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Full Length Research Paper

Ferriprotoporphyrin IX-*Combretum imberbe* crude extracts interactions: Implication for malaria treatment

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Leaves from *Combretum imberbe* traditionally used to treat symptoms of malaria in most parts of Southern Africa were studied for interaction of its crude methanolic, ethyl acetate and hexane extracts with hemin a product of hemoglobin degradation in 40% water-dimethylsulphoxide at pH 9, 7.4 and 5 using a spectrophotometric method. It has been established that hemin is the target of antimalaria quinolines thus, interaction of extracts with hemin may represent a crucial initial screening test to define efficacy. Observations were compared to results of interaction of hemin with quinine and artemisia as standards. The present results indicate that hemin complexed more strongly with methanolic extracts than with ethyl acetate and hexane extracts. The binding constants were pH-dependent. The present results are interesting because the crude extracts share a similar mode of action with quinine and artemisia with an isosbetic point at 352 nm. Methanolic extract showed better affinity for hemin than artemisia with complexation constants (K) of 1.011×10^4 and 0.612×10^4 M, respectively. However, quinine showed better affinity than methanolic extract with K of 1.101×10^4 M.

Key words: Ferriprotoporphyrin IX, *Combretum imberbe,* crude extracts, hemin.

INTRODUCTION

Malaria is becoming very difficult to treat everyday due to drug resistance of plasmodium (WHO, 2005; Egan, 2004), therefore calling for studies in alternative medication. Medicinal plants from folk remedies offer attractive options. It has been established that hemin is primarily involved in the anti-malarial activity of drugs (Tekwan and Walker, 2005; Dechy et al., 2002, 2003; Gong et al., 2001; Egan, 2002, 2004; Cointeaux et al., 2003). Thus, the interaction of herbal extracts with hemin may represent an initial crucial screening test to define efficacy. In the blood, *Plasmodium falciparum* use host's hemoglobin as a food source. This takes place in an acidic environment within the parasite called a food vacuole that has a pH in the range 5.0 to 5.6 (Spiller et al., 2002). Its proteolytic enzymes degrade hemoglobin and it use the amino acids derived from digestion for its biosynthetic needs. Hemoglobin degradation is an ordered process, which involves a number of proteases (Eggleson et al, 1999; Rosenthal et al., 2002). Denatured globin formed by plasmepsins is further degraded into small peptides by other proteases. A cysteine protease, falcipain, has been characterized from *P. falciparum (*Wu et al., 2003). It degrades denatured globin (Mpiana et al., 2007). Large quantities of free nonpoisonous heme groups are released from hemoglobin degradation (Tekwan and Walker, 2005; Macreadie et al., 2000). The formed heme is autoxidized into ferric form (hematin; aquaferriprotoporphyrin IX), which is highly toxic. It inhibits vacuolar proteases and damage parasite membranes (Berman and Adams, 1997). Detoxification of heme is therefore important for the survival and growth of malaria parasite (Meshnick, 2002). In the host, detoxification of heme is achieved by an enzyme heme

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oxygenase. It breaks heme to form biliverdin.

A second enzyme biliverdin reductase, converts biliverdin into bilirubin, which is further converted into a water-soluble conjugate and excreted through urine. Malaria parasite does not use this pathway for heme metabolism. In the food vacuole, heme is changed into hemozoin, a malaria pigment, which is a dimer of heme units linked through an iron-carboxylate bond (Pagola et al., 2000). Digestion of hemoglobin releases heme into the food vacuole, where it is oxidized to hematin. Heme is becoming another possible drug target and has been implicated in the mode of action of endoperoxide antimalarials, such as artemisinin and its derivatives (Tekwan and Walker, 2005; Robert et al., 2002). Hematin is said to be the target of chloroquine and other quinoline antimalarials. It has been shown that histidine-rich protein 2, (a histidine- and alanine-rich protein produced by *Plasmodium falciparum*) has been implicated as an enzyme or, more likely an initiator in the process of formation of hemozoin. Drugs like chloroquine and quinine mode of action has been shown to inhibit hemozoin formation through direct interaction with hematin (Kaschula et al., 2002), by displacing hematin from histidine-rich protein 2 (Pandey et al., 2006), or by preventing its binding to this protein. In this study a rapid screening method for investigating the ability of *Combretum imberbe* extracts to prevent hemozoin formation is followed.

