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RESEARCH ARTICLE

Antitheilerial Efficacy of Juglone, Buparvaquone and Oxytetracycline against Tropical Theileriosis in Naturally Infected Crossbred Cattle

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A B S T R A C T

Antitheilerial resistance and treatment failure are growing international concerns. Against this backdrop, the first study aimed to compare the efficacy of Juglone (a naturally occurring naphthoquinone) and buparvaquone against bovine tropical theileriosis during natural infection. A total of 60 adult cattle (2-4 years) were selected and divided into five groups, comprising 15 animals each. The animals of Group-I were administered two doses of buparvaquone (BPQ) at a dose rate of 2.5mg/kg body weight intramuscular, 48 hours apart. The cattle of Group-II were administered with BPQ and oxytetracycline (OXY) at the dose rate of 2.5 mg/kg (two doses at 48-hour intervals) and 20 mg/kg intramuscular 3 doses at 48-hour intervals, respectively. While infected animals of Group-III were treated with Juglone (JUG) at a dose rate of 0.48 mg/kg IV on alternate days for 7 days. Whereas, Group-IV acted as a healthy control. The hemogram revealed normocytic normochromic anemia in infected animals. A significant decrease of neutrophils along with decrease of total leukocytes and lymphocytes was noticed in recovered animals from all treated groups (Group I to III). After 21 days, the results of the therapeutic trial showed the highest efficacy of Group II (BPQ+OXY) (93.3%; 14/15), followed by Group III (JUG) (73.3%) and Group I (BPQ) (66.7%; 10/15) groups, three weeks after treatment. It can be concluded that BPQ+OXY had the highest efficacy; whereas the efficacy of Juglone is also comparable, this can be used as an alternative treatment against tropical theileriosis.

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INTRODUCTION

Theileriosis is an imperative tick-associated hemoprotozoan disease caused by the genus *Theileria*, responsible for considerable economic losses to livestock all over the world including Pakistan (Ben Said *et al.*, 2018; Rashid *et al.*, 2018; Zaman *et al*., 2022; Atif *et al*., 2022; Aslam *et al.*, 2023). Theileriosis, caused by *Theileria (T.) annulata* is known as tropical theileriosis, which is one of the most important piroplasm infecting bovines (Rashid *et al.*, 2018; El Damaty *et al*., 2022). It has been estimated that tropical theileriosis causes a 22-38% loss of milk production and a US $ 18,743.76 loss at the farm level (Gharbi *et al*., 2011; Rashid *et al.*, 2018). The imported and crossbred cattle are mainly affected while indigenous cattle are relatively resistant or less susceptible to infection (Amira *et al.*, 2018). The clinical disease initiates with fever, followed by restlessness, anorexia, anemia, swelling of the lymph node, accelerated respiratory and pulse rate, and sudden drop in milk production, followed by hemoglobinuria and death (Ben Said *et al.*, 2018; Ganie *et al*., 2019; Ullah *et al*., 2021). Anemia increases at the final stage and jaundice may occur (Ananda *et al.*, 2009).

Treatment is imperative because of its high morbidity and prolonged recuperative period (Hacılarlıoglu *et al*., 2023). Earlier various drugs have been tried against tropical theileriosis, such as buparvaquone (BPQ), a 2nd

generation hydroxynaphthoquinone, which is considered as a drug of choice against schizonts and piroplasms of *T. annulata* (Nene and Morrison 2016; Hines *et al.*, 2020). The efficacy of BPQ can be enhanced by combination with oxytetracycline; daily intravenous administration for 3 days. This has been proven effective against *T. annulata* schizonts during the acute phase of infection (Yousef *et al*., 2020a). Juglone (5-hydroxy-1,4-naphthaquinone), is a naturally occurring naphthoquinone found in the leaves, bark, roots, nut-hulls and wood of black walnut (*Juglans mandshurica maxim*) (Botanical Dermatology Database, 2021; Luan *et al.*, 2021). Juglone is a natural product and relatively safe. One study in dogs observed no adverse changes in heart rate, blood pressure and electrocardiogram. Nevertheless, histological examination revealed some effects on cell membranes leading to increased capillary permeability (Boelkins *et al*., 1968). It is evident that Juglone has a similar mode of action as buparvaquone which is the drug of choice for *T. annulata* (Marsolier *et al.,* 2015; Marsolier *et al.,* 2019; Gharbi *et al.,* 2020; Medjkane and Weitzman, 2020).

