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In vitro Efficacy of Extracts of *Cantharanthus roseus* and *Erythrina abbysinica* on *Babesia bigemina*

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ABSTRACT
This study was carried out using plants collected from Chikomba District in Mashonaland East Province of Zimbabwe, in July 2011, after interviews with the local people who had knowledge about plants used in the district to cure both malaria and tick-borne diseases. The suggested herbal plant samples were identified at the Government Herbarium in Harare and processed at the University of Zimbabwe. Bioassays were done at the Central Veterinary Laboratories of the Ministry of Agriculture in Harare, revealing that the plants had efficacy against Babesia bigemina. The in vitro studies revealed that the efficacy of plant extracts was similar to that of either of fansidar, chloroquine and berenil. fansidar, chloroquine are known antimalarial drugs whilst berenil is an antibabesial drug used to treat tick-borne diseases in cattle.
Key words: *Antibabesial; antimalarial; chloroquine; fansidar; berenil; synergistic.*

INTRODUCTION

Babesia is a malaria-like parasitic disease caused by infection with *babesia*, a genus of protozoa that infect red blood cells. After trypanosomes, *Babesia* are thought to be the second most common blood parasites of mammals and they can have a major impact on the health of domestic animals in warm areas. Human babesiosis has beent reported in recent times in the USA, Europe, Africa, Asia, and South America [1, 2].

Tick-borne diseases can cause paralysis, toxicoses, and severe allergic reaction in humans. There are similarities between transmission of malaria and that of babesiosis. Like the mosquito, the tick changes hosts during its life cycle, facilitating acquisition of pathogens that can be transmitted to the next host. Pathogens acquired by larval feeding are passed to subsequent life stages (trans-stadial transmission). The tick can feed on mammals, birds, and reptiles. It attaches firmly to the host and feed slowly, enhancing dispersal as the host moves in the environment. It can travel for miles while feeding on a bird, thus it may be carried between continents. The slow feeding is associated with the need to produce new cuticle to accommodate the ever increasing volume of blood it imbibes.

Like the mosquito, the tick's saliva, containing metabolites and excesses of fluids, is secreted back into the host, transmitting pathogens especially sporozoites to the host. Sporozoites infect red blood cells which may be identified and destroyed by the host's defence system, but may leave the host with anaemia if many red blood cells are infected. Often the host's immune system will begin destroying the uninfected red blood cells as well, a condition known as Immune-Mediated Hemolytic Anaemia with symptoms that include weakness, jaundice, fever, red or orange coloured urine. As with malaria, cells of the reticuloendothelial system in the spleen remove the damaged red blood result resulting in haemolytic anaemia. As with malaria, the primary immune response is a T-cell-mediated cellular immunity, and a secondary reactive polycyclonal hypergammaglobulinemia occurs because of excessive B-limphocyte reactivity [3, 4]. Babesiosis is further comparable with malaria in that (i) both cause haemolytic anaemia, renal failure, high fever, jaundice and hemoglobinuria; (ii) intraerythrocytic parasites are similar to plasmodium malaria except that *Babesia* form tetrads, but does not give hemozoin pigments in red blood cells and extracellular merozoites as observed upon Giemsa stain [5]. For ill patients, treatment is usually a two-drug regimen of quinine and clindamycin [1, 2]. The similarity of clinical features of babesiosis to those of malaria has necessitated the combination of antimalarials and antibabesiosis drugs to treat babesiosis in humans.

It is technically difficult to distinguish babesiosis and malaria and many have been treated for malaria when their problem was actually babesiosis. Some have been lucky to have their treatment regime re-evaluated when they did not respond to malaria treatment. Most have been dismissed to go and recuperate at home because doctors believed they had done enough to cure malaria, when in fact they should have been treating for babesiosis [5]. The similarity between babesiosis and malaria has enabled us to study malaria treatment drugs using babesia as a proxy.

INFORMATION ON PLANTS WHICH HAVE BEEN USED IN THIS STUDY

Catharanthus roseus **(Apocynaceae). Synonyms:** *Vinca rosea; Ammocallis rosea; Lochnera rosea.* Common Names: Periwinkle, Church flower, Cape winkle, Old maid, Rose periwinkle (English); Mufurauzi (Shona).

