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IN VIVO AND IN VITRO INVESTIGATION ON EFFECT OF SOLVENT EXTRACT OF Phyllanthus amarus ON Trypanosoma brucei brucei INFECTED RATS AND MICE

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ABSTRACT This study looked at the trypanocidal activity of *Phyllanthus amarus* (P.A) in *Trypanosoma brucei brucei* (T. b. b.) infected rat. Hexane and methanolic extracts of *Phyllanthus amarus* leaf and root were analysed in vitro for trypanocidal activity against *Trypanosoma brucei brucei* at a concentration of 10mg/ml. The methanolic leaf extract was strongly trypanocidal; 5mg/ml of the extract had similar trypanocidal activity with a standard drug (Diminazene aceturate), eliminating parasite motility at 5 minutes. The in-vivo activity of methanolic P.A extract showed that the immediate treatment has the most potent trypanocidal activity at ($P<0.05$); reducing the level of parasitaemia at day 4 when compared with the negative control. Furthermore, the effect of methanolic extract of P.A on T. b. b. infected wistar rats revealed a marked reduction in the hepatic enzymes viz ALT. C (43.18 ± 4.68), D (51.38 ± 3.08) and E (65.08 ± 5.06) when compared with the negative control (65.80 ± 1.57).ALT and ALP showed similar trend. Histological study showed an ameliorative activity of the methanolic *P. amarus* extract in the damaged tissues architecture especially in the hepatic cells caused by *Trypanosoma brucei brucei* infection. Therefore the leaf of *Phyllanthus amarus* should be added to the fodder of cattle and other ruminants as a curative measure against *Trypanosoma brucei brucei*.

KEYWORDS : Phyllanthus amarus, Trypanosoma brucei brucei, trypanocidal activity.

LITERATURE REVIEW

According to the World Health Organization (WHO, 2016). Human African trypanosomiasis, also known as sleeping sickness, is a vector-borne parasitic disease, caused by infection with protozoan parasites belonging to the genus *Trypanosoma*. They are transmitted to humans by tsetse fly (*Glossina* genus) bites which have acquired their infection from human beings or from animals harbouring human pathogenic parasites. Other parasite species and sub-species of the *Trypanosoma* genus are pathogenic to animals and cause animal trypanosomiasis in wild and domestic animals. In cattle the disease is called *Nagana*. Trypanosomiasis in domestic animals, particularly in cattle, is a major obstacle to the economic development of affected rural areas. This disease threatens millions of people in 36 countries in sub-Saharan Africa of which, many of the affected populations lives in remote rural areas with limited access to adequate health services, which complicates the surveillance and therefore the diagnosis and treatment of cases. In addition, displacement of populations, war and poverty are important factors that facilitate transmission. In 1998, almost 40 000 cases were reported, but estimates were that 300 000 cases were undiagnosed and therefore untreated. During the most recent epidemic, the prevalence reached 50% in several villages in Angola, the Democratic Republic of the Congo, and South Sudan. Sleeping sickness was the first or second greatest cause of mortality in those communities, even ahead of HIV/AIDS. But in 2009, after continued control efforts, the number of cases reported dropped below 10 000 (9878) for the first time in 50 years. This decline in number of cases has continued with 3796 new cases reported in 2014, the lowest level since the start of systematic global data-collection 75 years ago. The estimated number of actual cases is below 20 000 and the estimated population at risk is 65 million people.

The disease incidence differs from one country to another as well as in different parts of a single country. In the last 10 years, over 70% of reported cases occurred in the Democratic Republic of the Congo (DRC). The DRC is the only country that currently reports more than 1000 new cases annually and accounts for 85% of the cases reported in 2014 while Central African Republic is the only country that declared between 100 and 200 new cases in 2014. Other countries such as Angola, Burkina Faso, Cameroon, Chad, Congo, Côte d'Ivoire, Gabon, Guinea, Malawi, South Sudan, Uganda, United Republic of Tanzania, Zambia and Zimbabwe are reporting fewer than 100 new cases per year. However, countries like Benin, Botswana, Burundi, Equatorial Guinea, Ethiopia, Gambia, Ghana, Guinea Bissau, Kenya, Liberia, Mali, Mozambique, Namibia, Niger, Nigeria, Rwanda, Senegal, Sierra Leone, Swaziland and Togo have not reported any new cases for over a decade. Transmission of the disease seems to have stopped in some of these countries but there are still some areas where it is difficult to assess the exact situation because the unstable social circumstances and/or difficult accessibility hinder surveillance and diagnostic activities.