An emerging trend in buparvaquone resistance and treatment failure against Theileria is a growing concern in various countries like Iran (Sharifiyazdi *et al*., 2012), Egypt (Yousef *et al*., 2020b), Sudan (Chatanga *et al*., 2019; Salim *et al*., 2019) and Tunisia (Mhadhbi *et al*., 2015). Several molecular studies revealed point mutations in the cytochrome *b* gene, resulting in an alteration of amino acid sequence leading to buparvaquone resistance and treatment failure (Chatanga *et al.,* 2019, Salim *et al*., 2019; Yousef *et al*., 2020b). It is important to mention that juglone was only tested in *vitro* against *T. annulata*; it had never been tested in *vivo*. Keeping in view the above-mentioned scenario and limited antitheilerial drugs; it is imperative to search for other treatment options. The aim of this study was to compare the efficacy of juglone and buparvaquone during natural clinical infection in cattle under field conditions.

MATERIALS AND METHODS

Therapeutic study: Crossbred cattle presented at Veterinary clinics in and around Jhang City with anorexia, dullness, swelling of lymph nodes, anemia, fever (104-106^{o}F), and tick infested (Hyalomma) with packed cell volume (15.79-16.36%) and parasitized erythrocytes (16.2-17.35%) were included in the study. After screening, a total of 60 non pregnant crossbred Holstein Friesian cows (2-4 years) ranging in weight from 237-328 kg were selected (Holstein Friesian x Non-descriptive). The selected animals were divided into four groups, comprising 15 animals each. The confirmation of disease status and screening of selected animals were made based on clinical signs, peripheral blood smear (Atif *et al*., 2012), percent parasitized erythrocytes (Coetzee and Apley, 2006), and PCR positivity (Bilgic *et al.*, 2010).

The animals of Group-I (BPQ) were treated with buparvaquone (Butalex, ICI Pakistan LTD) at a dose rate of 2.5 mg/kg body weight intramuscular, 48 hours apart (Yousef *et al*., 2020a). The cattle of Group-II (BPQ+OXY) were administered with buparvaquone and oxytetracycline (Oxtra LA, Fatro, Fedagro) at a dose rate of 2.5 mg/kg body weight intramuscular, repeated after 48 hours and 20 mg/kg intramuscular, 3 doses with 48 hours interval, respectively (Yousef *et al*., 2020a). While infected animals of Group-III (JUG) were treated with Juglone (Sigma Aldrich®; CAS No. 481-39-0) @ 0.48 mg/kg IV on alternate days for 7 days. Whereas Group-IV acted as a healthy non-treated control. The powder of Juglone was immersed in a dimethyl sulphoxide (DMSO) solution. The solution was stored in airtight screw cap vials and kept in a desiccator until further used.

Evaluation of acute toxicity: Acute toxicity was evaluated according to the Organization of Economic Co-Operation and Development (OECD-423) guidelines available at https://ntp.niehs.nih.gov/iccvam/suppdocs/feddocs/oecd/oecd_gl423.pdf. For this purpose, twenty Swiss albino mice of either sex (25-30g) were used in 4 different groups. The mice were administered with a prepared solution of Juglone (Sigma-Aldrich®, MO, USA) at the dose rates of 1, 3, 4, and 5 mg/kg body weight via intraperitoneal injection. The animals were initially observed for 24 hours, then for the next 14 days for the manifestation of toxic effects and death. The major toxic effects listed for recording were convulsions, agility, and muscular tremors.

Monitoring the success of treatment trial: Hematological parameters: Percent parasitized erythrocytes (PPE), pack cell volume (PCV), hemoglobin (Hb), total erythrocyte count (TEC), Differential leukocyte count (DLC), and total leukocyte count (TLC) were measured as per the protocol described by Benjamin (1987), before treatment (on day zero) and posttreatment (7^{th}, 14 and 21^{st} days). The animals with significant improvement in clinical signs and significant reduction in parasitemia level were considered as cured.

