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Bicarbonate *In-Vitro* Effect on Beta-Hematin Inhibition by *Artemisia sieberi* Aqueous Infusion

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Abstract: Malaria is still considered the most threatening disease in Africa. *Plasmodium*; the malaria parasite, forms during its intra-erythrocytic stage a pigment called hemozoin, which acts as a protection shield against oxygen radical-mediated stress that leads to parasite's death. Many drugs targeting hemozoin formation such as chloroquine and amodiaquine, but recently strains of *Plasmodium* have gained resistance to such drugs. *Artemisia sieberi* stem and leaf water infusion extract compared with *A. sieberi* bicarbonate aqueous infusion were tested using a semi-quantitative *in-vitro* method based on the inhibition of ferriprotoporphyrin IX (FP) bio- mineralization developed by Deharo *et al.* to reveal the differences in antimalarial activity. Reversed phase preparative liquid chromatography coupled to Photo Diode Array (HPLC-PDA) detector was also used to explain this dissimilarity in antimalarial activity. We found that *A. sieberi* bicarbonate aqueous infusion inhibits the formation of β-hematin better than standard water infusion. The bicarbonate addition increases the extraction of more compounds as the chromatographic HPLC results revealed. Other Artemisia plants (*A. vulgaris* and *A. herba alba*) were also tested to explore any inhibition effects.

Key words: Malaria, Plasmodium, Hemozoin, *Artemisia sieberi*, bicarbonate, drug resistance.

1. Introduction

Despite the global efforts made to eliminate malaria, it is still the most prevalent serious infectious disease, caused by protozoan parasites of the genus *Plasmodium,* transmitted only by female *Anopheles Mosquitoes.* It is concentrated in the tropical areas mostly in developing countries. The majority of the mortality occurs in Africa where women and children are particularly at risk [1-4]. There are five identified species of this parasite causing human malaria, namely, *Plasmodium vivax, P. falciparum, P. ovale*, *P. malariae* and *P. knowlesi* [5, 6]. Only *P. falciparum* is known to be life threatening.

The *Plasmodium* resides inside the erythrocytes of the infected host during its unique life cycle. Once inside, it divides repeatedly eventually bursting the RBC's, liberating dozens of new parasites into the circulation. Each of these can invade another red cell and undergo the same cycle.

The malaria parasite digests hemoglobin for its biosynthetic requirements, resulting in accumulation of large amounts of monomeric free heme known as ferriprotoporphyrin (IX) (FePPIX) [7, 8].

The accumulation of ferriprotoporphyrin (IX) is highly toxic to the parasites and causes the generation of reactive oxygen species, which may induce oxidative stress leading to parasitic death [3]. The parasite uses a unique pathway of heme polymerization within the food vacuole at pH between 4.5 to 5.0 to avoid heme toxicity, forming a non-toxic, un-reactive, insoluble crystals called *Hemozoin* or "Malaria pigment" [8], with disastrous effects on health and immunity of humans. Heme molecules are connected

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to each other by a linkage in which iron of one hematin is linked to the propionic acid group of another and the structure is stabilized by hydrogen bonds.

A synthetic analogue to hemozoin called β-hematin is considered to be structurally and spectroscopically identical to purified hemozoin [9] and considered an important target in the search and finding of new antimalarial drugs [7, 9, 10].

The malaria parasites in many parts of the world have developed resistance to commonly used drugs such as quinine and artemisinin derivatives [11, 12]. This drug resistance of the parasites acts against malaria control and threatens the lives of millions around the world and represents a global challenge.

Plant sources of drugs have been used for medical purposes throughout history since they contain a quantity of metabolites with a great variety of structures and pharmacological activities. We had previously attempted to search for new antimalarial drugs, concentrating on the effect of *A. sieberi* extracts on the formation of β-hematin [13, 14]. In this study, we investigate the effectiveness of different sodium bicarbonate water extracts of the herb *A. sieberi* from Palestinian origin.

The genus Artemisia has always been of great pharmaceutical interest and is useful in traditional medicines for the treatment of a variety of diseases [15-17]. *A. sieberi* is a perennial shrub belonging to the family Asteraceae, it is generally widespread in mid to high latitudes [18].