C. imberbe Warwa*,* family Combretaceae grows widely in Southern African forests. In Zimbabwe, Zambia, Namibia and Mozambique it is used in folk medicine for treating various diseases such as malaria, diarrhea and bilharzia (Dan et al., 2010). Chewing *Combretum imberbe* leaves is regarded as a remedy for coughing or a bad cold (Ribeiro et al., 2010). Its ashes are used as toothpaste (Ribeiro et al., 2010). Angeh et al. (2007) reported antibacterial and anti-inflammatory activity of a triterpenoid extracted from *Combretum imberbe*. The interactions of hemin with selected herbal extracts obtained from *Combretum imberbe* leaves were investigated in water-dimethylsulphoxide mixture at pH 5, 7.4 and 9 using spectrophotometric method.

MATERIALS AND METHODS

All of the solvents used were of analytical grade purchased from Merck Co. (Germany). Hemin chlorides, Artemisinin, Tris (hydroxymethyl)-methylamine (Tris), and hydrochloric acid were purchased from Sigma Chemical Co. (St Louis, Germany). Quinine sulfate dihydrate was purchased from Fluka.

Plant collection

Plant leaves were collected from Chindunduma Mountains, Mashonaland Central, Zimbabwe, in December 2011. Selection was based on interviews with local communities. The plant specimens were identified by a taxonomist at Harare Botanical Garden and voucher specimen 2011/6 was deposited in the Chemistry Department of Bindura University (natural product section). Leaves were air-dried in the shade for two weeks and then ground into a fine powder using a wooden mortar and pestle.

Extraction

Ground Leaves of about 100 g were subjected to sequential cold extractions for 5 h using hexane, ethyl acetate and methanol. The extracts were decanted and filtered through Whatman No. 1 filter paper and the resulting filtrates were dried under reduced pressure at 40°C on a Bucchi rotary evaporator. The percentage yield for each sample was determined and the crude extracts were stored in a freezer.

Preparation of buffer solutions

Tris-HCl buffer solutions were prepared by mixing different amounts of 0.2 $mol.dm^{-3}$ Tris and 0.2 $mol.dm^{-3}$ HCl to give the required pH. For 100 ml of Tris-HCl buffer, 25 ml of Tris was mixed with 20.7 ml of HCl and diluted with distilled water to achieve a pH of 7.4. For pH 9, 25 ml of Tris was mixed with 2.5 ml of HCl and diluting it with distilled water to 100 ml. 25 ml of Tris was mixed with 30.6 ml of HCl and diluted with distilled water to achieve a pH of 5.

Water- Dimethylsulphoxide (DMSO) mixture

Water-dimethylsulphoxide (DMSO) mixture was adopted as a solvent of choice because it does not present some limitations with regard to the solubility of the reacting parties and dimerization process of hemin can be well controlled (Mpiana et al., 2007).Forty percent aqueous DMSO solutions (v/v) were prepared by mixing 40 ml of DMSO with 60 ml of corresponding buffer so that the final pH of the mixture was 5, 7.4 or 9.

Hemin solutions

Stock solutions, 306 µmol L^{-1} in concentration were prepared by first dissolving 10 mg of hemin in 20 ml of DMSO, followed by addition of 30 ml of buffer solutions. Stock solutions of hemin were refrigerated at 4°C and stored in the dark.

Quinine

Stock solutions (0.002 $mol.L^{-1}$) were prepared by dissolving 16.2 mg with 25 ml of acidic distilled water and then 10 ml of DMSO, followed by 15 ml of buffer.

Artemisinin solutions

Artemisinin stock solutions, (0.002 mol L^{-1}) were prepared by dissolving 14.1 mg with 10 ml of DMSO, followed by 15 ml of buffer.

Extract solutions

Extract stock solutions were prepared by dissolving 14.1 mg with 10 ml of DMSO, followed by 15 ml of buffer.

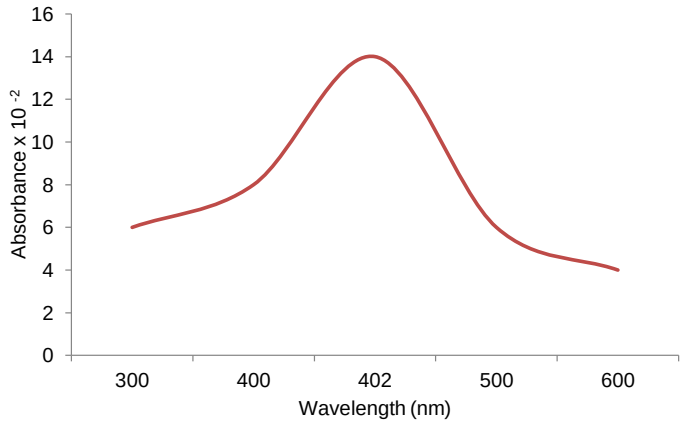

Figure 1. Spectra of hemin solutions in 40 % dimethylsulphoxide pH 5, temperature 36°C.