Chemotherapy is the most widely used means of controlling trypanosomiasis. The few registered trypanocides are often associated with severe side effects (Abdullahi, *et al*., 2010) and require lengthy parenteral administration, lack efficacy and are unaffordable for most of the patients (Legros, *et al*., 1985). Therefore there is urgent need to source for new, cheap and safe alternative chemotherapy against trypanosomiasis from natural origin.

In African countries where sleeping sickness is endemic, plants have traditionally been used for generations and are still widely used to treat this ailment with possible therapeutic activities, which have not been proved scientifically. The discovery of these potent antitrypanosomal extracts from plants has increased the great potentials of plant species to provide lead compounds for the development of new natural drugs for effective treatment of sleeping sickness (Legros *et al.,* 2002). Therefore, the present study investigated the in vivo and in vitro effect of solvent extract of *phyllanthus amarus* on *trypanosoma brucei brucei* infected rats and

mice.

2.0 MATERIALS AND METHODS

2.1 Plant Material

Fresh leaves of *P. amarus* were collected from Mkar community, Gboko, Benue State Nigeria. They were dried at room temperature until a uniform weight was obtained.

2.2 Preparation of the Plant Extract

Solvent extraction is the method of choice for the extraction. The plant materials, leaves and roots (50g) were subjected to 48 hours of sequential extraction with solvents of diverse polarities (hexane and methanol). Extraction was done first with n-hexane (300ml), followed by methanol for each sample. The extracts obtained were then filtered using Whatman No. 1 filter paper and filtrates concentrated in water bath at 40ºc. The extracts obtained were stored in the refrigerator at 4ºc for further investigation (Appiah, *et al.,* 2011).

2.3 Experimental Animals

Twenty two mice (males and females) weighing between 25-30g for each extract and 35 (male and females) of wistar albino strain weighing 120-150g were used for this study. The animals were obtained from Vector and Parasitological Department, Nigerian Institute for Trypanosomiasis Research (NITR), Kaduna, Nigeria. They were allowed one week acclimatization, after which they were reweighed and kept in well ventilated laboratory cages with 12 hours day and night cycles. The rats were maintained on a commercial poultry feed (Vital feeds) and drinking water *ad libitum*. The experiment protocol was followed as approved by Institutional Animal's Ethics Committee (IAEC) and animals care was taken in accordance with the guidelines of European convention for the protection of Vertebrate animals and other scientific purposes ETS-124 (European Treaty Series, 2005).

2.4 Acute Oral Toxicity Studies (LD_{50})

The acute oral toxicity was conducted using the limit test procedure. Twenty eight mice were grouped into seven groups of four per group. Extract administration was through orogastric intubations with varying doses of 100g, 200g, 500g, 1000g, 2000g, 3000g and 5000mg/Kg body weight of the methanolic leaf extract of *phyllanthus amarus* dissolved in distilled water. Animals were observed for mortality, signs of gross toxicity and behavioural changes (excitability, convulsions, lethargy and sleep) for 24 hours.

2.5 Experimental Design

The research work was organized into 3 phases.

Phase I: In-vitro Determination of Solvent Extracts

Four solvent extracts were examined for the most potent trypanocidal activity (methanolic leaf, methanolic root, hexane leaf and hexane root extracts).

Exactly 10mg of the different solvent extracts were weighed and dissolved in 1.0ml of phosphate buffered saline (PBS). Assessment of the in-vitro trypanocidal activity was performed in triplicates in well microtitre plates. In each well of the microtitre plates, 30µl of each extract was incubated at 37°C, with 50µl of the infected blood obtained from a donor rat with about 10^4 *T. b. b.* per ml of blood. For the control well, the 30µl of extract was replaced with PBS. Parasite count was monitored on a glass slide observed under a microscope at X400 magnification. The Motility of the parasites was observed at 5 minutes interval for 45 minutes.

Phase IIa: Designed to Compare the Potent Solvent Extract with a Standard Drug (Diminazene aceturate) at Different Concentrations.

