



***In vitro* Babeosis Assaying using *Acacia karroo* and *Dicoma anomala* Plant Extracts and Extract Fortified Antimalarial Drugs**

T.* Kazembe and J. Chinyuku

Department of Science and Mathematics Education, Faculty of Education, University of Zimbabwe, P.O.Box MP 167, Harare, Zimbabwe

*Author for correspondence Email: kazembet@rocketmail.com

ABSTRACT

*This study was carried out to extend a data base of plants which may be used to cure malaria and related diseases. The plants were collected from Mashonaland East Province in July 2011, processed in the Department of Chemistry, the University of Zimbabwe. Efficacies of *Acacia karroo* and *Dicoma anomala* ethyl acetate and methanol root extracts were evaluated at the Central Veterinary Laboratories, Ministry of Agriculture, Harare, Zimbabwe, revealed that the plants could be used to develop antibabesial agents. The extracts also enhanced in vitro efficacy of chloroquine against babesia bigemum. Based on the similarity of babesia and malaria, the successes could also be extended to malaria.*

Key words: *Acacia karroo; Dicoma anomala; plant extract fortified chloroquine; fansidar*

INTRODUCTION

The long history of the use of plants in African traditional healthcare systems, the indigenous knowledge, the historical success of plant-derived antimalarials (quinine, artemisinin), need for new drugs, and the accepted view that plant-derived drugs are environmentally friendly, dictates that efforts be refocused on developing plant-derived drugs for the different diseases that are a menace to humanity [1].

A number of alkaloids are used as drugs, for example quinine, derived from the bark of the tropical cinchona tree. Indians of South America long used cinchona bark to cure malaria, much as the Europeans used the willow tree bark to relieve pain and later on extracted aspirin from it. Europeans took the cinchona bark to Europe in the 1600s and used it like the Indians did. Quinine was purified in 1823 and soon replaced the cinchona bark as the standard treatment for malaria, but it was replaced by synthetic analogues in the 1930s. These offered fewer side effects and a more reliable supply. Quinine is, however, still used as the principal flavouring agent in tonic water, a beverage known for its ability to prevent malaria symptoms.

Cinchona bark also produces quinidine, used primarily to control abnormalities of the heart rhythm such as fibrillation, a series of rapidly quivering beats that do not pump any blood, and heart block, a condition in which electrical currents fail to coordinate the contraction of the upper and lower chambers of the heart.

Background information on the plants being evaluated in the current study

Acacia karroo (Fabaceae). Common names: Muunga (Shona); Mubayamhondoro (Shona); Sweet thorn (English); Mookana (Sotho); Mooka (Tswana); umuNga (Zulu; Xhosa; Ndebele) Soetdoring (Afrikaans).

Acacia karroo is native to southern Africa from southern Angola east to Mozambique, and south to South Africa, growing in coastal areas and riverbeds. It is one of Africa's most beautiful and useful trees. The bark is red on young branches, darkening and becoming rough with age. Sometimes an attractive reddish colour can be seen in the deep bark fissures. The leaves are finely textured. Abundant brilliant yellow flowers, typical of many *Acacias*, are sweetly scented, appearing in early summer, or after good rains, in a mass of yellow pompons. The seed pods are narrow, flat and crescent shaped, green when young becoming brown and dry [2-4].

Acacia karroo is a tree of open woodland and wooded grassland, growing to its greatest size in 800-900mm rainfall areas, but still grows well and thrives in very dry conditions, even in the karroo region of western South Africa. It requires deep soils which allow its roots to spread. The tree is

easily recognizable by its distinctive long white paired thorns and coffee coloured bark, both of which are very attractive. Its ability to fix nitrogen allows grasses and other plants to thrive in its shade. *Acacia karroo* is one of the fastest growing acacias, named after the Karoo region of South Africa, where it is common, and often the only tree found. It grows on deep, blackish nutrient-rich clay soils, and not on sand. It is the most widespread acacia in southern Africa. Zimbabwe is the largest producer of *Acacia karroo* gum. The plant's common name "Sweet thorn" is derived from this pleasant tasting gum eaten by people and animals. In dry areas, its presence is a sign of water, both above and underground, and also an indicator of sweet veld which is prized for the good grazing and fertile soils. The plant becomes invasive in overgrazed areas [2, 4].

