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Determination of phytochemicals and anthelmintic activity of *Rytigynia kigeziensis* Verdc extracts using *Eudrilus eugeniae* model

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Abstract

Helminthic infections are among the global public health problems. Due to high cost of standard drugs and resistance, rural dwellers use herbal preparations. However, efficacy and mechanisms of action remains elusive. In Uganda, *Rytigynia kigeziensis* is one of the commonly used anthelmintic herbs. The current study sought to determine the effect of phytochemicals in *R. kigeziensis* from the stem bark against E. eugeniae model for application as anthelmintic. The anthelmintic activity was carried out by time of paralysis and time of death at various at concentrations (15, 25, 50 and 100 mg/ml). Further LDH activity was examined to shade light on the possible mechanism of inhibition. All the results obtained were expressed as mean values ± SEM (n=3). The significance of difference between the means of albendazole and extracts concentrations were determined using One-way ANOVA on PAST version 3.15 and regarded significant at $P \leq 0.05$. The phytochemicals extracted were in the order ethanol> methanol> aqueous> ethyl acetate> chloroform whereby alkaloids, flavonoids, saponins, glycosides, steroids, anthraquionones and tannins were present in all the extracts. Phlobatannins were absent in all extracts while terpenoids were absent in ethyl acetate, reducing sugars and phenols were also not observed in chloroform extracts. The anthelminthic activity was dose dependent in all extracts in the order ethanol>methanol>ethyl-acetate>aqueous>chloroform extracts. The LDH activity was dose dependent and statistically significant. The results were statistically significant when compared against albendazole. The findings suggest synergistic effect *R. kigeziensis* stem bark extracts, and the plant may serve as a potential anthelminthic agent.

Keywords: *Rytigynia kigeziensis*; *Eudrilus eugeniae*; Anthelminthic; Phytochemicals; Glucose metabolism

1. Introduction

Helminthic infections are among the most common parasitic infections worldwide [1] and affect the poorest and most deprived communities in tropical and subtropical regions of Africa, including Uganda [2, 3]. The helminthes which infect the intestine are cestodes for example tape worms (*Taenia solium*), nematodes (*Ancyclostoma duodenale*), round worm (*Ascaris lumbricoides*), whipworm (*Trichiuria trichirus*) and trematodes or flukes (*Shistosoma mansoni* and *Schistosoma haematobium*) [4]. The main species of these parasites that infect and harbor in human beings are the roundworms, the whipworms and hookworms [5, 2, 6]. These soil transmitted helminthes (STH) are typically obligate organisms, dependent on vertebrate, and arthropod as hosts or both for survival, and the parasites may produce serious infections, which occasionally could lead to death. The STH infections occur when ingested food, and hands are contaminated with eggs or larvae [7]. The STH do not mainly subsist only in intestinal tract, but they are also found in tissue, as their larvae migrate towards them [5]. The helminthes do not directly contribute to mortality, but reduce the body's ability to absorb nutrients and vitamins resulting into malnutrition related diseases such as kwashiorkor, marasmus, anemia and pneumonia, as well as increased susceptibility to other infections [5]. Hence, the affected individuals are more likely to

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respond slowly to treatment which contributes to the development of complications of disease and ultimately weakens the affected individuals [7]. For children, especially school-age, helminthic infections result into stunted growth and poor cognitive development ability, and hence affect their academic performance; demotivating them from going to school, and as a result, they drop out of schools [2]. In adults, parasitic helminthes lead to reduced work productivity or adverse pregnancy outcomes in women [4].

In Western Uganda, people living around Bwindi Impenetrable National Park and the neighboring communities use several medicinal plants for the treatment of intestinal parasites. The commonly used plants include the stem bark of *Rytigynia kigeziensis,* and *Ocotea usambarensis* [8, 9]. The chemotherapeutic options available are expensive, limited availability, and increased resistance compared to the citizens. In addition, increased drug resistance. Thus, an alternative effective remedy which is readily available and cheap is needed to combat the helminthic infections. *Rytigynia kigeziensis* locally called *Nyakibazi* in *Bakiga*,- *Banyankole* and *Bafumbira*, belong to *Rubiaceae* or coffee family. This family has about 630 genera and 1300 species distributed worldwide especially in tropical and subtropical regions [8]. The plants have exhibited antimalarial, antimicrobial, antihypertension, antidiabetic, antioxidant and anti-inflammatory, anthelmintic properties. The phytochemicals found in most of these plants include alkaloids, tannins, flavonoids, saponins, terpenoids and anthraquinones [8]. Hence, the current study sought to determine the phytochemicals in *R. kigeziensis* stem bark extracts, and assess the anthelmintic activity using *E. eugeniae* model.

