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Phytochemical screening and anti-trypanosomal activity of methanolic seed extract of *Garciniakola* (Heckel) on *Trypanosoma brucei brucei* infected albino rats

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Abstract
African trypanosomiasis is a major disease of economic and public health importance caused by *Trypanosoma brucei brucei* that affects humans and livestock. The use of plant extract is fast becoming the choice method for the treatment of the disease. As a result, the current study investigated the *in vitro* and *in vivo* activity of methanolic seed extract of *Garcinia kola* on *Trypanosoma brucei brucei*. The *in vitro* assay was carried out by treating the parasites with 10, 5, 2.5, 1.3, 0.6 and 0.3 mg/ml of the extract. For the *in vivo* studies, the methanolic extract was administered orally at a dose of 200, 400 and 600 mg/kg body weight of rats 2 days post infection for 4 consecutive days. Parasitaemia and mean survival time were used as indices for monitoring the efficacy of the extracts. The phytochemical screening revealed the presence of alkaloids, flavonoids, terpene, quinines, saponins and tannins, with steroid having the highest concentration (31.13mg/100g) and quinone with the lowest concentration (0.08mg/100g).The *in vitro* screening showed antitrypanosomal activity at higher concentrations of the extract (5 and 10mg/ml) after the 60 minutes incubation period. Althoughin the *in vivo* study, the extract did not significantly decrease ($p>0.05$) parasitaemia of the infected rats, we presume this may have occurred due to inefficient absorption of the extract after oral administration. However, this study reveals that the extract has potential anti-trypanosomal activity *in vitro* and can be used to design drugs to reduce the global scourge of trypanosomiasis.

Keywords: antitrypanosomal, *Garcinia kola*, *Trypanosoma brucei brucei*, parasitemia.

Introduction
Trypanosomiasis, also known as sleeping sickness is an infectious disease of African origin that affects humans and animals. It is caused by the parasites *Trypanosoma species* and transmitted by the bite of infected tsetse flies (Abenga, 2014) [1]. *Trypanosoma congolense*, *vivax* and *brucei brucei* are the responsible for the disease in animals while the subspecies of *Trypanosoma brucei*, *Trypanosoma brucei gambiense* and *brucei rhodiense* are responsible for the disease in humans (Ogbole *et al*., 2016) [30]. Trypanosomes kill more than 3 million cattle annually and those animals that survive display low productivity due to the wasting effects of the disease (Hurse, 2001) [16]. In addition, the devastating effects of the disease affects the lives and livelihood of many rural farmers of sub-Saharan Africa that cannot afford good and quality health care services (Johnson and Omoniwa, 2014) [20].
Clinically, the disease is treated with melarsoprol, suramin, pentadimine and efflornithine that are very expensive and fast becoming impotent because of the emergence of resistant strains of the Trypanosomes. Current study is focused on the use of natural products from plants that could help to overcome the challenges associated with the conventional drugs used for the treatment of trypanosomiasis.
Many natural products of plant origin have been reported to have activities against different species of protozoan parasites including Plasmodium, Trypanosoma, leishmania and Entamoeba (Hoet *et al.*, 2004) [15]. Garcinia kola (G. kola) a species of flowering plant of the Clusiaceae or Guttiferae family found in the tropical rain forest region of West Africa is a plant known for its medicinal properties. The presence of bioactive compounds with high therapeutic properties in the seeds of the plant has been reported (Tona *et al.*, 1999; Okunji *et al.*, 2000; Farombi *et al.*, 2002; Farombi, 2003; Omwirhiren *et al.*, 2017) [41, 12, 14, 32]. Phytochemical compounds that have been isolated from G. kola include oleoresin (Onayade *et al.*, 1998) [33], tannins, saponins, alkaloids, cardiac glycosides (Ebana *et al.*, 1991) [7]. Two new