The tree is especially important as forage and fodder for domestic and wild animals, more popular with goats than with cattle. The flowers make it a very good source of forage for honey bees, giving mild and sweet honey of a very pleasant taste. The gum, bark and leaves have been used as a soothing agent and astringent for colds, conjunctives, wound healing and haemorrhage. The dried leaves are said to have antibacterial and antioxidant activities [2, 3]. Investigation of the aqueous extract of the stem bark of *Acacia karroo* Hayne at the University of Fort Hare in South Africa, revealed that the extract significantly reduced the formation of oedema as well as showed a good analgesic effect, giving a scientific basis to the traditional uses of the plant as wound poultices, eye treatments and cold remedies [5].

Acacia trees have been used medicinally, to treat fever since time immemorial. Several parts (mainly bark, root and resin) of *Acacia* are used to make incense for rituals, mainly in India, Nepal, and China. Smoke from *Acacia* bark is thought to keep demons and ghosts away and to put gods in a good mood. According to Easton's Bible Dictionary, the *Acacia* tree may be the "burning bush" which Moses encountered in the desert. Also when God gave Moses the instructions for building the Tabernacle he said to "make an ark, a table of acacia wood" [6].

The gum has been used medicinally as emollients and as pharmaceutical aids such as emulcifiers, stabilizers of suspensions and as additives for solid formulations. It has also been used to treat mouth ulcers and to treat diarrhoea, colds, dysentery, conjunctivitis and haemorrhage, The plant has antibacterial activity against *E. coli* and *staphylococcus aureus*.

Compounds which have been isolated from the heartwood: (2'S, 3'R)-3, 10-dihydroxy-9-O-(6'-hydroxy-7-O-methyl-2'-hydroxy-methyl-dihydrobenzofura-3-yl)dibenz-[b,d]-pyran-6-one and its 10-O-methylanalogue: 8-O-methylpiprosopin-4 β -ol; 8-methylfustin; and 7,8,3,4-tetrahydroxy-3'-methoxyflavone. These compounds have been reported to have antioxidant and antimutagenic activities and have also been reported to have antibacterial activity against the Gram positive bacteria such as *Bacillus aureus*, *Staphylococcus aureus*, *Enterococcus faecalis* and Gram negative bacterium *E. coli*. The phenolic acids, cinnamic, caffeic, p-coumaric, ferulic acids and chlorogenic acids from fresh fruit and leaves have been reported to have antilsterical activity [7].

Many of the compounds from *Acacia* are psychoactive in humans. Alkaloids found in acacia include dimethyltryptamine, 5-methoxy-dimethyltryptamine, N-methyltryptamine. The plant leaves, stem and roots are sometimes made into a brew, consumed for healing, ceremonial or religious uses [8].

An infusion of the roots is used by the Ndebele against general body pains, by Shona against dizziness, convulsions, gonorrhoea, and sometimes as an aphrodisiac. Roots are placed in chicken runs to reduce parasites. A decoction of the bark is used as an astringent, emetic and as an antidote to poisoning in cattle. The mucilage of the gum is used to relieve thrush in the mouth. A substance from the heartwood controls high blood pressure. The roots are used to drive away evil spirits [9].

Acacia karroo is used for diarrhoea, dysentery, cholera, fever, malaria. Although *Acacia karroo* is frequently used to treat fever and malaria, in vitro evaluation revealed that it's in vitro antimalarial activity was moderate. This might be due to the plants acting as antipyretics or may enhance the immune system rather than having direct antiparasitic activity or the plant could contain pro-drugs, non-active by themselves. The precursors of the active compounds have to be metabolized in vivo into active antimalarials.

While an infusion of the root of *Acacia karroo* is drunk as a remedy for malaria in Mozambique. The leaves are also used. In vitro tests in South Africa (2004) revealed a very high degree of antiplasmodial activity.

Root infusion of *Acacia karroo* is used to treat dizziness. Powders of fruits and roots are combined and used as local application against rheumatism. For convulsions, root infusion is drunk and also

used to wash the body. In South Africa the bark is used against diarrhoea and dysentery, the gum is used as emulscent and salve. In Zimbabwe, roots are used to kill external poultry parasites and fresh bark is boiled in water and given to goats and sheep to treat diarrhoea and intestinal parasites in South Africa [10-13].

