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Evaluation of Anti-theilerial Activity of A lipophilic Root Bark Extract of *Stereospermum kunthianum* against *Theileria Parva*

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Abstract
Naphthoquinones are common compounds found in several species of Bignoniaceae and exhibit anti plasmodial activity hence providing leads for discovery and development of anti theilerial drugs. This study has established the presence of naphthoquinones in the leaf, stem bark and root bark of S. kunthianum for both the hydrophilic and lipophilic extracts and established the anti theilerial activity of the lipophilic root bark extract. Sixteen unknown naphthoquinones in both the lipophilic and hydrophilic extracts of the leaf; stem bark and root bark of S. kunthianum were screened by Thin layer chromatographic technique. The lipophilic root bark extract of S.kunthianum caused death of macroschizonts of T.parva with 18 % mean percentage activity at a start experimental dose of 1 µg/L. The mean percentage activity against macroschizonts of T. parva increased with increase in the concentration of the lipophilic root bark extract of S. kunthianum. The in vitro Median Effective Concentration, EC_{50} of 1.585µg/L as compared to the reported EC_{50} of 0.3 µg/L for Buparvaquone, a known hydroxy naphthoquinone used in the treatment of ECF, was obtained for the lipophilic root bark extract of Stereospermum kunthianum against Theileria parvaby linear regression analysis.
Key words: Stereospermumkunthianum, Anti-theilerial Activity, Lipophilic,Root Bark Extract, Theileria Parva

INTRODUCTION

Evaluation of several works on the epidemiology and clinical specificities of East Coast fever (ECF) indicates that ECF is a very serious, often fatal, lympho-proliferative disease of cattle that causes major economic losses in Eastern, Central and Southern Africa (Marcellino *et al.*,2011; Farah *et al.*, 2012). The disease is caused by a single-celled protozoan parasite *Theileria parva*, which is transmitted by the brown ear tick, *Rhipicephalus appendiculatus* as it feeds on blood in cattle (Chenyambuga *et al.*, 2010).

Reports indicate that plants with good anti plasmodial activity are also very good candidates for development of anti theilerial drugs (Fieser*et al.*,1948; Kerdmanee*et al.*, 2001). The anti plasmodial drugs such as Chloroquine and Quinine

have been reported to exhibit anti theilerial activity with 50% inhibitory concentrations of 12 mM and 260 mM respectively (Kerdmanee *et al.*, 2001). This implies that one can begin the search for anti theilerial drugs by screening compounds or materials with anti plasmodial activity for anti theilerial activity. Research has further indicated that naphthoquinones are common compounds that exhibit anti theilerial activity hence providing leads for discovery and development of anti theilerial drugs (McHardy *et al.*, 1985; *Kinabo*, 1989).

This study aimed at establishing the presence of naphthoquinones in the leaf, stem bark and root bark of *S. kunthianum* and determine the anti theilerial activity of its lipophilic root bark extract. Whereas the lipophilic root bark extract of *S. kunthianum*has been reported to possess naphthoquinones with established anti-plasmodial activity (Onegi *et. al*, 2002, p.39), this has not been done for the leaf and stem bark extracts. The screening of naphthoquinones and anti theilerial activity of *S.kunthianum* adds to the ever increasing scientific data base of medicinal plants globally. The findings of this study therefore serves as a scientific contribution in the search for natural products of plant origin that can be used in drug discovery and development of new and cost effective drugs against East Coast Fever and other protozoan diseases.

EXPERIMENTAL

Collection and identification of plant material: The roots, stems and leaves of *S. kunthianum* were collected from Achwa Valley Ranch, Nwoya District (formally part of Amuru District) in Northern region of Uganda in the month of January 2013. The plant material was placed in a clean polythene bag, sealed and given an identification number. The collected plant material was labeled with the following details: Botanical name of the plant, location of collection, part that has been collected, date, time of collection and numbered.A herbarium specimen of the plant material was be prepared authenticated by an experienced Taxonomist, Mr. Adrian Kakooko and deposited as SK/MSC/2013 at the Natural Chemotherapeutics research Institute, Wandegeya for future reference purposes.

Preparation of the lipophilic root bark extract: The collected root bark of *S.kunthianum* was cleaned, air dried in dust free shade for 72 hours then ground with a mortar and pestle into fine powder which was then placed in air tight labeled container till required for use.