The percent parasitized erythrocytes were calculated using the formula (Coetzee and Apley (2006) after examining 1000 erythrocytes in five different microscopic fields (Neelam *et al*., 2017).

$$PPE = \frac{\text{No. of infected cells}}{\text{Total no. of cells counted}} \times 100$$

Blood smear examination: Five milliliters of blood sample was collected through jugular venipuncture from each cattle following aseptic conditions in a sterilized syringe in duplicate using a 16-gauge needle. The collected blood samples were transferred immediately into EDTA-coated vacutainers. A drop of blood was added to a clean glass slide and a thin blood smear was prepared as described by Benjamin (1987). The blood smears were fixed with absolute methanol for 30 seconds and stained with freshly prepared diluted Giemsa stain (5%) for 30 minutes. The smears were rinsed 3-4 times with tap water to remove extra stain and then air dried (Benjamin 1987). The blood smear slides were examined under oil immersion at 1000X magnification. The Theileria parasites were identified as described by the OIE manual (WOAH, 2020). Twenty microscopic fields were examined for the selected pathogen.

PCR: The genomic DNA was extracted with a DNA extraction kit (catalog No. K0782) by following the protocol as defined by the manufacturer (Thermo Scientific, USA). The amplification of specific genomic region of *T. annulata* was achieved by targeting the cytochrome b gene, utilizing Cytob1 (F- ACTTTGGCC GTAATGTTAAAC and R- CTCTGGACCAACTG TTTGG) primers in a PCR, as mentioned by (Bilgic *et al*., 2010). The reaction was achieved in an automatic thermal cycler and incorporated initial denaturation at 94°C for 3 minutes, followed by denaturation cycles (35) at 95°C for 50sec, annealing of primers at 55°C (50sec), extension of primers at 72°C (1min) and concluding extension occurred at 72°C (10 min). The PCR products were held at 4°C or stored at -40°C. A 1.5% agarose gel was used for electrophoresis from the extracted DNA and the gel was visualized with a UV illuminator.

Statistical analysis: The hematological parameters of therapeutic trials were compared using the t-test between the diseased animals and non-treated control as well. The standard error was calculated using MS Excel. The P-values <0.05 were considered as significant.

RESULTS

The current study outlined the treatment of *T. annulata*. All enrolled cattle were tick infested and manifested with clinical signs of anorexia, dullness, nasal discharge, accelerated respiratory and pulse rate, sudden drop in milk production, swelling of lymph nodes, anemia with temperature ranging from 104-106ºF, and black diarrhea. The Giemsa-stained blood smear microscopy revealed piroplasms. The percent parasitized erythrocytes ranged from 16-17% and 0.2-0.4% before and after treatment, respectively. While the animals of the negative control group tested negative.

Evaluation of toxicity: To evaluate the toxicity and dose, the ultrasonic extract of Juglone was dissolved in DMSO solution and administered at 1, 3, 4, and 5 mg/kg body weight (BW) in a set of five mice per group. It was observed that the highest and tolerable dose was 3 mg/kg body weight. There were no toxicities, but restlessness and itching were observed at the dose rate of 4 mg/kg while death was noticed in the group administered with a dose of 5mg/kg of Juglone extract. The dose administered in this study for therapeutic evaluation was 0.48 mg/kg body weight, which was six times smaller than the highest tolerable dose and this was safe in cattle. It was perceived that the highest tolerable dose was 3 mg/kg body weight. There was no toxicity, but restlessness and itching were observed at the dose rate of 4 mg/kg. Death of two out of five mice was noticed in the group administered with 5mg/kg of juglone extract. The dose administered in this study for therapeutic evaluation was 0.48 mg/kg body weight, which was six times smaller than the highest tolerable dose and proved safe.

Therapeutic study: In the current study, hemoglobin, PCV, and TEC were significantly lowered (P<0.05) in infected compared to healthy cattle, the decrease in these parameters resulted in anemia. The current study divulged a significant increase (P<0.05) in total leukocytes, neutrophils and lymphocytes. Whereas a non-significant increase of eosinophils and monocytes was noticed in infected animals. The animals of Group-I were treated with Butalex @ 2.5mg/kg BW intramuscular at two doses, 48 hours apart. It was revealed that 10 animals out of 15 diseased animals were recovered completely after the 21st day of treatment and indicated 66.7% recovery rate (Table 1) with closer to normal clinical and hematological parameters (Hb, 8.76± 0.26; PCV 22.89 ± 0.09; TLC 6.40 ± 0.16; neutrophil 55.03 ± 0.47; eosinophil 2.73 ± 0.21; lymphocytes 37.10 ± 0.11; monocytes 7.00 ± 0.32; TEC 5.13 ± 0.14; PPE 0.4 ± 0.00). The cattle of Group-II treated with buparvaquone @ 2.5mg/kg BW plus oxytetracycline @ 20 mg/kg showed 93.3% efficacy on the 21st post-treatment day with the recovery of 14 animals out of 15 and expressed hematological parameters close to normal (Table 2). Whereas, the animals of Group-III treated with juglone @ 0.48 mg/kg BW on alternate days (IV), showed 73.3% efficacy on the 21st day of treatment as evidenced by the recovery of 73.3% (11/15) (Fig. 1-2). There were significant differences among all experimental groups. Diarrhea was observed in three animals 4-5 hours after injection of Juglone, but animals recovered without any anti-diarrheal treatment (Table 2).

