](#); [Mills et al., 2015](#)).
8. At least 35% of the references of any submitted manuscript (for all types of article) should include scientific results published in the last five years.

-Examples (at the text- blue highlighted)

Abayomi (2000), Agindotan et al. (2003), (Kelebeni, 1983), (Usman and Smith, 1992), (Chege, 1998; Chukwura, 1987a,b; Tijani, 1993,1995), (Kumasi et al., 2001).

--Examples (at References section)

a) For journal:

Lucy MC (2000). Regulation of ovarian follicular growth by somatotropin and insulin- like growth factors in cattle. *Journal of Dairy Science*, 83: 1635-1647. DOI: XXX

Kareem SK (2001). Response of albino rats to dietary level of mango cake. *Journal of Agricultural Research and Development*. pp 31-38. DOI: XXX

Chikere CB, Omoni VT and Chikere BO (2008). Distribution of potential nosocomial pathogens in a hospital environment. *African Journal of Biotechnology*. 7: 3535-3539. DOI: XX

b) For symposia reports and abstracts:

Cruz EM, Almatar S, Aludul EK and Al-Yaqaout A (2000). Preliminary Studies on the Performance and Feeding Behaviour of Silver Pomfret (*Pampus argentens euphrasen*) Fingerlings fed with Commercial Feed and Reared in Fibreglass Tanks. *Asian Fisheries Society Manila, Philippine* 13: 191-199. [Link](#)

c) For edited symposia, special issues, etc., published in a journal:

Korevaar H (1992). The nitrogen balance on intensive Dutch dairy farms: a review. In: A. A. Jongebreur et al. (Editors), *Effects of Cattle and Pig Production Systems on the Environment: Livestock Production Science*, 31: 17-27. [Link](#)

d) For books:

AOAC (1990). *Association of Official Analytical Chemists. Official Methods of Analysis, 15th Edition*. Washington D.C. pp. 69-88. [Link](#)

Pelczar JR, Harley JP, Klein DA (1993). *Microbiology: Concepts and Applications*. McGraw-Hill Inc., New York, pp. 591-603. [Link](#)

e) Books, containing sections written by different authors:

Kuney M (1979). Pig Fattening. In: A. Alexiev (Editor), *Farm Animal Feeding*. Vol. III. Feeding of Different Animal Species, Zemizdat, Sofia, p. 233-243 (Bg). [Link](#)

In referring to a personal communication the two words are followed by the year, e.g. (Brown, J. M., personal communication, 1982). In this case initials are given in the text.

Nomenclature and Abbreviations

Nomenclature should follow that given in NCBI web page and Chemical Abstracts. Standard abbreviations are preferable. If a new abbreviation is used, it should be defined at its first usage. Abbreviations should be presented in one paragraph, in the format: "term: definition". Please separate the items by ";".

E.g. ANN: artificial neural network; CFS: closed form solution...

Abbreviations of units should conform to those shown below:

Decilitre	dl	Kilogram	kg
Milligram	mg	hours	h
Micrometer	mm	Minutes	min
Molar	mol/L	Mililitre	ml
Percent	%		

Other abbreviations and symbols should follow the recommendations on units, symbols and abbreviations: in "A guide for Biological and Medical Editors and Authors (The Royal Society of Medicine London 1977).

Papers that have not been published should be cited as "unpublished". Papers that have been accepted for publication, but not yet specified for an issue should be cited as "to be published". Papers that have been submitted for publication should be cited as "submitted for publication".

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3. For simple fractions, use the solidus (/), e.g. 10 /38.
4. Equations should be presented into parentheses on the right-hand side, in tandem.
5. Levels of statistical significance which can be used without further explanations are *P < 0.05, **P < 0.01, and ***P < 0.001
6. In the English articles, a decimal point should be used instead of a decimal comma.
7. In chemical formulae, valence of ions should be given, e.g. Ca²⁺ and CO₃⁻, not as Ca⁺⁺ or CO₃.
8. Numbers up to 10 should be written in the text by words. Numbers above 1000 are recommended to be given as 10 powered x.
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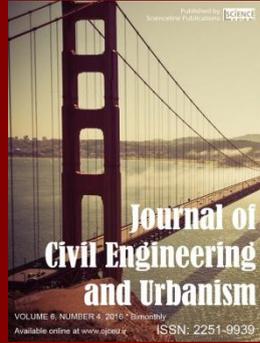
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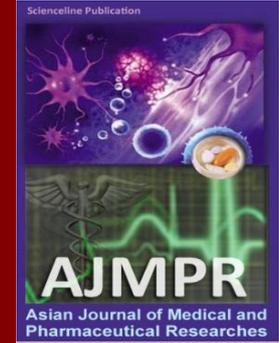
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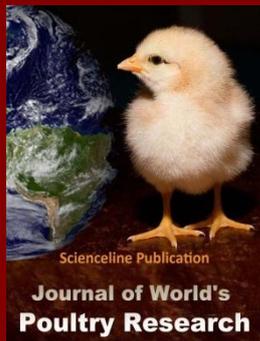
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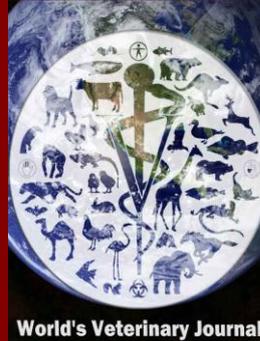
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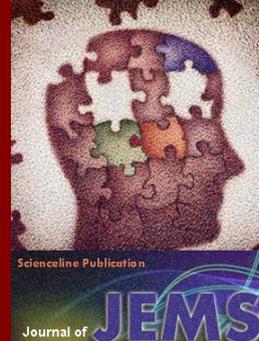
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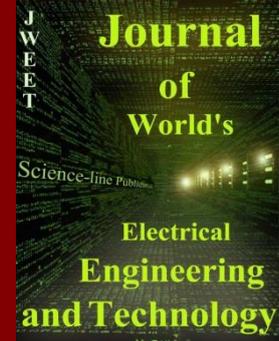
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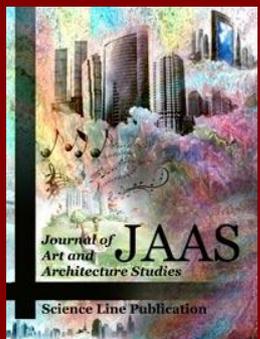
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