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ANTI-*TRYPANOSOMA BRUCEI BRUCEI* EFFECTS OF METHANOLIC AND CHLOROFORM EXTRACTS OF *ILEX KUDINGCHA* DRY LEAVES ON INFECTED SWISS ALBINO RATS

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ABSTRACT
Ilex kudingcha dried leaf extracted with methanol, chloroform and a combination of methanol with chloroform was investigated for its *in-vivo* activity against *Trypanosoma brucei brucei*, the causative agent of African animal trypanosomiasis in sub-Saharan Africa was tested on Swiss albino rats (male) infected previously with *T. brucei brucei* intraperitoneally. The leave extracts were administered 3 days post infection at peak parasitaemia level of104 trypanosomes/ml at doses of 200 and 400 mg/kg by oral administration once daily for 6 days. Parasitaemia, packed cell volume (pcv), mean survival time and change in body weight were used as indices for monitoring the efficacy of the extracts by comparing with the positive control (200mg of diaminazine aceturate) and negative control (2ml of water). All the extracts had LD_{50} greater than 5000mg/kg. There was no evidence of acute toxicity at the doses tested. The chloroform extract showed no effect on the parasitaemia level. The group treated with the methanol-chloroform extracts showed significant reduction in parasitaemia by 13.55±4.91 and increase in (pcv) by 12.00±0.57 ($p<0.001$). Body weight improvement by 177.73±7.51 and mean survival rate of 30.50±0.70 days were also observed in the groups treated with 400mg/kg methanol, and the chloroform-methanol extracts. There was a relapse of parasitaemia almost at the end of the work, which was due to trypanocidal drug resistance. The results obtained suggest pharmacological usefulness of *I. kudingcha* when extracted with methanol or methanol with chloroform. The chloroform extract alone shows no reduction in parasitaemia against *T. brucei brucei*, because it could not extract the active ingredient from the *I. kudingcha* which should act against the *T. brucei brucei*.

KEYWORD: Trypanosomiasis, *Ilex kudingcha*, Albino Swiss rats, methanol, chloroform.

INTRODUCTION

African trypanosomiasis is a flagellated unicellular protozoa disease of man and livestock. It is caused by trypanosomes of the genus *Trypanosoma* and family Trypanosomatidae. They are transmitted by tsetse flies (*Glossina* species), which are the vectors of trypanosomes. When an infected tsetse fly bites an animal, the parasites are transmitted in the saliva. Trypanosomes can also be spread by mechanical vectors including surgical instruments, needles, syringes and various biting flies such as horse flies (Tabanidae). Trypanosomes can be found wherever the tsetse fly vectors exist. Tsetse flies are endemic in Africa between latitude 15^0N and 29^0S, from the southern edge of the Sahara desert to Zimbabwe, Angola and Mozambique. They can also spread beyond the tsetse fly belt by transmission through mechanized vectors. Trypanosomes are also found in South and Central America and the Caribbean (Pinchbeck *et al.*, 2008). *Trypanosoma brucei gambiense* is the causative agent of Human Trypanosomiasis (AHT) known also as sleeping sickness; *Trypanosoma brucei* is the causative agent of African animal Trypanosomiasis (AAT) (Antia *et al*., 2009). The economic impact of trypanosomiasis in Africa are diverse and complex, with direct effects on animal production and human health, as well as indirect effects on settlement patterns, land use, animal husbandry and farming (Chanie *et al*., 2013).