DISCUSSION

Treatment of tropical theileriosis is imperative due to higher morbidity, long treatment, cost, production losses, and mortality. High mortality is linked to the ability of *T. annulata* to stimulate uncontrolled proliferation of infected leukocytes, consequently leading to severe cell destruction and death (Woods *et al*., 2021). This is the first study to conduct a chemotherapeutic trial in Pakistan to use an herbal drug, Juglone during a field clinical trial. Juglone has medicinal significance as it generates numerous *in vitro* and *in vivo* pharmacological properties including anti-inflammatory, antimicrobial, antioxidant, analgesic, anti-angiogenic, inhibiting Pin1, against endothelial dysfunction and anticancer (Marsolier *et al.,* 2015; Amira *et al.,* 2018; Marsolier *et al.,* 2019; Luan *et al.,* 2021).

Clinical signs observed during the clinical trial was anorexia, fever, anemia, swelling of lymph node, nasal discharge, accelerated respiratory and pulse rate, sudden drop in milk production, and 7% of animals expressed hemoglobinuria and tarry-like diarrhea. Anorexia was due to persistent fever whereas enlarged lymph nodes were noticed due to lymphoid hyperplasia in the acute stage of the disease. In the current study, hemoglobin, PCV, and TEC were significantly lowered in infected animals compared to healthy cattle. The decrease in these parameters resulted in anemia (Sarma *et al.,* 2016). The immune-mediated mechanism results in phagocytosis of schizont-infected RBCs (Abubakar *et al.,* 2019). The normochromic effect of anemia was noticed in diseased cattle due to toxic metabolites that affect bone marrow for erythropoiesis (Sarma *et al.,* 2016). The present study revealed a decrease in leukocytes; these changes are the indication of a defensive response and invasion of infected leucocytes in the lymphoid organs (Yousef *et al.,* 2020a). The increase in lymphocytes and monocytes are

Table 1: Summary of treatment response and recovery percentage.

Groups	Day 0	Day 7	Day 14	Day 21	Recovered/Total	Recovery (%)
BPQ*	0	3	7	10	10/15	66.7
BPQ+OXY**	0	6	11	14	14/15	93.3
Juglone	0	5	8	11	11/15	73.3

Table 2: Hematological parameters of *T. annulata* infection in cattle before and after treatment.

