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Anti-trypanosomal activities of polyherbal formulation of *Lawsonia inermis* and *Azadiractha indica* when combined with diminazene aceturate

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Abstract
Trypanosomosis is a disease complex affecting both human and animals. Chemotherapeutic approach to trypanosomosis is no longer befitting due to side effects associated with drugs and development of many resistant trypanosome worldwide. This study evaluated the polyherbal trypanocidal activities of *Azadiractha indica* and *Lawsonia inermis* when combined with diaminazene aceturate. Forty-five Wistar rats were randomly divided into nine groups. Groups: 1 (control), 2 (infected untreated), 3 (Diminazene), 4 (*Lawsonia inermis* (LI)), 5 (*Azadiractha indica* (AI)), 6 (LI+AI), 7 (LI+DA), 8 (LI+AI) and 9 (DA+LI+AI). All rats in groups (2-9) were infected with $3x10^6$ of *Trypanosoma brucei* per ml of blood. Result showed that both methanolic extract of *Azadiractha indica* and *Lawsonia inermis* contains important phytochemicals. Increased weight was observed in rats dosed with *L. inermis*+diminazene combination (6.3%) while *A. indica* treated rats presented significant ($P<0.05$) decreased weight. Survivability rate increased in most treated groups without noticeable relapse while 60% mortality was seen in group 8. Parasites were cleared within shortest time in the group treated with three combinations when compared to all other treatment groups. Haematological parameters were not significant ($P>0.05$) in most treatment groups compared to untreated control. This study concluded that both extracts reduced the level of parasitaemia in a transient phase and drug-extract combination cleared the parasitaemia within shortest time.

Keywords: *Azadiractha indica*, *Lawsonia inermis*, *T. brucei* and wistar rats

1. Introduction
Trypanosomosis is a disease complex caused by protozoan trypanosomes of the genus Trypanosoma that are transmitted by multiple variants of the tsetse fly (*Glossina* spp). It affects both human and animals. Approximately 50,000 people in 36 countries of Sub-Saharan Africa have African Trypanosomosis, which is caused by either *Trypanosoma brucei gambiense* or *Trypanosoma b1rucei rhodesiense* [1]. The modern history of this disease revolves around the identification of the causative organisms, mode of transmission, and therapeutic methods of controlling the disease. Reports have shown that the disease can be controlled chemically and biologically but not completely eradicated [3]. Human African Trypanosomosis (HAT) known as sleeping sickness is predominant among the rural populace in sub-Sahara Africa. The chronic form of the disease that occurs in West and Central Africa accounts for over 97% of current cases [4]. African Animal Trypanosomosis (Nagana) is usually caused by three of the available species of Trypanosomosis namely; *T. congolense*, *T. vivax*, and *T. brucei*. Reports have shown that all domestic animals are affected and the symptoms shown are fever, listlessness, emaciation, hair loss, ocular discharge, oedema, paralysis, and anaemia. The name ”N’gana” is a Zulu word that means “powerless/useless” which was derived from the eventuality of the disease as the animal becomes weak and unfit for work as the disease progresses [3]. In Nigeria, Trypanosomosis is a widespread disease of livestock and a major constraint to the economy of rural areas [5]. The increase in the costs of acquiring chemical fertilizers, agrochemicals, animal feeds, and synthetic drugs in developing countries like Nigeria, with a great increase in debt and acute poverty, has exerted enormous