A method described by Onegi *et al* (2002) was used to obtain naphthoquinones as a component of lipophilic root bark extract of *S. kunthianum*. Briefly, 100 grams of the powdered root bark material was sequentially extracted three times with equal parts of Petroleum ether (PE) and Ethyl Acetate (EOAC) at room temperature using cold maceration to afford a lipophilic extract. The weighed powdered plant material mixed with solvent was allowed to stand for 24 hours at room temperature (25 ^{0}C), shaking occasionally; liquid strained off; solid residue pressed, clarified by filtration; followed by evaporation and concentration. The lipophilic root bark extract was used in the bioassay. A stock solution was prepared and then serial dilutions made from this stock solution in µg/ L.

Determination of Naphthoquinones in Stereospermum kunthianum

The presence of naphthoquinones in the leaf, stem bark and root bark of *Stereospermum kunthianum* was established by means of Thin Layer Chromatographic technique described by Wagner, 1996).Briefly, the procedure involved preparation of extracts for TLC, reference solution, stationery phase and mobile phase and finally detection.One gram of each powdered plant materials (Dried leaf, stem bark and root bark of Stereospermum kunthianum) were sequentially extracted three times with equal parts of Petroleum ether (PE) and Ethyl Acetate (EOAC) at room temperature using cold maceration for 15 minutes to afford a lipophilic extract. Filtration was done using a membrane filter and 30 µl of the clear filtrate was pipetted for TLC analysis.

The reference Solution was prepared by dissolving 10mg of Buparvaquone obtained from Desbro Chemicals (Kenya) Limited in 1ml Methanol and 10 µl of this solution pipetted for TLC analysis. The stationary phase was silica gel $60F_{254}$ Precoated TLC plates (Merck, Germany) while the Mobile phase (Solvent system) was a solution of Toluene mixed with Formic acid in the ratio of 99:1.

Detection of Naphthoquinones was made by means of UV lamp. All naphthoquinones have been reported to show quenching in UV- 254 nm (Wagner, 1996). After spraying with 10%

Methanolic – Potassium hydroxide reagent, naphthoquinones show red florescence in UV-365 nm and red to red-brown colour (Wagner, 1996).

Hydrophilic extracts of the leaf, stem bark, root bark were separately obtained by maceration for 15 minutes over a water bath using water as a solvent. Filtration was done using a membrane filter and 30 µl of the clear filtrate was pipetted for TLC analysis as described above.

In vitro culture of Theileria parva:

Theileria parva has been shown to infect and transform B cells and T cells at similar frequencies in vitro (Baldwin*et al.*, 1988). The majority of parasitized cells in the tissues of infected cattle are alpha/beta T cells (Morrison *et al*., 1996).

The isolated B - lymphocytes were purchased and re-suspended in a tissue culture medium that consisted of RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum, 2 mM of l-glutamine, 100 units/ml of penicillin, 50 µg/ml of streptomycin, and 5×10^{-5} M of β-mercaptoethanol at a density of 3×10^{6}/ml.

T. parva tick derived stabilates were used for *in vitro* cultivation of *T. parva* as earlier described by Marcotty*et al.* (2004) and modified by Mbao*et al.* (2005). Briefly, *T. parva*sporozoitesstabilate obtained with assistance of researchers from the National Agricultural research organization, Kawanda were diluted in 96-well microtitration plates and then the isolated B - lymphocytes added to the wells. The plates were incubated for 10 days at 37 °C in 5 % CO_2 after which cyto-centrifuged samples were taken, giemsa stained and microscopically examined for *T. parva* macroschizonts.

Determination and Evaluation of Anti-theilerial activity

The following experimental set up was used to establish the anti-theilerial activity of the lipophilic root bark extract of *S. kunthianum*:

Group 1: This was a test group that consisted of the microorganism (*T. parva*sporozoitestabilate) plus different concentrations of the lipophilic root bark extract. This group was used to determine if the extracts are effective.

Group 2: This was a positive control group that consisted of *T. parva* plus known anti theilerial drug, Buparvaquone. This group was used to guarantee that the micro organism utilized was susceptible to known chemotherapeutic agents and not a resistant strain.

