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Original Article

Trypanocidal Activity of 50% Methanolic Extract of *Khaya Senegalensis* Tree Bark

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ABSTRACT

In our ongoing research for new leads to the development of trypanocide from medicinal plants, due to emerged resistant strains of trypanosomes, *Khaya senegalensis* tree bark was extracted with methanolic solvent. *K. senegalensis* tree bark at different concentrations ((250-1000 µg/ml) was screened against *Trypanosoma evansi* on Alsever's medium. Trypanosomes were suspended in Alsever's solution with inactivated bovine serum at 58 °C for 1 h. 180 µl of the medium was added to the MPE of *K. senegalensis* tree bark (20 µl) and incubated at 37 °C with 5% carbon dioxide for 4 h. On hourly basis, drops of the incubated mixture were observed under inverted microscope for antitrypanosomal activity. *In vitro* cytotoxicity of MPE of *K. senegalensis* tree bark at concentrations (1.56-100 µg/ml) was done on Vero cells grown in Dulbecco's Modified Eagle Medium (DMEM) at appropriate conditions. At the dose 250 µg/ml of 50% MPE of *K. senegalensis*, there was drastic reduction in trypanosomes count and at 1000 µg/ml at 5 h of incubation though no complete killing of trypanosomes in any of the ELISA plate wells was observed. But at 1000 µg/ml of 100% MPE of *K. senegalensis*, trypanosomes were not detected in the ELISA plate wells at 5 h of incubation. Significant trypanocidal activity was observed (P = 0.05 to 0.01). Both MPE of *K. senegalensis* and diminazineaceturate were cytotoxic to Vero cells in all concentrations except at concentrations of 1.56-6.25 µg/ml and 1.56-6.25 µg/ml. This result indicated that *K. Senegalensis* tree bark possessed trypanocidal compounds that may lead to discovery of new trypanocide and Alsever's medium supported the trypanocidal activity observed.

Key words: *Khaya senegalensis* tree bark, *Trypanosoma evansi*, Trypanocidal activity, Cytotoxicity test.

INTRODUCTION

Trypanosomiasis is a zoonotic disease that had resurged and causing lots of havocs to both animals and humans in regions of the world where the disease strives (WHO, 2012). It is an important blood protozoan parasite, which is caused by flagellate parasite of the genus *Trypanosoma* (Freiburghaus *et al.*, 1998; WHO, 2010). In Africa, the estimated losses as a result of the disease in agricultural production amounted to 3 billion pounds annually (Hursey, 2000). Resistance to current trypanocides is on the increase as reported in endemic regions globally where the disease is prevalent (WHO, 2012).

Different parts of *Khaya senegalensis* (Meliaceae) have been used in Malian traditional medicine as antiparasitic drug (Ahua *et al.* 2008).

Biological activities such as antimicrobial and antifungal by limonoids from *K. senegalensis* have been reported (Abdelgaleil *et al.*, 2005).

Previously, we reported antitrypanosomal activity of 100% of MPE of *K. senegalensis* tree bark on different medium (Shaba *et al.*, 2011)

Lots of research work in the areas of ethno pharmacology and ethno medicine revealed that several medicinal plants possess trypanocidal compounds that will lead to future potential trypanocides (Wurocheke and Nok, 2004; Shaba *et al.*, 2006, 2008, 2014 and 2015).

As a result of aforementioned problems militating on existing trypanocides, *K. senegalensis* tree bark extract was screened against *T. evansi* for possible antitrypanosomal activity.

MATERIAL AND METHODS

Plant Material

Khaya senegalensis (Juss) tree bark was obtained from herbarium, Department of Biological Science, Faculty of Sciences, Ahmadu Bello Universit Zaria, Nigeria.

Extraction

Twenty grams of *K. senegalensis* shade dried tree bark was pounded into powder with pestle and mortar and cold extracted twice with 200 ml of methanol (analytical grade) according to Stahl

(1969). The filtrates were dried at 37°C and stored at 4°C until used.

