ANTI-TRYPANOSOMAL AND CYTOTOXIC EFFECTS OF EXTRACTS FROM *COMMIPHORA SWYNNERTONII* BURTT 1935

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EXTENDED ABSTRACT

Application of trypanocides both curatively and prophylactically is a key component of African animal trypanosomosis (AAT) control methods. However, trypanocidal drugs are beset with many problems including drug toxicity and treatment failure due to widespread resistance of the parasites. Given little progress in vaccine development, there is a pressing need for discovery of new alternative trypanocidal molecules that can further be developed into trypanocidal drugs. Plants are one such source from which alternative trypanocidal molecules could be obtained. *Commiphora swynnertonii* is a member of the family Burseraceae which is reported to possess a number of compounds active against protozoa. This thesis provides a report on the anti-trypanosomal and cytotoxic effects of *Commiphora swynnertonii* extracts, and its potential as a source of lead molecules for development of alternative trypanocidal drug for trypanosomosis control. The main results are presented in two published papers and one submitted manuscript. The first paper presents the *in vitro* trypanocidal activity of *Commiphora swynnertonii* extracts on *Trypanosoma congolense* in which, the motility of *T. congolense* was evaluated after incubation for 20 minutes with ethanolic stem-bark and resin extracts at concentration of 2 mg/ml and 4 mg/ml. In the second study, *T. congolense* was incubated with the extracts at concentrations of 0.4 mg/ml and 2 mg/ml for 36 – 56 min after which 0.08 ml of the aliquots were inoculated intraperitoneally into mice to assess infectivity. In both studies, negative (phosphate buffered saline with glucose without the extract) and positive (diminazene diaceturate) controls were used. The findings showed that *C. swynnertonii* ethanolic stem bark extract caused complete cessation of *T. congolense* motility in 30 minutes at the concentration of 4 mg/ml. Resin extract had a delayed effect on the cessation of *T. congolense* motility observed after 90 and 100 minutes of incubation at concentrations of 4 mg/ml and 2 mg/ml respectively. The drug incubation showed that ethanolic stem bark extract reduced significantly ($P=0.000$) the infectivity of *T.*

congolense at concentration of 2 mg/ml compared to negative control and was not significantly ($P=0.897$) different from the positive control. It was concluded that, *C. swynnertonii* ethanolic stem bark extract possesses *in vitro* trypanocidal activity. The second paper presents the results of *in vivo* activity of *C. swynnertonii* ethanolic stem bark extract on *T. congolense* parasitaemia and its effect on immunological components in mice. Groups of mice infected with *T. congolense* were treated with the stem bark extracts at 1000 mg/kg, 1500 mg/kg and 2000 mg/kg, twice a day in one set and thrice a day in another setting for three days consecutively, and parasitaemia monitored. In the other setting, uninfected mice randomized in five groups were treated with the extract that was categorized as thorough mixed extract (TME) and supernatant extract (SE)) each at 500 mg/kg and 1500 mg/kg, in 8 hourly intervals respectively for three days consecutively. The groups that received the extracts (1000 mg/kg and 2000 mg/kg) at eight hourly intervals had drastically reduced parasitaemia ($P<0.05$). It was concluded that *C. swynnertonii* ethanolic stem bark extract possesses *in vivo* trypanocidal activity. On the other hand, SE at the dose of 1500 mg/kg significantly ($P<0.05$) reduced the percentage of peripheral lymphocytes. Both doses (500 mg/kg and 1500 mg/kg) of TME significantly ($P<0.05$) reduced lymphocytes percent while neutrophils and monocytes percent increased significantly ($P<0.05$). Histopathology of the spleen in the mice treated with 1500 mg/kg of SE and TME showed apoptosis of lymphocytes around the marginal zone and lymphoid follicles. Hence, it was concluded that *C. swynnertonii* ethanolic stem bark extract within anti-trypanosomal therapeutic dose range possesses cytotoxic effect on lymphocytes. The submitted manuscript provides the results on anti-trypanosomal activities of fractions and sub-fractions of *C. swynnertonii* ethanolic stem bark extract against *T. congolense*. Negative (phosphate buffered saline with glucose without the extract) and positive (diminazene diaceturate) controls were used. In addition, chromatographic techniques were employed to determine bioactive molecules in the sub-

fractions. The findings indicated that anti-trypanosomal activity in the fractions of aqueous, dichloromethane and petroleum ether were decreasing in that order. In this study, four terpenoids (borneol, geranylgeraniol, coronopilin and 4,8,13-duvatriene-1,3-diol) were detected and were considered to be likely responsible for trypanocidal activity of *Commiphora swynnertonii* ethanolic stem bark extract. Further studies to evaluate the *in vivo* trypanocidal potential are recommended. As a general conclusion, this study has shown that *C. swynnertonii* stem bark extract possesses *in vitro* and *in vivo* trypanocidal activity. The *in vivo* trypanocidal activity is probably affected by cytotoxic effect on lymphocytes at the therapeutic dosage. It was further found that borneol, geranylgeraniol, coronopilin and 4,8,13-duvatriene-1,3-diol could be responsible for observed trypanocidal efficacy of *C. swynnertonii* stem bark extract. Further studies to determine their therapeutic trypanocidal potentials are recommended.

DECLARATION

I, Yakob Petro Nagagi, do hereby declare to the Senate of Sokoine University of Agriculture that, this thesis is my own original work and that it has neither been submitted nor is it being concurrently submitted for a degree award in any other institution of higher learning.

..... 24/7/2018 .....

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The above declaration is confirmed

..... 24/07/2018 .....

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(2nd Supervisor)

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DEDICATION

This thesis is dedicated to my beloved wife Rehema Robert Nyaindi and my children Jackson and Baraka for their prayers, patience and encouragement during the study period. It is also dedicated to my mummy Joyce Nkondo Nagagi and to the memory of my late daddy Petro Nagagi for the groundwork of my education.

LIST OF PAPERS/MANUSCRIPT

1. Nagagi, Y.P., Silayo, R.S. and Kweka, E.J. (2016). Trypanocidal activity of ethanolic extracts of *Commiphora swynnertonii* Burtt on *Trypanosoma congolense*. BMC *Complementary and Alternative Medicine*, 16, 195. http://doi.org/10.1186/s12906-016-1191-0

2. Nagagi, Y. P., Silayo, R. S., Luziga, C. and Kweka, E. J. (2017). *In vivo* effect of *Commiphora swynnertonii* ethanolic extracts on *Trypanosoma congolense* and selected immunological components in mice. *BMC Complementary and Alternative Medicine*, 17, 275. http://doi.org/10.1186/s12906-017-1785-1

3. Nagagi, Y. P., Silayo, R. S. and Kweka, E. J. *In vitro* trypanocidal activities of fractions from the ethanolic stem bark extract of *Commiphora swynnertonii* against *Trypanosoma congolense*. Target journal, *Journal of Ethnopharmacology*, (status-submitted)

DECLARATION

I, Yakob Petro Nagagi, do hereby declare to the Senate of Sokoine University of Agriculture that the listed papers/ manuscript above that make this thesis summarize my independent work efforts. It is my own original work and will not be part of another thesis in the "Published Papers" format in any other institution. The format and writing style is according to the peer reviewed journal.

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LIST OF ABBREVIATIONS

AAT	–	African Animal Trypanosomosis
FAO	–	Food and Agriculture Organization
GC-MS	–	Gas Chromatography Mass Spectrometry
HAT	–	Human African Trypanosomosis
HPLC	–	High Performance Liquid Chromatography
IgG	–	Immunoglobulin G
IgM	–	Immunoglobulin M
Min	–	minutes
rpm	–	Revolution per minute
VSG	–	Variable Surface Glycoprotein
WHO	–	World Health Organization

CHAPTER ONE

1.0 Introduction

African trypanosomoses comprises a number of diseases caused by flagellate extracellular protozoan parasites of genus *Trypanosoma* that can survive in bloodstream and tissue fluids of both humans and animals. The disease is prevalent in the 37 African countries that are within the tsetse belt between the latitudes 14° N and 29° S (Franco *et al.*, 2014; Giordani *et al.*, 2016). In human, the disease is known as Human African trypanosomosis (HAT) or "sleeping sickness" that occurs in two forms, acute and chronic. The chronic form caused by *T. brucei gambiense*, is endemic in west and central Africa contributing to over 98% of the current cases while the acute form caused by *T. b. rhodesiense* is endemic in east and southern Africa accounting for the remainder of the cases (WHO, 2017). Despite the progress which has seen the prevalence being brought down with the number of cases fewer than 8000 per annum, HAT remains an important public health problem (Simo and Rayaisse, 2015). About 70 million people equivalent to 10% of the total population in the region (an area of 1.55 million km^2) are estimated to be at various HAT risk levels with most recent, transmission taking place in discrete endemic areas (hot spots) within the geographical distribution of tsetse fly (Simarro *et al.*, 2012).

In animals, the disease is known as African animal trypanosomosis (AAT) or "nagana" and is caused by *T. congolense, T. vivax* and *T. brucei brucei.* These parasites are transmitted mainly by the tsetse fly (*Glossina* species). They often cause a fatal disease mainly in cattle, sheep and goats but relatively mild infection in wild animals which serve as reservoir hosts. The symptoms of AAT in domestic animals include intermittent fever, anaemia, listlessness, emaciation, hair loss, exudates from the eyes, oedema, and paralysis (Taylor and Authiè, 2004). The affected animal weakens as the disease advances until it is not suitable for work and hence the name "N'gana", a Zulu word that means

"powerless/useless" (Steverding, 2008). The possibility of mechanical transmission of some trypanosome species has enabled the disease to spread beyond the tsetse belt of sub-Saharan Africa (Desquesnes and Dia, 2003; Baldacchino *et al.*, 2013). *Trypanosoma vivax* is now established cause of disease in cattle, causing high morbidity and mortality in South America (Batista *et al.*, 2007; 2009; 2012), and to a lesser extent in Asia and Europe (Desquesnes *et al.*, 2013). On the other hand, *T. evansi* causes another form of the disease "surra" which is a threat to livestock production particularly cattle, water buffaloes and camels across Asia and South America (Mekata *et al.*, 2009; Desquesnes *et al.*, 2013). The other important livestock trypanosome is *T. equiperdum* causing dourine an equine disease transmitted by coitus (Gizaw *et al.*, 2017).

1.1 The Impact of African Trypanosomosis

Human African trypanosomosis (HAT) is one of the neglected tropical diseases that affect rural development in sub-Saharan Africa. It mainly affects the poor people living in remote rural regions although it has also been reported in urban and peri-urban areas (Brun *et al.*, 2010). The economic burden of HAT is estimated at 0.56 million disability-adjusted life years (DALYs), representing the year of healthy life lost to disease that comprise aspects such as mortality, cost of surveillance, work force and income lost due to caring of the affected and the cost incurred by health facility to attend sick individuals (Fèvre *et al.*, 2008; Hotez *et al.*, 2014). The burden of the disease is further compounded by high level of under-reporting, a reduced chance of complete cure and increased risk of drug associated-adverse effects (Matemba *et al.*, 2010; Reid *et al.*, 2012).

On the other hand, AAT severely affects livestock production in rural areas of sub-Saharan region. It is estimated that 40 million cattle are at risk of acquiring AAT in the sub-Saharan Africa and 3 million die every year, leading to an economic loss of US$ 1.0 –

1.2 billion annually (Grady *et al.*, 2011). In addition, the presence of the disease in the tsetse infested areas has slowed down the exploration and invasion of large part of the African continent (Van den Bossche *et al.*, 2010). Apart from its effect on livestock distribution, benefits accrued to farmers through livestock keeping are hampered, culminating in less access to animal traction, nutrients, lower income from milk and meat sales, manure and liquid capital (Swallow, 2000). The total domestic product is estimated at US$ 4.5 billion per annum when secondary losses such as reduced manure and draft power are included (Grady *et al.*, 2011).

1.2 Classification of Trypanosomes

Trypanosomes are single-celled parasitic protozoa in the family Trypanosomatidae and the genus *Trypanosoma* (Stevens and Brisse, 2004). Although their cellular organization resembles that of eukaryotic cells, they are unique due to possession of kinetoplast formed by condensation of circular mitochondrial DNA (Brun *et al.*, 2010). These parasites have been known to evolve from ancestral insect flagellates that came to establish in mammalian host blood through various adaptive morphological and physiological changes. They are classified based on two criteria namely morphology in the vertebrate host and the mode of development in invertebrate host, whether in the anterior station (Salivarian) or posterior station (Stercorarian) (Hoare, 1972). They have been classified into a number of genera and subgenera as shown in Table 1.

1.2.1 Trypanosoma congolense

Trypanosoma congolense was first discovered by Broden in 1904 in the blood of sheep and donkey from then "Leopoldville" which is currently known as Kinshasa in the Democratic Republic of Congo (Hoare, 1972). It is the most economically important trypanosome that is pathogenic to ruminants, horses, pigs and dogs on the African

continent. More than 80% of AAT and losses in domestic animals in sub-Saharan Africa are due to *T. congolense* infection (Namangala and Odongo, 2013). *Trypanosoma congolense* is a monomorphic (12.1 – 17.6 μm) salivarian parasite (cyclic development in the mid-gut and mouthpart of tsetse flies) and lacks a free flagellum at any stage of development (Vickerman, 1969). Relative to other trypanosome species, *T. congolense* is a medium-sized parasite with a marginal sub-terminal kinetoplast (Fig.1). Unlike *T. vivax* and *T. brucei*, *T. congolense* occurs in the blood vessels only (Banks, 1978) except during development of infection at the site of inoculation where the parasite is found in the skin, extravascularly and localized draining lymphatics (Luckins and Gray, 1978; Luckins *et al.*, 1994).

In established infection, studies have shown uneven distribution of *T. congolense* in the host circulation, being mostly localized to the walls of capillaries and small vessels particularly of brain, heart and skeletal muscles (Maxie and Losos, 1977; Banks, 1978) and therefore, providing the possibility of passively damaging the cells to which they are attached to as a result of anti-trypanosome antibody and complement fixation (Banks, 1980).

Table 1: Trypanosome taxonomy

Phylum:	Protozoa
Order:	Kinetoplastida
Suborder:	Trypanosomatina
Family:	Trypanosomatidae
Genus:	*Trypanosoma*
	A. Section: Stercoraria
1. Subgenus:	*Megatrypanum*
Species	*Trypanosoma theileri*
2. Subgenus:	*Herpetosoma*
Species	*Trypanosoma lewisi, T. rangeli, T. musculi*
3. Subgenus:	*Schizotrypanum*
Species	*Trypanosoma cruzi*
	B. Section: Salivaria
4. Subgenus:	*Duttonella*
Species	*Trypanosoma vivax, T. uniforme*
5. Subgenus:	*Nannomonas*
Species	*Trypanosoma congolense*, T. *simiae, T. godfreyi*
6. Subgenus:	*Pycnomonas*
Species	*Trypanosoma suis*
7. Subgenus:	*Trypanozoon*
Species	*Trypanosoma brucei, T. evansi, T. equiperdum*

Source: McNamara *et al.*, (1994) and modified from Baral (2010)

1.2.2 The life cycle

More elaboration of the life cycle of *T. congolense* has been provided by Hoare (1972) and a recent developmental cycle in tsetse flies outlined by Peacock and colleagues (2012). In fact, two developmental stages namely trypomastigotes (comprising un-infective procyclics and the infective metacyclics) and epimastigotes are described in tsetse fly. The bloodstream trypomastigotes taken up by the fly differentiate into procyclics in the fly-midgut and grow in length. The procyclics move through peritrophic matrix reaching the proventriculus where they cease division and transform into trypomastigotes that are uniform in size and shape. These trypomastigotes migrate to the cibarium and proboscis

to differentiate into epimastigotes and finally metacyclic trypomastigotes. Some of these forms have extremely long or truncated posterior ends (Ibid). The infective metacyclics are very small and do not divide.

1.2.3 Trypanosome surface coat

Trypanosomes are covered by periodically changing antigenic surface coat of a single protein composed of a variant surface glycoprotein (VSG) (Mathews *et al.*, 2015; Pinger *et al.*, 2017). Only one such glycoprotein is expressed at a specific time. They also express different metacyclic-variable antigenic types (M-VATs), the phenomenon which plays a role in establishment of infection in a host (Luckins *et al.*, 1994). During the ascending phase of the infection in mammalian host the VSG is recognized by host immune system, triggering production of antibodies such as IgM and IgG. These antibodies often neutralize trypanosomes that have corresponding antigenic types leading to a decreased parasitaemia. In the meantime, a few of trypanosomes will have expressed a new variant antigenic type (VAT) shielding the new VSG-parasite from host effector cells and will continue to multiply until new antibodies are produced (Morrison *et al.*, 2009). This phenomenon is the kind of immune evasion that results in waves of parasitaemia. The switch has been found to occur either by transport of a variant surface glycoprotein gene to one of the 20 expression sites situated on different telomeres, or by silencing of an active telomere and activation of a telomere on another chromosome (Borst and Ulbert, 2001). Therefore, development of a conventional vaccine against trypanosomes is not feasible due to alternative expression and recombination of a repertoire of approximately 1,000 VSG-encoding genes (Stuart *et al.*, 2008).

1.3 Control of African Trypanosomosis

It is now more than 100 years of African trypanosomosis control that has relied on direct attack on the trypanosomes by use of chemotherapy and indirectly through tsetse control. Several tsetse control methods have been in use. They range from non-insecticidal to those involving use of insecticides (Leak, 1999). The non-insecticidal methods include selective shooting of wild animal hosts and bush clearing which have however been abandoned due to environmental concerns (WHO, 2017). Others are sterile insect technique (SIT) which was successfully applied to eradicate a tsetse fly species, *Glossina austeni* from Unguja Island in Zanzibar (Rozendaal, 1997; FAO, 2014) and entomopathogenic fungi which has attracted attention as a form of biological control (Maniania and Ekesi, 2013).

The use of insecticides to control tsetse flies had initially been based on ground spraying. Later, ultra-low volume application of non-residual insecticides in sequential aerial spraying (SAT) proved to be the most effective method (Leak, 1999). However, it has recently not been in use due to diminishing funding in tsetse control, and lack of political will of affected countries most of which are highly indebted (Feldmann *et al.*, 2005; Vreysen *et al.*, 2013). More recently introduced, is the cheap and easily applied animal bait technology whereby cattle are sprayed with residual insecticides that kill alighting tsetse flies. The cost of operating becomes even less when restricted application of insecticides on the belly and legs of the animal is adopted (Bourn *et al.*, 2005; Shaw *et al.*, 2013). The only limitation is that most tsetse infested areas are conserved or restricted areas which are legally cattle free (Malele *et al.*, 2011). Therefore, this method cannot be applied in game areas. An alternative is the use of artificial bait technology such as mechanical traps or insecticide-impregnated cloth targets, with attractant odours that mimic a natural host (Vale, 1993; Green, 1994). Despite being successful in reducing the number of tsetse flies (Vale *et al.*, 1988; Willemse and Takken, 1994), effective area-wide

tsetse control requires technical, logistic and financial support which are limited in most of the affected African countries (Omamo and d' Ieteren, 2003).

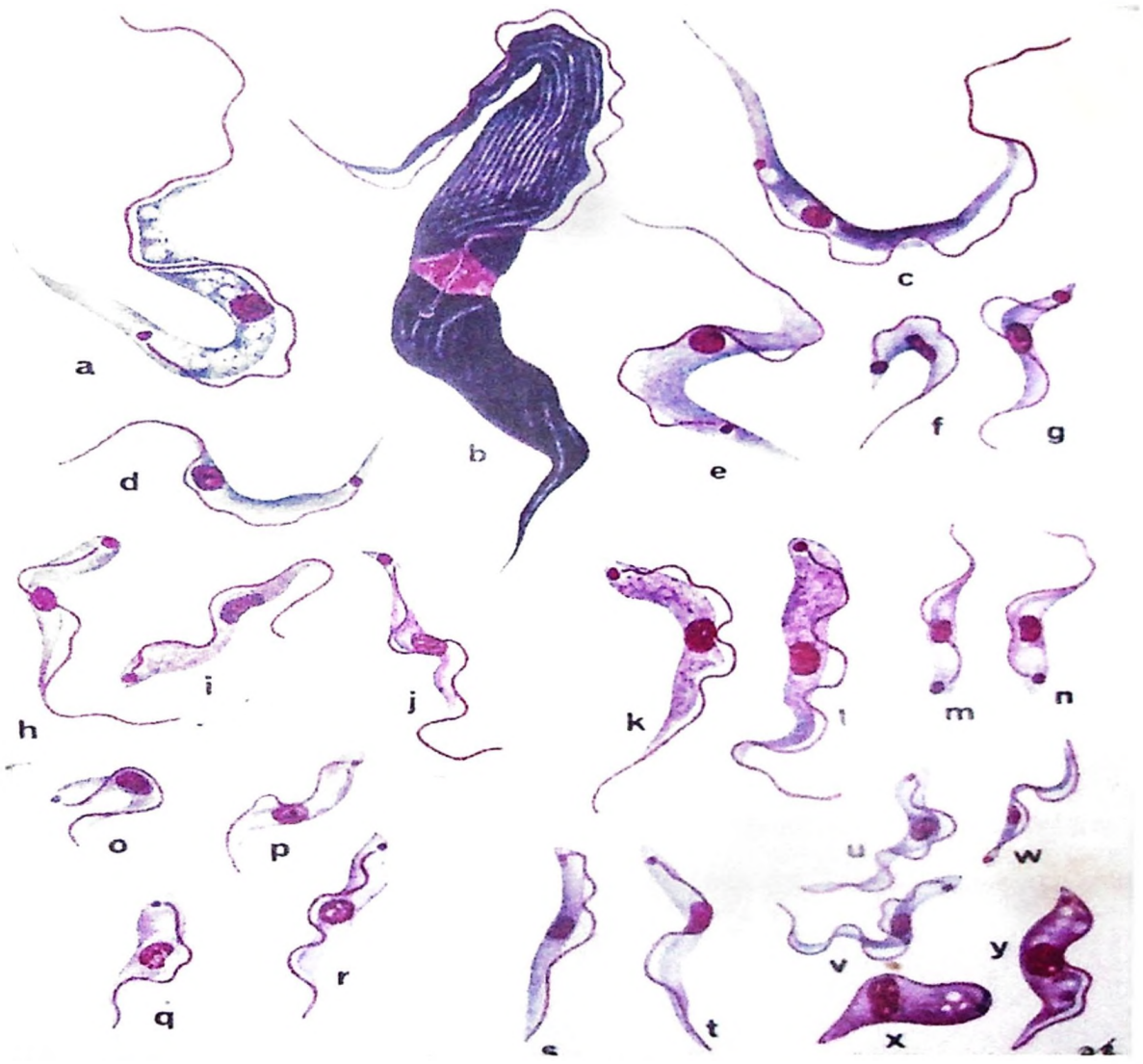

Figure 1: Morphology of type-species of Trypanosome subgenera

Source: Hoare, (1972), x 1800 Magnifications

STERCORARIA. a - *Trypanosoma theileri*, b - *T. ingens*, c - *T. conorhini*, d - *T. lewisi*, *T. rangeli*, f and g – *T. cruzi*; SALIVARIA. h, i and j - *T. vivax*, k and l - *T. caprae*, m and n - *T. uniforme*, o, p, q and r - *T. congolense*, s and t (also o – r) - *T. congolense* (=dimorphon), u, v and w (also o – r) - *T. simiae*, x and y - *T. montgomeryi*

On the other hand, chemotherapy and chemoprophylaxis has been a central component of animal trypanosomosis control for many decades (Giordani *et al.*, 2016). However, it is beset with the problem of delivery following the privatization of veterinary services in 1980s and 1990s in most African countries resulting in livestock keepers having to administer chemotherapy on their own in absence of veterinary professionals (Gauthier *et al.*, 1999). This, in some instances has led to misuse of trypanocides contributing to wide-spread treatment failure (Geerts and Holmes, 1998). Considering the problems of trypanosome resistance against available drugs for treatment of AAT, coupling with the highly needed safe and effective drugs for HAT, new trypanocidal agents are urgently needed.