Group 3: This group consisted of only *T.parva* on tissue culture medium to guarantee that the organism grows properly under the defined laboratory conditions thus help to distinquish poor growth from inhibition of growth.

Group 4: This was a negative control group and consisted of *T.parva* plus pure extraction solvents used in obtaining the lipophilic root bark extract of *S. kunthianum*. This group was to confirm that the extraction solvents had no inhibitory effect on growth of the organism.

The activity of the lipophilic root bark extracts of *S. kunthianum* was evaluated by counting the mean number of dividing cells in each slide, counting mean number of dead macroschizonts in 50 cells per slide and calculating the percentage of dead macroschizonts per cell in 10 cells per slide. The medium effective concentration of the extracts was then determined by regression analysis using the equation $y = a + b\,x$.

RESULTS AND DISCUSSION

Presence of naphthoquinones in S. kunthianum

The study revealed the presence of sixteen (16) unknown naphthoquinones in both the lipophilic and hydrophilic extracts of the leaf; stem bark and root bark of *S.kunthianum* that require to be isolated and their structures elucidated. The findings of phytochemical screening have been presented in Table1.

Table 1: General results for the screening of the hydrophilic and Lipophilic extracts of the leaf, stem bark and root bark of *S. kunthianum* for presence of Naphthoquinones using TLC

Plant part	Nature of extract	Compound detected	Distance moved from the spotting point (cm)	Rf value	Colour under UV 254 nm.	Colour under UV 365 nm
		Solvent	16	1	N/A	N/A
		Standard (Buparvaquone)	7.2	0.45	Yellow	Red
Leaf	Lipophilic extract	Un known 1LL	6.2	0.39	Yellow	Red
		Un known2LL	5.3	0.33	Blue	Not detected
		Un known 3LL	3.5	0.22	Green	Brown
		Un known 4LL	3.1	0.19	Yellow	Brown
	Hydrophilic extract	Unknown1HL	3.2	0.20	Blue	Red
		Unknown2HL	5.2	0.32	Blue	Not detected
		Unknown3HL	12.4	0.77	Yellow	Not detected
Stem bark	Hydrophilic extract	Un known 1HS	14.4	0.90	Yellow	Red
		Un known2HS	6.0	0.37	Blue	Not detected
		Un known3HS	7.5	0.47	Blue	Red
		Un known 4HS	6.2	0.38	Blue	Not detected
		Un known 5HS	5.4	0.34	Green	Brown
	Lipophilic extract	Un known1LS	3.4	0.21	Blue	Not detected
		Unknown2LS	5.3	0.33	Blue	Red
		Unknown3LS	6.1	0.38	Blue	Not detected
		Unknown4LS	11.8	0.74	Yellow	Red
		Unknown5LS	12.2	0.76	Green	Red
Root bark	Lipophilic extract	Unknown 1LR	6.5	0.41	Red	Red
		Unknown 2LR	5.3	0.33	Blue	Red
		Unknown 3LR	3.4	0.21	Blue	Not detected
		Unknown 4LR	1.3	0.08	Yellow	Not detected
		Un known 5LR	7.7	0.48	Yellow	Red
		Un known 6LR	12.5	0.78	Green	Brown
	Hydrophilic extract	Un known 1HR	1.7	0.11	Blue	Reddish brown
		Un known 2HR	3.2	0.20	Blue	Not detected

Earlier reports indicate that bioassay –guided fractionation of a lipophilic extract of the root bark of *S. kunthianum* led to the isolation of four novel naphthoquinones (Sterekunthals A-B, Pyranokunthones A-B) and one novel anthraquinone (Anthrakunthone) together with the known naphthoquinone pinnatal from *Kigelia pinnataDC* (Onegi *et al.*,2002). Thechoice of the solvent to use in the extraction of naphthoquinones is important as the lipophilic extract contained higher number of naphthoquinones (10) detected compared to the hydrophilic extract (6). See findings of the analysis as summarized in table 2. This therefore implies that, it is better to use lipophilic solvents to extract naphthoquinones as compared to hydrophilic solvents. The extraction yields for each of these naphthoquines may vary depending on solvent used.