Thin Layer Chromatography (TLC) Plates

Aliquots (0.2ml) of MPE *K. senegalensis* tree bark was applied on TLC plates, which were dried under room temperature and immersed inside the solvent systems in glass jar listed in the next subsection. This was done to detect, if any, the presence of bioactive constituents in applied MPE. After full development of plates in solvent systems, plates were dried at room temperature. Then, one set of TLC plates were immersed in iodine vapors in a glass jar. Second set of TLC plates was sprayed with Vanillin-sulphuric acid spray. Both media used facilitated the detection of bioactive constituents. This was carried out according to the method of Stahl, (1969).

Solvent Systems Applied.

The following solvent systems were tested to develop TLC plate, and to obtain a more suitable system for the extract (Stahl, 1969).

Chloroform/hexane/acetic acid (50:50:1)

Chloroform/ethyl acetate/acetic acid (50:50:1)

Methanol and chloroform (20: 80)

Test Organism

Trypanosoma evansi was obtained from the Division of Parasitology, Indian Veterinary Research Institute (IVRI), Izatnagar and was maintained in the laboratory by serial sub-passage in Swiss albino mice. The strain was routinely tested for virulence following the method Williamson *et al.* (1982)

In Vitro Trypanocidal Activity

In vitro trypanocidal activity was carried out on Alsever's medium (Talakai *et al.*, 1995). Trypanosomes were suspended in Alsever's solution with inactivated bovine serum at 58°C for 1 h. Trypanosomes concentration was 1x10⁶ parasites/ml. 180 µl of the medium was added to MPE of *K. senegalensis* (20 µl) and incubated at 37°C with 5% carbon dioxide for 5 h. On hourly basis, drops of the incubated mixture were observed under inverted microscope for antitrypanosomal activity.

The concentration of DMSO in the experiment had no deleterious effect by itself on host cells or parasites. 1% DMSO in distilled water was used as control (Young, 2000).

***In vivo* Infectivity Assessment**

In vivo infectivity of MPE of *K. senegalensis* tree bark was carried out after successful completion of anti-trypanosomal activity. Contents of microculture plate wells that contained reduced and apparently killed trypanosomes with MPE of *K. senegalensis* tree bark was inoculated (0.1ml mouse-1) into two groups of mice (six group-1) via intra-peritoneal, and observed for more than 60 days for parasitaemia (Woo, 1970; Igweh *et al.*, 2002).

***In Vitro* Cytotoxicity Test**

Cytotoxic effects of the MPE of *K. senegalensis* tree bark was determined according to the method described by Sidwell and Hoffman (1997). Vero cell line was grown in Dulbecco's Modified Eagle Medium (DMEM) (Sigma) Gibco, USA antibiotics (100 units penicillin, 100 µg streptomycin and 40 µg gentamycin) in 96-well flat bottom microculture plates (Nunc, Denmark). Each well received 100 µl of DMEM containing 5.10^5 cells/ml. The plates were incubated at 37 °C under 5% CO₂ for 48 h. After the formation of confluent monolayer, the medium was discarded and replaced with a fresh one. A high parasitaemic blood from mouse was diluted with DMEM to obtain a final parasite of 1×10^6 parasites/ml. Confluent monolayer of Vero cell was treated with serial dilutions of MPE of *K. senegalensis* (1.56-100 µg/ml) in triplicate and incubated under the same conditions described previously. After 24 h of incubation, the culture plate was observed for evidence of cytotoxic effects. The plate was incubated for 72 h and observed daily. It was repeated thrice. In each case, after the 72 h of incubation, the culture media of the incubated Vero cells were discarded. The adhered cells were stained with a drop of crystal violet in phosphate buffered solution. The plate was incubated for 24 hours at 37°C in an ordinary incubator. After 24 h of incubation, the culture plate was observed for evidence of cytotoxic effects.

Institute Committee on Welfare and Cruelty to Animals.