pressure to explore local resources to combat deficits and improve the quality of the life of the people and that of animals [6]. Additionally, the use of conventional chemotherapeutic treatment of Trypanosomosis is no longer appropriate due to unwanted side effects associated with the drugs and the development of many resistant trypanosomes in some parts of the world [7]. *Azadiractha indica* Linn is widely known for its cold-pressed seed oil, mainly used as an insecticide, but can also serve cosmetic, medicinal and agricultural uses [8]. Biologically it has munificent bioactive ingredients with diverse applications and these bioactive constituents are known to have arrays of pharmacological activities [6]. *Lawsonia inermis* Linn on the other hand, is known for its medicinal and cosmetic activities. It is used on hair to prevent lice and dandruff, treatment of liver and digestive disorders, reduction of leprosy tissue loss, diabetic foot, and ulcers [9]. Studies in Nigeria have shown that Trypanosoma infection is endemic in many parts of the country [10]. Reports on various livestock diseases occurring in many parts of Africa showed that trypanosomes are the major threat facing livestock production leading to huge economic losses [11]. Estimated losses in agricultural production due to trypanosomes is about three billion American dollars annually [12]. The only means to combat the menaces is through effective usage of drugs like chemotherapy and chemoprophylaxis. Chemotherapy is faced with problems such as limited choice of trypanocidal drugs, high cost, toxicity, and the emergence of drug-resistance trypanosome strains that have been reported [1]. No new trypanocidal drug has been introduced into the field since the 1960(s) [13]. This is partly because of cost involved in discovering and bringing new drugs to the market. This situation is due to huge capital required in developing new trypanocidal drugs which is usually not encouraging in countries where the political leaders did not have the will [14]. In the absence of new drugs, it is deemed necessary by several researchers to formulate new treatment protocols to improve the efficacy of existing drugs. The goals have been to elucidate the pharmacokinetics of trypanocides; formulate inexpensive, non-toxic, easy to administer chemotherapeutic drugs.

2. Material and Methods

2.1 Drug

A 2.36g sachet of Diminazene aceturate DA (Aether Centre® Beijing, China) was administered at 7mg/kg in the quadriceps using 23-gauge syringe and needle.

2.2 Plant Authentication and Preparation

The methanolic extract of *Azadiractha indica* and *Lawsonia inermis* were gotten ready made from the Department of Veterinary Pharmacology and Toxicology, University of Ilorin, Ilorin, Nigeria.

2.3 Ethics, Experimental Animals and Study design

Ethical approval was sought from Ethical committee on Animal use and care, University of Ilorin, Nigeria with approval code number, UERC/FVM/2021/020. Forty-five Adult Wistar rats weighing between 120-140 g obtained from the Laboratory animal unit of department of biochemistry University of Ilorin were used for the study. The rats were housed in clean cages in fly-proof house at room temperature and fed with standard commercial pelletized feed (Chikun feeds, Chikun Nig. Ltd) and provided with clean water ad libitum. The rats were allowed to acclimatize to the laboratory environment for one week before the experiment commenced. Rats were grouped into nine groups of five rats each and the extracts were administered through the oral route using oral gavage.

Each group was treated as follows: (n = 5 rats)

Group 1: (Negative control) was uninfected and was untreated.

Group 2: (Positive control) was infected but was not treated.

Group 3: Was treated only with Diminazene aceturate at 7mg/kg intra-peritoneally.

Group 4: *Azadirachtha indica* (AI) extract was administered orally at 200mg/kg daily for 14 days and DA was administered at 7mg/kg after infection.

Group 5: *Lawsonia inermis* extract was administered orally at 200mg/kg daily for 14 days and DA was administered at 7mg /kg after infection.

Group 6: *Azadirachtha indica* (AI) extract only was administered at 200mg/kg daily for 14 days.

Group 7: *Lawsonia inermis* was administered orally at 200mg/kg daily for 14 days.

Group 8: *Azadirachtha indica* (AI) extract and Henna extract were both administered orally at 200mg/kg daily for 14 days.

Group 9: *Azadirachtha indica* (AI) extract and *L. inermis* extract were both administered orally at 200mg/kg daily for 14 days and DA was administered at 7mg/kg after infection.

2.4 Trypanosoma Stock and Inoculation

Trypanosoma brucei was used in this study and was procured from Nigeria institute for Trypanosomosis and Onchocerciasis research institute, Vom, Plateau state, Nigeria. The dose of inoculum was estimated to be $3x10^6$ of *Trypanosoma brucei* per ml of blood using the "Rapid Matching method" described by Herbert and Lumsden (1976). The parasites were maintained by serial passage in rats. To infect the rat, 1ml of blood was collected from heavily infected rat and then mixed with 2ml of normal saline. The mixture was viewed under microscope at X40 magnification. The blood containing the parasites was then injected into rats intraperitoneally**.**

2.5 Weight measurement

The weights of the rats were monitored from first day and later on weekly basis using automated electronic scale (Sensor Disc Technology, London). To weigh a rat, a round plastic container was placed on the scale and tarred to zero following which the rat was dropped inside the container and subsequently weighed.