2. Materials and Methods

2.1 Materials

DMSO (Dimethylsulfoxide), purity 99.5% was obtained from Sigma Aldrich. Chloroquine diphosphate salt was obtained from Sigma. Glacial acetic acid was obtained from Fluka. Sodium acetate, purity 99% was obtained from Aldrich. Hemin chloride was purchased from Sigma and sodium bicarbonate 99.7-100.3% was obtained from Sigma Aldrich.

2.2 Plant Collection

A. sieberi samples were collected from Palestine and Jordan.

A.vulgaris was collected from Luxembourg, *A.herba alba* was collected from Morocco.

2.3 Extraction of Plant Components by Infusion

Stems and leaves of *A. sieberi* were separated and air-dried at room temperature. Stems were then cut into 0.3- 0.5 cm long fragments while leaves were ground into coarse powder. 2g of sample (either leaf or stem) were soaked in 150 mL of distilled hot water at 90 °C, left for 20 minutes at room temperature, then filtered using MN 615.Ø110 mm filter paper. The resulted solution concentration which is about 13.34 mg/mL was also used in the preparative HPLC separation.

The effect of bicarbonate was studied by dissolving 0.5g $NaHCO_3$ in 150 mL hot water, which was then used in the extraction process.

2.4 In vitro Semi-quantitative Test for Screening of Anti-malarial Activity

According to Deharo et al. [19], a mixture containing 50 μL of 0.5 mg/mL hemin chloride freshly dissolved in DMSO (dimethylsulphoxide), 100 μL of 0.5 M sodium acetate buffer (pH 4.4), and 50 μL of the tested potential anti-malarial drug solution or control, was incubated in a normal non-sterile 96-well flat bottom plate at 37 ºC for 18-24 hours. It is important that the solutions be added to the plate in this order. The plate was then centrifuged for 10 minutes at 4000 rpm. The supernatant was removed and the pH of reaction was measured. The final pH of the mixture should be between (5.0-5.2). The solution mixture in the wells were washed with 200 μL DMSO per well to remove free hemin chloride. The plate was centrifuged again, discharging the supernatant afterwards. The β -hematin remaining was then dissolved in 200 μL of 0.1 M NaOH to form an alkaline hematin that can be measured spectrophotometrically. Finally, the absorbance read at 405 nm using (Stat Fax 2100)

ELISA reader.

Ultra-pure water was used as negative control meanwhile chloroquine dissolved in ultra-pure water was used as positive control.

2.5 Separations of Plant Extract Using HPLC

Only *A. sieberi* stem extracts were analyzed using HPLC.

2.5.1 HPLC systems

The analytical HPLC system consisted of an Alliance 2695 HPLC equipped with 2996-Photo diode array (PDA) (Waters, Germany). Data acquisition and control were carried out using Empower ™ software. The Preparative HPLC system consisted of 3535 quaternary gradient module, equipped with 996 PDA detector (Waters, Germany).

2.5.2 Chromatographic conditions

The mobile phase was a gradient of acidic water at pH of 2.8 adjusted by phosphoric acid (eluent A) and ACN (acetonitrile) (eluent B). The gradient elution was set for a linear gradient starting from 90% of eluent A and 10% eluent B up to 100% of eluent B for 20 minutes. The HPLC analytical column was octadecyl silane C18 chemically bonded column (Waters XBridge, 4.6 × 150 mm, 5 μm). The flow rate was 1 mL/min. Before the analysis, the column was equilibrated with the starting mobile phase for about 7 minutes. The injection volume was 20 μl of 1mg/mL and the temperature of the column was at 25 ^{o}C.The wavelength was monitored using a photodiode array detector to extract the maximum wavelength of each separated peak. The major peaks absorption wavelengths were seen at mainly between 327 to 343 nm. Total run time of last eluting compound was about 16minutes. The HPLC Preparative experiments were run on ODS column (Agilent PrepHT C18, 22.2 × 250 mm, 10 μm). The same mobile phase and gradient conditions of the analytical method were utilized in the preparative-HPLC with the exception of the flow rate, which was 20 mL/minute, and the injection volume was 1000μL. Total run time of last eluting compound, was about 14minutes.