Most cases of trypanosomiasis are chronic, but acute disease, which may be fatal within a week. The initial signs of trypanosomiasis may be a localized swelling (chancres) at the site of the fly bite, but this usually remains unnoticed. The primary clinical signs are an intermittent fever, signs of anemia, lymphadenopathy and weight loss. Milk yield may be decreased in highly parasitized animal. Clinical signs include; edema, cardiac lesions, diarrhea, keratitis, lacrimation, appetite loss and other clinical signs as had been reported for African trypanosomiasis. Effects on reproduction include; abortions, premature births and prenatal losses, as well as testicular damage in males (Garner *et al., 2003).* Deaths are common among chronically infected animals that recover and clinically may relapse when stressed because the immune response of the animal is unable to completely eliminate trypanosomes and animals can become carriers of the parasite (Osorio *et al., 2008)*. An acute hemorrhagic syndrome has been reported among cattle infected with trypanosome in Africa. Affected animals have enlarged lymph nodes and signs of severe anemia, and they develop

widespread visceral and mucosal hemorrhage, particularly in the gastrointestinal tract (Albdrani, 2012; Magona *et al., 2008). Kudingcha* is a beverage tea consumed in China as an alternative to the more common ordinary green tea. The standard Chinese tea, from *Camellia sinensis*, is known as "cha" and this term is then applied to other herbs that are consumed in similar manner. Ku Ding describes this particular beverage tea: "Ku" means bitter, which aptly describes the initial test and "Ding" is a Chinese character that looks like a spike, depicting the appearance of the dried, long leave when they are twisted into a narrow nail-like piece; they can be formed into balls or roll. The herb name is often written as a single transliterated word "kudingcha" and it is sometimes misspelled without the "g" as kudincha". Ku Ding Cha in traditionally Chinese medicine is known to clear toxins from the blood. It has also been used to cure the common cold, rhinitis, itching eyes, conjunctival congestion and headache; it helps in digestion and alleviating the adverse effect to alcohol (Li *et al., 2011; Xu et al., 2001)*. Today, nearly 20 plants from different families with similarities in appearance, flavor and traditional usage in different areas of China are all named "kudingcha" (He *et al., 2003).* The large –leaved *kudingcha* was certified to be the original *kudingcha* species and has obvious antioxidant, anti-inflammatory, lipid metabolism hepatoprotective and anti-tumor activities (Wu *et al., 2008; Woo et al., 2001;* Nishimura *et al., 1999).* Over the last ten years *kudingcha* has been considered as a dietetic beverage and its gaining popularity with names like "beauty, slimming tea", longevity tea", green-golden tea and clearing –heat tea" (Wu *et al.,* 2008). *Kudingcha* was reported to have strong anti bacterial activities against *Salmonella auraus, Salmonella typhosa* and *B-Hemolytic streptococci*, as seen by the agar diffusion method. It could enhance the action of hypoxia tolerance, hypothermia tolerance, and sports tolerance in the mouse, where it was shown to have anti-stress activities (Dong *et al., 2012).* This work is aimed at determining the *in-vivo* anti-trypanosoma activity of the methanolic, Chloroform, and chloroform-methanol extracts in Swiss albino rats infected with *Trypanosoma brucei brucei.*

MATERIALS & METHODS

Sample collection and preparation

Plant collection

The air-dried leaves of *Ilex kudingcha* (a medicinal tea indigenous to China was imported from China and purchased at Oshodi market, Lagos, Nigeria.

Preparation of plant extract

Using a weighing balance (Sartorius electric weighing balance of make (800) 645-3108) made in New York, USA, 50g each of the powdery plant material was weighed and immersed in 500ml of chloroform, methanol, and chloroform-methanol mixture (50-50vol). The mixtures were left for 5 days with continuous stirring. The residues were sieved from the extracts, using filter guaze to remove coarse plant particles. The extracts were subsequently filtered of again, using sterile wartman filter paper into three clean beakers. The filtrates were concentrated to dryness at room temperature. The extracts were weighed and used in the preparation of the stock material for administration.

Laboratory Animals

Male Swiss albino rats bred at the University of Nigeria Nsukka were used in this study. They were kept in well ventilated metal cages. They were kept in standard conditions and allowed free access to growers feed and water. The animals were allowed a 2 week period of acclimatization before they were divided into six groups.