chromanols, garcioic acid and garcinal, together with σ tocotrienol were also reported to be isolated from G. kola (Terashima *et al.,* 2002) [40]. Other phytochemical compounds so far isolated from G. kola seeds are biflavonoids such as kolaflavone and 2hydroxybi-flavonols (Okunji *et al.,* 2000; Terashima *et al.,* 2002) [40]. Studies have shown that kolaviron, a natural biflavonoid from G. kola seeds possess the ability to protect against oxidation of lipoprotein in rats (Farombi and Nweokeafor, 2005) [13]. This activity which was demonstrated to be presumably by Fe^{2+} chelation and anti-oxidant activity might be of immense benefit in the management of African trypanosomiasis. It has been suggested that removal of excess iron through chelation could possibly prevent iron mediated injury to cells thereby reducing the pathology of anaemia and tissue damage associated with African trypanosomiasis (Ekanem *et al.,*2009; Johnson and Omoniwa, 2014) [23, 20]. Several medicinal plants are currently being investigated for their anti-trypanosomal properties in a bid to identify active components that can be used to combat the scourge of trypanosomiasis. *Garcinia kola (*Heckel) is one of the medicinal plants that is purported to have anti-trypanosomal effect. The plant is also reported to be useful for the treatment of diabetes, cough, laryngitis, infectious diseases, erectile problems, liver and lipid disorders among others (Iwu *et al*., 1990; Adegboye *et al*., 2008; Johnson et al., 2011) [18, 2, 19]. However, the active components of the plant and their mechanism of action is still unknown. Therefore, the present study seeks to identify the phytochemicals of *G. kola* seed and also investigate their *in vitro* and *in vivo* anti-trypanosomal activity.

Materials and Method

Collection of plant material and parasites: *Garcinia kola* seeds, purchased at Kaduna Central Market, Kaduna State, Nigeria, was authenticated at the Department of Biological science, Kaduna State University, Nigeria, by Mr. U. S. Gallah and assigned the Voucher No, 9501.

The parasite, *Trypanosoma brucei*, (strain *Trypanosoma brucei brucei*) was obtained from the Nigerian Institute for Trypanosomiasis Research Kaduna, Nigeria and was maintained via routine inoculation of normal albino rats.

Preparation of methanolic extract of *G. kola* seed: The seeds were peeled off and cut into smaller pieces, shade-dried and then ground into fine powder using mortar and pestle. About 80 g of the pulverized sample was soaked in 800 mL of methanol (analytical grade) for two (2) days at 25ºC.The extract was filtered with a clean muslin cloth and subsequently evaporated using a water bath at 40ºC. The jelly-like concentrates obtained was weighed and placed in sterilized sample bottle for storage in a refrigerator at 4ºC.

Phytochemical screening: The phytochemicals screening was carried out according the following methods: The total flavonoids content was estimated using the procedure described by Zhishen *et al (1999)*. Tannins content of the sample was estimated by the method of Siddhuraj and Manian (2007). Estimation of total saponins content was determined by the method described by Makkar *et al.*(2003). Steroids and terpenes were estimated according to the method described by Ejikeme *et al*. (2010).

Experimental animals: Eight (8) weeks old albino rats with average weight of 98.54 g obtained from the Animal house of Nigerian Institute for Trypanosomiasis Research (NITR), Kaduna were used for the study.They were housed in plastic cages with wood shavings as beddings and maintained on a commercial poultry feed (Fitzer, Nigeria),with access to clean water. They were acclimatized for two (2) weeks before commencement of the experiment.

Experimental design: Fifteen (15) albino rats were weighed and grouped randomly into five experimental groups that contained 3 rats each, for the *in vivo* study. The groups are as follows: Group 1 – Normalrats treated with 2% DMSO (normal control); Group 2– Trypanosome-infected rats treated with 2% DMSO (negative control); Group 3– Trypanosome-infected rats treated with the extract at a dose of 200 mg/kg body weight of rat; Group 4– Trypanosome-infected rats treated with the extract at a dose of 400 mg/kg body weight of rat; Group 5– Trypanosome-infected rats treated with the extract at a dose of 600 mg/kg body weight of rat. The treatment was done for 4 days consecutively.

Infection of animals: Blood from a highly parasitized mouse was obtained by cardiac puncture using a syringe and needle and transferred into an EDTA- coated sample bottle. This was diluted appropriately with normal saline to serve as inoculum. Healthy rats were infected intraperitoneally with 0.3 mL each of the inoculum that contains about10^6 trypanosomes/mL (Ene *et al.,* 2009) [9].

Determination of parasitaemia and antitrypanosomal activity of extract: Blood was collected from the tail of rats before and after treatment with the extract of *G. kola* and the level of parasitaemia was monitored *in vitro* and *in vivo* as described by Ene *et al*. (2014) [10]. The level of parasitaemia was expressed as log of the absolute number of parasites per mL of blood. Briefly, the *in vitro* anti-trypanosomal assay was performed in a 96 well plate seeded with the parasites. The parasites were treated with the extract and standard drug, veridium (positive control) and incubated at 37 ºC for 60 min. The negative control rats were treated with PBS-G. The motility of parasites was used as a basis for assessing the anti-trypanosomal activity of the treatments. For the *in vivo* study, parasitaemia was monitored every day until the last mortality was observed. The negative control group was treated with 2%DMSO.