2.5.3 Samples Preparation

The sample solution (13.34 mg/mL) was filtered using 0.45 μm PVDF membrane filter before injection to the preparative reversed phase HPLC. 1 mL of this solution was further diluted into 50 mL volumetric flask with pure water to bring the final concentration to 0.2 mg/mL. This solution was directly injected to analytical reversed phase HPLC-PDA.

3. Results and Discussion

Using the model of β-hematin inhibition screening potential antimalarial herbs is considered as an excellent tool to compare with the activity against the parasite [20-22], antimalarial *in vitro* test results are viewed in comparisons to positive and negative controls. In a previous work, we had already noticed that the addition of NaCl to the water used for the extraction enhanced the inhibitory effect [23]. Furthermore, we have completed another series of experiments using diluted solutions of sodium bicarbonate, which resulted in a spectacular tenfold increase in inhibitory action of β -hematin as seen in Figs. 1, 2 and Table 1. It is worthwhile mentioning that the concentration of the plant extract used in each experiment represents crude concentration, meaning that the active components concentration could be much lower.

Fig.1 shows the antimalarial activity of different dilutions of *A. sieberi* leaf water infusion compared to bicarbonate infusion. The absorption is inversely proportional to drugs efficiency, i.e., the lower the absorption is, the efficiency is better. Each result represents the average of 16 individual experiments. Fig. 2 shows the test results of *A. sieberi* stem water infusion compared to bicarbonate infusion. According to the semi-quantitative method used in this research, the absorption is inversely proportional to drug efficiency; the lower the absorption is the more efficient the drug becomes.

The herb *A. sieberi* has been used in folk medicine

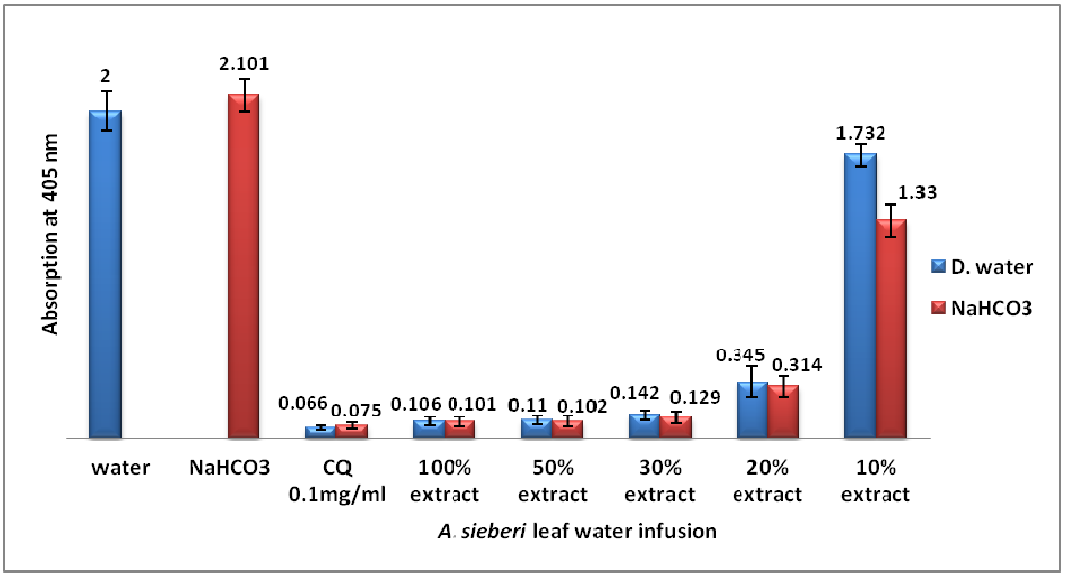


Fig. 1 Column diagram of potential anti-malarial *A. sieberi* leaf water infusion.