1.4 Plant Derived Molecules as Sources of new Chemotherapeutics

From time immemorial, plants have always been a frequent source of medicaments either in form of traditional preparations or as active principles. The continued use of plant-derived medicines in the current world is an indication of how beneficial they are, to human race. The development of sophisticated chemical analytical tools has permitted the isolation of many chemicals that have served as drugs or base for synthesis of many modern medicines (Veeresham, 2012; Yuan *et al.*, 2016). Drugs like aspirin, digitalis, morphine, quinine and artemisinin were originally isolated or synthesized from plant secondary metabolites. Due to diverse molecular structures of plant derivatives, new drugs from plant secondary metabolites will continue to increase (Cragg and Newman, 2013). For instance, among the 1073 new chemical entities of small molecules that were approved between 1981 and 2010, over 50% were derived or inspired by nature including plants (Newman and Cragg, 2012).

In the past, even before the advent of synthetic trypanocidal agents, local herdsmen in Africa were controlling trypanosomosis through different medicinal plants. Few cited examples are the use of decoctions from medicinal plants for treatment of trypanosomosis in ancient Egypt, Greece, Mediterranean, India, Assyria and China (Mbaya and Ibrahim, 2011). Moreover, plant materials such as stem bark of *Khaya senegalensis* and leaves of *Tamarindus indica* were administered either alone or mixed with materials from other medicinal plants in West Africa (Atawodi *et al.*, 2002). In East Africa on the other hand, *Adenia volkensii*, *Fagara chalybea* and *Salvadora persica* were plant species used (Assefa, 2017).

Subsequently, through study of pharmacopoeia, wisdom from village elders and traditional healers and natural instinct and progression of wild primates to utilize medicinal plants with anti-trypanosomal efficacy (Mbaya and Ibrahim, 2011); studies on anti-trypanosomal activity on a number of African medicinal plants were conducted to validate their medicinal use (Ibrahim *et al.*, 2014). In addition, such studies could lead to identification of chemical leads for development of new anti-trypanosomal agents. For this purpose, pioneering screening work on various plants have shown that many have promising *in vitro* and or *in vivo* trypanocidal activity potential (Kaminsky *et al.*, 1996; Freiburghaus *et al.*, 1997). The present study is one such effort towards the search of novel molecules that could serve as lead for trypanocidal drug discovery.

1.4.1 The genus *Commiphora*

The genus *Commiphora* belongs to family Burseraceae. The name *Commiphora* was derived from Greek words *Kommi* (meaning "gum") and *phero* (meaning "to bear") due to the fact that many species yield a fragrant "Oleo-gum-resin" following damage to the bark (Steyn, 2003). More than 1.6 million km^2 of tropical and sub-tropical East Africa is

dominated abundantly by *Commiphora* species in the *Acacia-Commiphora* woodlands. A few species are recorded outside African continent in a similarly dry habitat of Middle East, India, Sri Lanka and South America (Weeks and Simpson, 2006). They can readily be distinguished based on spininess, colour of bark, scent of sap, whether shrub or tree and their field appearance coupling with the habitat in which they occur (Bakari, 2013). The resin exudates have been used as perfume, incense or mummifying ointment and have been shown to be of tremendous medicinal values (Langenheim, 2003). Plant extracts from this genus have been used in indigenous medicine for treatment of wounds, pain, arthritis, fractures, obesity, parasitic infections and gastrointestinal diseases (Shen *et al.*, 2012). One interesting research output from this genus against parasites is Mirazid, a drug containing 300 mg purified resin extract of *C. molmol* in Egypt (Ibid). Mirazid has been shown to possess therapeutic effect on hepatic coccidiosis caused by *Eimeria stiedae* in domestic rabbits (Baghdadi and Al-Mathal, 2010), indicating that *Commiphora* species possesses anti-protozoan activity.

Throughout the literature, there is paucity of knowledge on the effect of *Commiphora* against protozoan parasites in the genus *Trypanosoma*. The available information is the *in vitro* trypanocidal activity of *C. emenii* and *C. kerstingii* against *Trypanosoma brucei brucei* (Ibrahim *et al.*, 2014). It was the aim of this study to expand this knowledge to *Commiphora swynnertonii* against *T. congolense*.

1.4.2 *Commiphora swynnertonii*

Commiphora swynnertonii was for the first time described in 1935 by a botanist Bernard D. Burtt. The plant name was given in honor of a naturalist and hunter Charles FM Swynnerton as appreciation to his great contribution to tsetse control and game conservation in Tanganyika territory (current name Tanzania) (McKelvey, 1973). The

plant is one of the *Commiphora* species that are exceedingly local in their distribution and has been reported in Simanjiro, Manyara, Dodoma, Kilimanjaro, Mwanza, Tanga and other parts of Tanzania (Bakari, 2013). Characteristically, it is very spiny, much-branched shrub, with a remarkable shining yellow-green bark and the leaves are directly attached to its base without a stalk (Fig. 2). In addition, the sap is watery and slightly pear-scented (Burtt, 1935). The plant is drought resistant and well adapted to its environment. A study by Bakari (2013) in Simanjiro district reported that the plant is used for fencing animal enclosures, as source of poles used as building material and income through selling of stem products mainly resin. Also, there was observed over-exploitation of the plant habitats, through mining, urbanization, overgrazing and other agricultural activities (Ibid).

Like any other plant species that are medicinally beneficial, extracts from *C. swynnertonii* have been reported to be of multifaceted range of medical and veterinary importance (Bakari, 2013; Kalala *et al.*, 2014; Mkangara *et al.*, 2014). This could be attributed to a number of secondary metabolites (phytochemical compounds) among which are terpenoids (Bakari, 2013). Terpenes are enormous group of natural products that have a wide range of biological activities against cancerous cells, bacteria, fungi, viruses and parasites (Wang *et al.*, 2005), with some reported as insect repellants (Müller *et al.*, 2009; Paluch, 2009). Over the past few years, hundreds of terpene-derived molecules have been shown to possess trypanocidal activity, some requiring only minor modification in order to improve their efficacy against the parasites (Lozano *et al.*, 2016). Therefore, this study aimed at exploring *C. swynnertonii* ethanolic stem bark extract as alternative source of trypanocidal molecules.

Figure 2: Characteristic appearance of *Commiphora swynnertonii*

A-leaves attached directly to base, **B**-much branched and yellow-green stem with exfoliates **C**-Spiny appearance

1.5 Host Immunity Support for Efficacious Chemotherapy

A functional immune system is essential for long lived multicellular organisms to survive in the world where they are sometimes challenged with single-celled pathogenic organisms. The immune system is one of the organism's most complex systems that is well tasked with protection against pathogenic organisms and other self-entities with deleterious effects (Mak and Saunders, 2006). Such function is not always successful because some pathogenic organisms such as trypanosomes are capable of evading the immune response leading to acute and/or chronic and debilitating diseases, something that has made the use of chemotherapeutic agents to aid warding off the infectious agent as necessity (Travis, 1991; Kaufmann, 2008). Experience has shown that immunosuppressed individuals have poor therapeutic responses (Berger and Fairlamb, 1992). In the case of malaria, patients with active and functional humoral immunity were comparatively more responsive to antimalarial drugs than non-immune (Mayxay *et al.*, 2001).

Similarly, a sound immune status of the host infected with trypanosomes can play an important role in successful chemotherapy. Trypanocidal drugs; suramin and

quinapyramine were reported to require an intact immune system for rapid removal of bloodstream trypanosomes (Berger and Fairlamb, 1992). Moreover, α-difluoromethylornithine (DFMO), a drug currently used for treatment of second stage of *T. b. gambiense* infection, was shown to clear infections in immunocompetent, but either partially or completely unable to cure infected immunocompromised mice (de Gee *et al.*, 1983; Bitonti *et al.*, 1984). Thus, interplay between therapy and antibody-dependent immune response is essential for trypanosome clearance. Normally, antibodies have their origin from lymphocytes particularly B cells. Studies have shown that marginal zone B cells, among other B cell types are evolutionary selected and maintained to facilitate prompt antibody responses (Amezcua-Vesely *et al.*, 2012). In the splenic white pulp, the immature B cells or immunoblasts will proliferate in response to antigenic stimuli, and mature to antibody (immunoglobulin)-producing plasma cells and migrate into the red pulp. Drug-induced down-regulation of lymphocytes and depletion of the marginal zone B cell would possibly suppress immune response and therefore, failure of parasite clearance from the affected host (Bitonti *et al.*, 1984; Elmore, 2006).

1.5.1 Cytotoxicity of plant extracts

Cytotoxicity is defined as ability of certain chemicals or mediator cells to destroy living cells either through induction of necrosis (accidental cell death) or apoptosis (programmed cell death) (Freshney, 2011). Host cell death can impair normal organ function(s) leading to observed associated signs and symptoms. Despite of the possible role of medicinal plants as source of trypanocidal molecules (Mbaya *et al.*, 2010; Sakirigue *et al.*, 2012), some secondary metabolites in the extract are toxic in nature (Mbaya *et al.*, 2007). For example, the toxicity of the decoction from the stem bark of *Butyrospermum paradoxum* was evaluated *in vivo* in rabbit and in rats (Mbaya and Ibrahim, 2011). Intra-peritoneal administration of the stem bark at doses (>80 mg/kg) produced behavioral changes,

morbidity and mortality in the rodents. The symptoms were dose dependent including anorexia, dehydration, depression, prostration, coma and death. Postmortem findings were congestion with oedema of the lungs, bronchi, bronchioles, kidneys and hepatomegally with focal necrosis of hepatocytes. In the case of *C. swynnertonii*, chicken administered with resin extract at doses of 750 and 1000 mg/kg had mild congestion, fatty degeneration and infiltration of mononuclear inflammatory cells around blood vessels at subcapsular level and around portal triad of the liver (Bakari *et al.*, 2015). Other lesions were cortical haemorrhages, medullary congestion, hydropic degeneration of cortical-tubular epithelium and glomerulus in the kidney (Ibid).

In the live animal, elevated plasma creatinine levels has shown to indicate damage of renal cortices and glomeruli while liver enzymes, the transaminases aspartate aminotransferase (AST) and alanine aminotransferase (ALT)) are specific markers for hepatocellular necrosis and are frequently used for determination of hepatocyte cytotoxicity (Thrall *et al.*, 2012). An increase in AST and ALT paralleled hepatocellular necrosis in the chicken administered fourteen days continuously with *C. swynnertonii* resin extract at 750 mg/kg (Bakari *et al.*, 2015). Similar correlations were noted between increased plasma creatinine and appearance of hydropic degeneration of cortical-tubular epithelium and glomeruli (Ibid). Therefore, this study aimed at establishing cytotoxic effect of *Commiphora swynnertonii* ethanolic stem bark extract on immunological components (leucocytes and spleen) and other organs such as liver and kidney in mouse model.

1.5.2 The role of marginal zone of the spleen

The marginal zone is a microanatomical site that borders the non-lymphoid red pulp and lymphoid white pulp of the spleen. It consists of marginal zone metallophilic macrophage that borders the lymphoid follicles and the outer marginal sinus layer. Peripheral to

marginal sinus is the thick outer ring comprised of dentritic cells, fibroblast, marginal zone macrophage and medium sized marginal zone B-cells (Cesta, 2006). The highly and specialized organization of the marginal zone enhances filtering of blood-borne pathogens, and initiation of innate and adaptive immune response (Zouali and Richard, 2011). The role of marginal zone B (MZB) cells in eliciting early but strong T-independent antibody response against trypanosomes-VSG coat has been studied (Shi *et al.*, 2004; Radwanska *et al.*, 2008). Following trypanosome infection, the MZB cells are activated to produce antibodies of IgM and IgG classes that mediate phagocytosis of trypanosomes by macrophage and Küpffer cells *in vivo*, contributing to parasite clearance (Taylor, 1998). However, some immunomodulatory drugs have frequently been found to cause decreased cellularity of the B cell-rich marginal zone, leading to deficits in T-independent antibody immune response (Elmore, 2006). In addition, trypanosome infection has also been reported to induce a significant B cell apoptosis and loss of the marginal zone, the phenomenon that hinders the host to mount anti-VSG antibody response (Radwanska *et al.*, 2008).

Previous studies on certain species of *Commiphora* have shown evidence of immunomodulatory activity (Haffor, 2010). It is worth noting that in chicken, the resin extract from *C. swynnertonii* was shown to significantly increase lymphocytes and monocytes count, thus indicating that it has ability to activate the defense mechanism (Bakari, 2013; Bakari *et al.*, 2015). There are no reports on previous studies in a mammal and therefore the present studies aimed at establishing the effect of *C. swynnertonii* ethanolic stem bark extract on leukocytes and spleen marginal zone within the range of anti-trypanosomal therapeutic response in mouse model.

1.5.3 Apoptosis

The term apoptosis refers to programmed cell death that is essential for normal embryonic development and tissue homeostasis (Ellis and Horvitz, 1986; Elmore, 2007). It can also be a result of normal physiological response to many stimuli, including irreparable DNA damage (Pistritto *et al.*, 2016). Previous reports have elucidated the mechanism of apoptosis, indicating a family of intracellular proteases, the caspases, directly or indirectly involved in morphological and biochemical changes characterizing the apoptotic process (Savitskaya and Onishchenko, 2015). This process leads to cell chromatin condensation (pyknosis), plasma membrane blebbing, cell shrinkage and nuclear fragmentations (karyorrhexis) that are eventually cleared by phagocytosis without causing any inflammatory response (Reed, 2000).

Plant extracts of *Linum persicum*, *Cirsium bracteosum*, *Dionysia termeana*, *Salvia macrocipho*, *Echinophora cinerea* and *Ferulago angulata* have been shown to induce apoptosis of lymphocytes through DNA damage (Amirghofran *et al.*, 2009). In another study, β-Caryophyllene from *Commiphora gileadensis* was found to cause apoptosis in lymphocytes tumor cell lines (Amiel *et al.*, 2012). Also, Bahashwan (2004) indicated that the higher the concentration of both *C. molmol* ethanolic extract and *C. gudiotti* hexane extract the better was the apoptotic induction in murine S180 sarcoma cells and HT1080 human fibrosarcoma cells. In the case of *C. swynnertonii*, LC_{50} values of the root-bark, stem bark, resin and leaf extracts against brine shrimp were found to be 3.5 µg/ml, 13.0 µg/ml, 15.0 µg/ml and 96 µg/ml respectively (Bakari, 2013). Studies have indicated that plant extracts with LC_{50} values below 20 µg/ml have a potential of causing cell death through induction of apoptosis (Moshi *et al.*, 2010; Vijayarathna and Sasidharan, 2012). However, previous studies have not shown indicated that *C. swynnertonii* has the potential of causing apoptotic effect. Therefore, this study aimed at providing information whether

C. swynnertonii extract at the anti-trypanosomal therapeutic dosage is associated with apoptosis.

1.6 General Methodology

1.6.1 Bioassay-guided fractionation

Fractionation is the term that refers to all separation processes that involves separation of extracts, using various analytical methods, into a number of fractions (Cannell, 1998). In its totality, bioassay-guided fractionation commence with the testing of an extract to confirm its biological activity, followed by separation of the compounds in matrix and testing of the fractions at each stage. Plant extracts contain various secondary metabolites with different polarities and bio-activity, the fact that provides room for separating them and analyzing the fractions to determine which contain the desired compound(s) (Ibid). Separation process can be achieved through liquid-liquid extraction and the contiguous elution by chromatography column that is artificially divided by the extractor, into fractions. Further separation can be achieved by means of electrophoresis and chromatographic techniques such as preparative chromatography and high-performance liquid chromatography (HPLC).

The presence of the compounds in the fractions can be identified using techniques such as Gas Chromatography Mass Spectrometry (GC-MS), and more precisely by Nuclear Magnetic Resonance (NMR) spectroscopy. The present study utilized GC-MS, HPLC and liquid-liquid extraction techniques to fractionate and identify the presence of terpenoids from sub-fractions of *C. swynnertonii* ethanolic stem bark extract. These methods were chosen because of accessibility and their ability to suit the purpose of this study. For instance, GC-MS has been shown to detect compounds through electron ionization to produce mass spectra and therefore, its use becomes feasible in absence of standard

substance and yet with the nearly identical accuracy to that with the standard (Nakashima and Hayashi, 2016).

1.6.1.1 Liquid – liquid extraction

This method employs the separation principle of liquid – liquid extraction where the sample of interest is distributed between two immiscible solvents. It follows the distribution law which states, if to a system comprising two essentially immiscible liquid layers, one adds a third substance which is soluble in both layers, then the substance will at equilibrium distribute itself between the two layers irrespective of the total amount of the substance present (Parris, 1984). Based on solvents used, the technique has advantage of concentrating metabolites of interest into one unit leaving the un-wanted into another compartment. The current study utilized this technique to fractionate the *C. swynnertonii* ethanolic stem bark extract into fractions which were then tested for anti-trypanosomal activity against *T. congolense*.

1.6.1.2 Column chromatography

Column chromatography is one of the most useful methods for separation and purification of both solids and liquids. It is synonymously named as solid – liquid chromatographic technique in which the adsorbent (stationary phase) is a solid and the mobile phase is a liquid. Several compounds have been employed as adsorbents and include alumina, calcium carbonate, calcium phosphate, magnesia and silica, the latter being the most commonly used (Snyder and Dolan, 2017). The separation principle is based on differential adsorption of the substance by the adsorbent. The selection of the solvent (mobile phase) is based on nature of both solvent and the adsorbent. Binding of the organic molecules on adsorbents vary depending on the intermolecular forces. The stronger the intermolecular force, the stronger the binding to the adsorbent and therefore

the longer the compound takes to go through the column and would give a good separation, and the reverse is also true.

The adsorbent is made into slurry with a suitable liquid and placed in a cylindrical tube that is plugged at the bottom by a piece of glass wool or porous disc. The mixture to be separated is dissolved in a suitable solvent and introduced at the top of the column and allowed to pass through the column. As the mixture moves down through the column the components are adsorbed at different regions (Fig. 3) depending on their adsorption properties. The component with greater adsorption power will be adsorbed at the top and the less adsorbed at the bottom. The different components can be desorbed and collected separately by adding more solvent at the top and this process is known as elution. The weakly adsorbed component will be eluted more rapidly than the other. The different fractions are evenly collected separately. Distillation or evaporation of the solvent from the different fractions would give relatively pure components (Cannell, 1998).

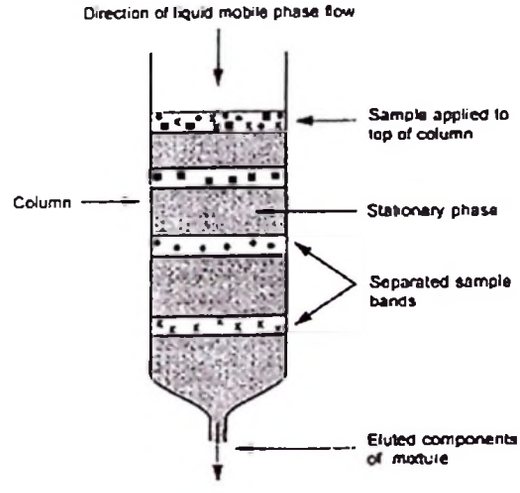

Figure 3: Schematic presentation of column chromatography with solid packing material (stationary phase) over which is passed a liquid (mobile phase) containing the sample

Source: Cannell, (1998)

1.6.1.3 High Performance Liquid Chromatography (HPLC)

HPLC is a separation technique that was developed in the early 1960s. It is a form of liquid chromatography where separation (or partition) occurs between a mobile phase and a stationary phase (the column packing). Recently, it has grown into an essential tool for the modern analytical laboratory and it has replaced gas chromatography (GC) for a variety of analyses (Mandal *et al.*, 2015). It is suited for separation of a wide range of chemicals including pharmaceuticals, foods and bio-chemicals (Bélanger *et al.*, 1997). Separation principles are similar to column chromatography except that the sample and the mobile phase are pumped into the stationary phase by an automatic injector to give a constant flow rate. It has a UV (Utraviolet) detector which is connected at the outlet end of the column. Its role is to monitor the column effluent in real time whereby each eluted compound is reflected as an individual peak. Thus, collection of the eluate, corresponds to individual peak. This technique was utilized in order to qualitatively analyze compounds that were detected in sub-fractions from *Commiphora swynnertonii* stem bark to ensure their validity.

1.6.1.4 Gas Chromatography Mass Spectrometry (GC-MS)

GC-MS is a combination of two techniques, the GC that can separate many volatile and semi-volatile compounds but not always selectively detect them, and MS that can selectively detect many compounds but not always separate them (Sneddon *et al.*, 2007). It is an indispensable technique for identification of drug and metabolites (volatile and semi-volatile) in pharmaceutical areas, molecular weight and elemental compositions in complex mixtures. Generally, it is an ideal technique for qualitative and quantitative determination of volatile and semi-volatile organic compounds in variety of samples (Ibid). Such compounds are plants' secondary metabolites (Iordache *et al.*, 2009; Hashmi *et al.*, 2013; Shah and Hossain, 2014). For this purpose, the identification of compounds

from various extracts is based on GC retention time on capillary column and computer matching of mass spectra with those of standards, and whenever possible by co-injection with authentic compounds (Hossain *et al.*, 2011).