The plant is native and endemic to Madagascar. In the wild, it is an endangered plant, mainly due to habitat destruction by slash and burn agriculture. However, it is also cultivated and is naturalized in subtropical and tropical areas of the world. It is an erect, bushy,evergreen shrub or herbaceous plant growing to a height of 1 meter, with a spread of 1 meter. It has simple opposite leaves which are exstipulate and petiolate. The inflorescence is racemous. The leaves are oval to oblong, 2.5 to 9 cm long and 1-3.5 cm broad, glossy green, hairless, with a pale midrib and a short petiole 1-1.8 cm long, arranged in opposite pairs. The flowers develop in auxiliary clusters of 2-3 petals and they can develop all year round giving rise to follicular fruits. There are 3 varieties of the plant: rose purple flowers, white flowers, white flowers with a red rose purple spot in the center, with a basal tube 2.5-3 cm long and a corolla 2-5 cm diameter with 5 petal-like lobes. The rose purple flowered variety is most cultivated because of its higher alkaloid content.

Catharanthus roseus has long been cultivated for herbal medicines and as an ornamental plant, as it is famous for its ornamental appeal. The Shona name is furauzi, meaning flower, probably due to its occurrence as a decorative plant. In traditional Chinese medicine, extracts of the plant have been used to treat numerous diseases, including diabetes, malaria and Hodgkin's disease. *C. roseus* can be dangerous if consumed orally. It can be hallucinogenic. Its alkaloids are hypotensive, sedative and have tranguilizing properties and are anti-cancerous. It helps in relieving muscle pain, depression of CNS and wasp stings. Rosinidin is an anthocyanidin pigment found in the flowers of *C. roseus* [6].

Most of the medicinal properties of the plant are due to compounds contained in the roots. Although *Catharanthus roseus* has toxic compounds, it continues being used medicinally because of its anticancer effects. The alkaloid vincristine and vinblastine compounds found in the plant have shown good results in treating lymphoma, leukemia, breast and lung cancer, hodgkin's disease, diabetes, rheumatism, menorrhaglia, and low blood pressure, using leaves, flowers, stem and roots. For example, a tea brewed from the bark or whole stem lowers blood glucose through raising insulin levels, has anti-inflamatory properties, and raises male potency [7].

Catharanthus roseus produces vinblastine and vincristine, two alkaloids which are used effectively for the treatment of white blood cell cancers. Vinblastine is especially useful against lymphoma (cancer of the lymph glands), while vincristine is used against the most common form of childhood leukemia [6, 8].

When researchers analysed *Catharanthus roseus* in the 1970s, they discovered it contained over 70 useful chemicals, the most interesting being vinblastine and vincristine. The vinca alkaloids stop the formation of chromosome spindles that are needed for cell division, which is the basis of its anticancer activity. Few plants have attracted as much scientific and medicinal research interest as has done *Catharanthus roseus*, grown commercially for its medicinal uses in Africa, Australia, India and southern Europe.

It contains more than 70 alkaloids mostly of the indole type. It has medicinal importance due to the presence of alkaloids like ajamalicine, serpentine and reserpine, which are well known for their hypotensive and antispasmodic properties. *Catharanthus roseus* is one of the few medicinal plants which have found mention in the folk medicinal literature as early as 2nd BC. Vincristine is an alkaloid derived from the flowering plant. Its use is limited because of its toxic effects, among them being neurotoxicity. The root bark of this plant contains the alkaloid alstonine which has been used traditionally for its calming effect and its ability to reduce blood pressure.

The following table illustrates the range of conditions for which the plant is effective.

Animal repellent activity	Antihyperglycemic activity	Antiascariasis activity
Antiinflamatory	Antimalarial	Antimitotic
Antibacterial	Antihypertensive	Antifertility
Antihypercholesterolemic	Antimutagenic	Antidiuretic
Antifungal	Antispasmodic	Antiviral
Cardiotonic	CNS depresant	Antitumor
Antispermatogenic	Hyperglycemic	Hypoglycemic
Hypotensive	Inotropic	Insect feeding deterant
Insect sterility induction	Insecticidal	Insulin
Larvicidal	Leukopenic	Smooth muscle relaxant
Spasmogenic	Uterine relazation effect	Uterine stimulant effect

Vinblastine and vincristine are all administered intravenously in their sulphate form. The solutions are fatal if they are administered any other way and can cause a lot of tissue irritation if they leak out of the vein. Although the two compounds are very similar in structure and have the same basic action, they have distinctly different effects on the body.

They are most commonly used in the treatment of Lymphomas, Acute lymphocytic leukemis, Neuroblastoma, Hodgkin's disease, Soft tissue sarcomas, Breast cancer and Multiple myeloma. Most recently, extracts from *C. roseus* have been shown to be effective in the treatment of various kinds of leukemia, skin cancer, lymph cancer, breast cancer and Hodgkin's disease.