Trypanosome inoculation and infection of animals

The parasite, *Trypanosoma brucei brucei (in-vitro culture)* was brought from the veterinary department of the University of Nigeria Nsukka. A 0.2ml volume of blood containing 10^4 trypanosomes/ml was inoculated intraperitoneally into two laboratory mice and transported to the Biology laboratory of Akanu Ibiam Federal Polytechnic Unwana. The development of trypanosomes in the infected mice was determined by wet film examination of blood obtained from the tail of the mice. After three days of post-infection, the donor mice showed signs of parasitaemia. After establishment of infection, the donor mice were subjected to cardiac puncture and blood was collected with an EDTA coated tube and immediately diluted with phosphate buffered saline. Then 0.2ml of blood collected from the donor mice and containing about 10^4 trypanosomes/ml was injected intraperitoneally into each of the rats previously acclimatized.

Acute toxicity

The acute toxicity was conducted on the methanol, chloroform and chloroform with methanol extracts in three phases using Swiss albino rats after one week of adaption. In the first phase, nine mice were divided into three groups of mice each. Each group was given 1900, 2600, 5000mg/kg body weight of the chloroform extract alone. The second phase of 9 mice was divided into three groups of 3 mice each. Each group was given 1900,2600,500mg/kg body weight of the methanol extract alone, while the third group of 3mice each were also given 1900,2600,500mg/kg body weight of chloroform with methanol. This was to determine the lethal dose (LD50) value of the extracts.

Each extract was dissolved in 10% of 80ml in sterile water and given orally, all animals were kept under strict observation for behavioral, and neurological changes such as alertness, coma, restlessness, diarrhea, lacrimation and convulsions for 24hrs with special attention during the first 4 hours. These observations continued for further 7days for any signs of overt toxicity. The lowest dose which killed one mouse and the highest dose which did not kill any mouse were noted and the geometric mean of these two doses gives $LD_{50.}$

Determination of Parasitaemia

Parasitaemia was monitored in the blood obtained from the tail of the mice (0.2ml of blood was collected from each mouse). The numbers of parasites were determined microscopically at x400 magnification using the "Rapid" Matching method (Herbert and Lumaden, 1976). The number of trypanosomes per microscopic field was then compared with the table of logarithmic values. The logarithm values of these counts obtained were converted to antilog to provide absolute number of trypanosome per ml of blood.

Determination of packed cell volume (PCV)

PCV was measured using wintrobe's method to predict the effectiveness of the test extracts in preventing hemolysis resulting from increasing parasitaemia associated with trypanosomiasis (Albert and Hussein, 2012). It was monitored on 4th and 7th day of treatment. Briefly blood was collected from the tail of each rat using heparinized microhaematocrit capillary tubes filled up to ¾ of their length. The tubes were then sealed immediately using a soap bar and centrifuged in a microheamatocrit for 5 minutes at 12000pm. After centrifugation, the heights of the red blood cell column were measured using hematocrit reader and compared to the total height of the column of the whole blood (Albert and Hussein 2012).

Determination of *In-vivo* efficacy of the plant exracts

In order to determine the effective dose, six groups of three rats each were distributed into six cages; group I, II, III, IV, V, and VI infected rats were treated with methanol extract, chloroform extract, chloroform-methanol extract, diminazene aceturate and distilled water. The drug administration was done intrapertoneally, with the extracts at a dose of 200 and 400mg/kg and diminazene aceturate (standard drug) at 1900mg/kg body weight per day. Group V the negative control was administered distilled water (ordinary water). Group VI was made up of 2 rats (positive control) which were neither infected nor treated with the extracts, diaminazane aceturate, nor distilled water. Each treatment continued for four weeks. Parasitaemia was monitored every two days under the microscope.

Mean survival time

Mortality was monitored daily and the number of days from the time of inoculation of the parasite up to the time of death was recorded for rat in the treatment and control groups throughout the follow up period for four weeks.