Serial dilution of the stock solutions of the extract using PBS was done to obtain concentrations, ranging from 2.5 to 10 mg/ml. while for standard drug; 25.3g/150ml concentration of Diminazene aceturate (DA) was used. Assessment of the *in vitro* trypanocidal activity was performed as in **Phase I** above.

Phase IIb: In-vivo Confirmatory Test

The treated blood (in-vitro) of all the groups were inoculated into wistar mice and the level of parasitaemia was monitored daily according to the method described by the Herbert and Lumsden (1976) using blood obtained from the tails under a microscope at X400 for (4days).

Phase III. In-vivo Study

Thirty (30) wistar rats (120-150g) were divided into six (6) groups of five (5) rats per group. Group B-E were intraperitoneally infected with 10^8 *T. b. b.* per 100g body weight. Treatment was carried out for 2 week, after which the animals were sacrificed and blood sample collected for Serum enzymes assay. ALT and AST were assayed using the method of Reitman and Frankel (1957) while ALP was estimated by the principle of Tietz (1995).

Histopathological Examination

The liver, kidney and spleen tissues were dissected out and washed on ice-cold saline immediately. A portion of these tissues were fixed in 10% neutral formal-saline fixation solution for histology study. The procedure described by Bancroft and Stevens (1982). The slides were viewed at magnification of x 400 and the photomicrographs taken.

2.6 Statistical Analysis of Data

Data obtained were expressed as Mean ± standard error of mean and analysed using the statistical package for social scientists (SPSS version 20) where applicable. Values at $P < 0.05$ was regarded as significant in comparison with appropriate controls.

3.0 RESULTS

Acute toxicity study of *phyllanthus amarus* Linn: The administration of *phyllanthus amarus* showed no apparent toxic symptoms or death in all the groups up to the dose of 5000mg/kg body weight as shown in [Table 1].

Table1. *Phyllanthus amarus* Administration at Different Doses for LD_{50} Assessment

Groups	Number of mice	Doses (mg/kg)	Mortality
A	4	100	Nil
B	4	200	Nil
C	4	500	Nil
D	4	1000	Nil
E	4	2000	Nil
F	4	3000	Nil
G	4	5000	Nil

In-vitro Determination of Trypanocidal Activity of Solvent Extract in Blood of Wistar Rats: The solvent extracts showed different degree of potency in motility activity against T. b. b at different time interval at dose of 10mg/ml, with the methanolic leaf extract showing the most potent.[Table 2].

Table 2. In-vitro Determination of Trypanocidal Activity of Solvent Extract in Blood of Wistar Rats.

Extract	5 minutes	15 minutes	25 minutes	35 minutes	45 minutes
Root hexane	||||	||||	|||	|||	||
Leaf hexane	|||	||	||	|	__
Root methanol	||||	|||	||	||	|
Leaf methanol	|	__	__	__	__

Key
|||| - very strongly motile
||| - strongly motile
|| - motile

| - weakly motile
__ - not motile

Comparative In-vitro Examination of the Methanolic Leaf Extract with Diminazene aceturate (DA) in Blood of Wistar Rats:

The extract showed highest activity in a dose dependent fashion eliminating parasite motility at 5 minutes of incubation as also observed in the standard drug (Diminazene aceturate). This is shown in [Table 3].

Table3.Comparative In-vitro Examination of the Methanolic Leaf Extract with Diminazene aceturate (DA) in Blood of Wistar Rats

Treatment	5 minutes	15 minutes	25 minutes	35 minutes	45 minutes
10mg/ml	__	__	__	__	__
5mg/ml	__	__	__	__	__
2.5mg/ml	|||	||		__	__
DA (25.3g/150ml)	__	__	__	__	__
Control	||||	||||	||||	|||	|||

Key
|||| - very strongly motile
||| - strongly motile
|| - motile
| - weakly motile
__ - not motile

Table 4 shows the examination of Parasite in Blood of Infected and Treated Rats. Parasitaemia was established at day 3 of infection in all groups. However, pre-treatment and immediate treatment groups showed significant decrease (P<0.05) in parasitaemia when compared with the negative control. Furthermore, immediate treatment showed the least case of parasitaemia at day 4 when compared with pre-treatment.