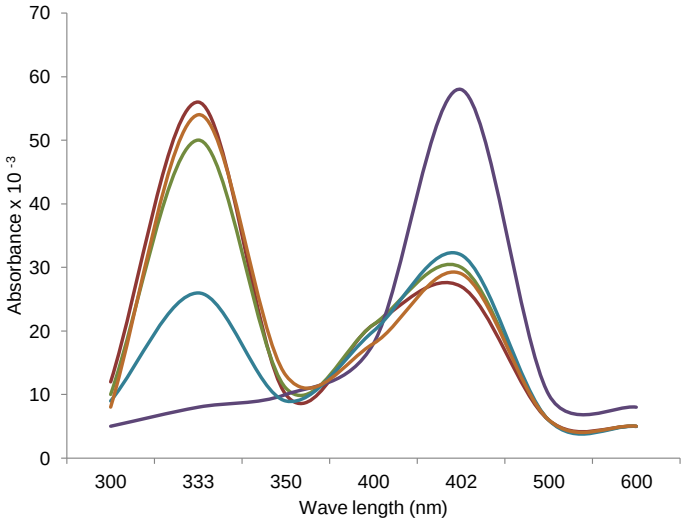

Figure 2. Hemin-drugs absorption spectrum after 10 h incubation pH 5, temperature 36°.

Hemin- drugs spectrophotometric titrations.

This was investigated in water-DMSO mixture at pH 5, 7.4 and 9 using spectrophotometric method. Titrations were carried out at the hemin characteristic Soret band at 402 nm by mixing a constant volume (0.3 ml) of hemin solution with various concentrations of quinine/ artemisinin / extract solutions. Before each measurement of absorbance, working solutions were incubated at 36°C for 10 h.

RESULTS AND DISCUSSION

Hemin was dissolved in 40% dimethylsulphoxide and it showed maximum wavelength at 402 nm (Figure 1). This was adopted as the working wave length.

The absorption wavelength at 333 nm is for derivatives of quinine, artemisia and extracts except for hexane extract. It can be seen that addition of quinine, artemisia and extracts modifies the hemin spectrum; however the peak maximums of hemin are still at about 402 nm. This reveals that the complexation does not involve significant changes in the structure of the porphyrin ring of the ferriprotoporphyrin IX. The results also show that quinine, artemisia and extracts produces an isosbetic point at 352 nm Figure 2. Titration of hemin by increasing amount of quinine, artemisia and extracts in mixed water-dimethylsulphoxide solution produced spectral changes shown in Figure 3 to 5. It can be seen that the hemin peak decreases with increasing total quinine, artemisia and extracts concentration except that for hexane extract. The results are similar to those observed on deuterohemin-quinine and hemin-chloroquine interactions in other mediums (Mpiana et al 2007; Tekwan and Walker, 2005; Gushimana et al., 1993).

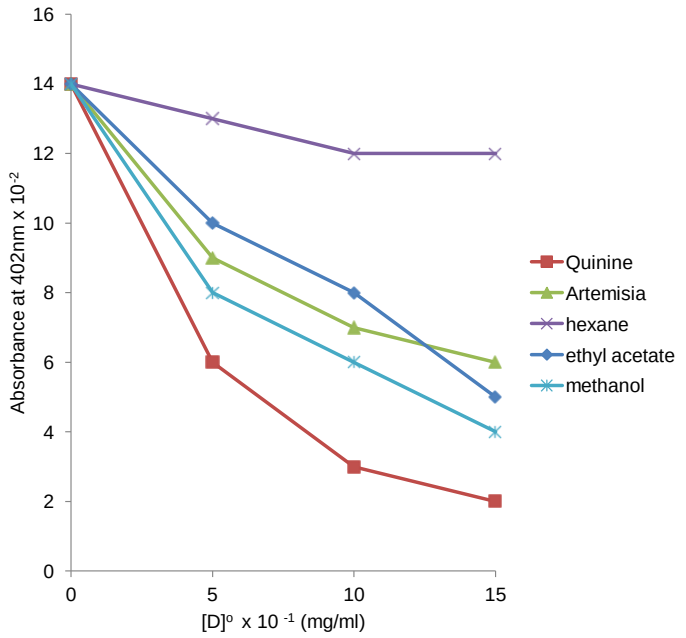

Figure 3. Absorbance of hemin at 402 nm at different concentrations of quinine, artemisia and extracts after 10 h incubation pH 5, temperature 36°C.