Indian Veterinary Research Institute Committee on Welfare and Cruelty to Animals received and approved application for the usage of mice in this research.

Statistical Analysis

Results of trypanocidal activity were expressed as mean \pm SEM. Statistical significance was determined by Sigma Stat (Jandel, USA).

RESULTS AND DISCUSSION

Current findings are presented in Tables (1-3).

Extraction

Methanol was appropriate in extraction of *K. senegalensis* powdered tree bark and bioactive constituents present in MPE of *K. senegalensis* were observed on TLC plate. This is similar to extraction of *Plumbago zeylanica* root bark and *Picrorrhiza kurroa* rhizomes (Shaba *et al.*, 2006 and 2015).

Solvent system

Out of four solvent systems tested in the analysis of thin layer chromatography (TLC) plates with applied aliquots of MPE of *K. senegalensis* tree bark, solvent systems, methanol/chloroform (20:80) was suitable than other solvent systems tested (plates not shown). On the TLC plates, different patterns of bioactive constituents were on display from MPE that were responsible for trypanocidal activity. This is similar to development of TLC plate of MPE of *Picrorrhiza kurroa* rhizomes (Shaba *et al.*, 2015).

***In vivo* Infectivity Test**

One group of mice inoculated with contents of ELISA plate wells with completely killed trypanosomes survived for more than 60 days. The other group inoculated with contents of ELISA plate wells with reduced trypanosomes count died of parasitemia. This result is comparable to *in vivo* infectivity test of MPE of *Plumbago zeylanica* root bark in which mice inoculated with contents of ELISA plate wells survived (Shaba *et al.*, 2006).

Table 1: *In vitro* trypanocidal activity of 50% methanolic extract of *Khaya senegalensis* tree bark against *Trypanosoma evansi* on Vero cell line

Concentration of plant extract in µg/ml	1 h	2 h	3 h	4 h	5 h
250	40.00±0.0	40.00±0.0	37.33±0.33	29.33±0.33	16.33±0.88
500	40.00±0.0	40.00±0.0	32.00±0.58	25.00±0.58	18.67±0.33
750	40.00±0.0	40.00±0.0	30.33±0.33	19.00±0.58	10.67±0.66
1000	40.00±0.0	40.00±0.0	28.67±0.33	12.67±0.66	8.000±0.58
Diminazineaceturate (50)	22.33±0.33	9.000±0.58	1.333±0.33	0.0±0.0	0.0±0.0
Positive control					
Control (Negative control)	40.00±0.0	40.00±0.0	40.00±0.0	40.00±0.0	40.00±0.0

Bioassay status: significant reduction of trypanosomes counts from concentration of 250 µg/ml and no complete killing of trypanosomes at 1000 µg/ml at 5th h of observation. An average mean parasites count of 37.67± 0.58 is statistically critical value. Average mean parasites counts from 37.67± 0.58 and below is significant between the treatment groups and negative control (P = 0.05 to 0.01).

Table 2: *In vitro* trypanocidal activity of 100% methanolic extract of *Khaya senegalensis* tree bark against *Trypanosoma evansi* on Alsever's medium

Concentration of plant extract in µg/ml	1 h	2 h	3 h	4 h	5 h
250	40.00±0.0	40.00±0.0	30.67±0.67	24.33±0.33	10.33±0.88
500	40.00±0.0	40.00±0.0	28.33±0.33	14.67±0.67	6.333±0.33
750	40.00±0.0	40.00±0.0	25.33±0.33	11.33±0.33	1.333±0.33
1000	40.00±0.0	40.00±0.0	19.33±0.33	1.333±0.33	0.0±0.0
Diminazineaceturate (50)	22.33±0.33	9.000±0.58	1.333±0.33	0.0±0.0	0.0±0.0
Positive control					
Control (Negative control)	40.00±0.0	40.00±0.0	40.00±0.0	40.00±0.0	40.00±0.0

Bioassay status: significant reduction of trypanosomes counts from concentration of 250 µg/ml and complete killing of trypanosomes at 1000 µg/ml at 5th h of observation. Average mean parasites counts of 37.67± 0.58 are statistically critical value. Average mean from 37.67± 0.58 and below is significant between the treatment groups and negative control (P = 0.05 to 0.01).