Data collection and analysis

Values of data obtained from the study were summarized as mean ± standard Error of Mean (SEM). Data analysis was performed using statistical Package for Social Science (SPSS). To compare results obtained from different groups. One way ANOVA were performed to determine statistical significance P values less than 0.05 were considered significant.

Phytochemical analysis

Qualitative analysis for alkaloids, flavonoids, tannins, saponins and glycosides were carried out, using the method described by Tyler and Herbagram, 1994.

Quantitative analysis of alkaloids, flavonoids, saponins, tannins, phenols and glycoside were carried out, using the method described by Williamson and Manach, 2005; Mattila and Hellstron, 2007.

RESULTS

The mean parasitaemia count of methanol-ethanol extract of *Ilex kudingcha* extract on Swiss albino rats treated with *T. brucei brucei*. Rats treated with methanol-chloroform extract of *Ilex kudingcha* at 400mg/kg dose had significantly low parasitaemia on day 6($p<0.01$), day 9 and day 13 ($p<0.01$).This is compared with the negative control group as seen in the graph. The positive control group (the diminazine group) showed clearance from day 6 to day 13. At day 15, both the control group and the extract groups relapse.

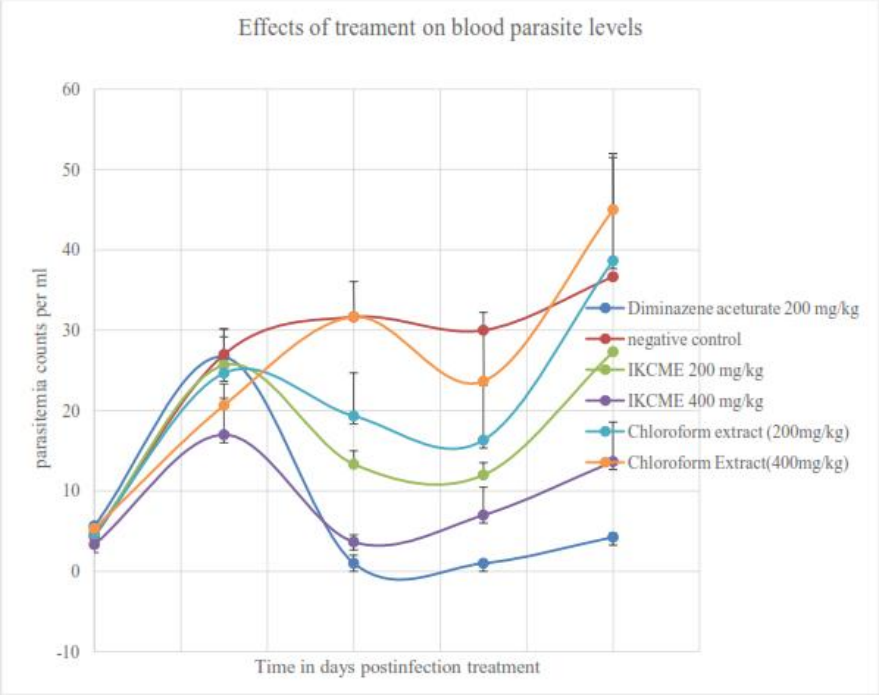

Table 1, The effect of the chloroform extract of Ilex *kudingcha* on the parasitaemia of albino rats infected with *Trypanosoma brucei brucei*

The *in-vivo* parasite count in albino rats experimentally inoculated with *Trypanosoma brucei brucei* and then treated with the chloroform extract of *Ilex kudingcha.*

Parasitaemia was observed on day three (3) post inoculation and motile stage was observed in all groups. Treatment with the chloroform extract at the doses 200 and 400mg/kg body weight for six days did not have much effect on the level of parasitemia as the motility stage slightly reduced in treated groups up to the day of post inoculation. However, the diminazene treated group showed no parasitaemia after treatment while the negative control increased in parasitaemia count as seen in the graph above.