Effect of Methanol Leaf Extract of *Phyllanthus amarus* on Serum Enzyme Activity of *Trypanosoma brucei brucei* Infected Wistar rats.

Table 4. **Examination of Parasite in Blood of Infected and Treated Rats**

Groups	Day 1 ($x10^6$ per ml)	Day 2 ($x10^6$ per ml)	Day 3 ($x10^6$ per ml)	Day 4 ($x10^6$ per ml)
A. Negative control	0.00 ± 0.00	0.00 ± 0.00	7.68 ± 1.66	114.86 ± 3.65
B. Pre-treatment	0.00 ± 0.00	0.00 ± 0.00	1.89 ± 0.82*	100.84 ± 5.96
C. Immediate treatment	0.00 ± 0.00	0.00 ± 0.00	0.58 ± 0.36*	8.59 ± 7.38* a
D. Post-treatment	187.43 ± 6.94* a	379.52 ± 7.31* a	1000.00 ± 0.00* a	1000 ± 0.00* a

*Result are expressed in mean ± SEM (n=5); *significant at P<0.05 compared with the Negative control; [a] significant at P<0.05 compared with pre-treatment*

In this work, ALT, AST and ALP activities were used to determine the protective effect of *Phyllanthus amarus* on T. b. b. infected wistar rats and were interpreted as follows;

Statistical evaluation revealed that for ALT levels, the extract recorded a significant (P <0.05) decrease for pre-treatment and immediate treatment groups, although there was no significant decrease in post treatment group; C (43.18 ± 4.68*) D (51.38 ± 3.08*), E (65.08 ± 5.06) when compare with negative control (65.80 ± 1.57). Similar trend were also observed in AST and ALP. See [Table 5].

Table 5. **Effect of Methanol Leaf Extract of *Phyllanthus amarus* on Serum Enzyme Activity of *Trypanosoma brucei brucei* Infected Wistar rats**

Treatment	Enzyme activity		
	ALT (Iu/l)	AST(Iu/l)	ALP(Iu/l)
A. Normal control	26.28 ± 2.80	72.10 ± 9.96	99.00 ± 12.93
B. Negative control	65.80 ± 1.57 [a]	120.86 ± 15.91 [a]	127.66 ± 3.26 [a]
C. Pre treatment	43.18 ± 4.68*[a]	91.06 ± 6.62*a	91.96 ± 9.23*
D.Immediate treatment	51.38 ± 3.08* [a]	105.34 ± 18.96*[a]	102.76 ± 2.12*
E. Post treatment	65.08 ± 5.06*[a]	118.18 ± 3.94[a]	122.18 ± 3.22* [a]
F. Extract	28.78 ± 7.36*	69.06 ± 17.82*	100.14 ± 10.25*

*Result are expressed in mean ± SEM (n=5); *significant at P<0.05 compared with the Negative control; [a] significant at P<0.05 compared with Normal control*

The monogram in figure 2.0 shows the histological examination of the liver, kidney and spleen of rats Infected with *T .b .b* and treated with methanolic leaf extract of *P. amarus.*

4.0 DISCUSSIONS

Natural products can exhibit trypanocidal activity by virtue of their inference with the redox balance of the parasites acting either on the respiratory chain or in the cellular defenses against oxidative stress. This is because natural products possess structures capable of generating radicals that may cause peroxidation damage to trypanothine reductase that is very sensitive to alterations in redox balance. It is also known that some agents act by binding to kinetoplast DNA of the parasite (Sepulvedda-Boza and Cassels, 1996).

4.1 In-vitro Effect of Solvent Extract of *Phyllanthus amarus* on *Trypanosoma brucei brucei*

This study evaluated the trypanocidal activity of *P. amarus* on rats infected with *T. b. b*. It was observed from the in-vitro work that the methanolic leaf extract has the most potent trypanocidal activity as shown in table 2. Further investigation with a standard drug (DA) at dose of 5mg/ml and 10mg/ml of the extract showed similar trypanocidal effect. This is shown in table 3.

4.2 Acute Oral Toxicity

From the acute toxicity study, there were no apparent signs and symptoms of neither toxicity nor death recorded at dose of 5000mg/kg body weight. This agreed with (Herbert and Lumsden, 1976, Ujah, *et al*., 2015)..