1.6.2 Laboratory based experimental studies

A laboratory experiment refers to experiment carried in a laboratory where conditions can be almost completely controlled. The set-up of the experiment is based on the knowledge of expected outcome(s) of an experiment, type of data to be generated and its analysis, and the knowledge of the subject matter under investigation (Kothari, 2004). Positive and negative controls are usually included. A positive control determines whether researchers' manipulation of the system/experiment produced the expected effect whereas negative control is meant for isolation of a change in the system or experiment to a single variable. Laboratory based experiments used in this study were *in vitro* (motility assays and drug incubation infectivity test (DIIT)) and *in vivo* experiments using mouse model. The *in vitro* experiments are experiments under which the effect of the treatment or test variable(s) is/are assessed outside the living organism and the results obtained can be used to explain the phenomena that can be observed in the natural living host. On the other hand, *in vivo* experiments are experiments at which the effect of treatment of test variable(s) is/are assessed or monitored in the living organism. *In vitro* experiments contribute to lessening the use of animals in scientific investigations on what takes place in the living organism but since no *in vitro* system has been designed to completely mimic the living system, *in vivo* studies are still needed to complement the *in vitro* system. The details of these *in vitro* and *in vivo* experiments are given in respective papers and the manuscript making this thesis.

1.7 Problem Statement and Justification

African trypanosomosis remains one of the most serious neglected tropical diseases with negative impact on the economic development in sub-Saharan Africa, affecting human health and their domesticated animals (Shaw, 2004). The use of trypanocidal drugs is the most relied-upon option in fighting these complex diseases (Holmes, 2013). In humans, four drugs (suramin, pentamidine, melarsoprol and eflornithine) are available to treat cases of trypanosomosis but their use is problematic due to their toxic effects (Kennedy, 2008). Despite of the recent hope on Nifurtimox – Eflornithine combination therapy (NECT) which has provided a relief in terms of reduced toxicity and cost, its administration remains relatively labor intensive and logistically complicated to implement (Babokhov *et al.*, 2013).

In animals prophylactic and curative trypanocidal drugs used against AAT are very often used indiscriminately by unsupervised livestock keepers resulting in complications that include toxicity due to overdosage, drug residues in animal products due to not observing recommended drug withdrawal periods and development of parasite drug resistance because of underdosage and not observing drug prophylaxis interval periods (Geerts *et al.*, 2001; Melaku and Birasa, 2013; Giordani *et al.*, 2016). Development of trypanosome drug resistance against the few trypanocides currently available for field use is the most serious issue facing control of AAT. The problem is further compounded by unwillingness of the industry to invest in research and development of new trypanocidal drugs (Kennedy, 2008). Due to drug resistance development, toxicity and associated adverse effects of the currently-available drugs, and the little prospect of developing vaccine against trypanosome infection as a result of parasite immune evasion (Horn, 2014), there is a pressing need for alternative solutions in trypanosomosis treatment.

Medicinal plants are important in the discovery of drugs for treatment of various ailments. Their secondary metabolites have proven as new lead sources of compounds in modern medicine. There are no previous studies on anti-trypanosomal activity of *C. swynnertonii.* However, other *Commiphora* species like *C. kerstingii* (Mikail, 2009) and *C. emenii* (Freiburghaus *et al.*, 1997) have been reported to possess *in vitro* trypanocidal activity potential. Therefore, the present study aimed at investigating the anti-trypanosomal potential of *C. swynnertonii* extracts as a step to future alternative source of trypanocidal drugs.

1.7.1 Objectives of the study

1.7.1.1 Main objective

To assess *Commiphora swynnertonii* as a source of lead molecules for development of new trypanocidal drugs against African Animal Trypanosomosis and Human African trypanosomosis.

1.7.1.2 Specific objectives

i. To investigate *in vitro* anti-trypanosomal activity of stem bark and resin extracts of *C. swynnertonii* against *Trypanosoma congolense*

ii. To evaluate the *in vivo* anti-trypanosomal activity of the most bio-active crude extract of *C. swynnertonii*

iii. To evaluate the toxicity of the crude extract of *C. swynnertonii*

iv. To determine the anti-trypanosomal compound(s) in the most bio-active sub-fractions derived from *C. swynnertonii* extract

1.7.1.3 Research questions

The following research questions are in response to the specific objectives;

i. What is the effect of the bark and resin ethanolic extracts of *Commiphora swynnertonii* on the motility of *Trypanosoma congolense*?

ii. Does the stem bark and resin ethanolic extracts of *C. swynnertonii* posess an *in vivo* anti-trypanosomal effect on *T. congolense*?

iii. What is the *in vivo* toxicity, if any, of the crude *C. swynnertonii* extract?

iv. What are the active chemical compounds from *C. swynnertonii* extract that have anti-trypanosomal activity?

1.8 Organization of the Thesis

This thesis is organized in five chapters that are preceded by an extended abstract which summarizes the objectives, methodology, principal research findings and the conclusion of this study. The first chapter covers an introduction and literature review on trypanosomes, their impact and control methods, plant derived molecules as source of new chemotherapeutics, host immunity support for efficacious chemotherapeutics and the general methodology. Finally in the first chapter is the problem statement and justification, study objectives and research questions. The second chapter gives results obtained under specific objective number one while the third chapter gives the results obtained to meet specific objectives two and three. The fourth chapter gives the results obtained under specific objective number four and the fifth chapter gives the conclusion and recommendations from this study.

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CHAPTER TWO

Trypanocidal activity of ethanolic extracts of *Commiphora swynnertonii* Burtt on *Trypanosoma congolense*

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Trypanocidal activity of ethanolic extracts of *Commiphora swynnertonii* Burtt on *Trypanosoma congolense*

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Abstract

Background: African trypanosomosis is the disease caused by extracellular protozoan parasites of the genus *Trypanosoma* transmitted by tsetse flies. The current study has evaluated the trypanocidal activity of *Commiphora swynnertonii* extracts on *Trypanosoma congolense*.

Methods: The effect of ethanolic stem bark and resinous extracts on motility of *T. congolense* was evaluated by *in vitro* study at concentrations of 2 mg/ml and 4 mg/ml. Then, trypanocidal activity was evaluated by drug incubation infectivity test using mice at concentrations of 0.4 mg/ml and 2 mg/ml. In both studies negative (without drug) and positive (diminazene diaceturate) controls were used.

Results: The *in vitro* study showed that, ethanolic stem bark extract of *C. swynnertonii* at concentration of 4 mg/ml caused complete cessation of motility for *T. congolense* in 30 min. However, resinous ethanolic extract had delayed effect on cessation of motility of *T. congolense* observed at 90 and 100 min post-incubation at concentrations of 4 mg/ml and 2 mg/ml respectively. The drug incubation infectivity test study depicted that ethanolic stem bark extract at concentration of 2 mg/ml significantly ($p = 0.000$) reduced the infectivity of *T. congolense* in mice. However, it did not vary significantly ($P = 0.897$) with group treated with diminazene diaceturate incubated mixture.

Conclusion: The current study has provided evidence that, ethanolic stem bark extract of *C. swynnertonii* possess trypanocidal activity against *T. congolense*. Based on these findings, further studies are recommended to determine its potential as a lead to trypanocidal drug discovery.

Keywords: Trypanocidal activity, *Commiphora swynnertonii*, *Trypanosoma congolense*

Background

African trypanosomosis also known as "sleeping sickness" in human or "nagana" in animals is caused by extracellular protozoan parasites of the genus *Trypanosoma* transmitted by tsetse flies [1]. While nagana has an appreciable contribution to low livestock productivity in rural areas [2], sleeping sickness is disabling and fatal disease that has however remained a neglected tropical disease contributing to rural underdevelopment [3–5]. About 70 million people distributed in approximately 1.55 million km^2 are at various risk levels of acquiring sleeping sickness in Africa [6]. On the other hand, nagana is known to reduce income accrued to livestock production up to 50 % and is distributed in approximately 10 million km^2 in Africa leaving many in abject poverty [2, 7]. In Tanzania, nagana is second to East Coast Fever (ECF) in causing cattle mortalities [8].

Nagana has been mainly controlled by the use of chemotherapy. However, chemotherapeutic options are very limited and currently available drugs for use are diminazene diaceturate and isometamidium chloride [9]. The later is a molecule developed by combining diminazene and homidium [10].

Plants have been the source of most active medical compounds for decades in Africa. *Commiphora swynnertonii* (family *Burseracea*) is one of the plant species

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reported to possess a multifaceted range of ethnobotanical use amongst Dorobo (sub-ethnic group of Masaai tribe) people in Tanzania [11]. It has been claimed to be used for treatment of sexually transmitted diseases, ulcers and wounds (cut wounds and burn wounds), recalcitrant ulcers, abscesses, swelling of legs, chesty cough and scabies [11]. Its resinous exudates are used for treatment of worm infestation and dental caries, cleansing bladder and control of insects such as ticks, lice, bed bugs and mange mites [11]. In fact, previous studies have established plenitude of knowledge about *C. swynnertonii* that have provided pragmatic documents to support its traditional use. Bakari and others [12] reported that resin and root ethanolic extract possess strong anti-microbial activity against *Streptococcus pyogenes, Escherichia coli, Bacillus subtilis* and *Candida albicans*. Chloroform leaf extracts have been shown to possess highest activity against *Vibrio cholera, Shigella flexineri* and *Cryptococcus neoformans* [13]. On the other hand, resinous ethanolic extract has also been shown to possess strong antiviral activity against Newcastle disease virus [14, 15], and anticoccidial activity on *Eimeria* species in chicken [16]. Additionally, essential oils of *C. swynnertonii* leaves have been shown to possess repellent effect on *Rhipicephalus appendiculutus* whereas the hexane and ethyl acetate bark extracts were reported to induce mortality to both nymphs and adults of *R. appendiculatus* [17, 18]. Notably, there is also a recent confirmation of strong acaricidal activity possessed by *C. swynnertonii* exudates [19].

Plant extracts have been found to have antitrypanosomal activity against trypanosomes in different plant extracts evaluated *in vitro* [20]. Considering the previous reports on *C. swynnertonii* extracts activity, there is none which directly underpins the basis of our current study. The basis of this study was; (i) information on its anticoccidial activity against *Eimeria* species which are protozoan parasites, (ii) evidence of *in vitro* trypanocidal activity of *C. kerstingii* in Nigeria [21] and (iii) hypothesis which was developed based on the literature evidence that sesquiterpenes are among the phytochemical compounds in *Commiphora* species [22] with various forms reported to possess trypanocidal activity *in vitro* and in vivo [23]. Therefore, this study presents the report on the evaluation of extracts of *Commiphora swynnertonii* Burtt against *Trypanosoma congolense* by *in vitro* and drug incubation infectivity test.

Methods

Plant materials

Stem pieces and resin of *Commiphora swynnertonii* were collected from Kitwai A village (4°05'42.00 S and 36°33' 34.42 E), Simanjiro district which is in the northern part of Tanzania. The plant specimen was submitted to National Herbarium of Tanzania, Tropical Pesticides Research Institute, Arusha (Specimen voucher Number CS..01). Confirmation was done by Mr. Emmanuel Mboya and plant specimen deposited at the herbarium. The collected plant materials were transported to Sokoine University of Agriculture for preparation, extraction and *in vitro* and in vivo testing.

Plant extract preparation

The resin was stored in a refrigerator at 4 °C while the stem bark peeled off and dried under shade for about 4 weeks. Dried bark was ground to fine powder using laboratory mill and stored in an airtight bag in cool dry room until used. Two hundred gram (200 g) of the ground stem bark was weighed and soaked in 400 ml of 99.9 % ethanol in a conical flask sealed with aluminium foil and left for 72 h in a dark place with occasional stirring after which it was first filtered using a piece of cotton wool in a funnel into a conical flask and then using Whatmann® filter paper No. 1. The obtained filtrate was put in beaker and concentrated under ceiling fan at room temperature. The resin was treated differently. After soaking, the ethanol was immediately evaporated at room temperature under ceiling fan. The resulting crude extracts were then stored at 4 °C in airtight bottles until used.

Two (2) grams of both resinous and stem bark extracts were weighed in a bijou bottle separately and each diluted with 10 mls of phosphate buffered saline with glucose (PBSG) (pH, 8.0) to make 200 mg/ml stock solution. Two other extract concentrations (10 mg/ml and 20 mg/ml) were prepared from stock solutions by serial dilutions using PBSG. The extract solutions were prepared just before use and labeled accordingly while the remaining stock solutions stored in a refrigerator at 4 °C until required.

Trypanosome stock

The trypanosome stock used in this study was a stabilate of putative drug sensitive strain *Trypanosoma congolense* originally isolated from Mikese, Morogoro. This strain is maintained by serial passage in Swiss albino mice at the Small Animal Unit of the Faculty of Veterinary Medicine, Sokoine University of Agriculture. Trypanosomes used to undertake the studies were from mice detected parasitaemic ($1.26–2.51 \times 10^8$ trypanosomes per milliliter of blood) 4–5 days post inoculation.

Determination of parasitic load

The parasitic load in mice was monitored in blood from the tail, pre-sterilized with methylated spirit. The number of parasites per milliliter was estimated microscopically at x400 magnification as per Herbert and Lumsden [24]. This involved counting of parasites per field in pure blood or blood diluted with buffered phosphate saline with glucose (pH, 8.0). Logarithm values of these counts

were obtained by matching with table of Herbert and Lumsden [24] and converted to antilog to provide absolute number of trypanosomes per milliliter of blood.

In vitro test for trypanocidal activity

In assessing the *in vitro* anti-trypanosomal activity, 5 μl from the prepared stock resinous and stem bark extracts (10 mg/ml and 20 mg/ml) were drawn and poured into separate labeled eppendorf tubes. For reference purposes, three other eppendorf tubes were included, one with 5 μl of PBSG without the plant extract and the other containing 5 μl of 10 mg/ml and 20 mg/ml diminazene diaceturate (Veriben®, Ceva Santé Animale, France). Test *T. congolense*–infected blood was collected from donor Swiss albino mice and diluted using PBSG to make estimated parasitic load of 3.16×10^7 trypanosomes per milliliter from which 20 μl were drawn and poured into the labeled eppendorf tubes including negative (PBSG only) and positive (with diminazene aceturate) control to make effective concentrations of 2 mg/ml and 4 mg/ml of resinous, stem bark extracts and veriben® respectively. These were gently mixed and allowed to stand in an incubator set at temperature of 37 °C for 20 min. Thereafter, about 2 μl of the test mixture were placed on separate microscope slide and covered with cover slips and parasite observed after every 5 min for motility. Cessation or reduction in motility of the parasites in extract treated blood compared to that of the parasite loaded control blood without extract was taken as a measure of trypanocidal activity [25, 26].

Drug incubation infectivity test

Experimental animals

Random-bred male and female, Swiss albino mice 2–4 months old, weighing 25–40 g were used in carrying out the drug incubation infectivity test (DIIT). They were randomly put into six groups kept in plastic cages with wood shavings as beddings and identified with picric acid markings. They were fed with broiler mash (finisher) and tap water provided adequately.

Reconstitution and incubation

Resinous and stem bark extract concentrations of 20 mg/ml and 100 mg/ml respectively were reconstituted from their 200 mg/ml stock solutions using PBSG. Diminazene diaceturate at 20 mg/ml to be used as treatment control was prepared from commercially available sachet (Veriben®, Ceva Santé Animale, France) using distilled water. Six eppendorf tubes were taken and labeled as G1, G2, G3, G4, G5 and G6. Thereafter, 10 μl from the reconstituted preparations with amount of resinous extract per ml being 20, 100 mg were put in eppendorf tubes G1, G2 respectively; stem bark extract 20, 100 mg in eppendorf tubes G3, G4 respectively; diminazene diaceturate 20 mg/ml in G5 while into tube G6 was put 10 μl of PBSG. This was followed by addition of 490 μl of diluted mouse blood containing 1.58×10^7 trypanosomes per milliliter prepared by diluting blood from donor mouse with PBSG at dilution rate of 1:8 and parasite concentration estimated by the method of Hubert and Lumsden [24]. Gentle mixing was done and incubated at 37 °C for 30 min.

Study design

Six groups each with five mice were inoculated intraperitoneally with 0.08 mls of incubation mixture (containing extract, diminazene diaceturate or PBSG and trypanosome suspension) as shown in Table 1. For logistical reasons the recorded incubation time varied from 36–56 min. Post inoculation monitoring of mice for parasitaemia by wet smear examination was carried out daily in the first week and thereafter 2–3 times a week for 7 weeks. At least 20 high power fields (x 400) were examined before considering a sample negative.

Data analysis

The infection rate post inoculation for the drug incubation infectivity test experiment was determined. The least significance difference (LSD = 0.05) was obtained by one way analysis of variance (ANOVA) using statistical package for social science (SPSS) version 16 (Chicago, SPSS Inc., USA).

Results

The motility of *T. congolense* after *in vitro* incubation with resinous and stem bark ethanolic extracts of *Commiphora swynnertonii* at concentration of 2 mg/ml and 4 mg/ml is shown in (Table 2). In this study, ethanolic stem bark extract at the concentration of 4 mg/ml caused complete cessation of the motility of *T. congolense* in 30 min. Meanwhile, at the concentration of 2 mg/ml, the trypanosomes were less motile. Complete cessation of motility was observed after 60 min of incubation. In contrast, the resinous extract at comparable concentrations as the stem bark imposed a minimal effect on trypanosomes. This varied from motile to less motile until complete cessation of motility was observed at 90 and 100 min for the concentrations of 2 mg/ml and 4 mg/ml respectively. Diminazene diaceturate imposed less motility

Table 1 Experimental groups, the type of treatment and the time elapsed before inoculation into mice

Group (G)	Extract	Concentration	Incubation Period (Min.)
1	Resinous	0.4 mg/ml	48
2		2 mg/ml	44
3	Stem Bark	0.4 mg/ml	36
4		2 mg/ml	52
5	Diminazene diaceturate	0.4 mg/ml	40
6	PBSG (control)	-	56

Table 2 Motility of *T. congolense* after *in vitro* incubation with various concentrations of *C. swynnertonii* extracts

Test Mixture	Post Incubation follow up (minutes)								
	25	30	40	50	60	70	80	90	100
Resinous 2 mg/ml	+++	++	++	++	++	++	+	+	-
4 mg/ml	++	++	+	+	+	+	+	-	-
Stem Bark 2 mg/ml	++	+	+	+	-	-	-	-	-
4 mg/ml	+	-	-	-	-	-	-	-	-
Veriben 2 mg/ml	+	+	-	-	-	-	-	-	-
4 mg/ml	+	-	-	-	-	-	-	-	-
PBSG (–ve control)	+++	+++	+++	+++	+++	+++	+++	++	++

Key: +++ Very motile, ++ Motile, + Less motile, – None motile

on the trypanosomes, complete cessation of motility was observed at 30 and 40 min at concentrations of 4 mg/ml and 2 mg/ml respectively. The trypanosomes however were very motile to being motile until towards the end of the follow up at 100 min in the negative control.

Results for *in vitro* trypanosomes drug incubation, followed by an assessment of infectivity to mice are summarized in Table 3. All the mice in group 1 were found to be infected at day 4 post inoculation. There was variation in infectivity of trypanosomes in mice in group 2. The infection was seen on 3rd day whereas in day seven, all the mice were infected. In group 3, infection was seen on the 2nd day and all the mice were found infected on day 4. Group 4 had one mouse infected at day 9 while the rest remained trypanosome free during the entire period of the experiment. None of the mice in group 5 had the infection. In group 6, infection was detected on the 2nd day and all the mice were infected on day 4. Nevertheless, infectivity of *T. congolense* in G4 was reduced significantly ($p = 0.000$) with respect to G6 (group inoculated with infective diluted blood without drug) (Table 4). However, there was no significance difference ($p = 0.897$) between G4 and G5.

Discussion

The findings of this study have demonstrated that, the stem bark extracts at concentration of 4 mg/ml caused complete cessation of motility of *T. congolense* within 30 min in the *in vitro* study of the ethanolic extracts of *C. swynnertonii*. This observation partly matches with that of a previous study whereby methanolic stem bark extract of *C. kerstingii* at concentrations of 2 and 4 mg/ml were both shown to cause complete cessation of motility on *T. brucei brucei* within 30 min. [21] The slight difference observed might be attributed to biochemical variations within species, geographical location, methods or mode of extraction, solvent used [27], and the season at which the plant material harvested [28]. On the other hand, the resinous extract had lower observed effect on the motility of *T. congolense* with cessation of motility observed at 90 and 100 min post incubation at concentrations of 4 and 2 mg/ml respectively. Among the extracts

Table 3 Proportions of mice positive for *T. congolense* Mikese parasitaemia after incubation with *C. swynnertonii* extracts

	Groups inoculated Number of mice infected/total mice present in the group					
Days post Inoculation	G1 (resinous, 0.4 mg/ml)	G2 (resinous, 2 mg/ml)	G3 (bark, 0.4 mg/ml)	G4 (bark, 2 mg/ml)	G5 (dimin. diacet., 0.4 mg/ml)	G6 (PBSG, drug free)
0	0/5	0/5	0/5	0/5	0/5	0/5
2	0/5	0/5	1/5	0/5	0/5	1/5
3	0/5	1/5	3/5	0/5	0/5	2/5
4	5/5	2/5	5/5	0/5	0/5	5/5
6	5/5	4/5	5/5	0/5	0/5	4/4
7	5/5	5/5	3/3	0/5	0/5	2/2
8	4/4	5/5	2/2	0/5	0/5	1/1
9	4/4	5/5	1/1	1/5	0/5	1/1
14	3/3	4/4	1/1	0/4	0/5	1/1
35	2/2	2/2	1/1	0/4	0/4	1/1
45	1/1	1/1	1/1	0/4	0/4	1/1
53	0	1/1	1/1	0/4	0/4	0

Table 4 Infectivity of *T. congolense* in mice following drug incubation infectivity test of *C. swynnertonii* extract

(I)	(J)	Mean			95 % Confidence Interval	
Groups treated	Groups treated	Difference (I – J)	Standard Error	Significance level	Lower Bound	Upper Bound
Groups treated	Groups treated	Difference (I – J)	Standard Error	Significance level	Lower Bound	Upper Bound
G4	G5	.02222	.17021	.897	-.3200-	.3645
	G6	-.71111	.17021	.000	-1.0533	-.3689

of *C. swynnertonii* previously studied, resinous extract was shown to possess strong antimicrobial activity compared with stem bark, root bark and leaves extracts [29]. In contrast, this study has found that the stem bark extracts exhibit more *in vitro* antitrypanosomal activity than the resinous extract. A study conducted from 37 extracts in Tanzania have reported that, stem bark extracts of *C. emenii* possess strong *in vitro* antitrypanosomal activity relative to the other plant parts (leaves and root bark) [30].