Drug	Parameters	0 day (Before treatment)	7th day	14th day	21th day	Healthy control
		Before treatment	Post-treatment			
BPQ**	Hb (g/dl)	5.37 ± 0.47	6.19 ± 0.40*	8.14± 0.27*	8.76± 0.26*	9.71 ± 0.22
	PCV (%)	16.31 ± 0.25	19.46 ± 0.40*	21.37 ± 0.24*	22.89 ± 0.09*	29.45 ± 0.02
	TEC (x 10^6/µl)	3.11 ± 0.09	3.88 ± 0.22*	4.57 ± 0.20*	5.13 ± 0.14*	6.55 ± 0.17
	TLC (x 10^3/µl)	10.80 ± 0.15	5.98 ± 0.24*	6.35 ± 0.21*	6.40 ± 0.16*	7.51 ± 0.15
	DLC (x 10^3/µl)					
	N	5.11 ± 0.82	3.02 ± 0.59*	3.49 ± 0.54*	3.54 ± 0.49*	4.82 ± 0.26
	E	0.34 ± 0.19	0.18 ± 0.04*	0.18 ± 0.05*	0.17 ± 0.04*	0.17 ± 0.02
	L	5.31 ± 0.71	2.61 ± 0.31*	2.51 ± 0.28*	2.37 ± 0.26*	2.32 ± 0.18
	M	0.79 ± 0.15	0.43 ± 0.09*	0.45 ± 0.04*	0.45 ± 0.03*	0.39 ± 0.01
	PPE (%)	16.20 ± 0.06	1.50 ± 0.04*	0.50 ± 0.01*	0.4 ± 0.00*	0.00 ± 0.00
BPQ+OXY**	Hb (g/dl)	4.92 ± 0.51	7.40 ± 0.35*	8.81± 0.29*	9.74± 0.24*	9.71 ± 0.22
	PCV (%)	15.79 ± 0.24	21.39 ± 0.25*	28.60 ± 0.17*	29.26 ± 0.06*	29.45 ± 0.02
	TEC (x 10^6/µl)	2.75 ± 0.14	3.56 ± 0.05*	4.86 ± 0.05*	6.09 ± 0.04*	6.55 ± 0.17
	TLC (x 10^3/µl)	11.00 ± 0.17	5.50 ± 0.21*	7.18 ± 0.21*	7.35 ± 0.16*	7.51 ± 0.15
	DLC (x 10^3/µl)					
	N	5.11 ± 0.82	2.98 ± 0.60*	4.22 ± 0.75*	4.50 ± 0.76*	4.82 ± 0.26
	E	0.41 ± 0.21	0.20 ± 0.05*	0.26 ± 0.06*	0.26 ± 0.03*	0.17 ± 0.02
	L	5.45 ± 0.84	2.14 ± 0.21*	2.62 ± 0.33*	2.50 ± 0.25*	2.32 ± 0.18
	M	0.81 ± 0.12	0.40 ± 0.07*	0.51 ± 0.08*	0.50 ± 0.05*	0.39 ± 0.01
	PPE (%)	17.35 ± 0.07	0.60 ± 0.01*	0.30 ± 0.01*	0.20 ± 0.01*	0.00 ± 0.00
Juglone**	Hb	5.23 ± 0.46	7.14 ± 0.34*	8.73 ± 0.27*	9.38 ± 0.25*	9.71 ± 0.22
	PCV	16.36 ± 0.22	20.38 ± 0.32*	25.26 ± 0.25*	26.33 ± 0.06*	29.45 ± 0.02
	TEC (x 10^6/ul)	2.90 ± 0.07	4.18 ± 0.03*	4.98 ± 0.03*	5.87 ± 0.12*	6.55 ± 0.17
	TLC (x 10^3/µl)	10.45 ± 0.30	5.18 ± 0.22*	6.74 ± 0.16*	7.23 ± 0.17*	7.51 ± 0.15
	DLC (x 10^3/µl)					
	N	4.90 ± 0.67	2.70 ± 0.59*	4.00 ± 0.64*	4.49 ± 0.69*	4.82 ± 0.26
	E	0.40 ± 0.32	0.19 ± 0.04*	0.25 ± 0.07*	0.27 ± 0.04*	0.17 ± 0.02
	L	5.02 ± 0.76	2.16 ± 0.09*	2.54 ± 0.34*	2.55 ± 0.23*	2.32 ± 0.18
	M	0.77 ± 0.08	0.38 ± 0.06*	0.49 ± 0.07*	0.50 ± 0.03*	0.39 ± 0.01
	PPE (%)	16.28 ± 0.05	0.70 ± 0.03*	0.30 ± 0.02*	0.30 ± 0.00*	0.00 ± 0.00

BPQ. Buparvaquone; OXY, oxytetracycline, Hb, hemoglobin; PCV, Packed cell volume; TLC, Total leukocyte count; DLC, Differential leukocyte count; N, neutrophil; E, Eosinophil; L, Lymphocytes; M, monocytes; TEC, total erythrocytes; PPE percent parasitized erythrocytes. Significant difference between time intervals* and significant difference between groups** (P<0.05).