Table 3: Cytotoxic effect of methanolic extract of *Khaya senegalensis* tree bark on Vero cell line compared to diminazineaceturate (Berenil)

Concentration of test material in µg/ml	Effects of test extract at various periods of incubation (24 h, 48 h, 72 h)						
	<i>Khaya senegalensis</i>	Berenil	<i>Khaya senegalensis</i>	Berenil	<i>Khaya senegalensis</i>	Berenil	Control
100	100%	66.6%	100%	100%	100%	100%	0
50	100%	33.3%	100%	100%	100%	100%	0
25	100%	0	100%	100%	100%	100%	0
12.5	0	0	100%	0	100%	33.3%	0
6.25	0	0		0	66.6%	0	0
3.13	0	0	0	0	0	0	0
1.56	0	0	0	0	0	0	0

MPE of *Khaya senegalensis* tree bark and diminazineaceturate were toxic to Vero cell line except at concentrations range of 3.13- 1.56 and 6.25- 1.56 µg/ml.

Same concentrations were used for diminazineaceturate (Berenil)

***In vitro* Trypanocidal Activity of Methanolic Plant Extract of *Khaya senegalensis* tree bark**

Trypanocidal activity of MPE of *K. senegalensis* tree bark at different concentrations ((250-1000 µg/ml) were as given in Tables (1-2). In these findings, at 250µg/ml of 50 % MPE of *K. senegalensis*, a drastic reduction in trypanosomes count was recorded and at 1000 µg/ml no complete killing of trypanosomes in ELISA plate wells at 5 h of incubation. But at

1000 µg/ml of 100% MPE of *K. senegalensis*, trypanosomes were not detectable in the ELISA plate wells at 5 h of incubation. Significant trypanocidal activity was observed (P = 0.05 to 0.01), which was statistically the same as diminazineaceturate (50 µg/ml) standard drug at 4 h of incubation. Trypanocidal activity was concentration-time dependent faction. Average mean trypanosomes counts from 37.67±0.58 and below is significant between the treatment

groups and negative control (P = 0.05 to 0.01). These results are comparable to *in vitro* screening of American plants extracts on *Trypanosoma cruzi* and *Trichomonas vaginalis*, *in vitro* anti trypanosomal activity of some medicinal plants used in the treatment of trypanosomiasis in Northern Nigeria with effectiveness at 8.3 mg/kg body weight and anti-trypanosomal activity of *Piper nigrum* L. (Black Pepper) against *Trypanosoma evansi* with varied anti-trypanocidal activities (Muella-Serrano et al., 200 and Shaba et al., 2012).

Trypanocidal activity of MPE of *K. senegalensis* tree bark may be due to already isolated compounds such as limonoids ((Abdelgaleil et al., 2005) and ability of extracts to intercalate DNA of trypanosomes that often kill them (Sepúlveda- Boza, 1996)

In Vitro Cytotoxicity Test

As shown in Table (3), MPE of *K. senegalensis* tree bark, and diminazineacetate were cytotoxic to Vero cells in all concentrations except at 1.56-3.13 and 1.56-6.25 µg/ml, respectively. Cytotoxic effects such as distortion, sloughing, swelling and dead of the affected cells were observed compared to normal cells. These cytotoxic effects observed are similar to Effects of *Terminalia arjuna* Bark extract on apoptosis of human hepatoma cell line (HEPG2) and MPE of methanolic extracts of *Moringa oleifera* tree bark and seed pods (Shaba et al., 2014).

CONCLUSION

In these current findings, *K. Senegalensis* tree bark possesses trypanocidal compounds, which is in line with previous report that may lead to discovery of new trypanocide and Alsever's medium supported the trypanocidal activity observed.

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