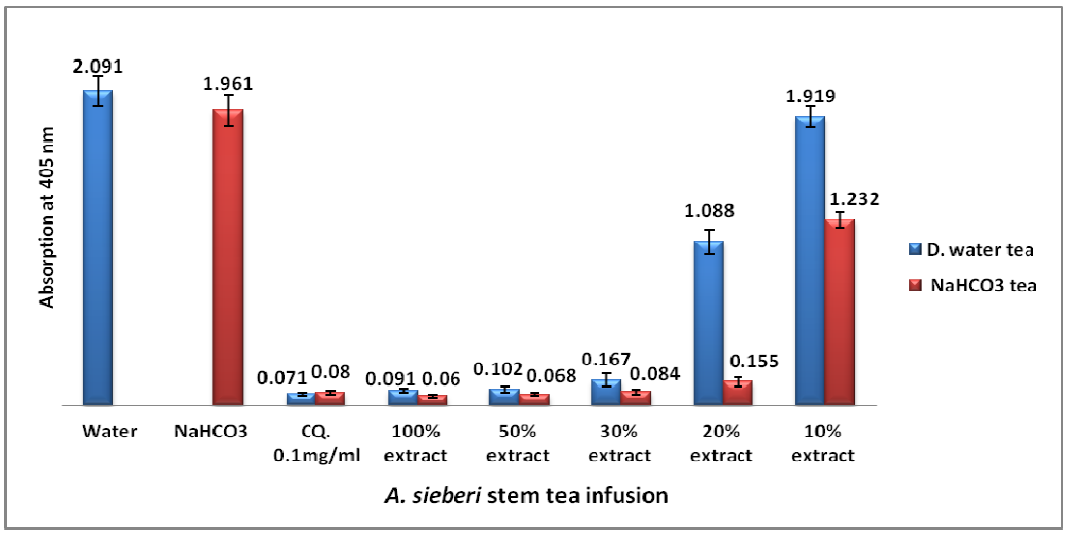


Fig. 2 Column diagram of potential anti-malarial A. sieberi stem water infusion extract.

Table 1 The efficacy of potential anti-malarial plants from different *Artemisia* species using different aqueous extracts (water infusion, water with bicarbonate).

Plant information				Average absorption at 405 nm					
Plant name	Plant origin	Plant part	Extract type	100%	50%	30%	20%	10%	5%
A. herba alba	Marocco	Whole plant	Water	0.105 ± 0.013	0.122 ± 0.027	0.16 ± 0.036	0.282 ± 0.061	1.663 ± 0.101	Eq.
A. herba alba	Marocco	Whole plant	+$NaHCO_3$	0.066 ± 0.007	0.062± 0.013	0.067± 0.019	0.063± 0.009	0.143 ± 0.025	0.608± 0.096
A. vulgaris	Luxembourg	Stem	Water	0.245 ± 0.022	0.471± 0.051	1.271± 0.09	1.857± 0.12	Eq.	Eq.
A. vulgaris	Luxembourg	Stem	+$NaHCO_3$	0.135 ± 0.017	0.187± 0.051	0.391± 0.091	0.615± 0.096	1.142 ± 0.11	Eq.
A. sieberi	Jordan	Stem	Water	0.337 ± 0.06	1.135± 0. 22	Eq.	Eq.	Eq.	Eq.
A. sieberi	Jordan	Stem	+$NaHCO_3$	0.065 ± 0.014	0.125± 0.028	0.786± 0.12	1.799 ± 0.1	Eq.	Eq.

with no reported toxicity [24]. The enhancement effect of antimalarial action of *A. sieberi* stem tea extract with sodium bicarbonate, may be due to the increase in solubility of some anionic compounds found in the herbal water extracts. Another possible explanation is the formation of complex in the extract that may prevent the formation of β- hematin.