Binding constant of hemin-drug complexes

The interaction between hemin (H) and antimalarial drug (D) can be described according to the equilibrium;

$$H + D \rightleftharpoons HD \quad (1)$$

If solutions are dilute, the association constant K of complex *HD* can be written as:

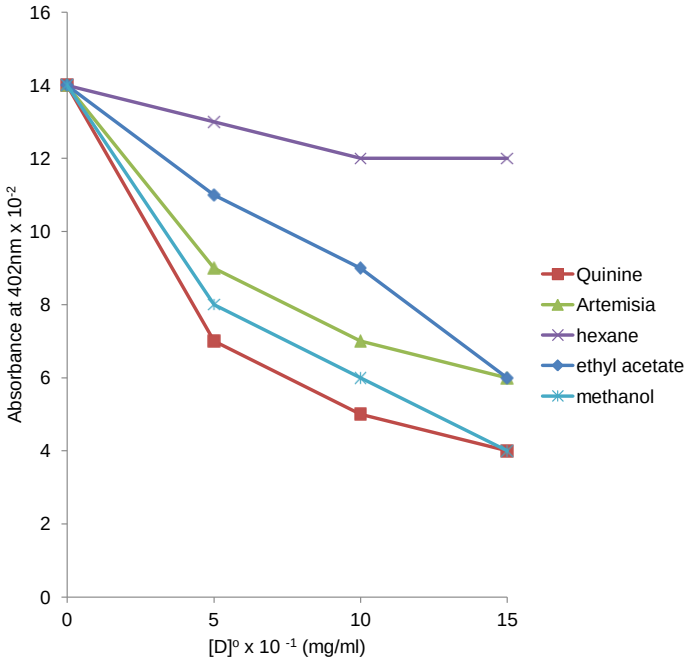

Figure 4. Absorbance of hemin at 402 nm at different concentrations of quinine, Artemisia and extracts after 10 h incubation pH 7.4, temperature 36°C.

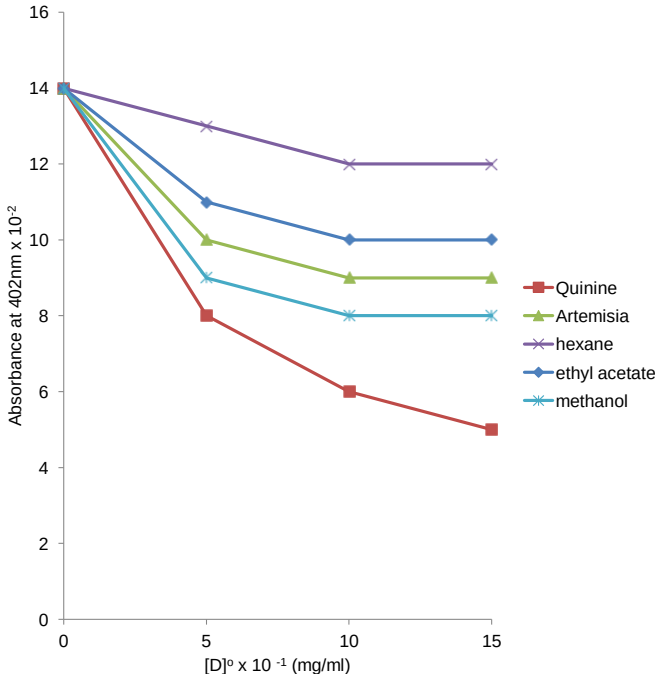

Figure 5. Absorbance of hemin at 402 nm at different concentrations of quinine, Artemisia and extracts after 10 hour incubation pH 9, temperature 36°C.

$$K = \frac{[HD]}{[H]_i[D]_i} \quad (2)$$

where [HD] is the concentration of complex and $[H]_i$ and $[D]_i$ are initial concentration of hemin and drug respectively.

It can also be seen that;

$$[H]_i = [H]_t + [HD] \quad (3)$$

where $[H]_t$ is the concentration of free hemin after time t Similarly for the drug the expression becomes

$$[D]_i = [D]_t + [HD] \quad (4)$$

where $[D]_t$ is the concentration of free drug after time t Combining equations 1 to 4 affords a quadratic equation:

$$[HD]^2 - ([H]_i + [D]_i + \frac{1}{K})([HD] + [H]_i[D]_i = 0 \quad (5)$$

One root of Equation 5 is given by;

$$[HD] = \frac{1}{2}[H]_i + [D]_i + \frac{1}{K} - \sqrt{([H]_i + [D]_i + \frac{1}{K})^2 - 4[H]_i [D]_i} \quad (6)$$

The optical density of the hemin-quinine, artemisia or extracts can be expressed as;

$$\frac{A}{d} = [H]\acute{\epsilon}_H + [HD]\ \acute{\epsilon}_{HD} \quad (7)$$

where *A* and *d* are the optical density and the light path, respectively, and $\acute{\epsilon}_H$ and $\acute{\epsilon}_{HD}$ are the molar extinction coefficients of hemin and its quinine, artemisia or extracts complex.