Drug incubation infectivity tests showed that after incubation with mixture of ethanolic stem bark extract at concentration of 2 mg/ml for 52 min the reduction of infectivity of *T. congolense* Mikese compared to negative control was highly significant ($P = 0.000$). However, it did not vary significantly ($P = 0.897$) with group treated with diminazene diaceturate incubated mixture. Therefore, from drug incubation infectivity test experiment it was confirmed that the stem bark extracts of *C. swynnertonii* possess trypanocidal activity while resinous extract had no activity at similar concentrations. However, our current study should never be extrapolated to mean efficacy in vivo due to metabolic processes which occur in multicellular organisms. Both techniques used in this study, exposed the trypanosomes to a specified drug concentration, thereby avoiding potential problems such as drug distribution, drug half-life and bioavailability of the drug, and the behavior exhibited by the trypanosomes in the host. [24].

The current study has added *C. swynnertonii* Burtt to the list of plant species of similar genus (genus *Commiphora*) that were previously tested *in vitro* and found to possess antitrypanosomal activity. Other plant species included are *C. kerstingii* Engl. in Nigeria [21], and *C. emenii* Engl. in Tanzania [30]. The results of this study concur with the assumptions made by Freiburghaus and others that, antitrypanosomal effects might be due to phytochemical compounds universally existing in a plant genus [30]. These include tannins, phlobatannins, terpenoids, flavonoids, cardiac glycosides, steroids, saponins and anthraquinones which were confirmed to be contained in *C. swynnertonii* by Bakari [29]. Nevertheless, the trypanocidal principles of the species previously tested and the one tested in this study are still unknown. Although phytochemical analysis of various species of *Commiphora* have shown sesquiterpenes, a diverse group of terpenoids as among the chemical compounds [22], with various forms of sesquiterpenes reported to possess trypanocidal activity *in vitro* and in vivo [23], interpretation of our results based on the biochemical compound(s) responsible for the valid trypanocidal activity from genus *Commiphora* awaits further studies using selected biochemical components.

Conclusion

The current study has provided the first indication that ethanolic bark extracts of *C. swynnertonii* possess trypanocidal activity. Considering the two extracts (stem bark and resinous) of *C. swynnertonii* evaluated in this study, the phytochemical compound(s) responsible for antitrypanosomal activity may be well placed in the stem bark extract than in resinous extract. Therefore, studies are in progress to determine the potential of stem bark extract of *C. swynnertonii* as a trypanocidal drug and or alternative source of lead compound(s) for trypanocidal drug discovery.

Abbreviations

DIT, Drug Incubation Infectivity Test; PBSG, Phosphate buffered saline with glucose

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Authors' contributions

YPN conceived and conducted the study. YP Nagagi, RS Silayo and EJ Kweka searched and reviewed the literature and wrote up the manuscript. All edited and revised the manuscript critically. All authors reviewed the work and approved it for submission.

Competing Interests

The authors declare that they have no competing interests.

Consent for publication

Not applicable.

Ethics approval and consent to participate

The study obtained ethical clearance by the research and publications committee of Sokoine University of Agriculture, Morogoro, Tanzania.

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CHAPTER THREE

In vivo effect of *Commiphora swynnertonii* ethanolic extracts on *Trypanosoma congolense* and selected immunological components in mice

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In vivo effect of *Commiphora swynnertonii* ethanolic extracts on *Trypanosoma congolense* and selected immunological components in mice

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Abstract

Background: The search for alternative trypanocidal compounds which can be available at affordable price is of paramount importance for control of trypanosomosis in human and animals. The current study evaluates the in vivo activity of ethanolic stem bark extracts on *Trypanosoma congolense* and selected immunological components in an inbred Swiss albino mouse model.

Methods: Groups of mice infected with *T. congolense* were treated with the stem bark extracts at a rate of 1000 mg/kg, 1500 mg/kg, and 2000 mg/kg, twice a day in one set and thrice a day in another setting for three days consecutively. Negative (infected and untreated) and positive (infected treated with diminazene diaceturate at 3.5 mg/kg) control groups were used. Levels of parasitaemia were monitored daily for the first 10 days and thereafter 2–3 times per week to the end of experiment. In the other setting, uninfected mice, randomized in groups were treated with the extract but categorized as: thorough mixed extract (TME) and supernatant extract (SE) each at 500 mg/kg and 1500 mg/kg, in 8 hourly intervals respectively for three days consecutively. Control group was administered with phosphate buffered saline with glucose at 0.1 ml/10 g in a similar manner as for the extract. Whole blood and spleen were taken 24 h after the last treatment for hematological and histopathological analysis.

Results: The groups that received the extracts at 8 hourly intervals drastically reduced the parasitaemia. The higher dose of SE significantly reduced the percentage of lymphocytes ($P < 0.05$). Both high and low dose of TME significantly reduced lymphocytes percent ($P < 0.05$) while percent of neutrophils and monocytes increased significantly ($P < 0.05$). Histopathological changes of the spleen in the mice treated with higher concentrations of the extract of *C. swynnertonii* were suggestive of lymphocytes toxicity.

Conclusion: The current study has provided evidence that, in vivo trypanocidal activity of ethanolic bark extracts of *C. swynnertonii* is probably affected by its negative effect on humoral mediated immune response. Further studies are recommended to determine its potential as an alternative source of lead compounds for trypanocidal drug discovery.

Keywords: *Commiphora swynnertonii*, *Trypanosoma congolense*, Immunity

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Background

African trypanosomosis is one of the many constraints that hinders Africa's struggle against poverty through livestock keeping. It is an important infection that greatly affects humans and livestock in 37 African countries of which 21 are the world's poorest countries [1]. The important species and subspecies, *T. brucei gambiense* and *T. b. rhodesiense* are known to cause Human African trypanosomiasis (HAT) [2] and *T. B. brucei, T. congolense, T. vivax* and *T. simiae* which are infectious to animals are known to cause Animal African trypanosomiasis (AAT) [3]. Despite the major reduction in the number of new cases in the recent past, HAT remains an important public health problem in the affected countries [4]. On the other hand, AAT is one of the major livestock production constraints leading to 34% of livestock keepers to subsist on less than 1.24 USD per day [5]. Therefore, control of AAT is imperative due to its massive impact on the livelihood of rural communities [6]. However, the current disease control tools which rely on extensive use of chemotherapeutic agents is outdated and blunted by overuse practices [7], leading to failure and or reduced efficacy against emergent trypanosomes resistant strains [7, 8]. The problem is compounded by lack of interest of industries to develop new drugs due to high costs of such endeavor versus expected return from poor affected African countries [9]. Nevertheless, there is a need for increased scientific interest in searching for new anti-trypanosome molecules that the industry could take up for further development into new trypanocidal drugs. One such source of candidate molecules are plants.

Plants remain to be essential in healthcare with earliest records dating back from around 2600 BCE documenting the uses of approximately 1000 plant-derived substances in Mesopotamia [10]. Currently, the advancement in scientific techniques has lead to isolation and identification of thousands of phytochemicals from plants. Many of these phytochemicals are leading sources for developing chemotherapeutic drugs against a number of diseases (infectious and non-infectious) [11–13]. *Commiphora swynnertonii* which belongs to the family Burseraceae is the most famous medicinal plant species in northern Tanzania. It has a number of medicinal purposes such as: treatment of sexually transmitted diseases, ulcers and wounds (cut and burn wounds), recalcitrant ulcers, abscesses, swelling of legs, chesty cough and scabies [14]. Its resinous exudates are used for treatment of worm infestation, dental caries, cleansing bladder and control of parasites such as ticks, lice, bed bugs and mange mites [14]. Indeed, previous studies have provided a number of information about *C. swynnertonii* to support its traditional medicinal use [15–20].

The ethanolic stem bark extract of *C. swynnertonii* has recently been shown to possess trypanocidal activity in vitro [21]. However, extracts effective in vitro are not necessarily active also in vivo [22]. Among the contributing factors include (i) active compounds in the extracts may be metabolized too quickly to a less active or inactive form, (ii) the efficacy of the extracts may rely on not disrupting the immune system or its ability to activate the defense mechanism in a way that facilitate clearance of the infectious agent.

The aim of the present study was to determine the in vivo activity of extracts of *Commiphora swynnertonii* Burtt in an inbred Swiss albino mouse model infected with an isolate of *Trypanosoma congolense*. In addition, the study, reports the effect of the extracts on circulating white blood cells and spleen in infected mice. Its implication on the antitrypanosomal efficacy is discussed.

Methods

Plant materials

Plant stem bark pieces were collected from Kitwai A village (04°05′42.00″S and 36°33′34.42″E), in Simanjiro district in Manyara region in Northern Tanzania. The plant specimen was submitted to National Herbarium of Tanzania, Tropical Pesticides Research Institute, Arusha (Specimen voucher Number CS-01). Confirmation of Plant species was done by Emmanuel Mboya, a plant taxonomist. The collected plant materials were transported to Sokoine University of Agriculture for preparation, extraction, in vitro and in vivo testing.

Plant extract preparation

The stem bark was peeled off and dried under shade for 4 weeks. The dried barks were ground to fine powder using laboratory mill (Christy Hunt Engineering Ltd., England) and stored in an airtight bag in a cool dry room until used. In one hand, five hundred grams (500 g) of the ground stem bark was weighed and soaked in 1000 ml of 99.9% ethanol in a conical flask sealed with aluminium foil and left for 72 h in a dark place with occasional stirring. On the other hand, fifty grams (50 g) of ground stem bark was weighed and soaked in 100 ml of 99.8% methanol in a similar manner as for the ethanol. Each mixture, was filtered using a piece of cotton wool in a funnel into a conical flask and then using Whatmann® filter paper No. 1. The obtained filtrates were put in a separate beaker and concentrated by evaporation under ceiling fan at room temperature. The resulting crude extracts were then stored at 4 °C in airtight bottles until used.

Sixteen (16) grams of stem bark extract was weighed into a bijou bottle and diluted with 80 mls of phosphate buffered saline with glucose (PBSG) (pH, 8.0) to make 200 mg/ml stock solution. Three other extract

concentrations (150 mg/ml, 100 mg/ml and 50 mg/ml) were prepared from stock solution by serial dilutions using PBSG. The remaining stock solutions were stored in a refrigerator at 4 °C until required.

Experimental animals

Male, random-bred, Swiss albino mice 2½ months old, with mean weight (mean ± SD) 29.71 ± 4.8 g were used in carrying out the studies. The mice were obtained at the Small Animal Unit of the College of Veterinary and Medical Sciences, Sokoine University of Agriculture.

Housing and husbandry

Handling of animals was done in accordance to OECD guidelines [23]. The mice were kept in plastic cages 275 × 160 × 130 mm, five mice per group with wood shavings as bedding and identified with picric acid markings. They were fed broiler mash (finisher) from local feed manufacturer and boiled tap water was provided adequately. They were given four days to acclimatize to the housing facility prior to treatment. The mice that participated on the in vivo activity of extract of *C. swynnertonii* on *T. congolense* study were continually provided with feeds and water adequately even after termination of the experiment. All sections of this report comply with ARRIVE guidelines for reporting animal research [24].

Trypanosome stock

The trypanosome stock used in this study was a stock of putative drug sensitive strain *Trypanosoma congolense* originally isolated from Mikese, Morogoro and maintained by serial passage in Swiss albino mice at the Small Animal Unit of the College of Veterinary and Medical Sciences, Sokoine University of Agriculture.

Monitoring of Parasitaemia

Parasitaemia in mice was monitored by microscopic examination of wet smear from mouse tail blood using the method described by Herbert and Lumsden [25]. This involved counting of parasites per field in pure blood. Logarithm values of these counts were obtained by matching with table of Herbert and Lumsden [25] and or converted to antilog to provide absolute number of trypanosomes per milliliter of blood.

Acute toxicity study

A study on acute toxicity was conducted according to OECD guideline for testing of chemicals using mice at a dose of 2000 mg/kg [23]. Five female Swiss albino mice received orally 2000 mg/kg of the ethanolic stem bark extract. After which they were continuously observed for 30 min then periodically during the 24 h with special attention given for the first four hrs and thereafter daily for a total of 14 days. This study indicated that there were no deaths and no visible signs of acute toxicity in the mice treated at the dose tested (2000 mg/kg) during the 14 days observation period.

In vivo activity of the extract on *Trypanosoma congolense*

A sum of 40 clinically health mice were inoculated intraperitoneally with approximately 3×10^5 trypanosomes. The mice had parasitaemia of approximately 2.5×10^5 trypanosomes per milliliter of blood on the third day. They were then assigned with numbers 1–40, and randomized using a RAND function in the Microsoft excel. This resulted into eight experimental groups (G1 – G8) of five mice each. The number of experimental groups and that of mice per group were derived based on standardized protocol recommended by Eisler and colleagues [26] with some modifications. The mice in G1 – G6 were administered with ethanolic extract of stem bark of *C. swynnertonii*. Those in G7 were treated as negative control while in G8 as positive control. A time line diagram for this experiment is shown in Fig. 1, a.

The dose and treatment regime was selected based on acute toxicity study and previous trial experiments. The extracts were thoroughly mixed before administration. The mice in G6, G4, G3 received the extract at a dose of 1000 mg/kg, 1500 mg/kg, 2000 mg/kg respectively, at 12 hourly intervals for three days consecutively. The mice in G5, G1, G2 received the extract at: 1000 mg/kg, 1500 mg/kg, 2000 mg/kg, at 8 hourly intervals for three days consecutively. The mice in G7 did not receive any treatment, while those in G8 were treated with (diminazine diaceturate (Veriben®, Ceva Santé Animale, France) at a dose of 3.5 mg/kg intraperitoneally as a single injection. Monitoring of parasitaemia was done daily for the first 10 days and thereafter intermittently 2–3 days per week until the 39^{th} day post inoculation. For any mouse that was found dead, was examined for postmortem changes, the spleen and any organ(s) with noticeable change were taken in 10% neutral buffered formalin for histopathology.

Analysis of the extract

Two hundred milligrams (200 mg) of methanolic extract was weighed and diluted with 2 mls of methanol (99.8%), the resulting solution was used for chemical composition analysis using gas chromatograph mass spectrometry (GC-MS), Agilent Technologies. The GC-MS conditions: helium as a carrier gas, 1.2 ml/min flow rate, 30 m column length, 0.25 mm internal diameter, 0.25 mm film thickness, ion source temperature 230 °C, injection mode-autoinjector with split-splitless, mass spectrometry detector inlet temperature at 250 °C, temperature of m/s quandrapole at 150 °C and pressure vacuum at 4.85×10^{-5} psi.

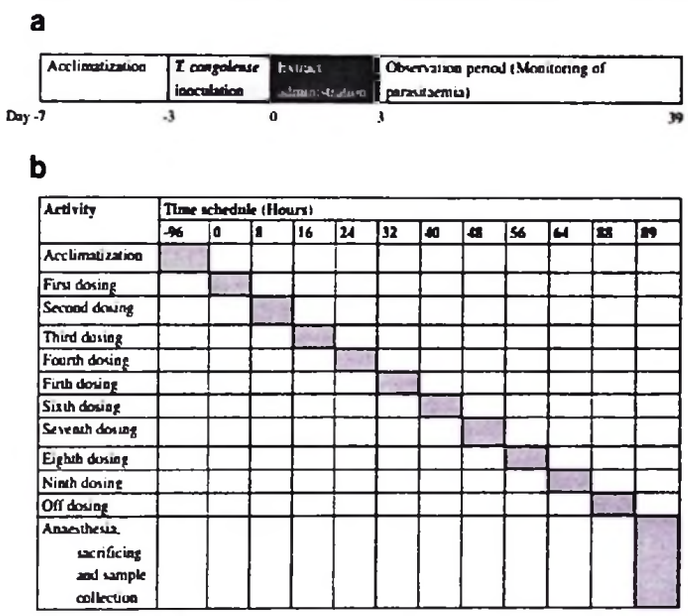

Fig. 1 Experimental timeline, **a** Timeline trend of major events for the experiment that was assessing the in vivo activity of *C. swynnertonii* extract on the parasitaemia of *T. congolense* in mice. **b** Timeline schedule extract administration for the experiment that was assessing the effect of *C. swynnertonii* extract on some immunological components in mice

Effect of extract on selected immunological components

To assess the effect of the extract on selected immunological components, another experiment was carried out. Twenty five clinically healthy mice were assigned numbers 1–25 and randomized as above into five groups (G9 – G13) of five mice each. The mice in G9 – G12 were administered with the ethanolic stem bark extract of *C. swynnertonii* while mice in G13 received PBSG only (control). A time line diagram for this experiment is shown in Fig. 1, b.

Mice in G9 and G12 were administered with 500 mg/kg, and 1500 mg/kg respectively of thoroughly mixed extract (TME). Mice in G10 and G11 were administered with 500 mg/kg, 1500 mg/kg of supernatant extract (SE) whereas G13 received 0.1 ml/10 g of PBSG only. The treatment was done at 8 hourly intervals for three days consecutively. Twenty four hours after the last treatment, the mice were weighed, anaesthetized and sacrificed. Immediately, blood from each mouse was collected in EDTA vacutainer tubes in ice packs,

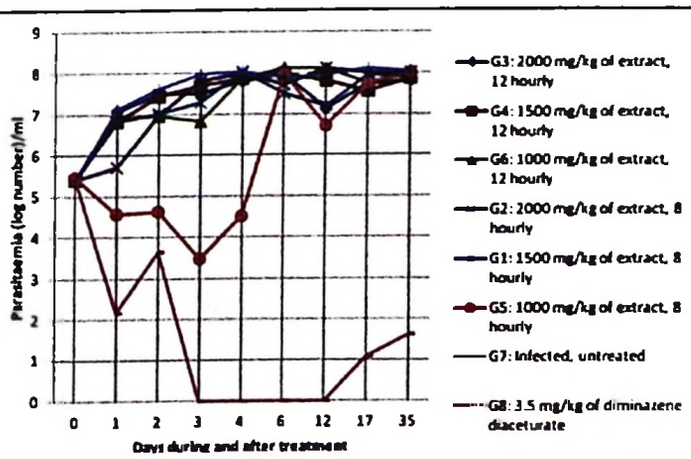

Fig. 2 Trend of the mean parasitaemia of *T. congolense* during and after treatment of extract of *C. swynnertonii*

spleen was excised and placed in bijou bottle containing 10% neutral buffered formalin. Later, total and differential white blood cell count was determined using an automatic haematological analyzer.

Hematoxylin and eosin staining

Organs with noticeable postmortem changes and the spleen were prepared for histopathology assessment. Three replicates from the liver, kidney and spleen sections of 5 μm per treatment were cut and processed by rapid manual tissue processing as described in Culling [27]. The processed sections were stained with hematoxylin and eosin (H & E) for histopathological observations.

Immunohistochemistry

Tissue preparation

Again, spleen tissues from mice treated with TME and SE of *C. swynnertonii* at a dose of 1500 mg/kg at 8 hourly intervals for three days consecutively were dissected. Spleen tissues from mice treated with 0.1 ml/10 g of PBSG only were also included. They were post-fixed with 4% paraformaldehyde (PFA; Sigma-Aldrich, St. Louis, MO) in 0.1 phosphate buffer (PB; pH 7.4) for 2 hrs at 4 °C before processing to paraffin wax and sectioning.

Immunostaining procedure

The procedure for immunostaining was done as described by Luziga and colleagues [28] with some modifications.

Table 1 Effect of extract of *C. swynnertonii* on parasitaemia in mice infected with *T. congolense*

	Parasitaemia level (logarithm number)/ml				
Days	G2	G1	G5	G7	G8
0	5.4 ± 0.00	5.4 ± 0.00	5.46 ± 0.134	5.4 ± 0.00	5.4 ± 0.00
1	5.7 ± 0.3*	6.84 ± 0.39	4.56 ± 2.56	7.14 ± 0.65	2.16 ± 2.96*
2	6.98 ± 0.38	7.02 ± 0.94	4.62 ± 2.60*	7.62 ± 0.34	3.66 ± 3.45*
3	7.8 ± 0.3	7.28 ± 1.08	3.48 ± 3.19*	7.98 ± 0.34	0.00 ± 0.00*
4	8 ± 0.35	8.03 ± 0.29	4.5 ± 2.55*	8.04 ± 0.25	0.00 ± 0.00*
6	7.7 ± 0.62	7.5 ± 0.42	7.98 ± 0.16	7.88 ± 0.29	0.00 ± 0.00*
12	8.1 ± 0.3	7.2 ± 0.55	6.72 ± 1.09	7.95 ± 0.17	0.00 ± 0.00*
17	8 ± 0.17	7.95 ± 0.17	7.74 ± 0.13	7.95 ± 0.30	2.16 ± 2.96*
35	8 ± 0.00	7.88 ± 0.29	8 ± 0.15	8.03 ± 0.15	3.18 ± 4.36

Values are mean ± STDEV; Day 0: the day treatment started; STDEV: standard deviation; G2, G1, G5: the groups that received ethanolic stem bark extract of *C. swynnertonii* at rate of 2000 mg/kg, 1500 mg/kg, 1000 mg/kg respectively, 8 hourly for 3 days; G7: infected-untreated group; G8: group that received diminazene diaceturate at rate of 3.5 mg/kg; Superscript "*" indicate significance at $P < 0.05$ compared to untreated group

The sections were deparaffinized in xylene then rehydrated through a descending ethanol series to phosphate-buffered saline (PBS). To inhibit endogenous peroxidase activity, sections were immersed in a 0.3% *v/v* hydrogen peroxide in distilled water for 30 min at room temperature followed by washing (3 × 5 min) in 0.01 M PBS, pH 7.4. Sections were then incubated with 10% normal goat serum in PBS for 1 h at room temperature to block non-specific binding. To detect single stranded DNA, a marker for apoptotic cells, sections were incubated with