4.3 In-vivo Effect of Solvent Extract of *Phyllanthus amarus* on *Trypanosoma brucei brucei*

Parasitological investigation revealed that, Parasitaemia was established at day 3 of infection in all groups. However, pre-treatment and immediate treatment groups showed significant decrease (P<0.05) in parasitaemia when compared with the negative control. Furthermore, immediate treatment showed the least case of parasitaemia after day 4 when compared with pre-treatment. The decreased in parasitaemia could be due to the presence of flavonoids and alkaloids which are known to have trypanocidal activity in a number of tropical plants (Sepulvedda-Boza and Cassels, 1996, Shrish, *et al*., 2011).

Enzyme study showed an elevation in the serum level of AST, ALT and ALP in the negative control group when compared with the normal control group. This may be as a result of established parasitaemia, (Hopp, *et al*., 1976) and the lyses of trypanosome resulting from the host's defense mechanisms (Nok, 2001). It was also indicated by Ngure *et al*., (2008) that measurement of the activities of various enzymes in the tissues and body fluids plays a significant role in disease investigation and diagnosis. The extract recorded a significant (p<0.05) decrease in AST level for pre-

treatment and immediate treatment groups, however, there was no significant decrease in post treatment group when compared with negative control. Similar trend were also observed in ALT and ALP. The mark decrease in the liver marker enzymes agreed with the studies carried out by other researchers on hepatoprotective effect of herbal plants such as Rooibos tea (*Aspalanthus linearis*) and *Moringa oleifera* leaf (Awobode, 2006).

4.4 Histology

The histological examination revealed that group B showed damage in liver, kidney and spleen consistent with lack of treatment. This may be due to the inhabiting mechanism of the parasite at established parasitaemia (Malomo, 2000), and might have led to the elevated levels of serum enzymes (Dacie and Lewis, 1999). Amongst groups C, D and E, group C showed the most destructive effect to the kidney tissues while group E showed the most destruction to liver tissues. Group A had slight effect on the tubules of the kidney and the lymphocyte hyperplasia seen in the spleen might be a normal body defense mechanism response compared to group F which showed slight effect on kidney glomerulus., the liver showed changes typical of normal body response and the spleen showed a higher normal body defense response than A. From this result, we can infer that the extract has ameliorative effect on the tissue damage caused by *T. b. b.* infection.

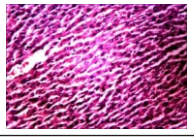
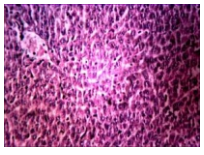
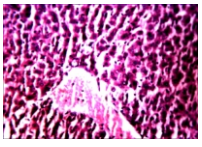
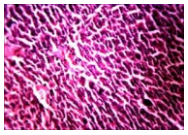
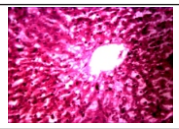
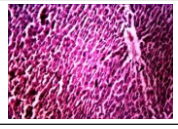
5.0 Conclusion

The acute toxicity study (LD_{50}) of methanolic leaf extract of *P. amarus* on oral administration, has no toxic side effect at the dose of 5000mg/kg body weight. And from the In vivo study, it was revealed, that the methanolic leaf extract has the most potent trypanocidal activity and it has close level of activity when compared with the standard drug (DA) at 5 minutes. This was further confirmed via in-vivo examination in mice and rat with immediate treatment, been the most effective. The plant also showed ameliorative activity on tissues as confirmed by histopathology. Therefore the leaf of *Phyllanthus amarus* should be added to the fodder of cattle and other ruminants as a curative measure against *Trypanosoma brucei brucei*.

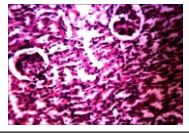
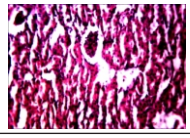
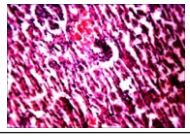
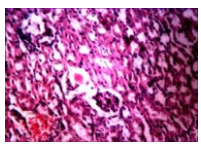
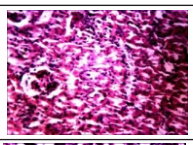
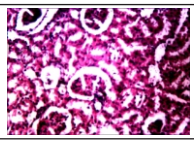
6.0 Recommendation

- An acute dermal toxicity study of *Phyllanthus amarus* methanolic extract, alongside the hematological and biochemical parameters should be assessed.
- Further in-vivo study of this plant extract on *T. b. b* infection may be examined at a longer period of time.
- Isolation, elucidation and characterization of fractions of this methanolic extract should be done in order to help identify the active fractions.