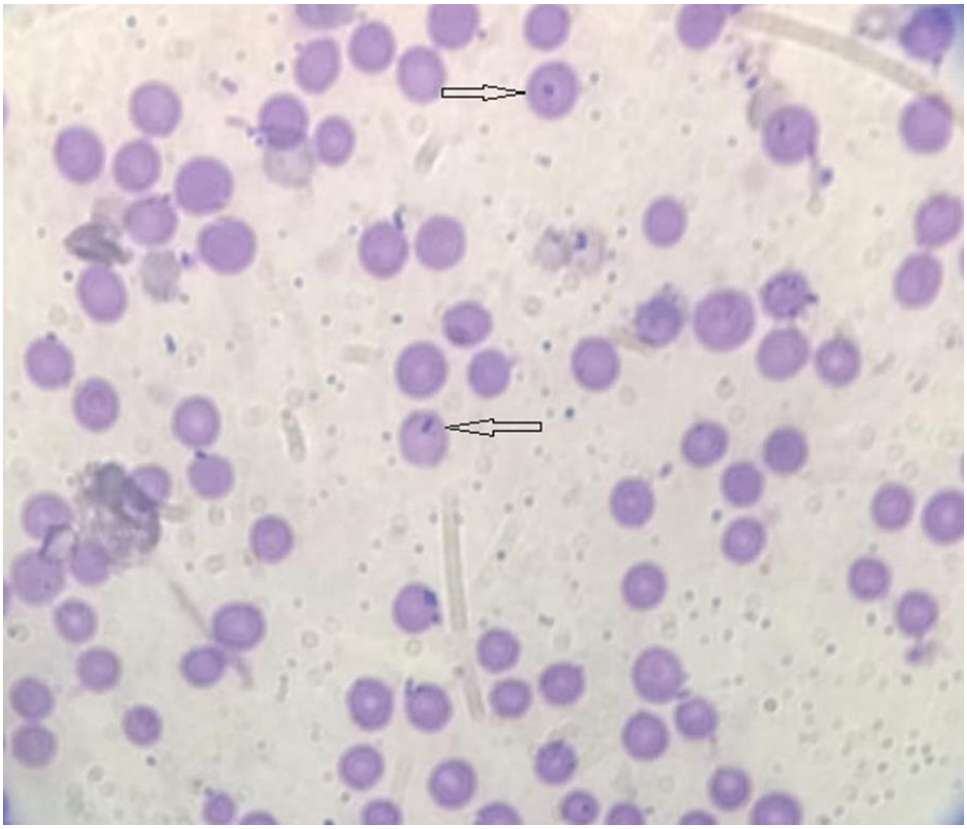

Fig. 1: *Theileria annulata* piroplasms in blood smear of a crossbred calf.