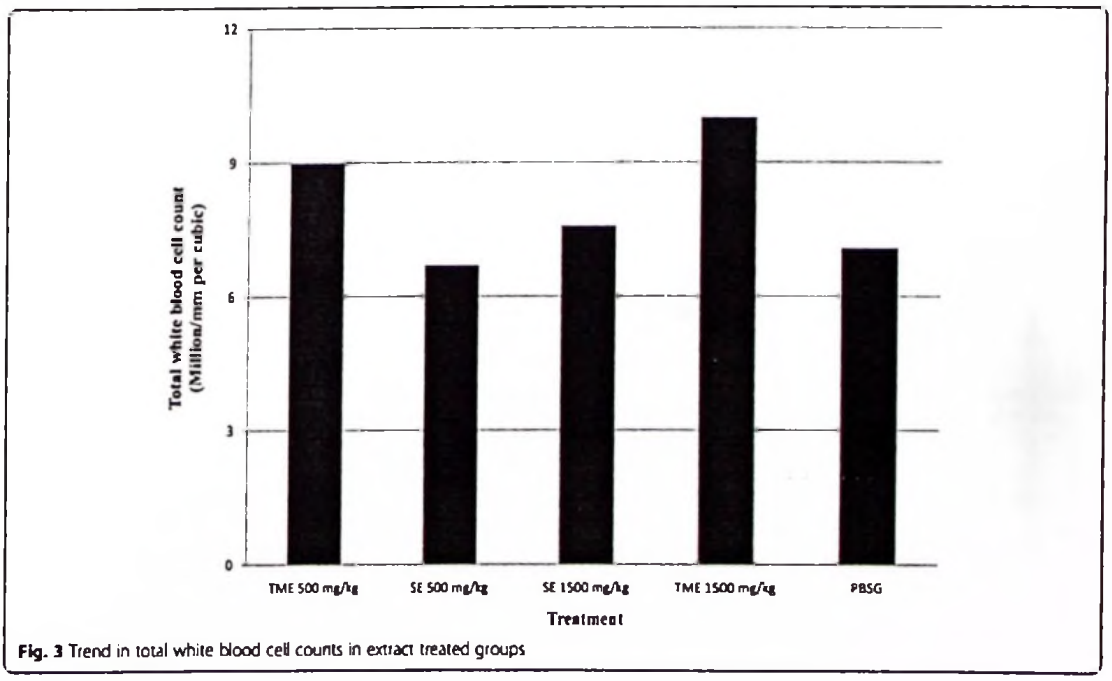

Fig. 3 Trend in total white blood cell counts in extract treated groups

Table 2 Effect of ethanolic stem bark extract of *C. swynnertonii* on differential white blood cells in mice

White blood cells (%)	Experimental groups				
	G9 (4)	G10 (5)	G11 (5)	G12 (3)	G13 (5)
Lymphocytes	66.7 ± 12.99*	77.08 ± 6.90	62.70 ± 21.09*	65.37 ± 9.52**	85.32 ± 5.67
Neutrophils	21.08 ± 7.0*	15.96 ± 5.23	20.96 ± 9.35	23.07 ± 4.69**	10.9 ± 3.64
Monocytes	9.23 ± 4.61*	2.94 ± 2.40	6.88 ± 9.18	7.5 ± 4.19*	2.68 ± 0.60
Eosinophils	2.75 ± 1.98	3.74 ± 2.10	9.16 ± 10.85	3.77 ± 1.18	1.02 ± 2.28
Basophils	0.25 ± 0.26	0.28 ± 0.19	0.3 ± 0.21	0.3 ± 0.26	0.08 ± 0.18

Values are mean ± STDEV; STDEV: standard deviation; G9, G12: groups treated with TME at rate of 500 mg/kg, 1500 mg/kg respectively; G10, G11: groups treated with SE at rate of 500 mg/kg, 1500 mg/kg respectively; TME and SE stands for thorough mixed extract and supernatant extract of ethanolic stem bark of *C. swynnertonii*; G13: group treated with PBSG at the rate of 0.1 ml/10 g. The *stand for significance with respect to negative control whereas *: $P < 0.05$, **: $P < 0.01$. The number of mice from which the data were obtained is shown in the brackets

polyclonal rabbit anti-ssDNA antibody (Immuno-Biological Laboratories Co., Ltd., Code No 18731) at a dilution of 2 μg/ml for 24 h in a dark, humid chamber at 4 °C. For the negative control, PBS was applied in place of primary antibody. Sections were washed (3 × 10 min) in PBS, before incubation with streptavidin-peroxidase conjugate for 30 min at room temperature. Visualization of binding sites was accomplished by incubating the sections for 3–5 min with a medium containing 0.05% 3,3-diaminobenzidine tetra-hydrochloride in 0.015% hydrogen peroxide and 0.01 PBS, pH 7.2 for 1–3 min at room temperature. The sections were counterstained with Mayer's hematoxylin for 30 s, rinsed for 15 min in running tap water and then dehydrated through a graded alcohol series, cleared and mounted in DPX. Immunolabeling was analyzed using a Olympus BH-2 microscope fitted with Olympus camera. For immunofluorescence labeling, the initial steps in processing tissues remained the same as for the streptavidin-peroxidase method. However, instead of incubating with streptavidin-peroxidase, the sections were incubated for 1 h at room temperature with Alexa Fluor® 488-conjugated goat anti-rabbit IgG (FITC) at a dilution of 1:100 (abcam). At the end of incubation, the sections were washed (3 x 5 min) in PBS and mounted, followed by visualization of the binding sites using fluorescence microscope.

Experimental outcomes

This study provides the in vivo activity of *C. swynnertonii* extract on *T. congolense* parasitaemia in a mouse model. The study also report on the effect of the extract on the white blood cells and the spleen as part of the components involved in the host immune response against pathogens.

Statistical methods

Data on levels of parasitaemia, total and differential white blood cell counts were presented as mean ± standard deviation. Statistical analysis was

Table 3 Effect of *C. swynnertonii* ethanolic stem bark extract on spleen

Treatments	White pulp			Red pulp
	Periarteriolar lymphoid sheath (PALS)	Lymphoid follicles	Marginal zone	
PBSG (0.1 ml/10 g)	Normal	Normal	Normal	Congestion
TME, 500 mg/kg	No change	No change	No change	Congestion
SE, 500 mg/kg	No change	No change	No change	Congestion
TME, 1500 mg/kg	No change	Marked reduction in cellularity and size, Apoptosis, pyknosis of lymphocytes and tingible body macrophage	Depleted	Cellularity and size severely reduced
SE, 1500 mg/kg	Apoptosis, white foci of variable sizes	-Apoptosis, -white foci of variable sizes, pyknosis of lymphocytes is evident in some of the foci. -Severely reduced in size	No change	Apoptosis, white foci of variable sizes
Infected, treated with 1500 mg/kg of extract but died on 3rd day	Apoptosis and white foci of variable size	-Apoptosis characterized by white foci and pyknosis of lymphocytes	A moderate widening	Congestion
Infected, treated with 2000 mg/kg of extract but died on 2nd day	No change	-Moderate apoptosis characterized with karyorrhexis of lymphocytes -Increased in size	A moderate widening	No change
Infected but not treated, died on 9th day post inoculation	No change	Severely reduced	No change	Increased cellularity and size

done using one way analysis of variance (One way ANOVA) in the statistical package for social science (SPSS) version 16 (Chicago, SPSS Inc., USA). Excel program was used to determine the trend in the levels of parasitaemia and total white blood cell counts in the respective treated groups.

Results

In vivo activity of the extract on *T. congolense*

The effect of the extract of *C. swynnertonii* on the parasitaemia of *T. congolense* during and after treatment is shown in Fig. 2. There were fluctuations in the parasitaemia of *T. congolense* in treated mice. Mice which received the extract at 8 hourly intervals had a moderately lower parasitaemia during therapy than those at 12 hourly intervals. The group, G5, which received the extracts at 8 hourly intervals had initially drastically reduced the parasitaemia. However, G2 (2000 mg/kg) was the only group that showed a significantly ($P < 0.05$) lower parasitaemia after the first day of therapy compared to negative control (Table 1). There was a significant ($P < 0.05$) reduction of parasitaemia in G5 (1000 mg/kg) on the 2^{nd}, 3^{rd} and 4^{th} day of therapy whereby some of the mice completely cleared the parasite as per observations by wet smear. Parasitaemia in G8 (diminazene diaceturate at 3.5 mg/kg) was significantly ($P < 0.05$) lower than the negative control until the 2^{nd} day post treatment. Thereafter, parasitaemia was not observed in blood by wet smear until the 17^{th} day when few mice had parasitaemia.

Effect of extract on selected immunological components

Effect of extracts on white blood cells

There was a slight elevation in the levels of the total white blood cell count (WBC) in the mice treated with the extract save for the group that received 500 mg/kg of SE (Fig. 3). The effects of the ethanolic stem bark extract of *C. swynnertonii* on differential white blood cell count in mice is shown in Table 2. The total WBC in mice treated with extracts did not vary significantly ($P > 0.05$) with the control (PBSG only). However, the percentage of lymphocytes decreased significantly in G9, G11 ($P < 0.05$) and G12 ($P < 0.01$) while that of neutrophils increased significantly in G9 ($P < 0.05$) and G12 ($P < 0.01$). Similarly, the percentage of monocytes was significantly ($P < 0.05$) higher in G9 and G12.

Effect of extracts on spleen and other organs

Macroscopic changes

The mice that were found dead had no noticeable macroscopic change in the heart, lungs, spleen and kidney. The liver had patches of brownish appearance on its surface while the intestines took the color of the extract which is greenish yellow appearance.

Histopathology (H & E)

The various changes on the histological sections of the spleen from the mice that were treated with different concentrations of the extracts of *C. swynnertonii* are summarized in Table 3. The main distinguishing features were observed in spleen from the mice treated with a higher dose of both SE (G11) and TME (G12). There was a presence of white foci of variable sizes in the white pulp which extended into the red pulp in G11 (Fig. 4, a). At higher magnifications apoptosis characterized with pyknosis of lymphocytes in some of the foci was evident (Fig. 4, b). In G12, a marked reduction in the size of the white and red pulp, the periarteriolar lymphoid sheath (PALS) had normal cellularity, lymphoid follicles and marginal zones were depleted (Fig. 5,

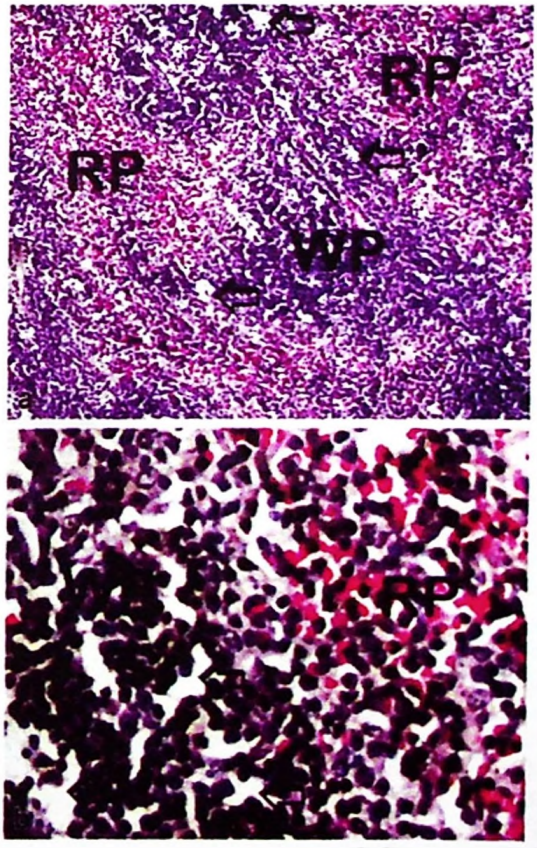

Fig. 4 Histological sections of spleen from the mice in G11 treated with SE of *C. swynnertonii* (SE, 1500 mg/kg), 8 hourly intervals for three days consecutively. There are *white* foci of variable sizes in the *white* and *red pulp* (*open arrows*) in (a). Pyknosis of lymphocytes in some of the *white* foci is evident in (b). Magnifications, (a) 100 x; (b) 400 x

a). At higher magnifications, there was moderate apoptosis with karyorrhexis and pyknosis of lymphocytes (Fig. 5, b). The spleen section from the control group had normal size and cellularity in the white and red pulp (Fig. 6).

The changes observed from the spleen section of the mouse that received 1500 mg/kg of extract but died on the third day of therapy had necrotic foci of variable sizes in the white pulp and a widening of the marginal zone (Fig. 7, a). In addition, apoptosis characterized by pyknosis of lymphocytes was observed (Fig. 7, b). The spleen section from the mouse that received 2000 mg/kg, 8 hourly intervals but died on second day of treatment showed widening of lymphoid follicles and marginal zone (Fig. 8, a). At higher magnifications apoptosis characterized by karyorrhexis of lymphocytes were evident (Fig. 8, b). Also,

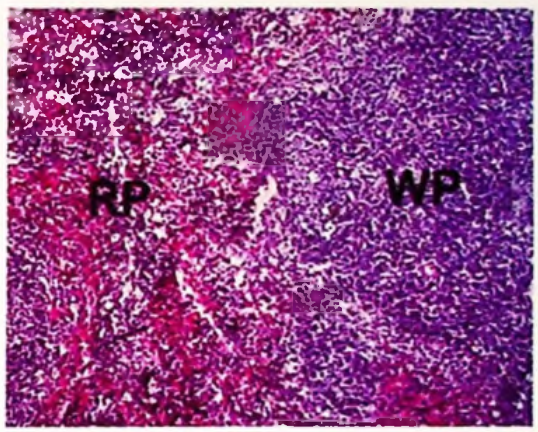

Fig. 6 Histological sections of spleen from the mice treated with PBSG at the rate of 0.1 ml/10 g as the extract at 8 hourly for three days consecutive for comparison with the mice treated with extracts of *C. swynnertonii*. The *red* and *white pulps* have a normal size and cellularity. In the red pulp there are discrete areas of red indicative of congestion. Magnifications, 100 x

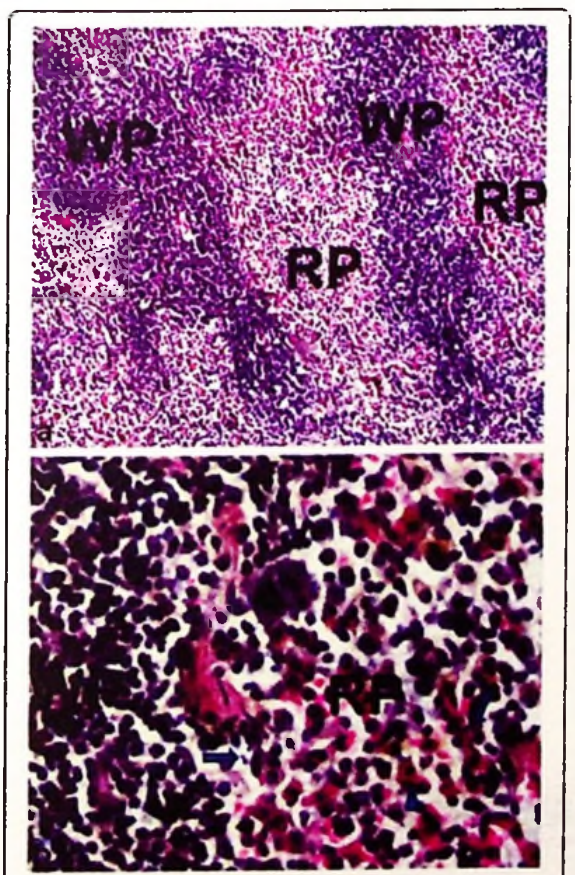

Fig. 5 Histological sections of spleen from the mice in G12 treated with TME of *C. swynnertonii* (TME, 1500 mg/kg), 8 hourly intervals for three days consecutively. In (a), the *white* and *red pulps* are markedly reduced in size; the PALS has normal cellularity, lymphoid follicles and marginal zone are depleted. There is moderate apoptosis with karyorrhexis (*arrows*) and pyknosis of lymphocytes in (b). Magnifications, (a) 100 x; (b) 400 x

the liver showed a marked cytoplasmic vacuolations of hepatocytes, some pyknotic hepatocytes and Küpffer cell hyperplasia (Fig. 9, a). The kidney had a mild hydropic degeneration of cortical-tubular epithelium and glomerulus (Fig. 10, a).

Immunohistochemical findings

Detection of immunoreactivity labeling of ssDNA, a marker of apoptotic cells was consistently observed from spleen sections of mice treated with SE (G11) and TME (G12) by both DAB peroxidase and secondary antibody (Alexa Fluor® 488-conjugated goat anti-rabbit IgG). The ssDNA was shown as a brown reaction resulting from DAB peroxidase reaction (Fig. 11 a, b and c). The reaction was shown to be severe in the red pulp and areas on the marginal zone of the white pulp, some of the reactions were also seen in the PALS and the lymphoid follicles (B and C). The PALS region of the white pulp of the spleen surrounding the central artery (black arrows) appeared darker as it is predominantly occupied by small lymphocytes in (A). However, from mice treated with PBSG at 0.1 ml/ 10 g as the extract at 8 hourly intervals for 3 days consecutive did not show any reaction (D). On one hand, severe apoptosis of lymphocytes was also confirmed by the secondary antibody as observed in the immunoflorescent images of the spleen in (Fig. 12). Immunoreactivity labeling indicative of apoptotic cells (white arrows) was largely seen at the periphery of the white pulp and extended in the red pulp (A). In PBSG treated mice, only few cells positive for ssDNA immunoreactivity were observed in the white pulp in (B).

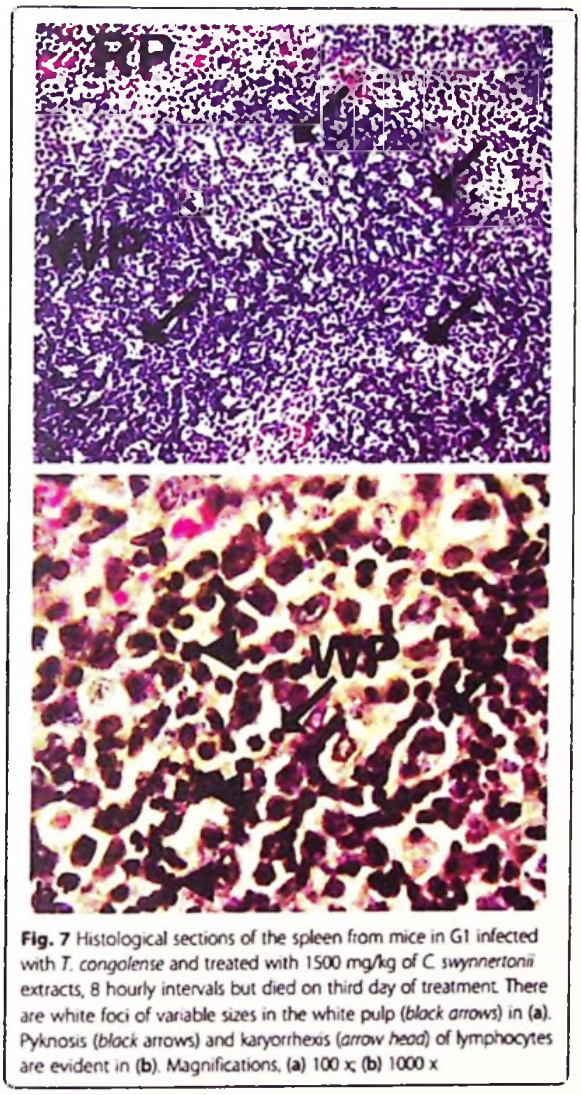

Fig. 7 Histological sections of the spleen from mice in G1 infected with *T. congolense* and treated with 1500 mg/kg of *C. swynnertonii* extracts, 8 hourly intervals but died on third day of treatment. There are white foci of variable sizes in the white pulp (*black arrows*) in (**a**). Pyknosis (*black* arrows) and karyorrhexis (*arrow head*) of lymphocytes are evident in (**b**). Magnifications, (**a**) 100 x; (**b**) 1000 x

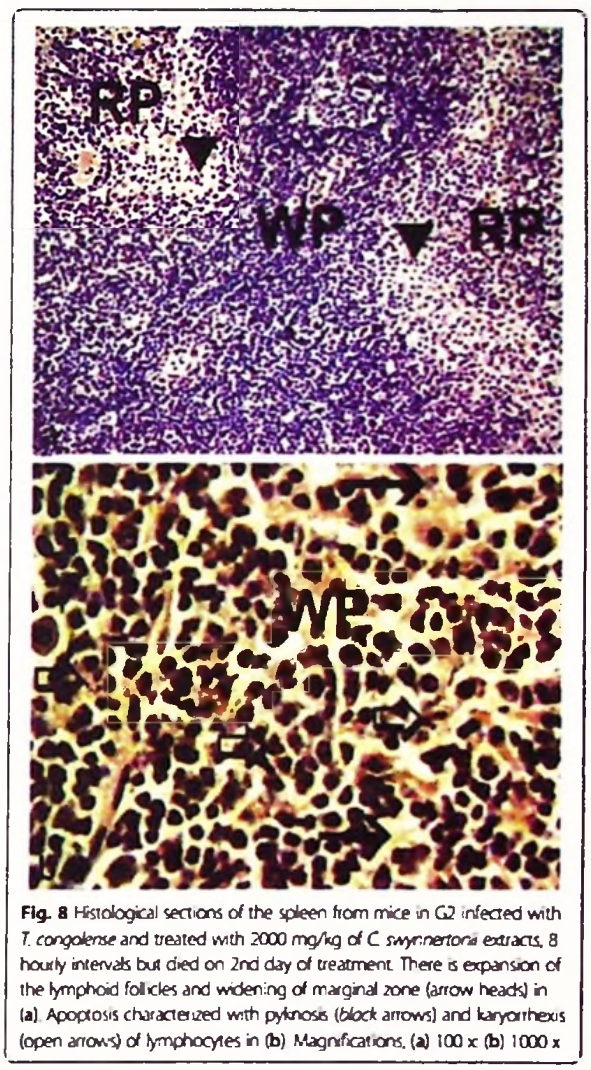

Fig. 8 Histological sections of the spleen from mice in G2 infected with *T. congolense* and treated with 2000 mg/kg of *C. swynnertonii* extracts, 8 hourly intervals but died on 2nd day of treatment. There is expansion of the lymphoid follicles and widening of marginal zone (arrow heads) in (**a**). Apoptosis characterized with pyknosis (*black* arrows) and karyorrhexis (open arrows) of lymphocytes in (**b**). Magnifications, (**a**) 100 x; (**b**) 1000 x

Chemical composition of methanolic extract

The presence of the components in *C. swynnertonii* methanolic stem bark is presented in Table 4 and Fig. 13.

Discussion

This study has evaluated the in vivo effect of ethanolic stem bark extract of *C. swyynertonii* on *T. congolense* and selected immunological components in a mouse model. There was considerable reduction in the levels of parasitaemia in the mice treated with the extract at 8 hourly intervals. The significant suppression of *T. congolense* at 2000 mg/kg a day post therapy and at 1000 mg/kg on day 2, 3 and 4 is an indication that the ethanolic stem bark extract of *C. swynnertonii* possesses antitrypanosomal activity. Similar observations using extracts from different plants have been observed by other workers [29, 30]. Therefore, the current results confirm our earlier report which had similar results [21]. In addition, the study shows that it was necessary to administer higher dose of the extract which was however administered three times per day at 8 hourly intervals to attain the observed effect. The better result following treatment at 8 hourly intervals could be affected by the short half-life of extract constituents thus being unable to persist long enough to exert a pronounced effect on the parasites [31]. Also, enzymatic inactivation of the active compounds and impaired absorption from the site of administration might lead to insufficient concentration and duration for any therapeutic effect at the target organs [32].