TABLE 1: PCV value of group treated with chloroform extract of *Ilex kudingcha*

Days of treatment	Extract (200mg/kg)	Extract (400mg/kg)	Diminazene (200mg/kg)	Negative control (2ml of water)
Day 4	11.66±1.20	11.00±0.58	9.66±0.33	10.33±0.67
Day 7	9.66±0.33	11.00±1.52	9.66±0.33	10.33±0.67

Table 1 shows the effect of the chloroform extract of *Ilex kudingcha* on packed cell volume (PCV) of infected albino rats with *T. brucei brucei.* Treating the rats with 200 and 400mg/kg body weight of the extract, the PCV values were maintained with slight changes during the course of treatment. The PCV value of diminazene treated group remained the same at day 4 and day 7 of treatment. The negative control group also maintained the same values during day 4 and day 7 of treatment.
IKCME 400 = *Ilex kudingcha* chloroform methanol extract 400mg/kg

TABLE 2: The PCV value of group treated with chloroform-methanol extract of *Ilex kudingcha*

DAYS	DA_{200}	NC (Water) 2ml	I_KCME_{200}	I_KCME_{400}
Day 4 of Treatment	10.00±0.57	10.33±0.66	10.00±0.57	10.44±0.22
Day 7 of Treatment	12.66±0.22	9.50±0.31	11.33±0.66	12.00±0.57
% changes PCV days 4-7	26.60	8.03	13.30	14.94

The mean PCV value of animals treated with 200 and 400mg/kg of the methanol with chloroform extracts of *Ilex kudingcha* was statistically significant at (P>0.01), higher PCV values were compared to the negative control group on day 7 of treatment (table 2)
Values are expressed in Mean ± S.E.M (n=5) performed with ANOVA followed by Turkey's post hoc multiple comparison test.
DA200 = Diminazine aceturate 200mg/kg = positive control
NC = Water 2ml = Negative control
IKCME200 = *Ilex kudingcha* chloroform with methanol extract 200mg/kg
IKCME400 = *Ilex kudingcha* chloroform with methanol extract 400mg/kg

TABLE 3: Comparison of the effects of chloroform-methanol extracts of *Ilex kudingcha* on parasitemia, PCV, body weight and mean survival time of *Trypanosoma brucei brucei* in Swiss albino rats

Plant	Extract	Dose	Mean Parasitemia	Mean PCV Value	Mean Body Weight	Mean surviv.
Ilex Kudingcha	Methanol- Chloroform	200mg/kg	27.33±10.26	11.33±0.66	154.76±19.71	11.50±9.19
		400mg/kg	13.66±4.91	12.00±0.57	177.73±71.51	30.50±0.70
Positive Control	Diminazine aceturate	200mg/kg	4.25±0.47	12.66±0.22	190.55±13.65	32.00±1.41
Negative Control	water	2ml	36.66±3.33	9.50±0.31	177.95±3.18	7.00±0.89

Table 3: shows the results of chloroform-methanol extracts of *Ilex kudingcha* on parasitemia, PCV, body weight and mean survival time of *Trypanosoma brucei brucei* infected intraperitoneally to Swiss albino rats.

TABLE 4: Quantitative analysis of methanol, chloroform, and methanol-chloroform extracts of *Ilex kudingcha*

Constituents	*Ilex kudingcha*		*Ilex kudingcha*
	Methanol	Chloroform	Methanol-chloroform
Alkaloid	++	++	+++
Flavonoid	-	-	--
Tannin	+++	-	++++
Saponin	+++	+++	++++
Glycoside	++	-	+++

++ = slightly present, +++ = highly present , - = absent

Table 4 shows that alkaloids, tannins, saponins and glycoside were present in the methanol extract, chloroform and methanol with chloroform extracts of *Ilex Kudingcha.* Flavonoid was absent in all the three solvents used for the extraction. Glycoside was absent in chloroform extract. Chloroform-methanol solvent extracted more of the active constituents than any other solvents.