2. Material and methods

2.1. Study design

The study was an experimental design and was carried out in Kampala International University according. African night crawler earthworm (*Eudrilus eugenia*) was used as a model organism, due to its anatomical and physiological resemblance with the intestinal round worm parasites of human beings. The phytochemicals in *R. kigeziensis* stem bark extracts were qualitatively assessed and documented.

2.2. Worm collection and authentication

The earthworms were obtained from the vermin-culture facility of Makerere University and maintained in moist, well aerated soil at room temperature in the Biochemistry Laboratory in a wooden box and fed on cellulose containing substrate made from cow dung [11]. The composting process of the substrates involved the periodic wetting by sprinkling with approximately 1 liter of water daily, turning of cow dung manure and soil under aerated conditions for several weeks prior to introducing earthworms.

2.3. Plant material collection and authentication

Fresh *Rytigynia kigeziensis* sample branches with leaves and fruits were collected from Nyabyondo village, Mitooma District, South-Western Uganda in the month of April. The specimens were identified by a registered botanist; Dr. Eunice A. Olet from Mbarara University of Science and Technology and voucher specimens (Evarist Asiimwe # 001) were deposited in the University herbarium.

2.4. Preparation of plant extract

The stem bark of *R. kigeziensis* was removed and thoroughly washed using running tap water to remove soil, adhered debris as well as epiphytic plants, and then finally washed with sterile distilled water. It was chopped into small pieces, and dried under shade for two weeks, then crushed well into fine powder using a laboratory mill (Retsch, 5657, Germany). The powder was stored in an air tight polythene bag pending extraction. 500 g of the powder was soaked in 1 litre of ethanol by cold maceration at room temperature for a total of 72 hours. The mixture was filtered using muslin cloth, and followed by filtration using Whatman No 1 filter paper. The filtrate of the plant material was concentrated in a rotary evaporator at 40°C, under reduced pressure to yield a thick crude extract. The same procedure was repeated for the aqueous, methanol, ethyl acetate and chloroform. The extracts were finally stored in sterile air tight bottles and kept in the refrigerator at 4°C till used.

2.5. Phytochemical analysis of *Rytigynia kigenziesis* extracts

The presence of phytochemicals such as alkaloids, flavonoids, tannins, phlobatannins, anthraquinones, terpenoids, phenols, steroids reducing sugars, glycosides and saponins according to [5] and [4].

2.5.1. Test for alkaloids (Mayer's test)

To 1 ml of ethanol extract, 2 ml of Wagner's reagent was added. Reddish brown colored precipitate was considered an indicator for the presence of alkaloids.

2.5.2. Test for flavonoids (Lead acetate test)

To 2 ml of plant ethanol extract, 5 ml of dilute ammonia solution was added followed by addition of concentrated H_2SO_4. Observation of a yellow coloration was taken as an indicator for the presence of flavonoids.

2.5.3. Test for saponins (Froth test)

To 2 ml of plant ethanol extract, 5 ml of distilled water was added and shaken vigorously. This was followed by addition of 3-4 drops of olive oil. Formation of stable foam was taken as an indicator for the presence of saponins.

2.5.4. Test for terpenoids (Copper acetate test)

To 5 ml of plant ethanol extract, 2 ml of chloroform and 3 ml of concentrated H_2SO_4 was added and formation of a reddish-brown coloration of the inter face showed positive results for the presence of terpenoids.

2.5.5. Test for phlobatannins (Precipitate Test)

To 5 ml of plant ethanol extract, 4 ml of 2% aqueous HCl was added and boiled, the deposition of a red precipitate was taken as evidence for the presence of phlobatannins.

2.5.6. Test for anthraquinones (Bontrager's test)

To 1 ml of plant ethanol extract, 2ml of dilute NaOH was added. Formation of blue green or red coloration indicated the presence of anthraquinones.