On the other hand, a dose of 2000 mg/kg failed to maintain its suppressive activity against *T. congolense* during therapy. High concentrations of all extracts of *C. swynnertonii* except the leaf extract have been

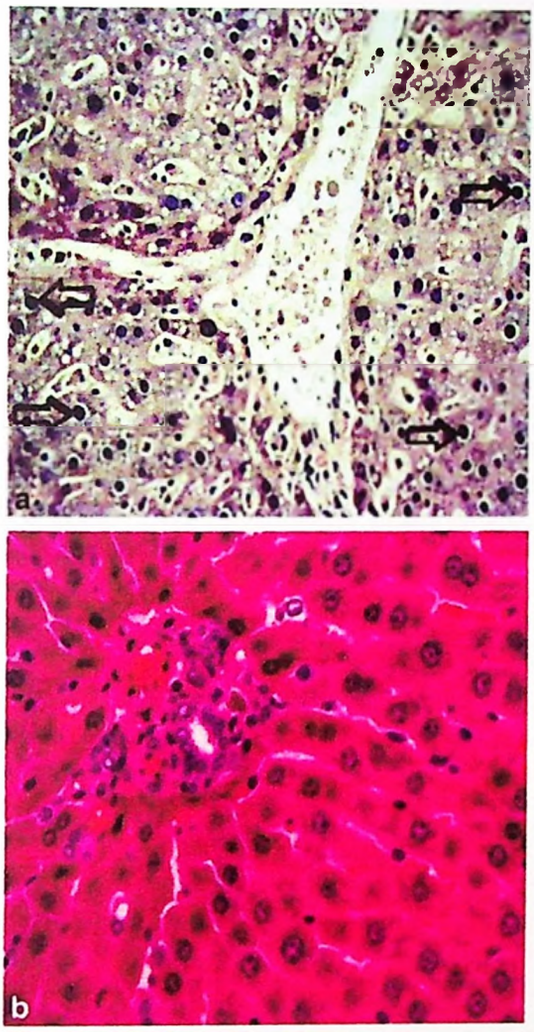

Fig. 9 Histological section of the liver from mice that received 2000 mg/kg, 8 hourly intervals but died on 2nd day of treatment. There is a marked cytoplasmic vacuolation of hepatocytes, some pyknotic hepatocytes (*black* arrows) and Küpffer cell hyperplasia in (**a**). Section (**b**) is for comparison, has normal hepatocytes and cellularity. Magnifications, (**a** & **b**) 400 x

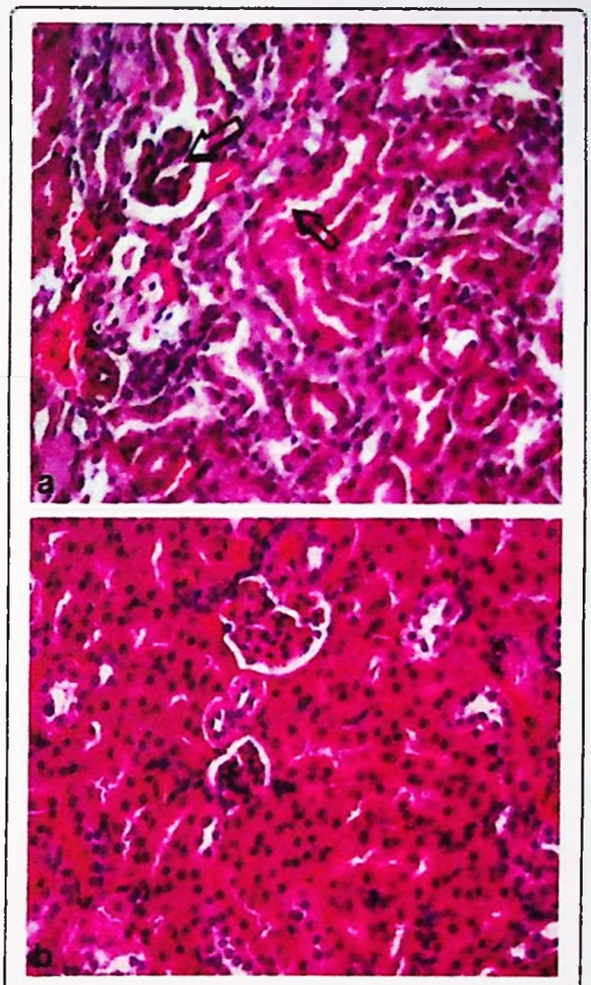

Fig. 10 Histological section of the kidney from mice that received 2000 mg/kg, 8 hourly intervals but died on 2nd day of treatment. There is a mild hydropic degeneration of cortical-tubular epithelium and glomerulus in (**a**). Section (**b**) is from normal kidney for comparison. Magnifications, (**a** & **b**) 400 x

suggested to acutely affect the biological systems [20]. Nevertheless, a dose of 1000 mg/kg provided an increase in therapeutic effect up until the end of therapy. Thereafter, levels of parasitaemia started to rise following cessation of therapy. This could be attributed to cumulative effect and tolerance by the mice. The extract could possess trypanocidal activity, cessation of therapy resulted in termination of trypanocidal effect of residual concentration. *T. congolense* has been found to localize the microvasculature of organs such as brain, heart and skeletal muscles [33, 34]. Hence, reappearance of parasitaemia in mice that showed complete clearance may be attributed to residual parasites from these hiding sites. Therefore, other factors such as antibody response are involved in facilitating total clearance of trypanosomes infection [35]. The extract contains many phytochemical compounds such as those presented in this study [20], the observed effect may be accounted by several possible mechanisms working separately or in concert [36, 37]. Diminazene diacteurate at 3.5 mg/kg cleared the trypanosomes in blood on third day post therapy though few mice relapsed on 17th day. This might probably be due to administered dose being subcurative in mice [38, 39].

The extracts induced a slight increase but an overall insignificant total white blood cell count in mice. However, TME at 500 mg/kg and 1500 mg/kg down modulated the percentage of lymphocytes whilst those of neutrophils and monocytes were up modulated. This finding differs

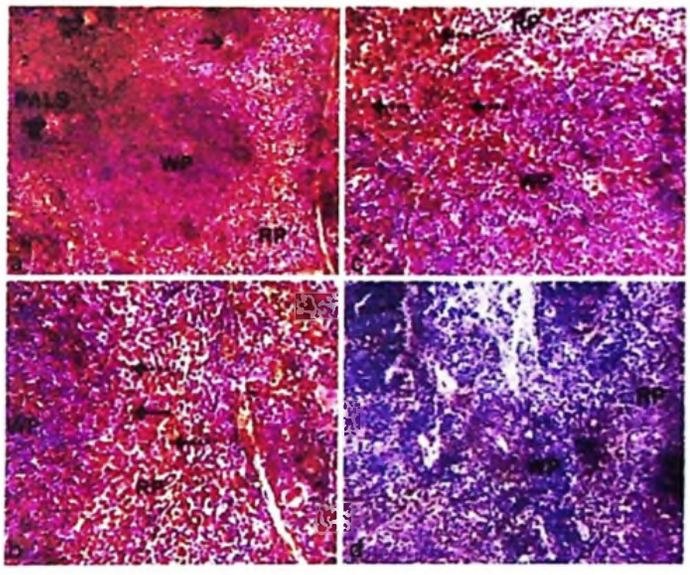

Fig. 11 Immunohistochemical images of the spleen section, from mice treated with TME at the rate of 1500 mg/kg, 8 hourly intervals for three days consecutively. In the white pulp, PALS region of the spleen surrounding central artery (*black* arrows) appear darker as it is predominantly occupied by small lymphocyte in (**a**). In (**b** and **c**), detection of ssDNA in apoptotic cells, a marker for apoptosis is shown as a *brown reaction* due to DAB peroxidase reaction (*dotted line arrows*). The reaction is severe in the *red pulp* and areas on the marginal zone of the *white pulp*, some of the reactions are also seen in the PALS and the lymphoid follicles. Image D is from mice treated with PBSG at 0.1 ml/ 10 g as the extract at 8 hourly for three days consecutive for comparison. Magnifications, (**a**) 100 x; (**b**, **c** and **d**) 400 x

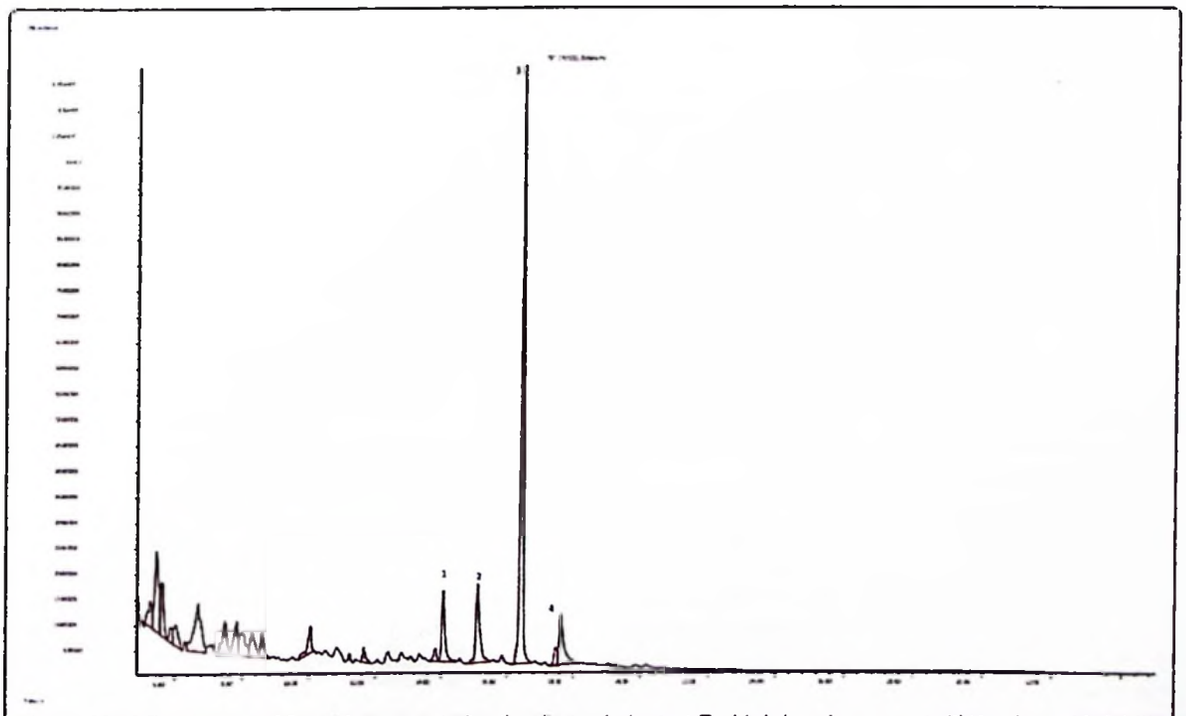

Fig. 13 GC-MS Chromatogram of *Commiphora swynnertonii* methanolic stem bark extract. The labeled number represents: (1) Hexadecane-1,2-diol, (2) 9-Eicosene, (E)-, (3) Borneol, (4) Dichloroacetic acid, tridecyl ester

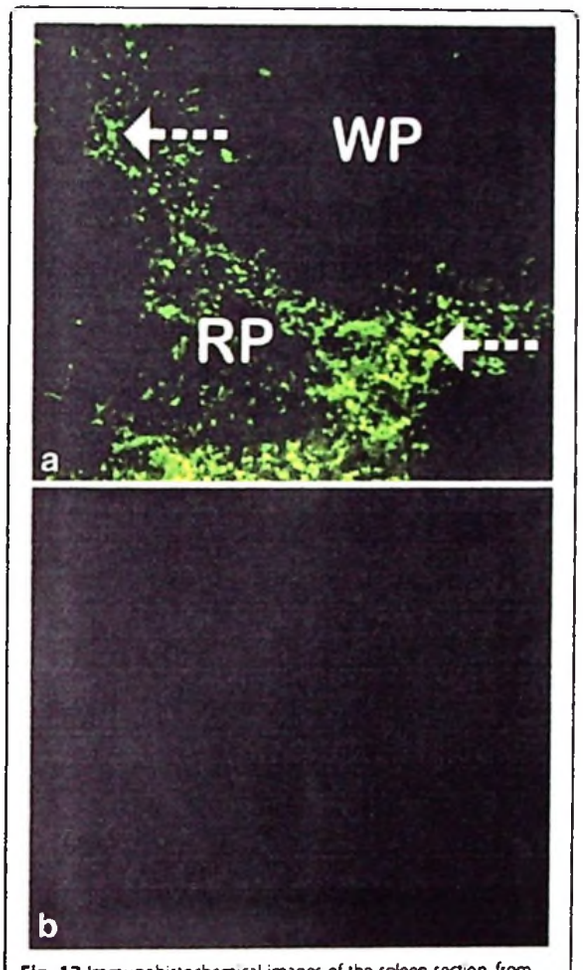

Fig. 12 Immunohistochemical images of the spleen section, from mice treated with TME at the rate of 1500 mg/kg, 8 hourly intervals for three days consecutively. Immunoreactivity labeling indicative of apoptotic cells (*white arrows*) largely seen at the periphery of the *white pulp* and extends in the *red pulp* in (**a**). Some cells positive for ssDNA immunoreactivity are also seen in the white pulp in (**b**). Magnifications, (**a** & **b**) 400x

Table 4 Major phytochemical components from *Commiphora swynnertonii* methanolic stem bark extract identified by GC-MS

Peak	Component	RT	% Composition
1	Undecanoic acid	5.287	2.269
2	beta.-D-Glucopyranose, 1,6-anhydro-	5.462	7.229
3	1,4-Benzenediol, 2-methoxy-	5.625	4.004
5	Methyl-alpha.-d-ribofuranoside	6.028	2.219
6	2H-Pyran-2-one, tetrahydro-4-hydroxy-6-pentyl-	6.689	8.084
7	Heptanoic acid	7.481	3.512
8	Benzoic acid, 4-hydroxy-3-methoxy-	7.829	3.190
9	endo-Borneol	8.018	3.415
10	alpha.-Santoline alcohol	8.292	2.911
11	Phenol, 3,4,5-trimethoxy-	8.595	2.070
15	Hexadecane-1,2-diol	14.076	5.405
16	9-Eicosene, (E)-	15.114	7.311
17	Borneol	16.466	35.852
19	Dichloroacetic acid, tridecyl ester	17.630	5.780

RT retention time

from Bakari and colleagues [40] who reported a significant up modulation of monocytes and lymphocytes in chicken. The difference might be attributed to species variations, mode of administration, the difference in dosage and the type of extract employed. Nevertheless, SE at 1500 mg/kg decreased the percentage of lymphocytes while the percentages of other leukocytes were insignificantly affected. This suggests that SE exhibits a relatively less effect on the immune system than TME. One of the evidence is the marked reduction in size of lymphoid follicles and depletion of the marginal zone, one of the changes imposed by immunomodulatory agent [41].

On the other hand, the mice that were not infected but treated with the extracts had mild to marked histological changes in spleen. At a low dose of both TME and SE, histological changes were indistinguishable from the control. A higher dose of SE induced apoptosis and white foci of variable sizes in the white pulp that extended in the red pulp. Such histological changes are clearly different from the one induced by the higher dose of TME which includes: a marked reduction of the size of white and red pulp compartments, the PALS had normal cellularity and size, lymphoid follicles and the marginal zone were almost depleted. This may provide an indication that TME at the higher dose could possess more toxic effect than the SE. One such evidence were observed in other organs from the mouse that received 2000 mg/kg, 8 hourly intervals but died on 2nd day of therapy. A marked cytoplasmic vacuolation of hepatocytes, some pyknotic hepatocytes, Küpffer cell hyperplasia and a mild hydropic degeneration of cortical-tubular epithelium and glomerulus were the characteristic features in the liver and kidney respectively.

Previous studies have stipulated that changes in the size and density of the PALS and or marginal zone, and a change in lymphoid follicles of the spleen is associated with exposure to immunomodulatory agent [42, 43]. It is clearly documented that apoptosis in the splenic white pulp as observed in this study at the higher dose of SE and TME is a typical feature of compounds that induce lymphocyte toxicity [44]. Such feature is coupled with a decrease in peripheral lymphocytes and down regulation of humoral mediated immunity [45]. Observations by Bakari [20] showed that antibody titre was inversely proportional to dosage of resinous extract of *C. swynnertonii*. Nevertheless, the depletion of lymphoid follicles and marginal zone at higher dose of TME and the mild

decrease at the low dose could be associated with deficit in T cell independent immune response [42, 46] which is essential in humoral response [47]. Thus, in the light of our findings, it is hereby hypothesized that, failure of crude extract of *C. swynnertonii* to clear the infection of *T. congolense* is attributed to its suppressive effect on humoral mediated immunity. To this effect, there is a sufficient amount of evidence to speculate that, besides its direct effect on the trypanosomes, the ethanolic stem bark extract of *C. swynnertonii* activated the cell mediated immune response thus down modulated the levels of parasitaemia but failed to clear the parasites due to its negative effect on humoral mediated immunity.

The role of immunity in facilitating clearance of trypanosome infection in host is well documented [35, 48, 49]. Considering trypanosome's ability of evading the immunity in favor of its survival in a host, switching to a different variant antigenic type (VAT) to avoid antibody mediated destruction [50, 51], It is less successful to attain therapeutic effect through administering crude extract of *C. swynnertonii*. However, our study indicates that ethanolic extract of *C. swynnertonii* could be a potential source of antitrypanosomal compound(s).

Conclusion

This study has provided evidence that even at its crude state, ethanolic extract of stem bark of *C. swynnertonii* possesses in vivo trypanocidal activity. Although the activity is probably impaired by its negative effect on the humoral mediated immune response, the extract of *C. swynnertonii* could still be a potential source of antitrypanosomal compound(s). It is suggested that fractionation of the phytochemical compounds to isolate the ones possessing trypanocidal activity could minimize the undesirable effect. Further studies are recommended to determine the potential of stem bark extract of *C. swynnertonii* as an alternative source of lead compound(s) for trypanocidal drug discovery.

Abbreviations

hr.: Hour; Min: Minutes; mm: Millimeter; PBS: Phosphate buffered saline; PBSG: Phosphate buffered saline with glucose

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Availability of data and materials

The datasets generated or analysed during the current study are not publicly available due to the undergoing research but are available from corresponding author on reasonable request.

Authors' contributions

YPN conceived and conducted the study under supervision of RSS. YPN, RSS and EJK searched and reviewed the literature and wrote up the manuscript. All edited and revised the manuscript critically. All approved the manuscript for submission.

Competing interests

Authors declare that they have no competing interests.

Consent for publication

Not applicable.

Ethics approval and consent to participate

The permission to conduct the current study was obtained from the research and publication committee of Sokoine University of Agriculture, Morogoro, Tanzania. Consent to participate was not applicable in this study.

Publisher's Note

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CHAPTER FOUR

In vitro trypanocidal activity of fractions from the ethanolic stem bark extract of *Commiphora swynnertonii* against *Trypanosoma congolense*

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Target Journal: Journal of Ethnopharmacology

Abstract

Ethnopharmacological relevance

Commiphora swynnertonii is a native species in the northern Tanzania. It is traditionally used for the treatment of fungal infection, sexually transmitted diseases, ulcers and wounds (cut and burn wounds), recalcitrant ulcers, abscesses, the swelling of the legs, chesty coughs and scabies.

Aim of the study

This study has identified bioactive molecules with trypanocidal activities from the ethanolic stem bark extract of *Commiphora swynnertonii* through bioassay-guided fractionation.

Materials and methods

Ethanolic stem bark extract from *C. swynnertonii* was suspended in distilled water and partitioned with dichloromethane and petroleum ether. Gas Chromatography Mass Spectrometry and High Performance Liquid Chromatography were used to determine the presence of molecules in the sub-fractions, followed by an *in vitro* anti-trypanosomal bioassay test. Positive (diminazene diaceturate) and negative PBSG (without the extract) controls were used.

Results

The effects of the Aqueous Fraction, Dichloromethane Fraction and Petroleum Ether Fraction on the motility of *T. congolense* at the tested concentrations were shown to decrease in that order. There was a significant reduction ($P<0.05$) in the motility of *T. congolense* in the test sub-fractions compared to negative control. Among the molecules identified in the test sub-fractions, coronopilin and 4,8,13-duvatriene-1,3-diol combination had significantly ($P<0.05$) stronger effect on the motility of *T. congolense*, with a complete cessation of motility being observed at 15, 25 and 45 minutes post-incubation at concentrations of 1, 0.5 and 0.25 mg/ml, respectively. Sub-fractions with coronopilin alone and coronopilin and hexadecane-1,2-diol combination had a delayed effect, which was observed at 50 minutes post-incubation at a concentration of 1 mg/ml.

Conclusions

This study has detected four terpenoids from the sub-fractions of ethanolic stem bark extract that could explain the trypanocidal activity of *Commiphora swynnertonii*. Further studies are needed to validate their trypanocidal potential.

Keywords: Trypanosomosis, *Trpanosoma congolense*, *Commiphora swynnertonii*, terpernoids, anti-trypanosomal effect.

Background

The importance of plant-derived secondary metabolites does not only lie in their pharmacological or chemotherapeutic effects but also in their provision of template molecules for producing new drug molecules (Seke and Mahomoodally, 2012). Important examples includes alkaloids from the bark of the cinchona tree (native to Latin America) and sesquiterpene lactones from *Artemisia annua* (native to China), which have contributed to the discoveries of quinine and artemisinin, respectively that are used for treating malaria (Cragg and Newman, 2013; Renslo, 2013). In addition, increased scientific interest in search of new anti-trypanosome molecules from plant sources in recent years (Olmo et al., 2015; Lozano et al., 2016) is one such endeavour. Trypanosomes, of which the most important are the tsetse-borne salivarian species, have immense economic impact in Africa, the only continent in which the vector tsetse is found. African trypanosomoses have direct effects on animal production and human health as well as indirect effects on human settlements, land use patterns and agricultural crops (Swallow, 2000; Oluwafemi, 2014). It is estimated that about 40 million cattle are at risk of acquiring African animal trypanosomiasis (AAT) in the sub-Saharan Africa and 3 million die every year, leading to an economic loss of US$ 1.0 – 1.2 billion annually

(James, 2007). The total domestic product lost is estimated at US$ 4.5 billion per annum when secondary losses such as reduced manure and draft power are included (James, 2007). Of all salivarian trypanosome species, *Trypanosoma congolense* is a major cause of AAT being highly pathogenic to a wide range of domestic animals including cattle, sheep, goats, horses, pigs and dogs (Namangala and Odongo, 2013). More than 80% of AAT and losses in domestic animals in sub-Saharan Africa are due to *T. congolense* infection (Namangala and Odongo, 2013). Chemotherapy is currently the primary means of combating the disease, but it faces a number of drawbacks, including failed and/or reduced efficacy against resistant trypanosome stocks and the toxicity of the trypanocidal drugs (Delespaux and Koning, 2007; Melaku and Birasa, 2013). The problem is further compounded by the uncertainty of vaccine development and unwillingness of the pharmaceutical industry to develop new compounds as a result of high costs versus expected returns from the poor affected African countries (Kennedy, 2008). The aim of the present study was to contribute to the search for new molecules from plant sources for developing new trypanocides.