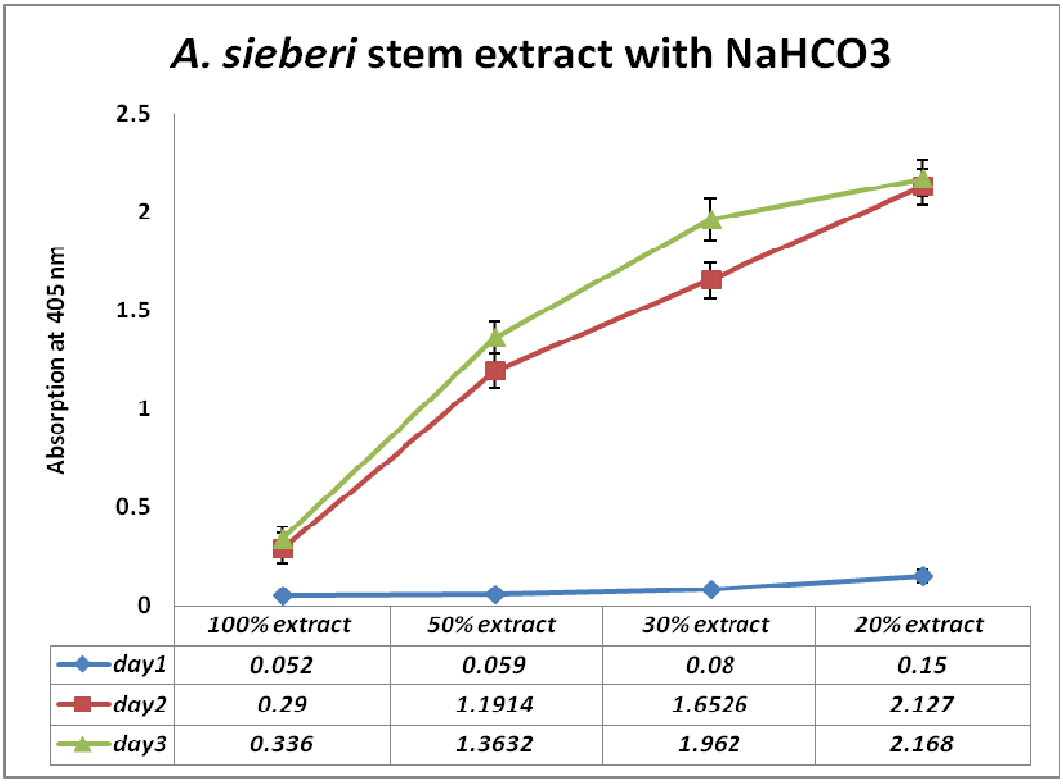
Bicarbonate is known to have a wide spectrum of beneficial biological effects; not excluding its biological action by the pH buffering properties of bicarbonate.

A comparison between the water and sodium bicarbonate infusions was done also using different *Artemisia* species from different origins. Table 1 summarizes the difference in β -hematin inhibitory effect of different *Artemisia* plants aqueous infusion compared to bicarbonate infusion.

Results obtained in Table 1 showed that the bicarbonate effect is not specific for *A. sieberi*, as other species like *A. vulgaris, A. herba alba* gave the same effect when their sodium bicarbonate infusions were tested. That emphasizes that this effect must be given more attention.

As to the activity, it appears that time and alkaline conditions have a deteriorating effect where oxidation occurs, see Fig. 3. Therefore, it is essential to prepare fresh infusions rather than working with extracts even left for short time.

Under the same chromatographic conditions and concentrations, analytical reversed phase HPLC experiments of water and bicarbonate solutions were conducted in an attempt to understand the bicarbonate solution antimalarial superiority in comparison to the water solution. Ten µl of water and bicarbonate crude mixture was injected successively to analytical HPLC at a flow rate of 1 mL/min at a monitoring λ of 327 nm. The result is shown in Fig. 4. It revealed 5 major compounds and 12 other minor peaks at 325 nm (Fig. 4). The first three major peaks that eluted at 4.85, 7.35 and 7.55 minutes were noticed to share close UV-Visible maxima using photodiode array detector. This observation may indicate they share similar chromophoric functionally (Fig. 6).



	100% extract	50% extract	30% extract	20% extract
day1	0.052	0.059	0.08	0.15
day2	0.29	1.1914	1.6526	2.127
day3	0.336	1.3632	1.962	2.168

Fig. 3 Effect of time on the efficiency of potential anti-malarial drug *A. sieberi* stem (Palestine) water infusion extract.