2.6 Parasitaemia Assessment and Prepatent period

The presence of parasites in the blood of infected rats was monitored from the second day post inoculation. Estimation of parasitaemia was conducted using the method Herbert and Lumsden (1976) [15]. A number of field (10-15) of each drop blood or incubated media and parasites in triplicate were counted using glass slides under inverted microscope (X400). An average mean trypanosome count was taken as number of trypanosomes per field.

2.7 Data analysis
All data obtained from the study were expressed as mean ± SD. The differences between the groups were analysed by one-way analysis of variance (ANOVA) followed by Dunnett's post-hoc multiple comparison test using GraphPad Prism statistical package, San Diego, California, U.S.A (www.Graphpad.Com). Statistical estimates were made at confidence interval of 95%. Probability Values less or equal to 0.05 ($P<0.05$) were considered significant.

3. Results
3.1 Phytochemical analysis
Phytochemical analysis of methanolic extract of *Azadiractha indica* Linn and *Lawsonia inermis* Linn are shown in table (1) and (2) below revealing presence of various Phyto-constituents

Table 1: Phytochemical constituent of (*Azadiractha indica* Linn)

Component	Test	Observation	Scoring
Alkaloid	Dragendorffs	Brownish-red colour	+
Tannins	Ferric chloride	Deep red colour	+
Flavonoid	Pew's	Red colour	+
Saponin	Frothing	Persistence foam	+
Glycosides	Salkowski's	Reddish brown	+

*The phytochemical constituents of *A. indica* Linn leaves.

Table 2: Result of Phytochemical screening of *Lawsonia inermis* Linn

Test	Crude Methanol Extract
Saponins	++ve
Tannins	++ve
Flavonoids	++ve
Cardiac glycosides	++ve
Terpenoids	+ve
Steroids	+ve
Anthraquinones	+ve
Alkaloids	+ve

Interpretations-ve: Absent, +ve: Present, ++ve: Abundantly present

3.2 Weight gain
Table (3) showed the percentage weight changes in *T. brucei* infected rats treated with methanolic extract of *A. indica* Linn, *Lawsonia inermis* Linn leaves, diminazene and diminazene-extract combination for 14 days. All values are expressed in mean ± standard deviation. Percentage weight gain of rats in the group dosed with *L. inermis* + diminazene combination increased (6.3%) when compared to untreated control. Diminazene alone treated group showed a considerably weight gain (8.7%) after day 14 when compared with other treatment group and the negative control. *A. indica* treated group presented significant ($P<0.05$) decreased in weight after 14days treatment when compared to all other treatment groups and the two control groups. *L. inermis*+ *A. indica* group showed a significant ($P<0.05$) decreased weight when compared to untreated control. All treatment groups showed improved weight after 14days treatment when compared with untreated control (Positive) as stated in table [3].

Table 3: Weight gain of *T. brucei* infected rats treated with methanol extract of *Azadiractha indica, Lawsonia inermis* and diminazene aceturate

Days/Group	Day 0	Day 7	Day 14
Negative control	178±18.3	185±19.4 (3.7%)	191.0±21.6 (6.5%)
Positive control	170.4±5.68	174±8.701 (2.1%)	147.0±16.08* (-15.9%)
Diminazene (DA)	160.6±19.5	167±22.2 (4.1%)	175±27.2 (8.7%)
L. inermis (LI)	156±14.5	158±17.5 (1.4%)	149±23* (-4.9)
A. indica (AI)	181.0±21.8	179±21.2 (-1.0%)	151±12.3* (-19.5%)
LI + AI	161±14.9	168±15.5 (4.8%)	138±18.2** (-16.8%)
DA+LI	175.0±6.52	179.0±8.51 (2.2%)	186±9.36 (6.3%)
DA+AI	179±16.9	174±22.9 (-3.2%)	184±31.1 (2.5%)
DA+LI+AI	170±15.4	176±14.5 (3.6%)	174±13.8 (2.3%)