The genus *Commiphora* (family Burseraceae) is largely African, comprising more than 150 plant species, with only eleven having been recorded outside the continent (Burtt, 1935; Shen et al., 2012). This genus is distributed throughout tropical and subtropical regions, especially in north-eastern Africa, southern Arabia and India (Shen et al., 2012). Investigations on a number of *Commiphora* species have identified more than 300 secondary metabolites and crude extracts with several pharmacological effects (Shen et al., 2012). For example, *Commiphora swynnertonii* extracts alone have a number of pharmacological effects such as treatment of sexually transmitted diseases, ulcers and wounds (cut and burn wounds), recalcitrant ulcers, abscesses, the swelling of the legs,

chesty coughs and scabies (Kalala et al., 2014). Its resinous exudates are used to treat worm infections and dental caries, for cleansing the bladder and control of parasites such as ticks, lice, bed bugs and mange mites (Kalala et al., 2014). Additionally, *C. swynnertonii* extracts have been shown to possess activity against a range of bacteria and fungi species (Bakari et al., 2011; Mkangara et al., 2014), and therapeutic effects in chickens that were experimentally infected with *Eimeria* species (Bakari et al., 2013) and Newcastle disease virus (Bakari et al., 2012; Bakari, 2013). Furthermore, a recent study has shown that the ethanolic stem bark extract possesses trypanocidal activity *in vitro* (Nagagi et al., 2016) and *in vivo* (Nagagi et al., 2017). However, given the amount of information presented in previous studies on *C. swynnertonii* extracts, less is known about its molecules.

Terpenes are the major group of secondary metabolites and one of the phytochemicals in *C. swynnertonii* (Bakari, 2013). In the recent past, hundreds of terpene-derived molecules have been reported to possess trypanocidal activity, some requiring minor modification in order to improve their efficacy against various *Trypanosoma* species (Lozano et al., 2016). Therefore, this study through bioassay-guided fractionation presents the active terpenoids in *C. swynnertonii* ethanolic stem bark extract that are responsible for trypanocidal effect on *T. congolense*.

Methods

Plant Materials

Stem bark pieces were collected from Kitwai A village (4°05′42.00″ S and 36°33′34.42″ E), Simanjiro district in the Manyara region of northern Tanzania. A plant specimen was submitted to the National Herbarium of Tanzania, Tropical Pesticides Research Institute,

Arusha (Specimen voucher Number CS-01). The confirmation of the plant species was performed by a plant taxonomist.

Extraction

The stem bark was peeled off and dried in the shade for four weeks. After that, the dried barks were ground into a fine powder using a laboratory mill and stored in an airtight bag in a cool dry room until use. Five hundred grams (500 g) of the ground stem bark was weighed and soaked in 1.0 litre of 99.9% ethanol in a conical flask sealed with aluminium foil and left for 72 hours in a dark place with occasional stirring. The extract was then filtered through a piece of gauze in a funnel into a conical flask, after which it was dispensed into several 50 ml centrifuge tubes and centrifuged (Zentrifugen-Universal 320, Itettich, Germany) at 4,000 rpm for 10 minutes. The resulting supernatant was filtered using Whatmann® filter paper No. 1, and the filtrate was poured into a round flask and concentrated to dryness (11.32 g) under reduced pressure using a rotary evaporator (Rotavapor R 110, Laboratoriums-Technik AG, Switzerland) with a water bath temperature of 40 °C.

Trypanosome stock

The trypanosome stock used in this study was a putative drug-sensitive *Trypanosoma congolense* stock obtained from the Small Animal Unit at the College of Veterinary Medicine and BioMedical Sciences, Sokoine University of Agriculture where it has been maintained by serial passage in Swiss albino mice after its original isolation from a cow at Mikese, Morogoro. For the present studies, the stock was further maintained by serial passage in Swiss albino mice at the Division of Livestock and Human Diseases Vector Control, Tropical Pesticides Research Institute, Arusha.

Determination of the parasitic load

The parasitic load in mice was monitored by microscopic examination of wet smears from mouse tail blood using the method described by Herbert and Lumsden (1976). This approach involved counting the parasites per field of a wet blood smear. Logarithmic values of these counts were obtained by matching the values with a Herbert and Lumsden table and/or converted to antilog values to provide the absolute number of trypanosomes per millilitre of blood (Herbert and Lumsden, 1976).

Data Analysis

The least significant (0.05) effect of the fractions and sub-fractions on motility of *T. congolense* was obtained by Kruskal Wallis test and comparison between treatments done using pairwise Mann-Whitney U test. Both tests were done using statistical package for social science (SPSS) version 16 (Chicago, SPSS Inc., USA).

Fractionation and GC-MS analysis

The ethanolic extract of *C. swynnertonii* stem bark is likely to contain tannins, polyphenols, flavonols, terpenoids, sterols and alkaloids (Cowan, 1999). Previous studies have shown that dichloromethane can selectively extract the less polar terpenoids (Ankli et al., 2000; Wetungu et al., 2014). On the other hand, petroleum ether has been recorded to extract mostly alkaloids, steroids, essential oils, flavonols, phenols and triterpenoids (Singh, 2008). Therefore, an attempt was made to isolate terpenoids from the ethanolic stem bark extract of *C. swynnertonii* by partitioning with dichloromethane and petroleum ether. Ethanolic stem bark extract of *C. swynnertonii* in a round flask was re-suspended with 150 ml of distilled water and partitioned with an equal volume of dichloromethane and the remaining aqueous solution was again partitioned with an equal volume of

petroleum ether. Each solution was concentrated to dryness under reduced pressure using the rotary evaporator with the temperature of the water bath at 40 °C (for dichloromethane and petroleum ether solutions) and at 55 °C for the aqueous solution, leading to a dichloromethane fraction (DF) (3.73 g), a petroleum ether fraction (PF) (2.93 g) and an aqueous fraction (AF) (3.55 g).

Twenty (20) milligrams from each fraction was weighed and separately dispensed into an eppendorf tube, and each was diluted with 1 ml of phosphate-buffered saline with glucose (PBSG) (pH, 8.0) to make 20 mg/ml stock solutions. Two other concentrations, 10 mg/ml and 5 mg/ml, were prepared from stock solutions by serial dilution using PBSG. Each of the fractions was assessed for their *in vitro* anti-trypanosomal activity using the previously described method (Nagagi et al., 2016) with some modifications.

For each concentration, 5 µl was drawn and added to a separate eppendorf tube. Control tubes were, a negative containing 5 µl of PBSG (without the drug) and a positive with 5 µl of 20 mg/ml diminazene diaceturate (Veriben®, Ceva Santé Animale, France). A mouse infected with *Trypanosoma congolense* Mikese stock with parasitaemic load of antilog value 8.4 as per Herbert and Lumsden chart and table (Herbert and Lumsden, 1976) was humanely killed by head stunning using a piece of iron rod followed with decapitation (Close et al., 1996). Blood was immediately collected in EDTA tube, a 0.35 ml was measured and diluted with 2.42 ml of PBSG to make an estimated parasitic load of antilog 7.4 equivalent to 3.16×10^7 trypanosomes per millilitre. After that, 450 µl was added into each of the different prepared concentrations including the negative and positive controls. The mixtures were left to stand for two minutes at room temperature, after which an amount 2 µl was applied to separate slides and each covered with a cover slip. The

motility of trypanosomes was assessed every 5 minutes using a light microscope at a 400x magnification, for a total of 70 minutes. The cessation or reduction in motility of the parasites in the test mixtures with respect to that of the parasite-loaded control without the extract was considered as a measure of trypanocidal activity (Atawodi et al., 2003; Ene et al., 2014).

Afterwards, the aqueous and dichloromethane fractions were subjected to further fractionation using column chromatography packed with silica gel. Gas Chromatography Mass Spectrometry (GC-MS) (Agilent Technologies) and High-Performance Liquid Chromatography (HPLC) were used to determine the presence of molecules in the sub-fractions.

Briefly, 1.2 g of the aqueous fraction was poured into a silica gel column measuring 30 x 1.2 cm that was packed with 15 g of silica gel (60–120 mesh). Sub-fractions 1–47 of 0.5–1 ml were collected when the column was eluted with an n-hexane: ethanol gradient (99.9%). Similarly, 1 g of dichloromethane fraction was eluted with n-hexane with the gradual introduction of dichloromethane to yield 1–20 sub-fractions in another silica gel column measuring 26 x 0.9 cm that was packed with 10 grams of silica gel (60–120 mesh). Based on the GC-MS (with helium as a carrier gas, 1.2 ml/min flow rate, 30 m column length, 0.25 mm internal diameter, 0.25 mm film thickness, ion source temperature 230 °C, injection mode-autoinjector with split-splitless, mass spectrometry detector inlet temperature at 250 °C, the temperature of the m/s quadrapole set at 150 °C and a pressure vacuum at 4.85 x 10^{-5} psi) analysis, the sub-fractions were combined as A, B, C, D, E and F. Moreover, sub-fraction F was again poured into another silica gel column packed with 15 g of silica gel and eluted with n-hexane: dichloromethane to yield

10 sub-fractions. Based on the GC-MS (under the conditions given above) analysis, they were combined as F1 and F2.

Each of the combined sub-fractions was injected separately into the HPLC (solvent delivery system, Varian 9012; variable wavelength UV-VIS detector, Varian 9050; wavelength 254; flow rate 0.30; and Pressure 327 psi) and eluted with methanol and acetonitrile at a 50:50 ratio and the collected sub-fractions were qualitatively determined by GC-MS analysis. After that, the combined molecules were concentrated by evaporating the solvent to dryness using nitrogen gas from a nitrogen generator.

***In vitro* trypanocidal activity of sub-fractions**

An evaluation of *in vitro* trypanocidal activity was performed on the sub-fractions B, C, E, F1 and F2 in three replicates. Stock solutions for B, C, E, F1 and F2 were prepared in eppendorf tubes by weighing 2 mg each and diluting in 1 ml of PBSG to yield a concentration of 2 mg/ml. Two other concentrations (1 mg/ml and 0.5 mg/ml) were made from each of the stock solutions by serial dilution with PBSG and labelled accordingly. After that, 5 µl from the prepared concentrations (2 mg/ml, 1 mg/ml and 0.5 mg/ml) of B, C, E, F1 and F2 were drawn separately and poured into their respectively labelled eppendorf tubes. Subsequently, separate eppendorf tubes, one containing 5 µl of PBSG (without the extract) and the other 5 µl of 2 mg/ml diminazene diaceturate (Veriben®, Ceva Santé Animale, France) were prepared as controls. Then, three drops of *T. congolense*-infected blood was collected from donor Swiss albino mice with parasitaemic load of antilog 8.4, and diluted using 1 ml of PBSG to make an estimated parasite load of 3.16×10^7 trypanosomes per millilitre. From the diluted blood, 5 µl was drawn and dispensed into the labelled eppendorf tubes, including the negative control containing the

PBSG (without the extract) and the positive control diminazene diaceturate, to make effective concentrations of 1 mg/ml, 0.5 mg/ml and 0.25 mg/ml for the sub-fractions and 1 mg/ml for the diminazene diaceturate. These samples were gently mixed and allowed to stand for 2 minutes. Thereafter, 2 µl of the test mixture was placed on a separate microscope slide and covered with a cover slip and the parasites observed every 5 minutes for motility. The cessation or reduction in motility of the parasites in the test mixtures with respect to that of the parasite-loaded control without the extract was considered as a measure of trypanocidal activity (Atawodi et al., 2003; Ene et al., 2014).

Results

The motility of *T. congolense* following *in vitro* incubation with various concentrations of the test fractions mixtures is shown in Table 1. There was a significant reduction ($P<0.05$) in the motility of *T. congolense* incubated with fraction mixtures and across the tested concentrations. However, the reduction in the motility of *T. congolense* did not vary significantly ($P>0.05$) between test fractions of aqueous, dichloromethane and petroleum ether at 0.22 mg/ml and between the aqueous and dichloromethane test fractions at 0.11 mg/ml. Similarly, no significant differences ($P>0.05$) were observed between dichloromethane test fraction at 0.11 mg/ml and the aqueous test fraction at 0.055 mg/ml, petroleum ether test fraction at 0.11 mg/ml and the lowest concentration (0.055 mg/ml) of the aqueous and dichloromethane test fractions. Also, between petroleum ether test fraction at 0.055 mg/ml and PBSG (without the extract) at which the motility of *T. congolense* remained high up until when the test experiment was terminated at 70 minutes. The motility of *T. congolense* in the petroleum ether and dichloromethane test fractions at 0.11 mg/ml and 0.055 mg/ml, respectively was not significantly ($P>0.05$) different from that in the positive control test mixture of diminazene diaceturate at 0.22 mg/ml.

A summary of combined sub-fractions after fractionation and GC-MS analysis of aqueous and dichloromethane fractions is presented in Table 2. Among the compounds identified in aqueous fraction were borneol (Figure 1), coronopilin (Figure 2), and geranylgeraniol (Figure 3) while in dichloromethane fraction there were coronopilin (Figure 2) and 4,8,13-duvatriene-1,3-diol (Figure 4).

The motility of *T. congolense* after *in vitro* incubation with various concentrations of the sub-fractions containing the identified compound(s) is presented in Table 3. There was a significant reduction ($P<0.05$) in the motility of *T. congolense* in the test sub-fractions C (coronopilin) and F1 (coronopilin and 4,8,13-duvatriene-1,3-diol) at concentrations of 1 mg/ml, 0.5 mg/ml and 0.25 mg/ml when compared to negative control (PBSG without the extract). Sub-fraction E (coronopilin and hexadecane-1,2-diol) significantly ($P<0.05$) reduced the motility of *T. congolense* at concentrations of 1 mg/ml and 0.5 mg/ml. The reductions in motility of *T. congolense* by sub-fractions C and E at concentrations of 1 mg/ml and 0.5 mg/ml as well as F1 at 0.25 mg/ml were not significantly ($P>0.05$) different from that caused by diminazene diaceturate (DA) at concentration of 1 mg/ml. Both C (coronopilin) and E (coronopilin and hexadecane-1,2-diol) had a delayed effect on motility of *T. congolense* with the complete cessation of motility being observed after 50 minutes of incubation.

Sub-fraction F1 (coronopilin and 4,8,13-duvatriene-1,3-diol) at 1 mg/ml had a pronounced effect on the motility of *T. congolense* that was significantly ($P<0.05$) greater than that produced by C and E at similar concentrations. The complete cessation of motility of *T. congolense* was observed after 15, 25 and 45 minutes of incubation at concentrations of 1, 0.5 and 0.25 mg/ml, respectively. On the other hand, effects of sub-fractions B and F2 on

the motility of *T. congolense* were not significantly different (*P>0.05*) from that of PBSG (without the extract) test mixture in which the *T. congolense* motility remained high up until when the test experiment was terminated.

Discussions

This study has evaluated the *in vitro* trypanocidal activity of fractions and sub-fractions of *Commiphora swynnertonii* ethanolic stem bark extract. The trypanocidal activity of *C. swynnertonii* ethanolic stem bark extract could be attributed to the presence of four terpenoids that include borneol, coronopilin, geranylgeraniol and 4,8,13-duvatriene-1,3-diol from the aqueous and dichloromethane fractions. Previous studies have reported borneol (Mulyaningsih et al., 2010) and geranylgeraniol (Menna-Barreto et al., 2008) to possess trypanocidal activity. In addition, the present study has shown that the reduction in *T. congolense* motility by sub-fraction F1 (coronopilin and 4,8,13-duvatriene-1,3-diol) at the lowest concentration of 0.25 mg/ml was comparatively similar (*P>0.05*) to 1 mg/ml of sub-fractions C (coronopilin), E (coronopilin and hexadecane-1,2-diol) and DA (diminazene diaceturate). This phenomenon provides an indication that both coronopilin and 4,8,13-duvatriene-1,3-diol possess *in vitro* trypanocidal activity against *T. congolense* and that their action is synergistic. Other clear evidence is the pronounced and complete cessation of *T. congolense* motility after 25 and 45 minutes that was observed at 0.5 and 0.25 mg/ml respectively, in contrast to coronopilin alone and the combination of coronopilin and hexadecane-1,2-diol. A previous study has identified coronopilin as being among the molecules from ethanolic extracts of *Parthenium hysterophorus* flowers that have *in vitro* and *in vivo* trypanocidal activity (Talakal et al., 1995). Therefore, the relatively stronger effect on the motility of *T. congolense* by the combination of the

coronopilin and 4,8,13-duvatriene-1,3-diol is an indication that 4,8,13-duvatriene-1,3-diol could also possess trypanocidal activity.

It was found in the present study that, the effects of the fractions (obtained by partitioning the *Commiphora swynnertonii* ethanolic stem bark extract) on the motility of *T. congolense* at the tested concentrations were decreasing in the order AF, DF and PF. This is supported by the insignificant ($P>0.05$) variation of motility of *T. congolense* in aqueous test fraction at 0.055 mg/ml and in dichloromethane test fraction at 0.11 mg/ml. In addition, the reduction in *T. congolense* motilities in aqueous and dichloromethane test fractions at their lowest concentration (0.055 mg/ml) were comparatively similar ($P>0.05$) to that observed in petroleum ether test fraction at higher concentration of 0.11 mg/ml. When *T. congolense* motilities in the test mixtures (petroleum ether and dichloromethane test mixtures at 0.11 mg/ml and 0.055 mg/ml) were compared with that in the positive control (diminazene diaceturate at 0.22 mg/ml) mixture there was no statistically significant difference ($P>0.05$). Therefore, from this observation, AF had a superior *in vitro* reduction effect on the motility of *T. congolense* over the other fractions including the positive control (DA). This has two implications. First, the major compounds with trypanocidal activity from ethanolic stem bark extracts of *C. swynnertonii* dissolve in polar solvents. This finding is consistent with our previous study, in which a significant *in vivo* trypanocidal effect was observed when mice were treated with a supernatant extract with PBSG as the diluent (Nagagi et al., 2017). Second, the superior effect of AF on the motility of *T. congolense* over DF and PF could be attributed to an additive or synergistic effect from borneol, geranylgeraniol and coronopilin on single or multiple target sites associated with a physiological process of the parasite (Feyera et al., 2014). For example, borneol has been shown to induce caspase activity, which suggests that apoptosis could be

the primary mechanism of action (Mulyaningsih et al., 2010). Likewise, geranylgeraniol has been reported to cause mitochondrial swelling and the disruption of several organelles and the kinetoplast DNA network (Menna-Barreto et al., 2008). Although it is a potent inducer of apoptosis (Ohizumi et al., 1995; Masuda et al., 1997), these effects are suggestive of cell death events that occur through autophagic pathway (Menna-Barreto et al., 2008). Thus, its precise mechanism of action remains a subject of further studies. Currently, there is paucity of information on the mechanism of action of coronopilin against trypanosomes. However, a study on leukaemia cell lines has shown that coronopilin could induce apoptosis through DNA damage, a loss of mitochondrial membrane potential and release of cytochrome c, and the activation of the caspase-3 pathway (Cotugno et al., 2012). Studies that would determine its exact mechanism of action against trypanosomes are needed.

This study has shown that coronopilin and 4,8,13-duvatriene-1,3-diol combination and to some extent hexadecane-1,2-diol could contribute to the trypanocidal activity of dichloromethane fraction (DF). There is no previous report on the medicinal values of 4,8,13-duvatriene-1,3-diol since its first identification (Chang and Grunwald, 1976). Thus its mechanism of action cannot be speculated and requires further studies.

The limitations of this study includes, failure of the column chromatography to completely separate discrete sub-fractions containing closely related compounds. For instance, fractionation using column chromatography was unable to separate coronopilin from 4,8,13-duvatriene-1,3-diol probably due to shortness of column length. In addition, using PBSG as diluent could possibly partial dissolved the molecules in petroleum ether fraction

(PF) and contributed to its low activity. Therefore, studies utilizing non-polar solvents are recommended to determine the activity of non-polar terpenoids against trypanosomes.

Conclusions

This study has identified four terpenoids (borneol, coronopilin, geranylgeraniol and 4,8,13-duvatriene-1,3-diol) that could be responsible for trypanocidal activity of *Commiphora swynnertonii* ethanolic stem bark extract. It was shown that a relatively low dose at which coronopilin and 4,8,13-duvatriene-1,3-diol were detected had comparatively high *in vitro* effect on the motility of *T. congolense* than that produced by diminazene diaceturate, the phenomenon that indicated that both coronopilin and 4,8,13-duvatriene-1,3-diol could possess trypanocidal activity. Therefore, there is a need to undertake further studies to determine the *in vivo* trypanocidal activities of coronopilin and 4,8,13-duvatriene-1,3-diol.

Abbreviations

DNA: Deoxyribonucleic acid

FAO: Food and Agriculture Organization

DF : dichloromethane fraction

PF: petroleum ether fraction

AF: aqueous fraction

PBSG: phosphate-buffered saline containing glucose

GC-MS : Gas Chromatography Mass Spectrometry

HPLC: High-Performance Liquid Chromatography

Declarations

Ethics approval and consent to participate

This study was granted ethical approval by the Research and Publications Committee of Sokoine University of Agriculture, Morogoro, Tanzania. These experiments followed proper guide to the care and use of experimental animals recommended by FAO.

Consent for publication

Not Applicable

Availability of data and material

The datasets used and or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

YPN conceived and conducted the study under the supervision of RSS. YPN, RSS and EJK searched and reviewed the literature and wrote up the manuscript. All the authors edited and revised the manuscript critically. All the authors reviewed the work and approved it for submission.