TABLE 5: Results on quantitative analysis of the phytoconstituents of the leaf extract of *Ilex kudingcha*

Constituents	*Ilex kudingcha*		*Ilex kudingcha*	
	Methanol	Chloroform	Methanol-chloroform	
Flavonoid	-	-	-	-
Alkaloid	35.36±0.49	25.33±0.38	35.36±0.49	25.33±0.38
Saponin	38.28±0.24	25.63±0.32	38.28±0.24	25.63±0.32
Tannin	0.20±0.22	-	0.20±0.22	
Glycoside	3.27±0.16	-	3.27±0.16	

Values are expressed as mean ± SEM

DISCUSSION

The *Ilex kudingcha* has a folkloric medicinal uses. The research on this plant extract is as a result of high level of resistance developed by trypanosomes against trypanocides (Mbaya *et al.*, 2007). Based on the results of acute toxicity obtained and compared with existing results, the plant extracts had shown LD_{50} greater than 200mg/kg. thus since *I. kudingcha* has been proved to be efficacious in treating many ailments, the experimental determination of this good safety margin would justify that the plant is safe at the dose levels (200, and 400mg/kg) used in this study which is an additional proof for the edicinal value of the plant in folk medicine for the treatment of infectious diseases (Heck and De Mejia, 2007). The results obtained during the monitoring period had shown that the higher dose (400mg/kg) of the methanol-chloroform extracts of *Ilex kudingcha* exhibited appreciable anti-trypanosamal activity by reducing the level of parasitaemia (13.66±4.91) as compared to the increase in the negative control group (36.66±3.33) and prolonging the life span of the test animals beyond that of the negative control at day 15 post infection (Mann *et al., 2009).* In addition, the findings of this study had shown that the plant extracts did not completely eliminate the parasites from the blood of the infected rats, but only reduced the level of parasitemia to a reasonable level. Several researchers made similar observations on reduction in parasitaemia (Ibrahim *et al., 2012;* Wurochekke and Nok 2004; Ogbadpyi *et al.*, 2011). Among the groups treated with diaminazine aceturate, there was no parasite development from day 7 to 12. Although relapse occurred in all the rats approximately on days 13-15 of treatment. Similar observations were made by (Ibrahim *et al., 2012;* Miruk *et al., 2008; Osorio et al., 2008;* Afewerk *et al., 2000).* The relapse of parasitaemia might be due to trypanocidal drug resistance by trypanosomes or the ability of *T. brucei brucei* to sequester in small vessels and capillaries of the heart, skeletal and other tissues, hiding from the effects of the extracts of *Ilex kudingcha* (Mbaya *et al., 2007; Osorio et al., 2008).* The study on packed cell volume (PCV) shows that there were fairly consistent with the observations made on parasitemia, when treated with *I. kudingcha* extracted with methanol-chloroform. There was an improvement in the PCV value. From day 7 post infection, the PCV value of the group treated with methanol-chloroform extract increased to12.00±0.57 while infection caused significant drop in the PCV value in a negative control group and the group treated with chloroform extract alone (9.50±0.3; and 9.66±0.33) respectively. The low PCV value in the infected group may be due to acute hemolysis and as a result of the growing infection. In addition, infections with trypanosomes result in increased susceptibility of red blood cell membrane to oxidative damage. Reactive oxygen species generated by trypanosomes can also attack red blood cell (RBC) membranes induce oxidation and subsequently hemolysis. This phenomenon subjects RBC to massive erythrophagocytosis by an expanded and active mononuclear phagocytic system (MPS) of the host resulting in anaemia (Albert and Hussein, 2012). The infected rats treated with the diaminazine aceturate showed significant improvement in PCV. This is because the drug was able to eliminate the parasites from the blood to a considerable level within 7-13 days. According to the