Weight gain, significant from normal * $P<0.05$, ** $P<0.01$

3.3 Relative organ weight
Table (4) showed the percentage relative organ weight in *T. brucei* infected rats treated with methanolic extract of *Azadiractha indica* Linn, *Lawsonia inermis* Linn leaves, diminazene and diminazene-extract combination for 14days. Kidneys of rats treated with *L. inermis*, *Azadiractha indica* and *L. inermis-A. indica* combination increased significantly ($P<0.01$) when compared to both control groups. The weight of the liver did not show any significant alteration when compared to normal control. Spleen gave contrasting result by showing a significant increase ($P<0.001$) relative weight in groups *L. inermis*, *A. indica* and *L. inermis-A. indica* combination when compared to negative control. Just like the above treatment groups, positive untreated control presented a significant increased spleen when compared to other treatment group and untreated control. There is no major alteration in the relative weight of the heart in all the groups except *L. inermis -A. indica* combination that showed significant increased weight. The relative weight of the testes is not altered in all the treatment groups compared to untreated control.

Table 4: Relative organ weight of *T. brucei* infected rats treated with methanol extract of *Azadiractha indica*, *Lawsonia inermis* and diminazene aceturate

Organs /Group	Kidney	Liver	Spleen	Heart	Testes
Negative control	0.49±0.11	3.41±0.74	0.33±0.10	0.33±0.18	1.65±0.44
Positive control	0.67±0.03	3.60±0.70	1.28±0.06***	0.50±0.06	1.83±0.06
Diminazene (DA)	0.55±0.13	3.30±0.34	0.34±0.09	0.33±0.04	1.55±0.07
L. inermis (LI)	0.88±0.17*	3.70±0.61	1.49±0.33***	0.57±0.06	1.72±0.63
A. indica (AI)	0.89±0.13*	4.30±0.18	1.15±0.21**	0.58±0.20	1.90±0.77
LI + AI	0.98±0.17**	4.54±0.47	1.13±0.47**	0.60±0.11*	1.90±0.66
DA+LI	0.62±0.18	2.94±0.39	0.42±0.10	0.35±0.09	1.80±0.08
DA+AI	0.53±0.058	3.02±0.28	0.30±0.10	0.28±0.03	1.80±0.10
DA+LI+AI	0.79±0.17	2.93±0.49	0.40±0.10	0.3±0.00	1.89±0.29

All values are express in mean ± standard deviation
Organ weight significant from normal control, * $P<0.05$, ** $P<0.01$, *** $P<0.001$

3.4 Parasitaemia

The parasites were detectable in the blood of all infected rats following inoculation of *Trypanosoma brucei.* Parasites were detectable by 72hours post infection in all the experimental rats. The level of parasitaemia persistently increased in all groups and reached (25 $X10^8$ trypanosomes/ml). Following treatment, parasites were considerably low in the three combinations (*A. indica*+ *L. inermis*+DA) within 72 hours post treatment. DA+HE p resented a significant decreased parasitaemia just like diminazene after 72hours treatment. However, DA+*A. indica* presented a marked decreased parasitaemia even lower than diminazene treated group after 72hous treatment. 1 week post treatment showed that the three combinations cleared (*A. indica*+ *L. inermis*+DA) the parasites better when compared with the use of diminazene alone. All other treatment group reduced the level of parasitaemia in a transient phase. At day 14, there is a considerable reduced parasitaemia in all the treatment groups. The extract combination with diminazene has cleared the parasitaemia while extract only (*A. indica* and *L. inermis*) significantly reduced the level of parasitaemia (Figure 1).