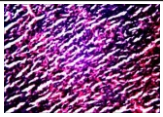
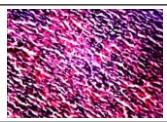
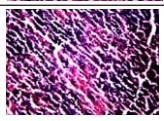
Histogram of Hepatic Cells of rats Infected with *T .b .b* and Treated with Methanolic Leaf Extract of *P. amarus*.

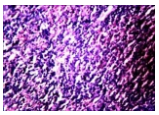
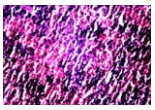
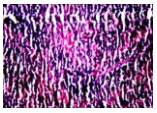
GROUP	Liver	Result
A: Normal control		NORMAL HEPATOCYTES WITH SLIGHT BLOOD CONGESTION H and E stained x 400
B: Negative control		INTENSE KUPFER CELL HYPERPLASIA WITH MODERATE FOCAL NECROSIS H and E stained x 400
C: Pre-treatment		MODERATE HEPATOCELLULAR NECROSIS WITH SLIGHT VASCULAR CONGESTION AND KUPFER CELL HYPERPLASIA H and E stained x 400
D: Immediate treatment		MODERATE HEPATOCELLULAR NECROSIS WITH SLIGHT VASCULAR CONGESTION AND KUPFER CELL HYPERPLASIA H and E stained x 400
E: Post-treatment		INTENSE HEPATOCELLULAR NECROSIS WITH VACOULATIONS H and E stained x 400
F: Extract		KUPFER CELL HYPERPLASIA WITH SLIGHT YMPHOCYTE HYPERPLASIA H and E stained x 400

Histogram Showing Effects of Methanolic Leaf Extract Administration of *Phyllanthus amarus* on Kidney cells of *T. b. b.* Infected Wistar Rats

GROUP	KIDNEY	Result
A: NORLMAL CONTROL		SLIGHT TUBULAR NECROSIS H and E stained x 400
B: NEGATIVE CONTROL		MODERATE GLOMERULAR AND TUBULAR NECROSIS WITH LYMPHOCYTE HYPERPLASIA H and E stained x 400
C: PRE-TREATMENT		INTENSE TUBULAR AND MODERATE GLOMERULAR NECROSIS AND BLOOD CONGESTION H and E stained x 400
D: IMMEDIATE TREATMENT		SLIGHT TUBULAR AND GLOMERULI NECROSIS WITH BLOOD CONGESTION AND SLIGHT LYMPHOCYTE HYPERPLASIA H and E stained x 400
E: POST-TREATMENT		MODERATE TUBULAR AND SLIGHT GLOMERULI NECROSIS WITH SLIGHT LYMPHOCYTE HYPERPLASIA H and E stained x 400
F: EXTRACT		SLIGHTLY GLOMERULAR NECROSIS H and E stained x 400

Histogram of Spleen Cells of rats Infected with T .b .b and Treated with Methanolic Leaf Extract of *P. amarus*.

Groups	Spleen	Result
A: NORMAL CONTROL		MODERATE LYMPHOCYTE HYPERPLASIA H and E stained x 400
B:NEGATIVE CONTROL		MODERATE LYMPHOCYTE HYPERPLASIA AND SINUSOIDAL CONGESTION H and E stained x 400
C:PRE-TREATMENT		INTENSE LYMPHOCYTE HYPERPLASIA H and E stained x 400

D: IMMEDIATE TREAMENT		MODERATE LYMPHOCYTE HYPERPLASIA WITH SINUSOIDAL CONGESTION H and E stained x 400
E: POST-TREATMENT		MODERATE LYMPHOCYTE HYPERPLASIA WITH SINUSOIDAL CONGESTION H and E stained x 400
F: EXTRACT		INTENSE LYMPHOCYTE HYPERPLASIA H and E stained x 400