2.5.7. Test for steroids (Salkowski's test)

To 0.5 ml of plant ethanol extract, 2 ml of acetic anhydride was added followed by 2 ml H_2SO_4.The colour change from violet to blue or green in samples was considered to indicate the presence of steroids.

2.5.8. Test for phenols (Ferric chloride test)

To 1ml of plant ethanol extract, 2 ml of distilled water was added followed by a few drops of 10% aqueous ferric chloride solutions. The formation of blue or green colour indicated the presence of phenols.

2.5.9. Test for tannins (Gelatin test)

To 1 ml of plant extract, 1 ml of 0.008 M potassium ferric cyanide was added followed by 1-3 drops of 0.02 M ferric chloride. Formation of a blue-black coloration indicated the presence of tannins in the extract.

2.5.10. Test for glycosides (Legal's test)

To 5 ml of plant ethanol extract, 2 ml of glacial acetic acid was added followed by one drop of ferric chloride solution. This is followed by addition of 1 ml of concentrated H_2SO_4. A brown ring or violet or greenish ring indicated presence of glycosides.

2.5.11. Test for reducing sugars (Benedict's test)

To 2ml of plant ethanol extract, 2ml Benedict's reagent was added and heated gently. Orange red precipitate was considered as an indication for the presence of reducing sugars.

2.6. Anthelmintic activity

2.6.1. Standard drug preparation

Albendazole was used as the standard drug [12, 13] into different concentrations (15, 25, 50 & 100 mg/ml) that were used, and were prepared freshly for each experiment. Normal saline was used as control.

2.6.2. Preparation of experimental animals

A total 252 adult *E. eugeniae* worms measuring averagely 7.0 cm in length (using a ruler), and weighing 1.0g. The groups and treatments are depicted in Table 1. All experiments were carried out at room temperature, in triplicates. The starvation was prevented by immersing the worms in normal saline solution [4].

Table 1 Groups and worms involved in the study

Groups	Treatments (15, 25, 50 & 100 mg/ml)
1	Normal saline (NS)
2	NS+ Albendazole
3	NS + DMSO + Aqueous extract
4	NS + DMSO + Methanol extract
5	NS + DMSO + Ethanol extract
6	NS + DMSO + Ethyl acetate extract
7	NS + DMSO + Chloroform extract

2.7. Anti-helminthic activity

The anthelmintic activity assay was carried out as described by [14]. Briefly, Observations were made for the time taken for paralysis and death of individual worm. The average time was finally calculated recorded for each group.

2.7.1. Determination of time of paralysis

The time of paralysis was noted when no movement of any sort was observed except when the worms were shaken vigorously [4, 5].

2.7.2. Determination of time of death

The time of death of the worms in minute was recorded after ascertaining that worms could not move when shaken vigorously, worms not showing any motility were picked out, in case of revival in motility, the observed worms were counted as alive; otherwise, they were counted as dead [5, 4].

2.8. LDH enzyme assay

2.8.1. Tissue homogenate preparation

The tissue homogenate preparation was done as described by [15]. The earthworm tissue weighing 2.7g from the ethanol extract treated group of worms was crushed using the sterile mortar and pestle. The crushed tissue was mixed with 10ml of phosphate buffer at pH 7.2 in 15ml test tubes. The test tubes containing the mixture were centrifuged at 2,500 rpm for 10 minutes. The supernatant was collected and kept on ice (4^0C) until used.

2.8.2. Lactate dehydrogenase activity test

The activity LDH was assayed using the Bio Vision clinical chemistry analyzer according to [15]. Lactate dehydrogenase (LDH) activity takes advantage of the fact that while reduced coenzyme NADH absorbs light at 340 nm, oxidized NAD+ does not. A difference in absorption behavior of NAD+ and NADH is between 300 and 400 nm results from changes in the nicotinamide ring during oxidation or reduction. To measure the LDH activity, a solution containing lactate and NAD+ is placed in a cuvette, and absorption is recorded at a constant wavelength of 340 nm. Briefly 1.0ml of working reagent and the buffer were pippeted into pre-warmed cuvette at 250C. The spectrophotometer was adjusted to zero using water at 340nm. 0.05ml of the supernatant was added and the mixture was warmed again to 250C for five minutes. The cuvette mixture was transferred into the spectrophotometer and absorbance (A1) was recorded after 30 seconds. Absorbance (A2) was also read and recorded after 1 minute. Using the difference in absorbance (A2-A1) the LDH activity calibrated by the following formula;

$$\text{Enzyme activity (U/L)} = \frac{(A2 - A1) \times 1.050 \times 1000}{1 \times 6.22 \times 0.050 \text{ ml}}$$

$$= (A2 - A1) \times 3376$$

Where:

(A2-A1) = Change in absorbance
1.050 = Total reaction volume in ml
1000 = Conversion of U/ml to U/L
1 = Light path in cm
6.22 = Millimolar absorptivity of NADH
0.050 = Sample volume in ml

2.9. Data analysis

The results obtained were expressed as mean values ± SEM (n=3) in each group between the albendazole and extracts and subjected to statistical analysis using One-way ANOVA on PAST version 3.15 and $P \leq 0.05$ was regarded significant.

2.10. Ethics and consent to participate

Approvals for the research were obtained from the Kampala International University - Institution Research and Ethics Committee (KIU-IREC). Earthworms are not vectors of any known disease to human health thus safe and suitable for use in the study. The recommended number of worms was used in this study. Afterwards the study guidelines for disposal of laboratory used animals were followed.

3. Results

3.1. Phytochemical screening

Various plant extracts of *R. kigeziensis* showed presence of phytochemicals as shown in Table 2, and were in the order ethanol> methanol> aqueous> ethyl acetate> chloroform. It is worth noting that ethanol extract revealed a high concentration of alkaloids, flavonoids, saponins and tannins. However, moderate presence of phenols and glycosides was noted, while terpenoids, anthraquinones and steroids were in trace amounts and Phlobatannins, were completely absent in the stem bark ethanol extract. On the other hand, only tannins and phenol were present in high amounts while alkaloids, saponins, steroids terpenoids, anthraquinone, reducing sugars and flavonoids were only moderately present and the rest were in trace amounts in methanol extract. Ethyl acetate extract from the stem bark possesses high concentration of alkaloids, flavonoids, steroids, glycosides and phenols with tannins being the most prominent. The chloroform extract had only flavonoids, alkaloids and terpenoids present in moderate amounts. While phenols, reducing sugars and phlobatannins were completely absent.

Table 2 Phytochemicals in *Rytigynia kigeziensis*

Phytochemicals	Aqueous	Methanol	Ethanol	Ethyl acetate	Chloroform
Alkaloids	++	+	+++	++	++
Flavonoids	+	++	+++	++	++
Saponins	++	++	++	+	+
Glycosides	++	+	++	++	+
Steroids	++	++	+++	++	+
Tannins	+++	+++	+++	+++	+
Terpenoids	+	++	+	-	++
Phlobatannins	-	-	-	-	-
Anthraquinones	++	++	+	+	+
Reducing sugars	++	++	++	+	-
Phenols	+++	+++	+++	++	-

Key: Highly present (+++) moderately present (++) Traces present (+) absent (-)

The presences of eleven phytochemicals were tested in extracts of five solvents of varying polarity. Ethanol extract contained more of *R. kigeziensis* phytochemicals than other solvents extracts.

3.2. Anthelminthic activity

3.2.1. Time of paralysis

The time of paralysis was determined and recorded in triplicate and data was expressed as mean values ± SEM. The time of paralysis was found to be dose dependent. 15mg/ml showed the highest activity whereas 100mg/ml with least time of paralysis for albendazole and the extracts used. The concentrations with same letters (aa, bb, cc and dd) indicate significant difference when compared to albendazole at the same concentration while concentrations with single letter (a, b, c and d) were not statistically significant when compared to albendazole at the same (Fig 1). The results were considered statistically significant at $P < 0.05$.