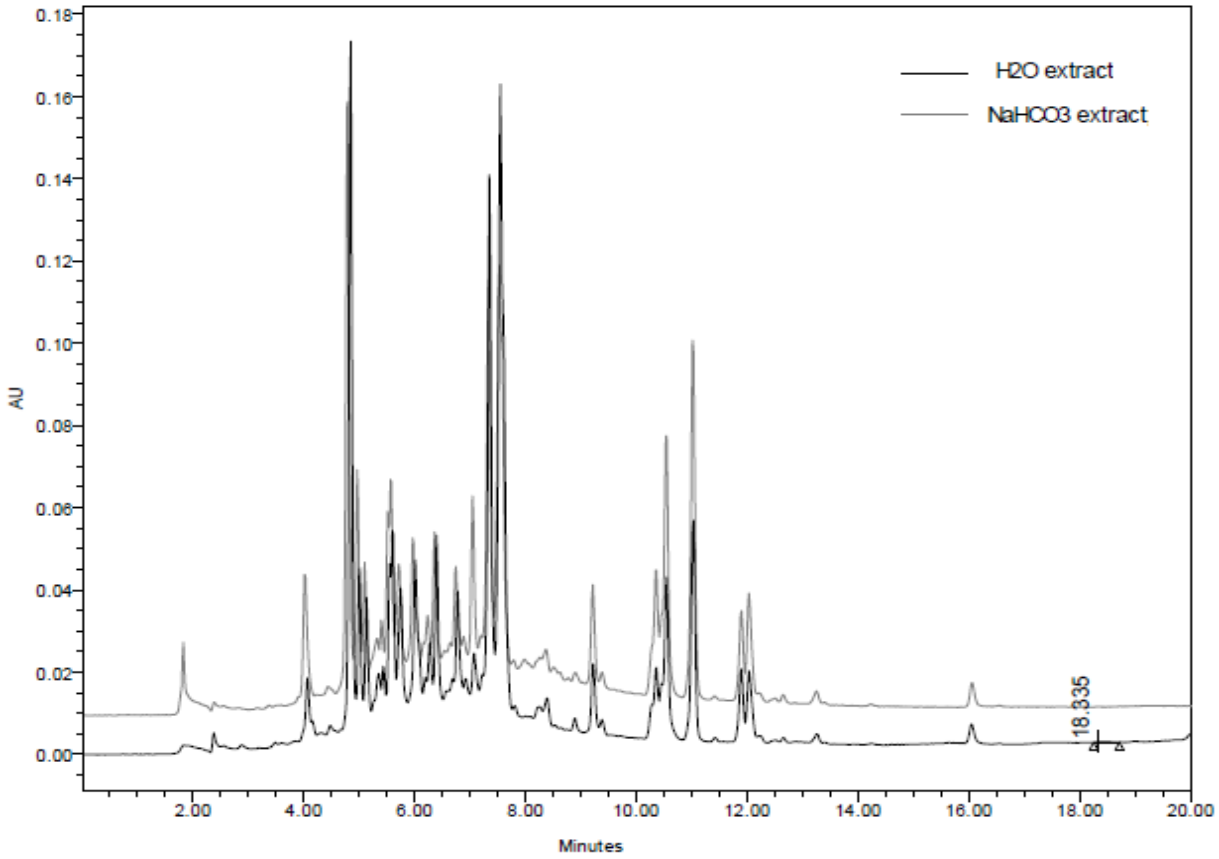


Fig. 4 Overlaid chromatograms of the analytical RP-HPLC of water and bicarbonate crude solutions.

Fig. 5 shows the overlaid preparative HPLC chromatograms of water and bicarbonate at 327 nm. 1 mL of both the water and the bicarbonate solution were directly injected into a preparative reversed phase HPLC at a flow rate 20 mL/min and a monitoring λ of 327 nm. The chromatographic profiles in Fig. 5 are much more significant in comparison to the analytical ones since the amount injected is about 2,000-fold greater. It is obvious that the bicarbonate sample contains more compounds in comparison to water sample.

Injection volume was 1,000 µL, the flow rate was 20 mL/min and the monitoring λ was at 327 nm.

The overlaid UV-Visible spectra of the major eluted peaks from the extract mixture is depicted in Fig. 6. The first two major peaks that eluted at 4.85, 7.35 minutes (Fig. 4) were noticed to share similar UV-Visible maxima, namely at 327.9 nm, while the peak eluted at 7.55 minutes was having a maximum wavelength at 329.1 nm.