***Dicoma anomala* Sond. (Asteraceae). Common Names: Fever bush, Stomarch bush (English); Chifumuro (Shona); Hloenya, Mohasetse (Sotho); Inyongana (Swazi, Xhosa); Isihlamakhondlwana, Umuna (Zulu).**

Dicoma anomala is an erect, sub-erect or prostrate, decumbent, perennial herb bearing aromatic semi-woody tubers at the base of a woody subterranean stem. It may have few or many erect stems arising from a woody rootstalk. The stems are branched, yellowish, trail along the ground and over surrounding plants and rocks. They are thinly covered with hairs. The leaves are simple, narrow, positioned alternately on the stem 4 to 5cm apart and 5mm wide, stalkless, linear or narrowly lanceolate. The upper surface of the leaves is glabrous, olive green, sometimes grey, rough, with a prominent central vein along which the leaf folds inward. The lower surface is white hairy (velvety) with a faintly uneven margin. Flower heads are terminal, cup or cone-shaped, cream to pinkish-white, with a fluffy top. Flowering occurs from November to July, peak flowering period being in February and March [1, 14, 15].

Dicoma anomala is a grassland species widely distributed in sub-Saharan Africa, and morphologically diverse. It is not a threatened species but users of this widely used plant should be informed of the dangers of over-utilization. The plant is widely distributed in sub-Saharan Africa, resulting in pronounced morphological variety, growing in the summer rainfall areas, in stormy grasslands, hillsides, or flat grasslands in savannah, or dolerite or sandy soils, at altitudes ranging from 165 to 2075m.

The genus name *Dicoma* was derived from Greek words *di* meaning two and *kome* meaning tuft of hair, referring to the double row of pappus bristles. The species name *anomala* is Latin, meaning irregular or deviating from the normal [1, 16].

Herbal medicines can alter the activity of drug-metabolizing enzymes and transporters, potentially resulting in herb-drug interactions. Drug interactions can result in therapeutic failure due to inhibition or induction of metabolism or transport. *Dicoma anomala* have been reported to be moderate inhibitors [17].

Dicoma anomala is used as a root decoction for blood disorders, colic, diarrhoea, dysentery, toothache, fever, malaria, coughs and colds, haemorrhages, ulcers, dermatosis, venereal diseases, labour pains, intestinal parasites, stomach pains, toothache, purgative for intestinal worms, an ingredient for preservation of medication. The plant parts are used for coughs, respiratory conditions and the root can be chewed to induce vomiting when there is suspicion that poisoned food has been ingested [1, 16, 17].

These uses can be linked to pharmacological properties: antibacterial, anti-helminthic, antiviral, anti-plasmodial, anti-spasmodic, wound healing, analgesic, anti-inflammatory. Gelfand et al [11] reported the antibacterial and anti-inflammatory properties of the extracts of *Dicoma anomala*. In vitro anticancer activity of extracts of *Dicoma* species have been reported [18, 19] and confirmed by phytochemical investigations which revealed the presence of acetylenic compounds, phenolic acids, flavonoids, sesquiterpene lactones, triterpenes, phytosterols, asymmetrical sesquiterpene dimers with potent anti-plasmodial and anticancer properties [1].

The main active constituent of *Dicoma anomala* was identified as dehydrobrachylaenolide, a eudesmanolide-type sesquiterpene lactone, demonstrating an in vitro IC₅₀ of 1.865 M against chloroquine sensitive strain (D10) of *Plasmodium falciparum*. Synthetic analogues confirmed the requirement for the presence of α -methylene lactone in the eudesmanolide for activity. This feature is absent in the artemisinins or the quinines, suggesting a different mode of activity from those of the other two types [20]. The traditional medicinal uses of these two plants appear to be supported by their reported phytochemistries.

METHODOLOGY

Acacia karroo and *Dicoma anomala* roots and a branch of each were obtained from Manyene village, Chikomba District in July 2011, following interviews with local people. 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°C. The extracts were combined, re-dissolved in MeOH, homogenized, and the MeOH removed at the rotary evaporator, yielding 1.1050g *Acacia karroo* extract as a grey gum, and 0.8020g *Dicoma anomala* 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°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 *Acacia karroo* root extract and 1.3756g of an oxblood coloured gum of *Dicoma anomala* 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. Parasitized blood was drawn intravenously 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°C. The cattle annul temperature was regularly monitored using a clinical thermometer and inoculated with berenil and imizol if 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 [21, 22]. 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 [23].

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 [23].

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 the each root.

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

Kazembe & Chinyuku

1.0ml of parasitized blood with 1.0ml of each of the herbal extracts at the 3 concentrations. The experiment was repeated with lower volumes of drug and/or herbal concentrations. Reducing the volume of herbal extract to 125 L being added to 1.0ml of parasitized blood maintained at 37°C 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°C. Heparinized blood is blood that contains heparin to prolong the life span of blood cells to about 3 days.