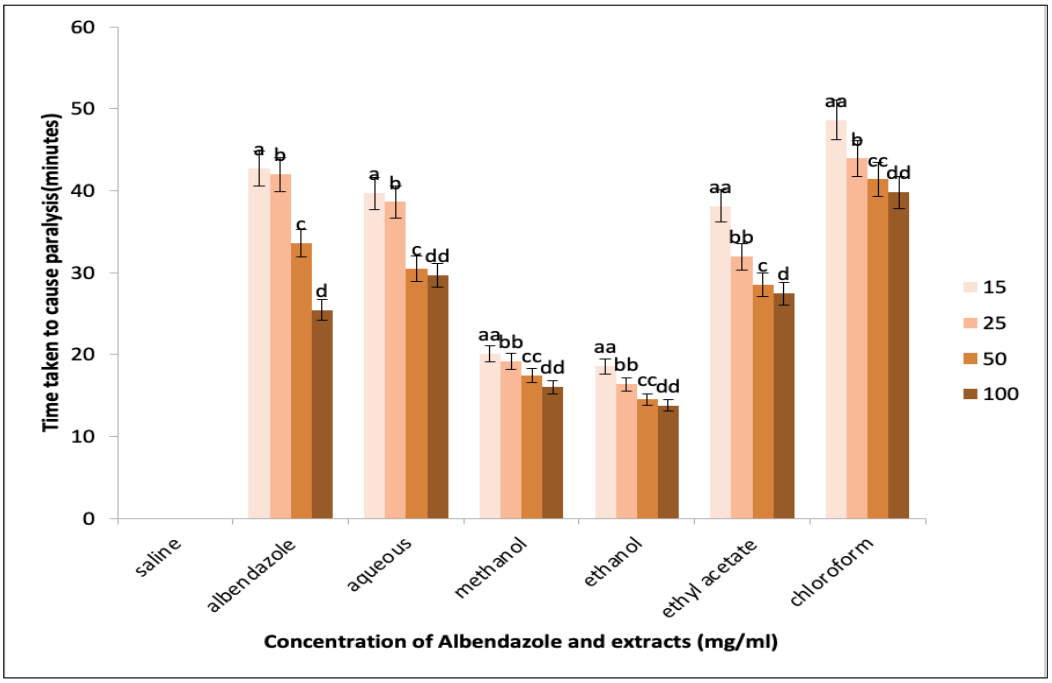

Figure 1 Time of paralysis (minutes) of worms at varying concentrations of solvent extracts compared to albendazole (mg/ml)

3.2.2. Time of death

The time of death was determined and recorded in triplicate and data was expressed as mean values ± SEM. The death time of worms was also observed to be dose dependent throughout the extracts as well as albendazole (Fig. 2). The concentrations with same double letters (aa, bb, cc and dd) indicate significant differences, when compared to albendazole at the same concentration while concentrations with single letter (a, b, c and d) were not statistically different when compared to albendazole.

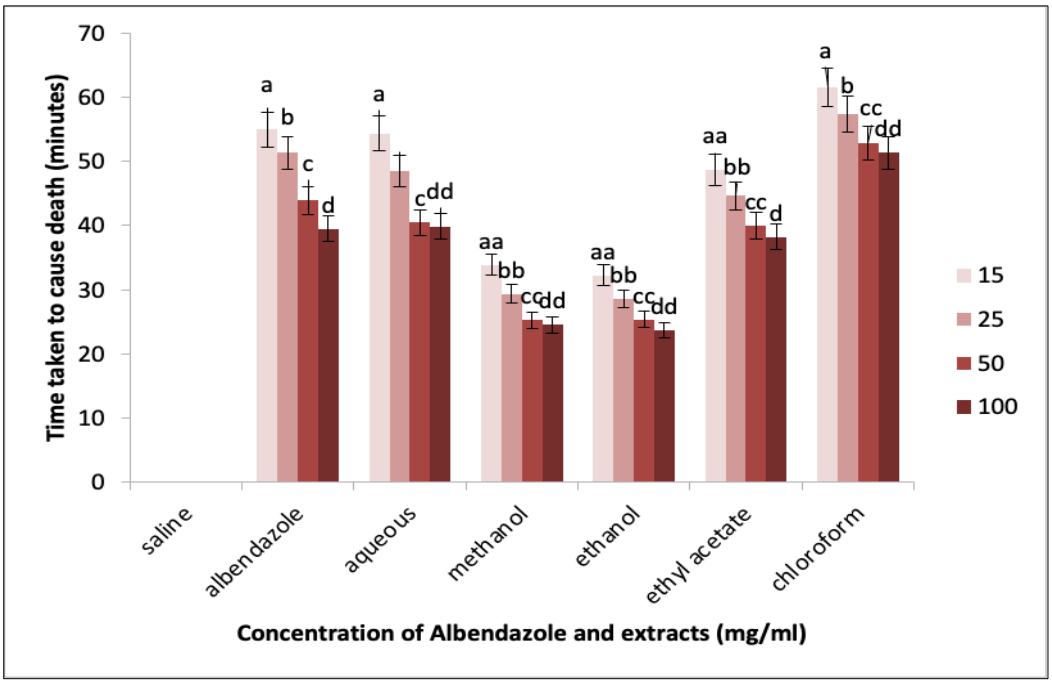

Figure 2 Time of death (minutes) of worms in varying concentrations of albendazole and solvent extracts (mg/ml)