Table 2: Nature of extract of Stereospermum kunthianum and presence of Naphthoquinones as screened by TLC at UV 365 nm by Plant part

Colour under UV 365 nm	Compounds detected as per Nature of extract used*		Total number
	Hydrophilic	Lipophilic	
Brown	1	3	4
Not detected	4	5	9
Red	3	7	10
Reddish brown	2	0	2

*Pearson chi2 (3) = 3.8657 Pr = 0.276

The rate at which compounds migrate up a silica gel plate during TLC analysis of a sample depends on their polarity (Watson, 1999). In a given length of time, the most polar compound moves the least distance up the plate while the least polar moves furthest (Watson, 1999). In the present study, it was observed that 18 of the 25 un known compounds in the leaf, stem bark and root bark of *S. kunthianum* detected by TLC were found to be more polar than the standard compound Buparvaquone (See table 3). Further analysis indicate that majority of these compounds were found in the lipophilic root bark extract. As summarized in Table 1 above, there were ten unknown compounds in lipophilic extracts of *Stereo kunthianum* which were more polarthan Buparvaquone comparable to seven in the hydrophilic extracts. This collaborates earlier reports that have indicated that Several naphthoquinones are very poorly water soluble and lipophilic molecules, a fact frequently associated to a low bioavailability of drugs with a dissolution rate-limiting step to absorption (“*Chapter 3: Materials and methods”).*

Table 3: Polarity of unknown compounds detected in *S. kunthianum* as compared to Buparvaquone

Polarity as compared to BPQ*	Number of compounds
Less polar	7
More polar	18

* Categorization based on distance moved up the silica gel.

Bapelaa *et al.* (2007) have reported that “the type and level of naphthoquinones vary between different plant species and variation in bioactivity is often encountered between different parts of the same plant.” The presence of phytochemical compounds can vary depending on the geographical location, age of the plant, plant part being investigated, the nature of the extract, season of collection of plant material among other factors (Evans, 2009). The likelihood of variation in the content of naphthoquinones in different parts of *S. kunthianum* therefore needs to be investigated.

Anti theilerial activity of Stereospermum kunthianum

The lipophilic root bark extract of *S. kunthianum* caused death of macroschizonts of *T.parva* with 18 % mean percent activity at a start experimental dose of 1 µg/L. This shows that the lipophilic root bark extract of *S. kunthianum* was very potent given that even in such a crude form, it still showed activity against *T. parva* at such a low a concentration as 1 µg/L (See Table 4) .

The mean percent activity against macroschizonts of *T. parva* increases with increase in the concentration of the lipophilic root bark extract of *S. kunthianum*. In fact, doubling the concentration of the lipophilic root bark extract of *S. kunthianum* from 1 µg/L to 2µg/L increased its activity against macroschizonts of *T.parva* by 3.056 times. However, doublings of concentration of this same extract from 20µg/L to 40µg/L and from 40 µg/L to 80µg/L increased the activity by only 1.0625 times and 1.0353 times respectively implying that doubling of concentration at lower doses increases

the activity more folds as compared to increases of the concentration at higher dosage.

A tenfold increase in concentration from 2 µg/L to 20 µg/L resulted in an increased activity of the lipophilic root bark extract of *S. kunthianum* against macroschizonts of *T. parva* by 1.454 times (68.75% increase in activity) although this was lower than observed activity at doses 40µg/L and 80µg/L of the extract. The macroschizonts were almost eliminated at higher concentrations of 40µg/L and 80µg/L with the lipophilic root bark extract of *S.kunthianum* showing 85% and 88% activity respectively. This implies that the extract is more active at these concentrations although the toxic effects of the extract need to be investigated as well at these particular concentrations.

Table 4: Effect of Lipophilic root bark extract of *Stereospermum kunthianum* on the macroschizonts of *Theileria parva*

Concentration in µg/L	Total number of cells examined per slide	No. of cells with alive macroschizonts per slide	No. of cells with dead macroschizonts per slide	Mean Percent Activity* (%)
0	50	50	0	0
1	50	41	9	18
2	50	17	33	66
10	50	12.5	37.5	75
20	50	10	40	80
40	50	6.6	42.5	85
80	50	6.0	44	88

*****Pearson chi2 (25) = 30.0000 Pr = 0.224;
Kendall's tau-b = 1.0000

The percentage of cells that were infected by *Theileria parva* following treatment with the lipophilic root bark extract of *S. kunthianum* decreased with an increase in the concentration of the extracts (See Table 5). The number of viable cells counted after 10 days following treatment with the lipophilic root bark extract of *S. kunthianum* was higher and almost double that observed after 48 hours at similar concentrations. Maximal activity was achieved at the concentration of 80µg/L where the extract almost eliminated the infection of the cells for both 48 hours and 10 days after its administration.