Combining Equations 3, 6 and 7 yield the following equation;

$$A = A_i + \frac{1}{2}d\Delta\acute{\epsilon}[H]_i + [D]_i + \frac{1}{K} - \sqrt{([H]_i + [D]_i + \frac{1}{K})^2 - 4[H]_i [D]_i} \quad (8)$$

where A_i is the molar extinction of hemin solution at $[D]_i$ =0, and ($\Delta\acute{\epsilon} = \acute{\epsilon}_{HD} - \acute{\epsilon}_H$) is the difference of the molar extinction coefficients between hemin complex and free hemin. The basic data are initial concentrations of hemin $[H]_i$ and quinine, artemisia or extracts $[D]_i$ and the corresponding optical absorption of hemin (A). Given this data (parameters) more importantly the equilibrium constant *K*, can be obtained according to Equation (8). Microsoft Origin 6.1 package computed K values for quinine, artemisia or extracts at different pH as shown in Table 1.

The complexation of ferriprotoporphyrin IX with the drugs plays the role of bringing back the hemin into solution such that it is prevented from polymerization.

Table 1. Binding constant of hemin- quinine, artemisia or extracts complexes at various pH.

pH	*K* (10^4 M)				
	Hemin-quinine	**Hemin- artemisia**	**Hemin- methanol extract**	**Hemin- ethyl acetate extract**	**Hemin- hexane extract**
5	1.101	0.612	1.011	0.410	0.010
7.4	1.103	0.611	1.010	0.401	0.010
9	0.501	0.610	0.408	0.311	0.010

Accumulation of hemin in the food vacuole will result in the death of the parasite (Mpiana et al., 2007; Meshnick, 2002). The capability of extracts to complex with hemin will inhibit the formation of hemozoin (hematin). The extract that has a greater affinity with hemin maintains more hemin in solution and is thus more effective. This means that methanolic extracts (K = 1,011 x 10^4M at pH 5) show the highest efficiency, followed by ethyl acetate extract (K = 0.410 × 10^4M at pH 5). Hexane extract revealed no affinity for hemin hence less effective. It can also be seen that values *of K* are pH-dependent. The dependence may be due to acidic-basic equilibrium influence on electrostatic interactions between hemin and the drugs (Steele et al., 2002; Bienvenu, 2007).

The decrease in absorbance of the hemin band can be as a result of two possible processes, either addition of micro molar concentrations of drug inducing aggregation of hemin or the changes may show drug association with hemin (Bienvenu, 2007). A large decrease in the absorbance of the band may indicate aggregation; equally large decreases may be caused by formation of p-p (donor-acceptor) complexes (Egan et al., 2000, Egan and Marques 1997). Generally, spectral changes of iron porphyrins in the visible region vary depending on the conditions of solvents and pH and the nature of interacting species. The decrease of hemin absorbance is dependent on the drug concentration. For this study dilution experiments showed that Beer's law is strictly adhered to in the concentration range studied thus providing no evidence of hemin aggregation within this concentration range (Mpiana et al., 2007)

The most plausible explanation for these spectral changes therefore, is the presence of drug-hemin association. Another feature on the titration curves Figure 2 is an appearance of an isosbetic point around 352 nm. Such behaviour indicates an equilibrium establishment between two species (Bilia et al., 2002). These spectral changes reveals progressive disruption of delocalized -electron system of the hemin tetrapyrrole ring. Similar results have been observed with *Momordica foetida* extracts (Froelich et al., 2007) and medicinal plant extracts (Steele et al., 2002).

Conclusion

In the light of the present results it has been shown that hemin, a product of hemoglobin degradation complex with crude extract of *Combretum imberbe*. It was observed that hemin complex more strongly with methanolic extract than with ethyl acetate and hexane extract, and binding constants were pH-dependent. The present results show that *Combretum imberbe* has a potential of becoming a future medication for malaria treatment. It shares a simillar mode of action as the highly successful quinolines drugs such as quinine. Future studies may focus on isolation identification and toxicity studies of the bioactive compounds.

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