Statistical analysis: The data obtained from the study were expressed as mean ± standard deviation of mean (SD). Data analysis was performed using Statistical Package for Social Science (SPSS), version 20.0 (Inc. Chicago IL, USA). One way ANOVA was performed to determine statistical significance difference at 95% confidence interval.

Result and Discussion

Table 1: Quantitative Photochemical Componentsof seed extract of *Garcinia kola*

Phytochemicals	Concentration (mg/100g)
Alkaloid	2.30±0.05
Flavonoid	2.05±0.03
Terpenes	3.05±0.03
Tannines	0.35±0.03
Saponins	2.47±0.04
Quinones	0.08±0.00
Anthraquinones	1.12±0.02
Steroids	31.13±1.00

Data in duplicate: mean ±S.D

Table 2: *In vitro* anti-trypanosomal activity of metholic extract of *G. kola* against *trypanosome brucei brucei*

Concentration (mg/mL)	Trypanosome count (Mean ± SEM)	% mortality
10	0.00±0.00[a]	100
5	0.00±0.00[a]	100
2.5	6.00±1.15[b]	74.3
1.25	5.33±0.33[b]	71.2
0.625	8.33±0.67[c]	64.3
0.3125	8.67±0.66[c]	62.8
Positive control	0.00±0.00[a]	100
Negative control	23.3±1.45[d]	0.00

Superscript: a, b, c and d indicates statistical differences

Table 3: Effect of methanol seed extract of *Garcinia kola* on parasitaemia of *Trypanosoma brucei brucei* infected rats.

Groups	Days post inoculation				
	2	3	4	5	6
1	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
2	0.00±0.00	2.67±1.15	38.7±24.1	98.0±42.4	-
3	0.13±0.23	4.00±2.00	78.0±55.7	171.3±73.3	-
4	0.67±1.15	4.22±5.05	102.0±133	96.0±45.3	-
5	0.00±0.00	2.72±2.21	25.3±11.5	104.0±34.9	-

Values are given as mean ± standard deviation (mean ±SD)

Table 4: Percentage (%) mortality of rats infected with *Trypanosoma brucei brucei* treated with various doses of methanolic seed extract of *Garcinia kola*

Groups	% mortality					M.S.T (days)
	Day 2	Day 3	Day 4	Day 5	Day 6	
1	0	0	0	0	0	>14[a]
2	0	0	0	33.3	100	5.7±0.6[b]
3	0	0	0	0	100	6.0±0.0[b]
4	0	0	0	33.3	100	5.7±0.6[b]
5	0	0	0	0	100	6.0±0.0[b]

M.S.T= mean survival time, subscript: a and b indicates statistical differences. In each column, mean values with the same superscripts have no statistical significant difference (p> 0.05).

Despite the enormity of the health and economic implication of African trypanosomiasis, current chemotherapeutic options are very limited and very expensive (Legros *et al*., 2002). Therefore, there is a need for more potent and cheaper alternatives that could be used to manage the debilitating disease. Some plants have been reported to be very potent in the treatment of trypanosomiasis (Asuzu and Chineme, 1990; Nok, 2002; Mergia *et al.,* 2015; Nwodo *et al.* 2015) [5, 27, 25, 28]. The present study investigated *in vitro and in vivo* anti-trypanosomal activity of the methanolic extract of the seeds of *Garcinia kola*. The phytochemical screening of the methanolic seed extract of *G. kola* reveals the presence of alkaloids, saponins, flavonoid, terpene, tannine, steroids, anthraquinone and quinone (Table 1). From the phytochemicals screened, steroid had the highest concentration (31.13mg/100g) while quinone has the lowest concentration (0.08mg/100g). This finding is in agreement with previous study by Adesuyi *et al.* (2012) [3] that reported that *G. kola* seed contained 0.342, 2.471, 0.645 and 2.041% of tannins, saponins, alkaloids and flavonoids respectively. Findings from present study indicate the the presence of these phytochemicals may have contributed to the trypanocidal activity observed in the current study.