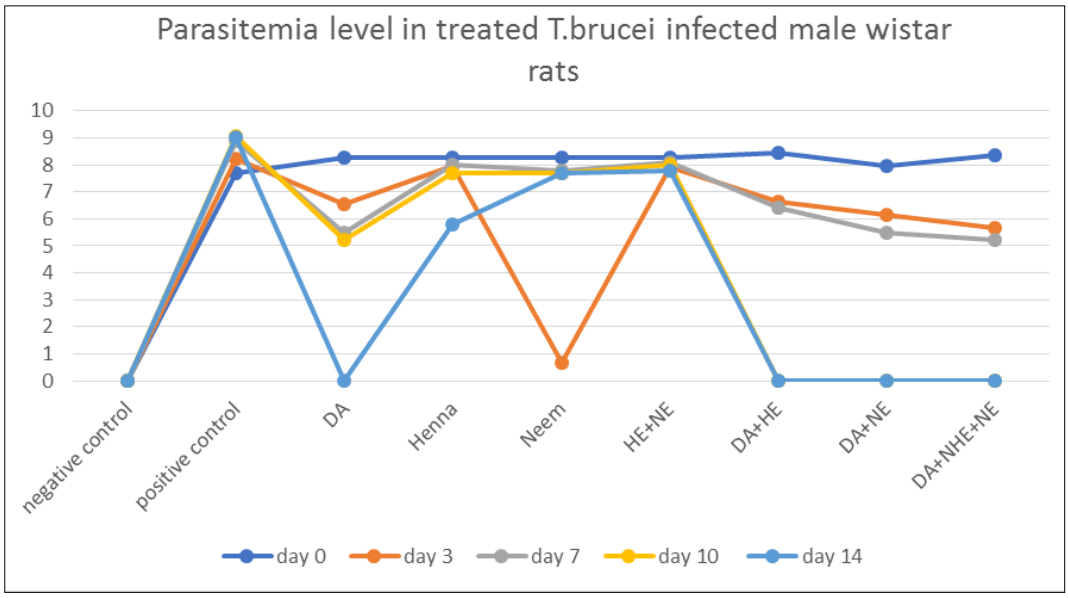

Fig 1: Parasitemia level in treated *T. brucei* infected male Wistar rats

3.5 Survivability and mortality rate

Table (5) below showed the survivability and mortality rate of the rats infected with *T. brucei*. All the rats treated with diminazene aceturate alone, *L. inermis* alone and diminazene-*L. inermis* combination survived till 14days without noticeable relapse until sacrificed. There was 60% mortality (2/5) in group treated with diminazene and *A. indica* combination. The survival rate in *A. indica* alone is about 90% just like the three combinations.

Table 5: Survivability and mortality rate of *T. brucei* infected rats treated with methanol extract of *Azadiractha indica*, *Lawsonia inermis* and diminazene aceturate

Group/Days Rx	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Negative control	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Positive control	0/5	0/5	0/5	0/5	0/5	0/5	¼	0/4	0/4	0/4	0/4	0/4	0/4	0/4
Diminazene (DA)	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
L. inermis (LI)	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
A. indica (AI)	0/5	0/5	0/5	0/5	0/5	0/5	¼	0/4	0/4	0/4	0/4	0/4	0/4	0/4
LI + AI	0/5	0/5	¼	0/4	0/4	0/4	0/4	0/4	0/4	0/4	1/3	0/3	0/3	0/3
DA+LI	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
DA+AI	0/5	3/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2
DA+LI+AI	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	¼	0/4	0/4	0/4	0/4

3.6 Haematology results

The PCV of all treatment groups were within normal range, however, using the uninfected group as a reference, all other groups were relatively low compared to this group but none showed any significant alteration. Reading the leucogram, all groups when compared to untreated control group A had a normal WBC count but group treated with *A. indica* and *A. indica*-*L. inermis* combination presented significant changes ($P<0.01$ and $P<0.05$) respectively. Infected and untreated control groups showed had mild significant increase in neutrophils count ($P<0.05$) when compared to all treatment groups and uninfected control.

4. Discussion

Phytochemical compound present in plant is usually linked to medicinal and pharmacological activities. Some of the bioactive substances that are derived from plants are flavonoid, alkaloid, carotenoids, tannins antioxidants and phenolic compounds. The qualitative phytochemical screening of the methanolic extract of *Azadiractha indica* Linn and *Lawsonia inermis* Linn leaves used in this study indicated the presence of tannins, alkaloid, flavonoid, saponin and glycosides. This conforms with previous reports of Tahiliani and Kar 2000 [16] who ascertain that medicinal plant possesses various phytochemical compounds. Saponin, tannins, flavonoids and cardiac glycosides were abundantly present in both plants. Saponin are often bitter to taste leading to reduce palatability but it is ironically known to enhance nutrient absorption and smooth digestibility in animals [17]. Tannins are plant polyphenol with extensive anti-oxidant activities. Flavonoids possess significant health benefits due their antioxidant activities linked to functional hydroxyl groups that scavenge free radical and chelation of metallic ions [18]. Cardiac glycosides are natural drug with primary effects on the heart inform of benefit (cardiotonic) and toxicity (heart poisons). Cardiac glycoside is beneficial when it increases the force of contraction of the cardiac muscle during arrythmias and cardiac failure [19]. The outcome of percentage weight gain in this study showed that *L. inermis* leaves improve the percentage weight gain after 14days (15.1%) when compared to *A. indica* leaves (0.5%) but the observed weight gain in negative control group improved (26%). This decrease in percentage weight gain may be as a result of presence of saponin and tannins in the two plants which produce anti-nutritive effects thereby reducing feed consumption. This is in accordance with the work of Addisu (2016) [20] that reported that plant containing tannin and saponin reduces feed consumption.