Table 5: Effect of the lipophilic root bark extract of *S. kunthianum* on the infection of cells following treatment

Concentration in µg/L	Number of viable cells ($x10^4$/ ml) 48 hrs before treatment with extract	Percentage of cells infected by T. parva after 48 hrs and 10 days following treatment with the extract.	
		48 hrs after (% of cells infected)*	10 days after (% of cells infected)*
0	10	100	100
1	10	67	30
2	10	45	25
10	10	42	20
20	10	35	10
40	10	12	5
80	10	10	2

*Pearson chi2 (20) = 24.0000Pr = 0.242

There was a decrease in the mean number of diving cells per slide following treatment with the lipophilic root bark extract of *S. kunthianum*. The activity of the extract on diving of cells perfectly

increased with an increase in the concentration of the extract(See table 6). This shows that lipophilic root bark extract of *S.kunthianum* has the capacity to interfere with the proliferation of lymphocytes exhibited by *Theileria parva.* Studies have indicated that *Theileria parva* is transmitted by ticks of the genus *Rhipicephalus*, which inject cattle with infectious sporozoites. The sporozoites are said to invade lymphocytes in blood of host cattle and then develop into multinucleated macroschizonts that then progresses down in two pathways. One pathway involves the transformation of B or T Lymphocytes into proliferating cells, with the multinucleate macroschizont dividing between daughter lymphocytes. The second pathway generates free "merozoites" that subsequently infect erythrocytes, in which they form "piroplasms" that can then be taken up by blood feeding ticks (Cynthia *et al.*, 2011; Kerdmanee, 2001; Morrison *et al.*,1996).

Table 6: Effect of Lipophilic root bark extract of *Stereospermum kunthianum* on the number of dividing cells per slide

Concentration µg/L	Number of dividing cells/ slide Binucleated*	Multinucleated*	Mean total Number of dividing cells/ slide*
0	32	19.5	51.5
1	26	18	44.0
2	20	15	35.5
10	19	11	30.0
20	13.5	12	25.5
40	10	7	16.5
80	4	2	4.00

*Pearson chi2 (25) = 30.0000Pr = 0.22

The activity exhibited by the lipophilic root bark extract of *S. kunthianum* against *T.parva* as observed in this study was most likely due to the naphthoquinones present in this plant material. Earlier works have indicated that naphthoquinones are common compounds that exhibit anti theilerial activity hence providing leads for discovery and development of anti theilerial drugs (McHardy et al., 1985; *Kinabo*, 1989).

CONCLUSION

This study has established the presence of naphthoquinones in the root bark, leaf and stem bark of *Stereospermum kunthianum* for both the hydrophilic and lipophilic extracts. The results of this study have further revealed that the lipophilic root bark extract of *Stereospermum kunthianum* is active against *Theileria parvain vitro* with a median effective concentration, EC_{50} of 1.585 µg/L as compared to the reported EC_{50} of 0.3 µg/L for Buparvaquone, one of the hydroxylated naphthoquinones that is active against Theileria parva both in vitro and in vivo.

AREAS FOR FURTHER RESEARCH

Further research should be conducted in the following areas:

i. There is need to carry out bioassay guided isolation and characterization of the naphthoquinones present in *S. kunthianum.*

ii. An *in vivo* study should be conducted to confirm the in vitro anti theilerial activity of *S. kunthianum* established in this study and also determine the compounds responsible for the activity with their mechanism of action.

iii. Acute, sub acute and chronic toxicity testing of the plant materials (leaf, stem and root bark) used in the study should be done to establish/ confirm safety of the plant.

Ethical considerations

The study was approved by the ethics committee of Makerere University college of health sciences. The communities where the plant material was collected needs to be sensitized on the usefulness of *Stereospermum kunthianum* and its conservation to avoid over harvesting.

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