The application of easily degradable plant compounds is considered to be one of the safest methods to control insect pests and vectors than the use of synthetic pesticides. In a study to monitor the effect of plant extracts on larvae and pupae of *Aedes aegypti* mosquitoes, *Lantana camara* and *Catharanthus roseus* appeared to be better vector control agents than the synthetic xenobiotics [9].

Erythrina Species

Erythrina is a genus of flowering plants in the pea family, Fabaceae. The genus contains about 130 species, distributed in tropical and subtropical regions worldwide. The generic name is derived from the Greek word, erythros, meaning red, referring to the flower colour of some of the species. The name coral tree is used as a collective term for these plants. Many species of *Erythrina* have bright red flowers, and the growth of the branches may resemble the shape of sea coral. Most species have legume-type fruits (pods), containing one or more seeds. *Erythrina* trees are used widely in the tropics and subtropics as street and park trees, especially in drier areas. In some places, such as Venezuela and Bengal, the plants are used as shade trees for coffee or cocoa crops, and as "frame" trees for vines to grow up on.

Some of the trees are used as floral emblems, for example Cockspur Coral tree is the national flower of Argentina and Uruguay. The coastal coral tree (*E. caffra*) is the official city tree of Los Angeles, California. The state trees of Merida and Trijillo in Venezuela are *E. poeppigiana* and *E. fusca*, respectively. Yonabaru, Okinawa Prefecture, and the Pathum Thani Province have the Indian coral tree, *E. variegata*, as their floral emblems. Zumpahuacan in Mexico derives its name from Nahuatl, "place of the Erythrina Americana. In Vietnam, people wrap nem (a kind of fermented pork) using the leaves of E. variegate.

Erythravine is tetrahydoisoquinoline alkaloid from *Erythrina mulungu* and is thought to have anxiolytic properties. The seeds of at least 1/3 of the species contain potent Erythrina alkaloids, some of which are used for medicinal and other purposes by indigenous peoples. They are all toxic to some degree and some of them cause fatal poisoning. The main active compounds in this genus are alkaloids, such as scoulerine, erysodin, crysovin and the putative anxiolytic erythravine. Except for ornamental purposes, growing, selling or possessing *Erythrina* is prohibited by Lousiana State Act 159 [10, 11].

***Erythrina abyssinica* Lam, ex DC**

Erythrina abyssinica Lam, ex DC (Synonym: *Erythrina tomentosa* R. Br. Ex A. Rich.) Common names: Lucky-bean tree (English); Munhimbiti (Shona); Mutete (Shona); Mutiti (Shona); Mutsiti (Shona); Red hot porker tree (English); Umgqogqogqo (Ndebele). The plant is common and native to Zimbabwe. It is a small to medium sized tree of wooded grassland, as well as open-wooded and rocky hillsides. The plant's typically rounded crown is usually leafless at the time of flowering. The

leaflets of the 3-foliate leaves are almost as broad as they are long, hairy when young and sometimes with scattered prickles on the underside of veins.

The plant flowers in spectacular terminal racemes which are scarlet-red. Fruits are woody pods which are strongly restricted between seeds. The seeds are often used to make necklaces. They contain a poison which can be lethal if injected into the bloodstream, but has no effect if swallowed as a whole.

The specific name *abyssinica* is derived from Abyssinia (Ethiopia). The plant grows in wooded grassland, open-wooded and rocky hillsides, at an altitude ranging 800-1750 m, and flower in July-October. In Africa it is distributed in the Democratic Republic of Congo, Ethiopia, Sudan, Uganda, Kenya, Tanzania, Malawi, Mozambique, Zambia and Zimbabwe [6, 10- 11].

Erythrina abyssinica is a native deciduous tree of savanna Africa, used in Ethnoverterinary treatments of babesiosis or Red Water. Its conservation can be promoted as a nitrogen fixing agroforestry tree as well as an ornamental tree in public parks [12]. Flavonones have been isolated from the stem bark [13]. The plant is widely used to treat pneumonia, Sexually Transmitted Diseases, and Prostate cancer. However, in a study to screen some Kenyan medicinal plants for antibacterial activity, the root and bark which are used by traditional healers did not show any antibacterial activity [14].

Antimalarial drugs, usually in combination have been used to treat Babesia in people because of the similarities of the two disease, but with limited success [1, 2]. Hence it is important to work towards development of drugs for babesiosis cure, especially in people.