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Table 1: Motility of *Trypanosoma congolense* following *in vitro* incubation with various concentrations of the test fractions

Test Mixture (mg/ml)	Fractions	Post-incubation follow up (minutes)														Kruskal Wallis test
		5	10	15	20	25	30	35	40	45	50	55	60	65	70	Mean Rank
0.22	AF	0	0	0	0	0	0	0	0	0	0	0	0	0	0	36.00*
	DF	0	0	0	0	0	0	0	0	0	0	0	0	0	0	36.00*
	PF	1	1	0	0	0	0	0	0	0	0	0	0	0	0	43.29*
0.11	AF	0	0	0	0	0	0	0	0	0	0	0	0	0	0	36.00*
	DF	2	2	1	1	1	1	0	0	0	0	0	0	0	0	61.50*
	PF	3	3	3	3	2	2	2	1	1	1	1	0	0	0	96.25*
0.055	AF	3	3	2	1	1	1	1	1	1	1	0	0	0	0	81.61*
	DF	3	3	3	3	3	2	2	2	1	1	1	1	0	0	103.57*
	PF	3	3	3	3	3	3	3	3	2	2	2	2	1	1	123.71
0.22	DA	2	2	2	2	2	2	1	1	1	1	1	1	1	1	97.93*
PBSG		3	3	3	3	3	3	3	3	3	3	3	3	3	2	136.64

Key: 3: Very motile, 2: Motile, 1: Less motile, 0: Not motile

AF: Aqueous fraction; DF: Dichloromethane fraction; PF: Petroleum ether fraction; DA: Diminazene diaceturate; PBSG: Phosphate Buffered Saline with Glucose; Superscript "*" indicates significance at $P<0.05$ when compared to PBSG

Table 2: A summary of combined sub-fractions after GC-MS analysis

Fraction type	Fractionated fractions		Weight (mg)
	Label	Combined fractions	
Aqueous fraction	A	9–10	ND
	B	12–28	584.225
	C	30–44	138
	D	46–47	ND
Dichloromethane fraction	E	2–7	54.78
	F	9–20	ND
	F1	2–5	488.8
	F2	7–10	276.2

Key: A: borneol; B: 1,1'-bis(cyclooct-2-en-4-one); C: Coronopilin; D: Geranylgeraniol; E: Coronopilin and Hexadecane-1,2-diol; F: 6,8-Dimethyl-non-4-enedionic acid, Dimethyl ester; Hexadecane-1,2-diol; Pentadeca-2,3,6,9,12,13-hexaen-8-one, 2,5,5,11,11,14-hexamethyl; Coronopilin and 4,8,13-Duvatriene-1,3-diol; F1: Coronopilin, 4,8,13-Duvatriene-1,3-diol; F2: 6,8-Dimethyl-non-4-enedionic acid, dimethyl ester; Hexadecane-1,2-diol; Pentadeca-2,3,6,9,12,13-hexaen-8-one, 2,5,5,11,11,14-hexamethyl; ND: Not determined

Table 3: Motility of *T. congolense* after *in vitro* incubation with various concentrations of sub-fractions

Test Mixture (mg/ml)	Fraction	Post-incubation follow up (minutes)														Kruskal Wallis test
		5	10	15	20	25	30	35	40	45	50	55	60	65	70	Mean Rank
1	B	3	3	3	3	3	3	3	3	2	2	2	1	1	1	138.93
	C	3	3	2	2	2	2	1	1	1	0	0	0	0	0	76.96*
	E	3	2	2	1	1	1	1	1	1	0	0	0	0	0	63.07*
	F1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	28.43*
	F2	3	3	3	3	3	3	3	3	3	3	3	3	3	3	180.50
0.5	B	3	3	3	3	3	3	3	3	3	3	3	2	2	2	163.89
	C	3	3	3	3	3	2	2	2	1	1	1	1	1	1	113.96*
	E	3	3	2	2	2	1	1	1	1	1	1	1	0	0	83.07*
	F1	2	2	1	1	0	0	0	0	0	0	0	0	0	0	39.93*
	F2	3	3	3	3	3	3	3	3	3	3	3	3	3	3	180.50
0.25	B	3	3	3	3	3	3	3	3	3	3	3	3	3	3	180.50
	C	3	3	3	3	3	3	2	2	2	2	2	2	2	2	136.21*
	E	3	3	3	3	3	3	3	3	3	2	2	2	1	1	147.25
	F1	3	3	2	2	2	2	1	1	0	0	0	0	0	0	74.00*
	F2	3	3	3	3	3	3	3	3	3	3	3	3	3	3	180.50
1	DA	2	2	2	2	1	1	1	1	1	1	0	0	0	0	63.29*
PBSG		3	3	3	3	3	3	3	3	3	3	3	3	3	3	180.50

Key: 3: Very motile, 2: Motile, 1: Less motile, 0: Not motile

B: 1,1'-bis(cyclooct-2-en-4-one); C: Coronopilin; E: Coronopilin and Hexadecane-1,2-diol; F1: Coronopilin, 4,8,13-Duvatriene-1,3-diol; F2: 6,8-Dimethyl-non-4-enedionic acid, dimethyl ester, Hexadecane-1,2-diol; Pentadeca-2,3,6,9,12,13-hexaen-8-one, 2,5,5,11,11,14-hexamethy; PBSG: Phosphate Buffered Saline with Glucose; Superscript "*" indicates significance at $P<0.05$ compared to PBSG (untreated control)

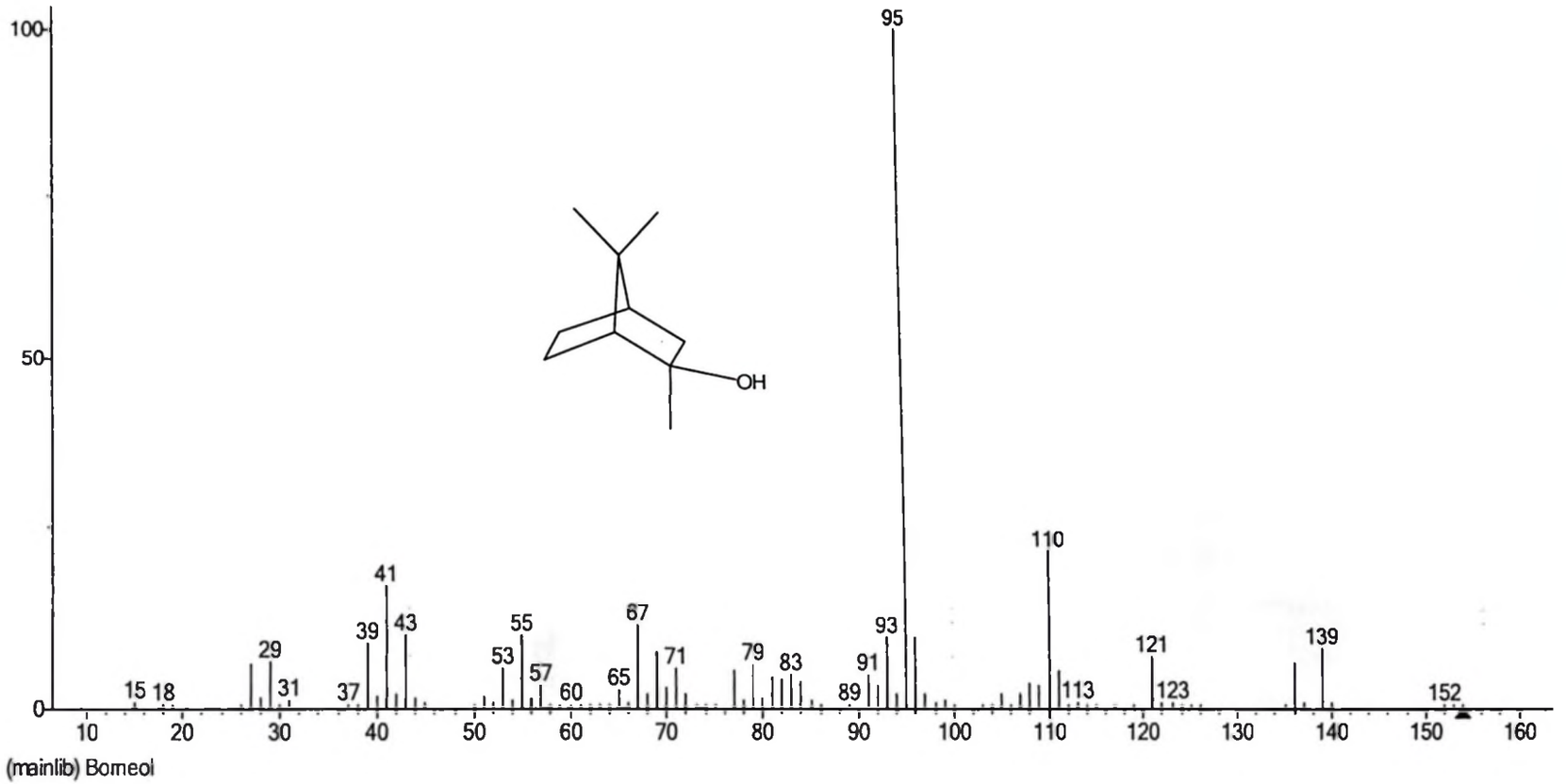

Figure 1: The chemical structure of Borneol

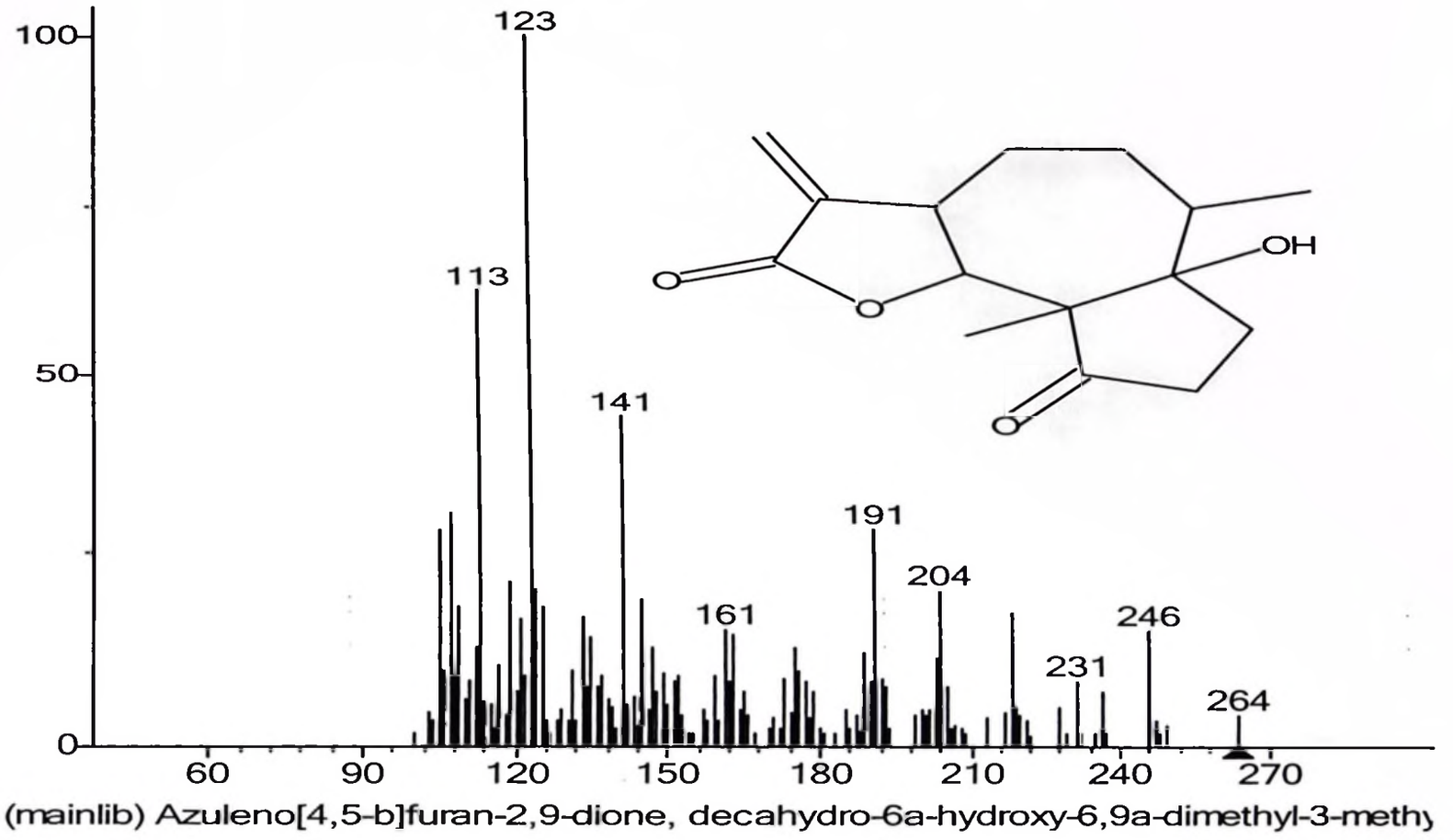

Figure 2: Chemical structure of Coronopilin

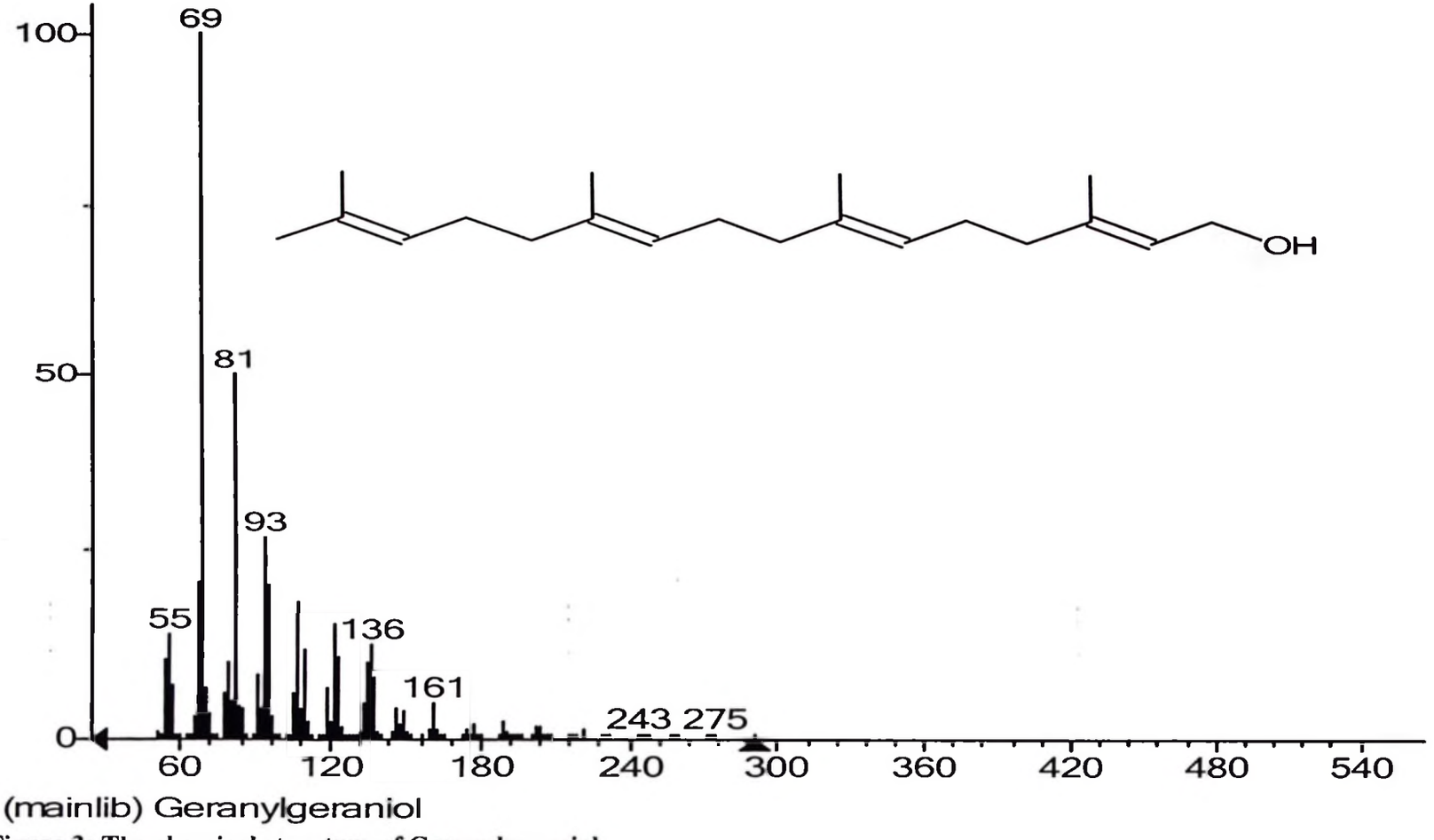

Figure 3: The chemical structure of Geranylgeraniol

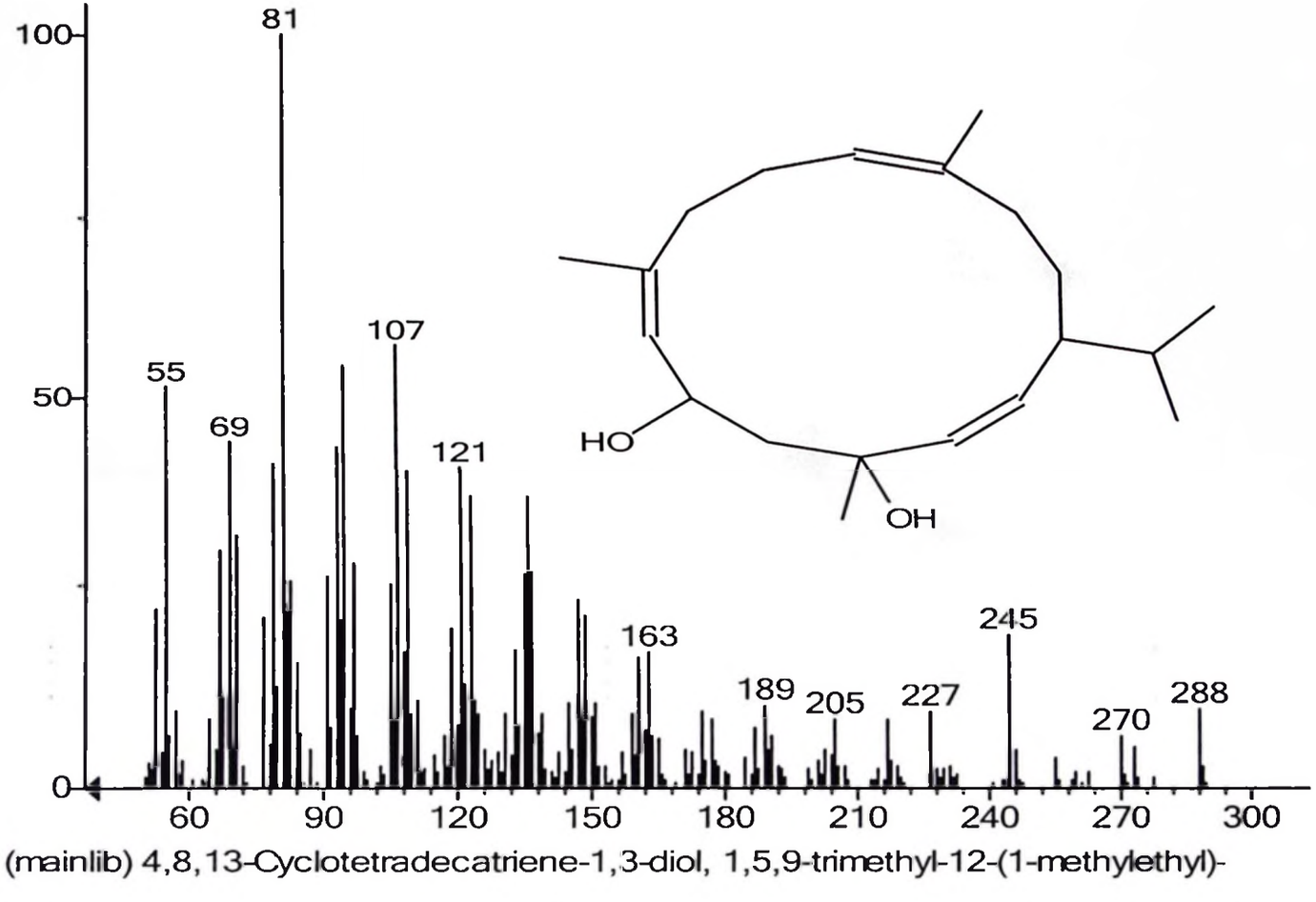

Figure 4: The chemical structure of 4, 8,13-duvatriene-1,3-diol

CHAPTER FIVE

5.0 CONCLUSIONS AND RECOMMENDATIONS

The findings of this study have contributed to showing the potential of *Commiphora swynnertonii* as a source of lead trypanocidal molecules. For the first time *Commiphora swynnertonii* ethanolic stem bark extract has been shown to possess anti-trypanosomal activity demonstrable *in vitro* and *in vivo*. Relatively high doses of *Commiphora swynnertonii* ethanolic stem bark extract were required to clear parasitaemia in infected mice which nevertheless relapsed after cessation of the therapy. In addition, the study has shown that *Commiphora swynnertonii* ethanolic stem bark extract causes a decrease in lymphocytes and depletion of splenic marginal zone in uninfected mice. This provides evidence that supports the theory that down-regulation of humoral immunity contributes to *Trypanosoma congolense* relapse after treatment of infected mice with the extract. From the study, it can also be concluded that relatively high doses of *Commiphora swynnertonii* ethanolic stem bark extract that were within anti-trypanosomal therapeutic dose range in mice, induce cytotoxic effect on lymphocytes, hepatocytes and kidney epithelial cells. Despite the demonstrated cytotoxic effects of the *Commiphora swynnertonii* extract, it is recommended that further studies be undertaken on the extract to isolate and characterize trypanocidal molecules that are less toxic and/or further modified to make them effective and safe for treatment of trypanosomosis.

From the fractionation studies on *Commiphora swynnertonii* ethanolic stem bark extract, it is concluded that fractions and subfractions obtained using polar solvents possess relatively stronger *in vitro* trypanocidal activity, and therefore molecules responsible for trypanocidal activity in *Commiphora swynnertonii* might well dissolve in polar solvents. However, it is recommended that studies be conducted using organic solvents that dissolve

well the non-polar molecules to find out whether it can improve trypanocidal activities of fractions from the non-polar solvents.

From the GC-MS analysis of the sub-fractions, it can be concluded that trypanocidal activity of *Commiphora swynnertonii* ethanolic stem bark extract is partly explained by presence of geranylgeraniol, borneol and coronopilin that were detected and have previously been shown to be trypanocidal. Additionally the studies have shown for the first time that 4,8,13-duvatriene-1,3-diol has trypanocidal activity that is synergistic to that of coronopilin. However, detection of these molecules in the sub-fractions was based on GC-MS and HPLC analysis at which the purity of the sub-fractions was not considered, and therefore, it is recommended that further studies be conducted to validate the detected compounds in *Commiphora swynnertonii* extract. More advanced techniques such as preparative chromatography and nuclear magnetic resonance (NMR) should be used to isolate, purify, identify and characterize them.

It is now more than 20 years of anti-trypanosomal research from plant sources in Africa. There are yet realistic molecules that are to be subjected to vigorous clinical trials leading to useful agents for chemotherapy of African trypanosomoses. Most studies have ended either *in vitro* and or *in vivo*. Factors involved in such setback could be lack of advanced analytical tools/techniques as such observed in this study. It is now high time to build research capacity in Africa in order to turn the promising anti-trypanosomal compounds into useful product(s) just as what has been achieved in malaria chemotherapy in which the plant derived artemisinin is in current use for treatment.