Numerous *in vitro* and *in vivo* studies conducted on the antitrypanosomal activities of the class of compounds listed above reported the potential of each class of compounds in killing or inhibiting the growth of wide ranges of trypanosomes (Yabu *et al*., 2013; Johnson and Omoniwa, 2014) [45, 20]. It has been well established that flavonoids and flavonoid-derived plant natural products are effective antioxidant (Nwodo *et al.*, 2015) [28]. Antioxidants have been shown to protect cells against the damaging effects of reactive oxygen species, such as singlet oxygen, superoxide, peroxyl radicals, hydroxyl radicals and peroxynitrite (Pieta, 2000). An imbalance between antioxidants and reactive oxygen species results in oxidative stress, leading to cellular damage. Cellular damage and consequently anaemia which is characterized by a significant reduction in PCV is one of the major pathologic effects of trypanosome infection (Ekanem *et al*, 2009; Johnson *et al.*, 2011; Johnson and Omoniwa, 2014) [8, 23, 19, 20]. The antioxidant property of flavonoids in G. kola promises to be of great benefit in the fight against this debilitating disease. Furthermore, the trypanocidal activity of a number of plants is attributed to the highly aromatic flavonoids they contain. Earlier, we observed the presence of flavonoid in the methanolic extract of *G. kola* which we presume that it contributes to the bioactivity of the extract (Johnson *et al.*,2011; Ogbadoyi *et al.*,2011) [19, 29]. Although *in vitro* activity of crude extract may need further verification, it remains one of the best tools widely used in bioassay guided identification of active components in plants (Wurochekke *et al*., 2014) [43].

In the *in vitro* assay, the trypanocidal activity of the extract and standard drug (veridium) increased significantly ($p<0.05$) as the concentration of extract was increased (Table 2). Similarly, the mortality increased as the concentration of the extract increased. The extract effectively inhibited the motility of *Trypanosoma brucei brucei* and at a concentration of 5 and 10 mg/mL of the extract, a 100% mortality was observed *in vitro* (Table 2). Therefore, the observed *in vitro* antitrypanosomal activity of *Garcinia kola* might be attributed to either the individual class of compounds present in the extract, or to the synergistic effect that each class of compounds exert to give the observed biological activity (Mergia *et al*., 2014) [24]. Natural products can generate free radicals that causes peroxidative and DNA damage (Atawodi *et al*., 2003) [6]. The extract of *G. kola* may have exerted its trypanocidal activity through peroxidative and DNA damage and this corroborates previous studies (Ogbadoyi *et al.,* 2011; Johnson and Omoniwa, 2014) [29, 20]. Earlier reports has shown that The antitrypanosomal property of alkaloids has been suggested to be due to DNA intercalation in combination with protein biosynthesis inhibition while the trypanocidal activity exhibited by the saponin fraction may be as a result of cytotoxicity. Saponins are natural glycosides which possess a wide range of pharmacological properties including cytotoxic activity. Their cytotoxic effects have been suggested to be due to either apoptosis inducement or non-apoptotic cell death stimulation. Cell death may be as a result of mechanisms like - stimulation of autophagic cell death, decrease in NO production in cells, or cytoskeleton integrity disassembly (Merschjohann *et al.,* 2001; Podolak *et al.,* 2010; Mann *et al.,*) [26, 37]. The doses administered to rats did not significantly decrease ($p>0.05$) the level parasitemia (Tables 3 and 4). Even though the *in vitro* screening shows the extract has trypanocidal activity, the *in vivo* studies does not confirm this fact. This we believe may be due to insufficient absorption of the extract after administration. Impaired absorption of drugs is known to affect the efficacy and therapeutic effect of drugs (Mann *et al*., 2009) [23]. Therefore, it is very imperative to

improve absorption of the extract and also understand the factors that affect the potency of the extract. It is also possible that the active component may need some form of activation as observed in many clinically used drugs. This study may actually lead to an increased potency of the seed extract of *Garcinia kola*.

However, our study has shown that the extract has potential anti-trypanosomal activity and this finding corroborates previous reports and justifies the traditional use of *Garcinia kola* in the treatment of trypanosomiasis.

Conclusion

Trypanosomiasis is a disease of humans and livestock that affects the livelihood of sub-Saharan Africans in rural settlements (Fairlamb, 1982) [11]. The search for a potent drug that could help reduce the scourge of African trypanosomiasis remains elusive. Natural products are fast becoming the choice method for the treatment of the disease because they are safer and cheaper. The current study reveals that the extract of *G. kola* seed contains components that can effectively destroy the parasite and reduce mortalities associated with African trypanosomiasis. This findings no doubt is indeed an encouragement for the development of present and future African chemotherapy that promises succor to a region that has suffered from the debilitating effects of trypanosomiasis and consequently improve the quality of life. Further collaborative study in this area intend to focus on the isolation and spectroscopic characterization of the bioactive ingredients in G. kola which may serve as novel compounds in the quest for the development of new, affordable and more effective antitryponocidal therapies.

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