REFERENCES

1. WHO Media Centre (2016) http://www.who.int/mediacentre/factsheets/fs259/en/.
2. Abdullahi, M., Oluwaseyi, R.I., Abdulfatai, T.A., Chidiebere, U., Ekpenyong, E.U., Isaac, I.O., Mohammed, S.S., Dauda, R.I., Yusuf, A.Y., Adamu, Y.K., and Emmanuel, O. O. (2010). In Vivo Antitrypanosomal Effects of Some Ethnomedicinal Plants from Nupeland of North Central Nigeria. Afr J Tradit Complement Altern Med. 2011; 8(1): 15–21.
3. Legros, D., Ollivier, G., Gastellu-Etchegorry, M., Paquet, C., Burri, C., Jannin, J., Buscher, P. (1985). Treatment of human African Guttering W.E. Existing chemotherapy and its limitations. Br Med Bull. 41:162–168. [PubMed]
4. Legros, D., Ollivier, G., Gastellu-Etchegorry, M. et al. (2002). Treatment of human African trypanosomiasis—present situation and needs for research and development. Lancet Infect Dis. 2: 437–440. [PubMed].
5. Appiah, R., Opong, A.K.., Nyarko, D., Dodoo, F.N., Gyang, K.A., Koram and KAyisi, (2011). Antiplasmodial Activity of Extracts of Tridaxprocumbens and Phyllanthus amarus In vitro Plasmodium Falciparum Culture Systems.
6. European treaty series (2005). European Convention for the Protection of Vertebrate Animals and other Scientific Purposes, ETS-124
7. Herbert, W.J and Lumsden, W.H. (1976). Trypanosome brucei: A Rapid "Matching" Method for Estimating the Host's Parasitaemia
8. Reitman, S. and Frankel, A.S. (1957). Clinical approach to enzyme assay. Am J. Clin. Pathol. 28:53-63.
9. Tietz, N.W. (1995) Clinical Guide to Laboratiry Tests. 3rd Edition. W.B Sunders. Philadephia PA. 518-519.
10. Bancroft, J.D and Stevens (1982). Theory and Practice of Histological Techniques. Churchill Livingstone, Einburgh. 41-120.
11. Sepulveda-Boza, S and Cassels, B.K. (1996). Plant Metabolites Active Against Trypanosoma cruzi. Planta Med. 62:98-105
12. Shirish, S., Pingale, Shrikant, S. and Shewale (2011). Acute toxicity study of phyllanthus amarus. International journal of Pharmaceutical Sciences Review and Research, Volume 9, issue 1, Article-014.
13. Hopp, K.H., Cunningham, L.V., Bromel, M.C., Schermester, L.J. and Wahba, K.S.K. (1976). In vitro Antitrypanosomal Activity of Certain Alkaloids against Trypanosoma lewisi. Lloydia 39: 375- 377.
14. Nok, A.J. (2001). Azanthraquinone Inhibits Respiration and In vitro Growth of Long Slender Blood stream Forms of T. congolense. Cell Biochem.Func. 20:205-212
15. Ngure, R.M., Ndungu, J.M., Ngotho, J.M., Nancy, M.K., Maathal, R.G., Gateri, L.M. (2008). Biochemical changes in the plasma of vervet monkeys (Chlorocebusaethiops) experimentally infected with Trypanosoma brucei rhodesiense. Journal of Cell and Animal Biology 2(7): 150-157
16. Awobode, H.O. (2006). The Biochemical Changes Induced by Natural human African trypanosome infections. African Journal of Biotechnology 5 (9) : 738-742
17. Malomo, S.O. (2000). Toxicological Implication of Ceftriaxone Administration in rats. Nig. J. Biochem. Mol. Biol., 15(1): 33-38.
18. Dacie, J.V. and Lewis, SM. (1999). Practical Haemology, 7th Edition ELBS with Churchill Livingston, England. 106;345
19. Ujah, O. F., Berinyuy, E.B., Edeh, F. O and Ujah, R.I. (2015). Phytichemical Screening and Combined Effect of Ethanolic Leaf Extracts of Phyllanthus amarus and Psidium guajava in Streptpzotocin Induced Diabetic Rats.Asian J. OF Biochemical and Pharmaceutical Research. Vol.5 Issue 2.