3.2.3. LDH activity

LDH activity was observed to decrease with increase in concentration between ethanol extract and albendazole. The LDH activity was 196.245±0.2808164 µmol/min, 183.6267±0.1423335 µmol/min, and 191.8967±0.756668 µmol/min for normal saline, albendazole and ethanol extract respectively at concentration of 15mg/ml as shown in Fig. 3. 15mg/ml treated worms had the highest LDH activity while 100mg/ml treated ones had a lower activity. The concentrations with different letters (b) indicate significant difference activity of LDH when compared to albendazole, whereas letters (a) were not statistically different.

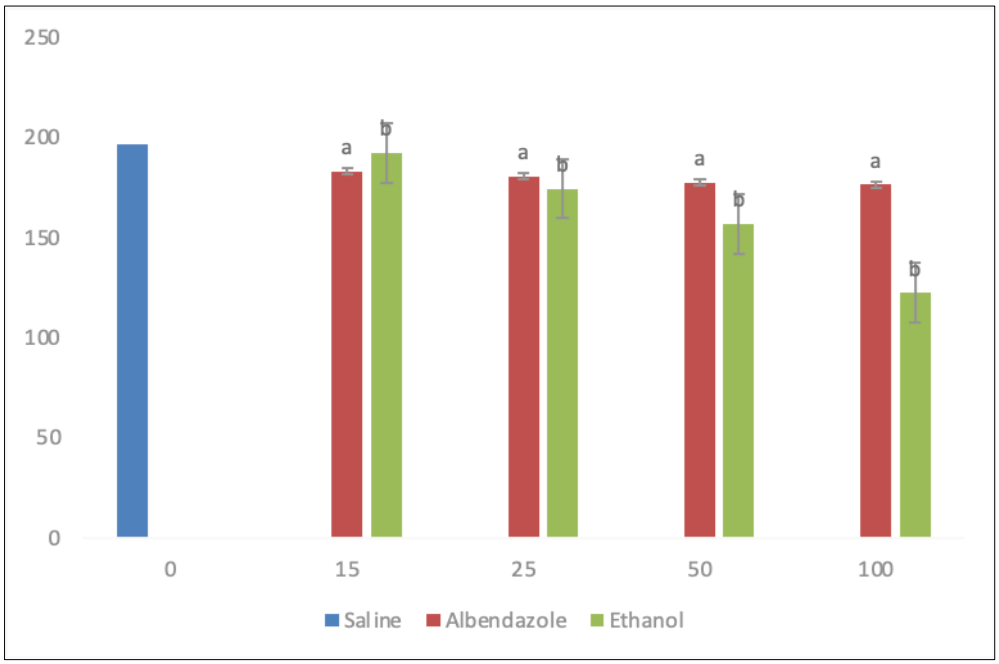

Figure 3 LDH activity against concentrations of Albendazole and *Rytigynia kigeziensis* ethanol extract

4. Discussion

4.1. *Rytigynia kigeziensis* plant phytochemicals

The presence of various phytochemicals by various solvents, are in line with previous studies [10] indicating that *Rytigynia nigerica* has tannins, saponins, reducing compounds, steroids, and flavonoids. A closely related *Rytigynia umbellulata* showed alkaloids, tannins, saponins, reducing compounds, and flavonoids. The aqueous extract had high concentrations of phenols, tannins, flavonoids, alkaloids, glycosides steroids, anthraquinones, terpenoids, and reducing sugars. The phytochemicals which are water soluble (tannins and phenols) were found to be more abundant and a few were moderately present. The metabolites soluble in less polar solvents were present in little amounts or even absent. The presence phytochemicals in the aqueous extract were also observed by [4, 16, 17] in the study of the anthelmintic potential *Xylopia aethiopica* and *Monodora myristica* in Nigeria. Further, by comparing different solvents, ethanol extracts possess more bioactive compounds as it is easier to penetrate the cellular membrane to extract the intracellular ingredients from the plant material. The methanol extract exhibited little amounts of most phytochemicals as compared to ethanol extract, this could be due methanol being more polar than ethanol and as result extracting less amounts of non-polar phytochemicals than ethanol [17], ethyl acetate being less polar in nature, whereas chloroform being the non-polar property on chloroform and hence being less able to enter the plant cell and extract out phytochemicals.