METHODOLOGY

Catharanthus roseus areal parts and *Erythrina abyssinica* roots and a branch were obtained from Manyene village, Chikomba District in July 2011, as a result of interviewing local people with knowledge of traditional medicines. The plant parts were taken to the National Botanical Gardens (National Herbarium), Harare, for identification. The samples were dried in the shade for two weeks, chopped to small pieces with a small axe, ground to powders using a motorized laboratory grinding mill in the Chemistry Department at the University of Zimbabwe, and the powders accurately weighed into 50g samples. The powders were exhaustively extracted with ethyl acetate (50 g powder, 500ml EtoAc, 24h x 3), decanted, filtered and the EtoAc recovered using a rotary evaporator at 40^{o}C. The extracts were combined, re-dissolved in MeOH, homogenized, the MeOH removed at the rotary evaporator, yielding 1.1050g *Catharanthus roseus* extract as a grey gum, and 0.8020g of *Erythrina abyssinica* root extract as a greenish-yellow coloured gum. The extracts were stored in the fridge until used. The residues were then exhaustively extracted with MeOH (500 ml, 24h x 3), decanted, filtered and the MeOH recovered using a rotator evaporator at 50^{o}C. The extracts were combined, re-dissolved in MeOH, homogenized, the MeOH removed at the rotary evaporator, yielding 2.117g of a grey gum of the root extract *of Catharanthus roseus* and 1.3756g of an oxblood coloured gum of *Erythrina abyssinica* root extract. The extracts were stored in the fridge until used.

All sensitivity tests were carried out at the Central Veterinary Laboratories using blood from cattle infected with 1993 *Babesia bigemina* Rusape Field Strain. Paratisized blood was drawn intravernously from the neck of cattle using a 500ml syringe, and the blood immediately stored in 10 x 100ml heparinised sample tubes and kept in a water bath incubator maintained at 37^{0}C. The cattle annul temperature was regularly monitored using a clinical thermometer and inoculated with berenil and imizolif their condition deteriorated.

Determination of Sensitivities of the extracts against *Babesia bigemina*

Babesia bigemina and *Plasmodium falciparum* are intraerythrocytic and structurally similar. Antimalarials have been used to treat babesial diseases with a degree of success. The sensitivity tests were based on microscopic examination of parasites as well as the condition of the red blood cells after exposure to a drug. Random sampling was used to estimate the parasite population. The average of individual estimates was used to evaluate the drug's potency at different concentration levels, whilst excluding bacteria, rejecting slides that were contaminated with bacteria.

Measurement of Packed Cell Volume

Packed Cell Volume (PCV) is the ratio of live red blood cells to the total volume of blood sample. The PCV was determined by centrifuging heparinized blood in a capillary tube at 1000 RPM for 5

minutes, separating the blood into 2 layers, and measuring the length of each layer. PCV determines whether the drugs used killed parasites only, or both the parasites and blood cells. Agents that kill both parasites and blood cells lead to anaemia, among other complications.

Microscope Slide Preparations

The materials include Giemsa stain, 100% methanol, bibulous paper and a microscope withx100 oil immersion lens, a 10x10 grid eyepiece and microscope immersion oil. The slides were fixed in 100% methanol for about 3 minutes and rinsed in tap water. Fresh solution of 10% Giemsa stain in distilled water was added and the slides were left to dry for about 30 minutes, and the slides rinsed in tap water and dried thoroughly using bibulous paper. A light microscope with a 100x100 magnification was used to observe the parasites, red blood cells and white blood cells.

Parasites estimation

The slides were viewed under oil immersion with a 100x objective. Parasitemia was estimated by counting the number of infected cells. A 10x10 grid square in the eye piece facilitated counting. An even-blood-smear yields about 100 red blood cells per 10x10 grid. A one infected blood cell in a 10x10 grid would be about 0.1 parasitemia. An average-of-10 fields are counted and the average taken to obtain a representative estimate [15].

Preparation of Standard Solutions of berenil, fansidar and chloroquine

Exactly 0.0200g of each of the 3 powders and each of the EtoAc and MeOH extracts was weighed using an electronic balance and dissolved in 20ml distilled water, giving 0.0010g/ml of each drug, which was then halved and the volume made up with distilled water, giving 0.0005g of each. This was further halved and made up to give 0.00025g/ml for each of berenil, fansidar, chloroquine, EtoAc and MeOH extracts of each plant sample.