Table 1. Efficacy of EtoAc Extracts on *Babesia bigemina*

Sample	Conc.	parasitemia	PCV / 25	Efficacy	RBC cond
<i>Acacia karroo</i>	0.0010g/ml	0.14	14	effective	intact
	0.0005g/ml	0.14	15	effective	intact
	0.00025g/ml	0.14	14	effective	Intact
Dicoma anomala	0.0010g/ml	0.25	14	effective	Intact
	0.0005g/ml	0.14	15	effective	Very intact
	0.00025g/ml	0.17	13	effective	Fairly intact
Fansidar	0.0010g/ml	0.17	15	effective	Intact
	0.0005g/ml	0.10	15	v. effective	Intact
	0.00025g/ml	0.10	7	v. effective	haemolysed
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 / 25	Effective	RBC cond
<i>Acacia karroo</i>	0.0010g/ml	0.00	18	v. effective	Intact
	0.0005g/ml	0.00	19	v. effective	Intact
	0.00025g/ml	0.07	18	v. effective	Intact
Dicoma anomala	0.0010g/ml	0.07	0	v. effective	haemolysis
	0.0005g/ml	0.10	10	v. effective	haemolysis
	0.00025g/ml	0.00	8	v. effective	haemolysis
Fansidar	0.0010g/ml	0.10	15	effective	Intact
	0.0005g/ml	0.10	15	v. effective	Intact
	0.00025g/ml	0.10	7	v. effective	haemolysed
Water		3	15	No effect	Intact
Blood		4	18	No effect	Intact

Table 3. Results of fortification of chloroquine with extract

Sample	Sample	parasitemia	Effective	PCV/25	RBC
5 <i>L. D. anomala</i>	120 L Chloroquine	0.077	v. effective	14	Intact
10 <i>L. D. anomala</i>	115 L Chloroquine	0.10	v. effective	14	Intact
15 <i>L. D. anomala</i>	110 L Chloroquine	0.11	v. effective	14	Intact
5 <i>L. A. karroo</i>	120 L Chloroquine	0.063	v. effective	14	Intact
10 <i>L. A. karroo</i>	115 L Chloroquine	0.13	v. effective	14	Intact
15 <i>L. A. karroo</i>	110 L Chloroquine	0.067	v. effective	14	intact
Chloroquine	125 L Chloroquine	0.14	Effective	9	h/lysis
Blood	125 L blood	0.25	No effect	21	No effect

Tables 1 and 2

The MeOH extracts were more effective than the EtoAc extracts. The *Acacia karroo* MeOH extract was more red blood cell friendly than the EtoAc extract, as revealed by the PCV. On the basis of PCV and the red blood cell conditions, *Dicoma anomala* methanol extract was very unattractive, causing extensive haemolysis.

The ethyl acetate extracts of the two plants were less sensitive than fansidar, but caused less damage to red blood cells. The methanol extracts were more effective than fansidar. The methanol extract of *Acacia karroo* cleared parasites from blood, did no harm to red blood cells, as indicated by PCV and the condition of the red blood cells. PCV results indicate that blood treated with the methanol extract of *Dicoma anomala* was identical to blood to which nothing had been added, PCV=18.

Table 3

Chloroquine treatment gave a parasitemia equalling 0.14, accompanied by haemolysis as indicated by PCV and the appearance of red blood cells. Treatment with chloroquine to which extracts had been added improved the efficacy of chloroquine as indicated by parasitemia for both *Dicoma anomala* and *Acacia karroo*. Fortified chloroquine left the blood cells intact as indicated by PCV and the appearance of red blood cells. Thus fortification appears to have inhibited blood haemolysis by chloroquine. This selective inhibition is important in that the haemolysis was inhibited, but the efficacy was not.

A case is, thus, made for the development of extracts of *Dicoma anomala* and *Acacia karroo* either as outright curing agents or to fortify chloroquine and other synthetic antimalarial drugs.

ACKNOWLEDGEMENTS

Drs Mudyavavana and Hodobo, Mr Jomane and Mr Kambeva of the Central Veterinary Laboratory for allowing the work to be done in their laboratory and the assistance given during the study.