4.2. The *Rytigynia kigeziensis* anthelmintic activity

4.2.1. Time of paralysis and death

The anthelmintic activity found to decrease in a dose-dependent manner concur to many studies, that albendazole (15mg/ml) caused paralysis and death at 42.730 ± 1.710 and 54.958 ± 1.388 minutes respectively. This is in agreement with the study of [5] during which the concentration of albendazole (15mg/ml) caused paralysis resulting death at 43±1.60 and 55±1.60 minutes respectively [4]. Additionally, the same concentration would paralyze earthworms and died at 32.0±0.87 and 38.87±0.65 minutes respectively. Albendazole acts by destroying the cytoskeletal structure (microfilaments, microtubules and β-tubulins) of the worm, by preventing its assembly, which disrupts the glucose uptake, leading to glycogen depletion, reduction in ATP (energy) generation, and consequently the worm becomes paralyzed and finally dies [6]. It's evident that the presence of saponins identified in the current study, and which have been shown, to increase cell permeability by combining with membrane associated sterol [5] could be responsible for disorganization the cell membrane structure, possibly leading into free entry and exit of materials in and out of the cell. Further, leading to easy entry of other phytochemicals, linkage of intracellular enzymes, and as a result some cellular metabolic activities are either increased or reduced and others completely inhibited. On the other hand, phenols contained in the extract (niclosamide, oxyclozanide and bithionol) may act by uncoupling the mitochondrial reaction involved in electron transport for ATP generation [19, 20]. This could make the worm weak, thus causing paralysis and eventually death. The study alludes that alkaloids, also present in very high amounts in the extract and shown to interfere with the nervous system could be involved in the current revelations [5], whereas tannins which have been shown to precipitate glycoproteins, on the cuticle receptors or channels, and thus interfere with several metabolic activities either directly and indirectly. Taken all together, the study suggests the presence of all these metabolites, could have been involved could work singly or in combination, and accountable for the observed anti-helminthic activity.

4.2.2. LDH Activity

The LDH activity decreased with increase in concentration, which agree to previous studies [21]. The observations could be due to the interference by saponins affecting glucose transportation across the cell membrane. This is supported by the previous studies, whereby worms living in oxygen limited supply, like in fluids, as an adaptation to such an environment, carry out more anaerobic respiration than aerobic [22, 23, 24]. Lactate dehydrogenase enzyme catalyzes reduction of pyruvate by NADH to form lactate, plays a vital role in the anaerobic generation of energy from glucose as well as in the synthesis of glucose from lactate, suggesting its importance in parasitic organisms. In addition, excellent utilization of LDH, converts pyruvate to lactate and oxidizes the reduced form of nicotinamide adenine dinucleotide (NADH) to NAD^+, key in ATP synthesis [25, 26, 27]. Hence, the tannins contained in *R. kigeziensis* extracts may have inhibited LDH enzyme leading to energy reduction generated by glycolysis which concur to [18, 21], whereby *Illicium verum* fruit and *Artocarpus heterophyllus* seed with tannins, were found to be active against *Haemonchus contortus*, and due to low energy, the worms' survival processes such transmission of impulses were interfered leading to paralysis and death, as observed in this study.

5. Conclusion

In this study it was established that *R. kigeziensis* is rich in anthelmintic phytochemicals that have synergistic effect on the STH. However, further studies to isolate individual phytochemicals, toxicity, synergistic studies and *in-vivo* investigations needs to be performed to shade more light on the possible application of the plant for anti-helminthic activity.

Compliance with ethical standards

Acknowledgments

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Disclosure of conflict of interest

None declared conflict of interest.

Statement of ethical approval

Approvals for the research were obtained from the Kampala International University - Institution Research and Ethics Committee (KIU-IREC).

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