The sensitivity and haemolytic properties of each of the standards and each of the extracts at the 3 concentrations above were assessed using the level of parasitemia and PCV as the measurable parameters. The initial test to verify the general sensitivities of the extracts consisted of mixing 1.0ml of parasitized blood with 1.0ml of each of the herbal extracts at the 3 concentrations (Table). The experiment was repeated with lower volumes of drug and/or herbal concentrations (Table). Reducing the volume of herbal extract to 125 L being added to 1.0ml of parasitized blood maintained at 37^0C using a float water bath. All the parasitized blood test samples were stored and used in heparinized sample tubes. Unused blood for the day was stored in a fridge at 4^0C. Heparinized blood is blood that contains heparin to prolong the life span of blood cells to about 3 days.

RESULT AND DISCUSSION

The methanol extract of *Cantharanthus roseus* were slightly more efficacious than the ethyl acetate extracts and were comparable with the fansidar solution, as revealed by their parasitemia figures. Apart from one entry for the methanol extract, the PCV figures for the two extracts were identical and comparable with the figures for fansidar. Fansidar itself had a bad figure which was possibly an outlier. The red blood cells appeared intact after treatment with either of the ethyl acetate extract of the methanol extract. The PCV figures and the appearance of the red blood cells indicated that the extracts did not cause red blood cell haemolysis, but fansidar caused haemolysis (Tables 1 & 2).

Parasitemia indicated that the methanol extract of *Erythrina abbysinica* was more effective than the ethyl acetate extract, and both of them may be considered effective. However, the effectiveness of the methanol extract was closer to that of fansidar. The PCV figures indicated that the ethyl acetate extract was closer to fansidar. The methanol extract caused extensive haemolysis and the appearance of the red blood cells confirmed this (Table 2).

Fortification of chloroquine with the extracts of the two plants indicated that the extracts were compatible with chloroquine and possibly had a synergistic relationship with the synthetic drug, leading to a slightly improved efficacy to chloroquine, leading to no negative effects on the efficacy, the PCV, or the appearance of red blood cells due to chloroquine treatment.

Babesiosis, a disease caused by tick bites is similar to malaria and has been described as the malaria of the United States of America. Antimalaria drugs have been used to cure Babesiosis because of the similarity of the two diseases. In this study we have used extracts of plants which are used to treat malaria in traditional medicine, the hypothesis that the information obtained will

be useful in the study of antimalarial drugs as well, since *Babesia* are closely similar to *Plasmodium falciparum.* It is hoped that the fortification studies with chloroquine will be applicable to studies directed at curbing malaria resistance to chloroquine.

Table 1. Efficacy of EtoAc Extracts of *Cantharanthus roseus* and *Erythrina abbysinica* on *Babesia bigemina*

Sample	Conc.	parasitemia	PCV out 25	Efficacy	RBC appearance
C. roseus	0.0010g/ml	0.14	14	effective	intact
	0.0005g/ml	0.14	14	effective	intact
	0.00025g/ml	0.13	14	effective	intact
E. abbysinica	0.0010g/ml	0.14	0.0	effective	haemolysis
	0.0005g/ml	0.14	17	effective	Very intact
	0.00025g/ml	0.17	15	effective	Very intact
Fansidar	0.0010g/ml	0.15	15	effective	intact
	0.0005g/ml	0.10	15	v. effective	v. intact
	0.00025g/ml	0.10	7	v. effective	haemolysis
Water		3	15	No effect	intact
Blood		4	18	No effect	intact

Table 2. Efficacy of MeOH Extracts on *Babesia bigemina*

Sample	Conc.	parasitemia	PCV out 25	Efficacy	RBC appearance
C. roseus	0.0010g/ml	0.20	14	effective	intact
	0.0005g/ml	0.10	14	v. effective	intact
	0.00025g/ml	0.14	8	effective	intact
E. abbysinica	0.0010g/ml	0.07	8	effective	haemolysis
	0.0005g/ml	0.125	9	effective	haemolysis
	0.00025g/ml	0.14	8	effective	intact

Table 3. Results of fortification of chloroquine with extract

Sample	Sample	parasitemia	Efficacy	PCV/25	RBC appearance
5 L C. roseus	120 L Chloroquine	0.080	v. effective	14	Intact
10 L C. roseus	115 L Chloroquine	0.080	v. effective	14	Intact
15 L C. roseus	110 L Chloroquine	0.080	v. effective	14	Intact
5 L E. abbysinica	120 L Chloroquine	0.080	v. effective	14	Intact
10 L E. abbysinica	115 L Chloroquine	0.081	v. effective	14	Intact
15 L E. abbysinica	110 L Chloroquine	0.092	v. effective	14	intact

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