REFERENCES

1. Becker, J. V. W.; Van der Merwe, M. M.; Van Brummelen, A. C.; Pillay, P.; Crampton, B. G.; Mmutlane, E. M.; Parkinson, C.; Van Heerden, F. R.; Crouch, N. R.; Smith, P. J.; Mancama, D. T.; and Maharaj, V. (2011) In vitro antiplasmodial activity of *Dicoma anomala* subsp. Gerrardii (Asteraceae): identification of its main active constituent, structure-activity relationship studies and gene expression profiling. *Malaria Journal*, 10:295.<http://www.malariajournal.com/content/10/1/295>
2. Wikipedia (2011) Wikimedia Foundations, Inc.<http://en.wikipedia.org/w/index.php?title=acacia-karoo&oldid=430020561>
3. University of Arizona (2010) *Acacia karroo*, UA Campus Arboretum Species, University of Arizona, College of Agriculture and Life Sciences, P. O. Box 210036.
4. Aubrey, A. (2002) *Acacia karroo* Hayne, South African National Biodiversity Institute www.plantzafrika.com
5. Adedapo, A. A.; Sofidiya, M. O.; Masika, P. J.; Afolayan, A. J. () Anti-inflammatory and analgesic activities of the aqueous extracts of *Acacia karroo* stem bark in experimental animals. National Centre for Biotechnology Information, US National Library of Medicine 8600 Rockville
6. Eaton's Bible Dictionary
7. Nyila, M. A. (2010) Antilisterial Bioactivity and/or biofilm formation by compounds from *Acacia kiarroo*. University of Pretoria. upetd.up.ac.za/thesis/available/etd-06222011.../02chapter2.pdf
8. Wikipedia (2012) *Catharanthus roseus*. Wikimedia Foundation, Inc"http://en.wikipedia.org/w/index.php?title=Catharanthus_roseus&oldid=492575222"
9. Rico (2012) Kew Scientific Publications, Retrieved 17 May 2012
10. Anold, H.;-J; Gulumian, M (1984) Pharm acopoeia of traditional medicine in Venda. *Journal of Ethnopharmacology*, Volume 12, pp 35-74
11. Gelfand, M; Mavi, S. M; Drommond, R. B; Ndemera, B. (1985) The traditional medicinal practitioners in Zimbabwe. Mambo Press, Gweru, Zimbabwe. 411p
12. Kling, H. (1923) *Die Sieketrooster*, Van de Sandt de Villiers, Cape Town.
13. Chavunduka, D. M. (1976)
14. Hyde, M. A; Wursten, B. T & Ballings, P. (2012) Flora of Zimbabwe: Species information: *Dicoma anomala* <http://www.zimbabweflora.co.zw/specification/species.php?species-id=161880>, retrieved 25 May 2012
15. Kinkel, J. (2012) *Dicoma anomala*, Operation Wildflower- rescuing indigenous vegetation, Designed by Shape5.com
16. Mnengwane, J. and Koekemoer, M. (2007) National Herbarium, Pretoria. National Biodiversity Institute, South Africa.
17. Gwaza, L; Wolfe, A. R; Benet, L. Z; Guglielmo, B. J; Chagwedera, T. E; Maponga, C. G; and Masimirembwa, C. M. (2009) In vitro inhibitory effects of *Hypoxis obtusa* and *Dicoma anomala* on cyp450 enzymes and p-glycoprotein. *African Journal of Pharmacology*, Volume 3 (11) pp 539-546.
18. Steenkamp, V; Mathivha, E; Gouws, M. C; Van Rensburg, C. E. (2004) Studies on antibacterial, antioxidant and fibroblast growth stimulation of wound healing remedies from South Africa. *Journal of Ethnopharmacology*, 95: 353-357.
19. Steenkamp, V. and Gouws, M. C. (2006) Cytotoxicity of six South African medicinal plant extracts used in the treatment of cancer, *South African Journal of Botany*, 72: 630-633.
20. Alkaloid Medical Uses (2012) Net Industries and its Licensors Alkaloid - Medical Use - Bark, Quinine, Cinchona, and Reserpine - JRank Articles <http://science.jrank.org/pages/228/Alkaloid-Medical-use.html#ixzz1vPcdRmQh>
21. Homer, M. J., Anguilar-Delfin, I, Telford III, S. R, Krause, P. J., and Persing, D. M. (2000) Babesiosis. *American Society for Microbiology Clinical Microbiology Reviews*, Microbiol. Reviews, Volume 13 Number 3 pp 451- 469. Doi: 10.1128/CMR.13.3. 451- 469.2000 Clin.<http://www.dpd.cdc.gov/dpdx/HTML/Babesiosis.htm>
22. American Lyme disease Foundation (2006) Research Education Preventive American Lyme Disease Foundation, Inc.
23. Warhurst, D. C., Williams, J. E. 1996 ACP Broadsheet No 148. July 2006. Laboratory Diagnosis of Malaria. *Journal of Clinical Pathology* 49; 533-538.