results of the phytochemical analysis, the methanol extract of *Ilex kudingcha* showed positive test for the presence of alkaloid, glycosides, saponins, and tannins but tested negative for flavonoids, while the chloroform extract showed positive for only alkaloids and saponins but negative for flavonoids, glycosides, saponins, and tannins. The combined extracts of chloroform-methanol also showed positive test for alkaloid, glycosides, saponins and tannins in larger concentrations but showed negative for flavonoids. Saponin which has the highest level of concentration has been reported to posses wide spectrum of antimicrobial and parasitic activity. Its mechanism of action is thought to be by interaction with parasite membrane sterols, proteins and phospholipids (Williamson and Manach, 2005). One of the molecular actions of tannins is by complexing proteins through the so-called non –specific forces such as hydrogen bonding and hydrophobic effects, as well by covalent bond formation (Taylor, 2000). Therefore, the observed anti trypanosomal activity of *Ilex kudingcha* might be attributed to their individual class of compounds, or to the synergistic effects that each class of compound exerts to give the observed biological activity. Hence, further in-depth investigation should be carried out to understand this better. It is also important to note that the chloroform alone could not extract flavonoid, tannin and glycoside. That is why there was no reduction in parasitaemia when the rats were treated with the extracts of *Ilex kudingcha* extracted with chloroform. Therefore, there was also a drop in weight and PCV values on those rats. From the research work, it is deduced that chloroform is not a good solvent in extracting *Ilex kudingcha*, because it could not extract the active components like tannin, saponin and glycoside. The suppressive effect of the extracts against trypanosome infection can further be inferred from the weight status of the treated animals. At day 15 post treatment, animals that received 400mg/kg dose of the methanol-chloroform extracts of *Ilex kudingcha* gained weight as compared to the negative control group and the chloroform extract group. This shows that as a result of reduction in parasitaemia and prevention of drop in PCV by the

extracts, the physical body weight of the treated rats improved. They were therefore, more able to resist weight loss that is usually associated with trypanosomiasis. Due to the significant drop in PCV level of the negative groups as a result of high parasitaemia level, their appetite decreased and the animal losses weight. There was consumption of the fat reserves but there were also severe degenerative changes of the muscle cells and other tissue cells, and there is an increased breakdown of protein in muscles, leading to atrophic degeneration. The decreased supply of oxygen because of the anemia also is an important factor (Rahman, 2005). Methanol extract, shows the same effect of parasitic reduction, increase in PCV, increase in survival rate and weight gain, as seen in methanol-chloroform extract. Therefore, reporting the result of methanol extract will be the same as repeating the result of methanol-chloroform extract which will be a repetition. From this study, methanol is confirmed to be the main solvent responsible for the extraction of the active ingredients from the dry leaves of *Ilex kudingcha.* Chloroform could not do much in extracting the active component from the Ilex *kudingcha* dry leaves as to cause parasitic reduction in the Swiss albino rats infected with *Trypanosoma brucei brucei*.

CONCLUSION

The work demonstrated good safety margin of the crude extracts of *Ilex kudingcha* against *T. brucei brucei* in Swiss albino rats suggesting their ethanopharmacological usefulness and efficacy. The crude extract exhibited high *in-vivo* activity at higher dose. However, the extract was not effective enough to eradicate the parasites completely. The study revealed that the methanol-chloroform extract of *Ilex kudingcha* has promising effect in reducing parasitaemia by 13.66±4.91, increasing PCV by 12.00±0.57, increasing body weight by 177.73±71.5 and prolonging the survival time by 30.50±0.70 days in *T. brucei brucei* infected Swiss albino rats. Chloroform extract of *I. kudingcha* alone was not effective in extracting the active ingredient from the plant (*I. kudingcha).* Methanol-chloroform extract has the same effect as methanol extract.

RECOMMENDATION

The current study has established dry leaves of *Ilex kudingcha* as a potential candidate for anti trypanosomal activity which can be considered as a potential source for the search of new drugs against African Animal Trypanosomiasis (AAT). Further research work is also recommended on the plant *Ilex kudingcha.*

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