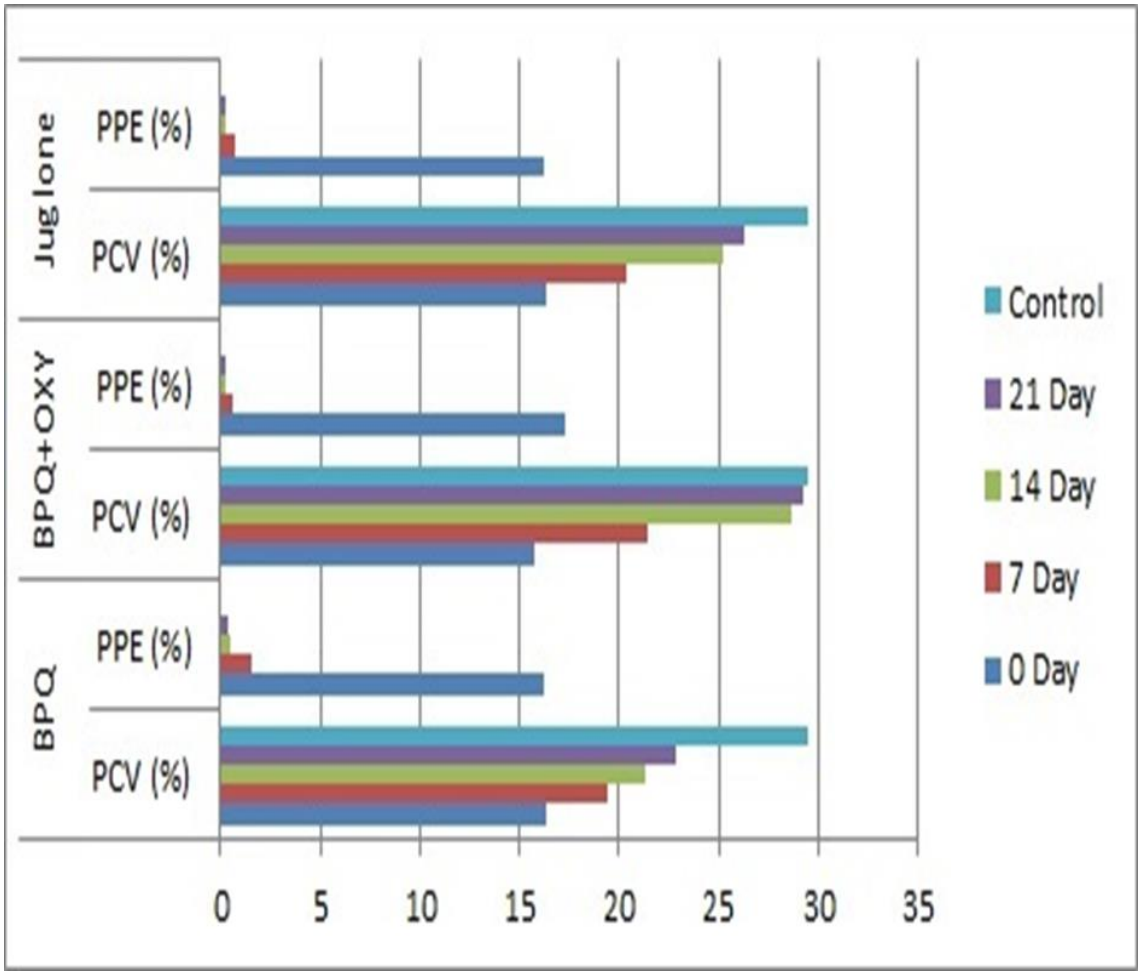

Fig. 2: Comparative efficacy of Juglone, buparvaquone with oxytetracycline against tropical theileriosis in crossbred cattle. PPE (percent parasitized erythrocytes); PCV (Packed Cell Volume).

the indication of a compensatory mechanism against invaded protozoa. Furthermore, earlier studies were partially in accordance to our results, they mentioned a significant decrease in neutrophils and an increase of leukocytes, lymphocytes, monocytes, and eosinophils as compared to healthy controls (Sarma *et al.,* 2016; Yousef *et al.,* 2020a). Leukopenia might be due to the degree of the destruction of white blood cells by the Theileria parasite. Nevertheless, neutropenia results due to the production of toxic metabolites by *T. annulata* infection in hemopoietic system especially in the bone marrow (Mbassa *et al*., 1994; Al-Emarah *et al*., 2012).

In this study, we observed that the combination of oxytetracycline and buparvaquone was the most efficient drugs against tropical theileriosis. A single dose of buparvaquone at the dose rate of 2.5 mg/kg IM is the drug of choice for bovine tropical theileriosis but this drug is costly and resource-limited farmers cannot afford. Our results are in agreement with previous studies, as they reported successful treatment with buparvaquone against acute theileriosis in cows (Azhahianambi *et al*., 2021), but in chronic cases, no response to the drug was seen. Likewise, Mbwambo *et al.* (2006) documented that imminent treatment was effective after observing the clinical signs. Furthermore, Nagar and colleagues depicted that the combination of buparvaquone and oxytetracycline had an 86.66-100% cure rate against theileriosis in cattle (Nagar *et al*., 2020).

In the current investigation, the successful treatment was achieved by Juglone against clinical theileriosis. due to the inhibition ability of peptidyl-prolyl isomerases of *Theileria* parasite (TaPin1). Therefore, Juglone eliminate the theilerial parasites through their anti-protozoal, anti-oxidant and immunomodulatory properties (Marsolier *et al*., 2015). There is an emergence of some resistant strains of *T. annulata*, which represent a momentous danger for livestock production in enzootic countries. Specific point mutations in isolates of *T. annulata* lead to buparvaquone resistance due to their exclusive association with resistant *T. annulata* isolates (Chatanga *et al.,* 2019). In the absence of an alternative to naphthoquinones, it was crucial to generate novel research for the development of new theilericide drugs using other biological products such as juglone (Juglone (5-hydroxyl-1,4-naphthoquinone), a phenolic compound present in walnuts.

Conclusions: Our findings on the therapeutic trial concluded that a combination of buparvaquone and oxytetracycline showed the highest activity. At the same time, Juglone along with oxytetracycline can be a promising alternative to naphthoquinone. The information may assist in filling the knowledge gaps about treatment failure. However, further studies should be conducted to evaluate its efficacy at variable dosages.

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Authors contribution: FAA and MUN conceived the idea, wrote the original draft, and utilized software. MUN and SM contributed to formal analysis. FAA, TR, SM and IA were involved in visualization, writing review and editing. FAA involved in acquisition of funding, provision of resources and supervision.

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