Organomegaly is a sign of disease and abnormality resulting from drugs and other toxic agents. Standard and accepted table defining organomegaly has yet to be established [21]. Relative organ-body weight ratio is an important index of inflammation, atrophy and hypertrophy [22]. The relative organ weight of this study showed significant increase in spleen and kidney of extract treatment groups. Liver, heart, kidneys and testes were not altered when compared with normal control group. This inference showed that two plants may have slight nephrotoxic activities.

This study showed that methanolic extract of *Azadiractha indica* and *Lawsonia inermis* leaves possess trypanocidal activities against *T. brucei* infected rats. This observation is as a result of significant reduction in parasitaemia level in extract treated groups when compared with the untreated positive control. This observation follows the reports of Omoja *et al.* (2011) [23] and Atawodi *et al.* (2003) [24], both confirmed the trypanocidal activities of *Azadiractha indica* and *Lawsonia inermis* respectively. Diminazene-extract combination showed improve efficacy by clearing the parasites within short time (day 5) unlike when use alone (day 7). Diminazene-*L. inermis* combination showed improved synergistic activities improving the weight, survivability rate and decreased mortality.

Diminazene-*A. indica* combination present contrasting synergistic activities because most of the treated rats showed decrease weight, decrease survivability rate and increase mortality. This observation contradicts the report of Omoja *et al.* (2011) [25] that confirmed the synergistic effect of diminazene aceturate and *A. indica*. Observed variation may be solely attributed to the dosage employed by Omoja *et al.* (2011) [25] (diminazene aceturate-7mg/kg and *Azadiractha indica* -125mg/kg) unlike this study where diminazene aceturate-7mg/kg and *Azadiractha indica*-200mg/kg was used.

Anaemia is a common clinical sign in animals infected with Trypanosomosis, however, in this study, putting the parameters of a detailed erythrogram together, i.e., PCV, MCV, MCHC of all the groups, the result signified that all infected groups showed no sign of anaemia, clinically. On the contrary, using the PCV of the negative control group, there is a relative mild decrease of no statistical significance in the PCV of all the groups with diaminazene alone and diaminazene-extract combination (DA+LI+AI) having values that are very close to normal. This is in contrast with the findings of Ngure *et al*. (2009) that said, infected lab animals given *A. indica* extract only have their PCV increased significantly when compared to infected ones given water only which was ascribed to enhanced resistance of erythrocyte haemolysis [26]. Total Leucocyte count of all the treatment groups exhibited non-significant alteration when compared to uninfected control. Varying levels of leucocytosis was seen in *A. indica* and its combination when compared to *L. inermis* and its combination (table 4), this inference implies that *L. inermis* had a better modulatory activity in controlling leucocytosis. Neutrophilia and eosinophilia were seen in all groups with no Diminazene aceturate in their therapy, which indicated that the combination of diminazene aceturate with either or both plants is synergistic in maintaining levels of white blood cells in *T. brucei* infected rats.

5. Conclusion

From the above study, it can be concluded that *Azadiractha indica* and *Lawsonia inermis* possess transient trypanocidal activities against *T. brucei* infected male Wistar rats. The extracts also improve weight gain and survivability rate. There is a good synergistic activity with diminazene aceturate which is a known trypanocidal drug. *Lawsonia inermis* showed better synergistic effect than *Azadiractha indica*. The combination of the two extracts with Diminazene aceturate presented a better effect against *T. brucei*, while the combination of the two extracts alone presented an effect with less efficacy.

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