It was noticed that extract acquired pale yellow color upon dissolving in water while the sample treated with bicarbonate turns to greenish color immediately. The amount of the main compounds in pure water and bicarbonate solution were quite similar except that of the latter eluting peaks at 10.5, 11, 11.9 and 12 minutes. The UV-spectra of these four compounds are depicted in Fig. 7.

We noticed the same UV-Vis spectral pattern in our previous investigation [23].

The subtle difference of the bicarbonate peak area may be attributed to the role of $NaHCO_3$ in converting the less water-soluble phenolic acids to their corresponding conjugate water-soluble sodium bases. The conjugated basic forms are more polar and thus better soluble in water. Accordingly the total phenolics recovery most probably increased and their β-hematin inhibition efficacy elevated. Moreover, it has

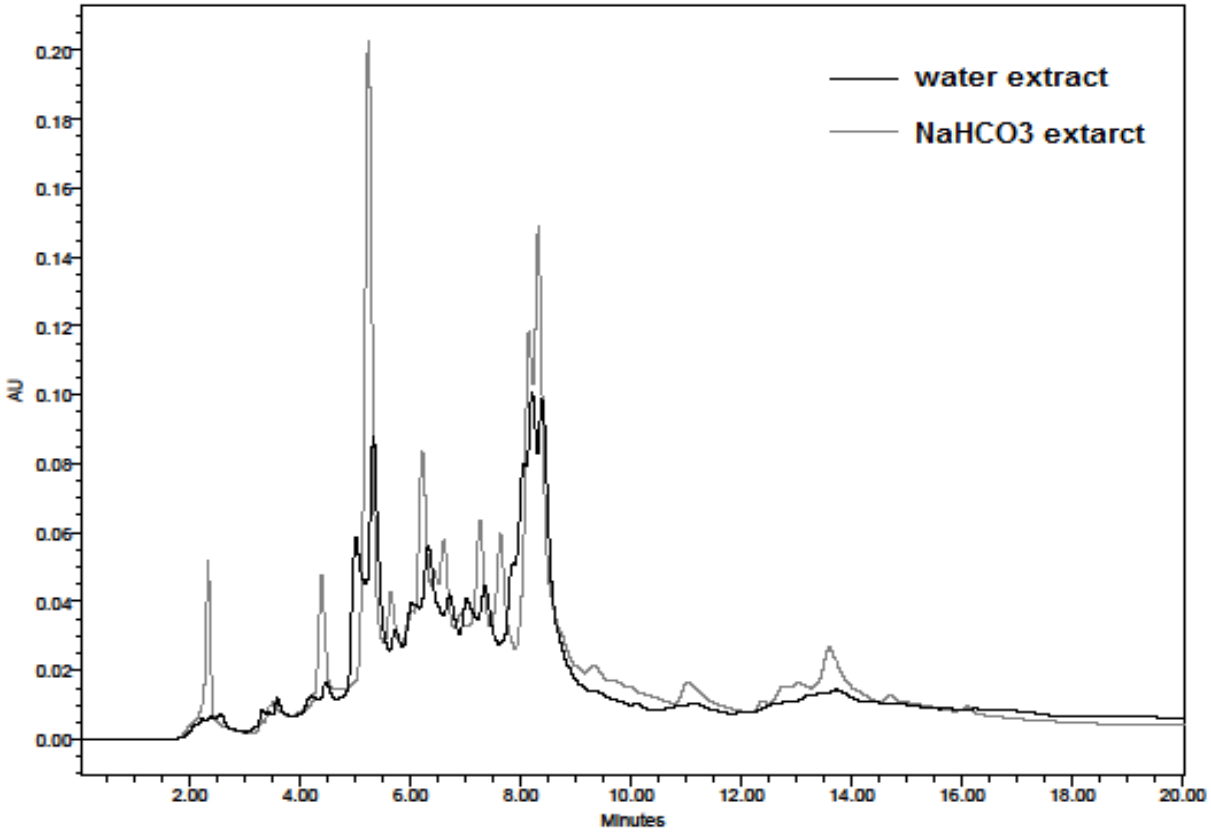


Fig. 5 Overlaid chromatograms of the preparative HPLC chromatogram of water and bicarbonate crude solutions.

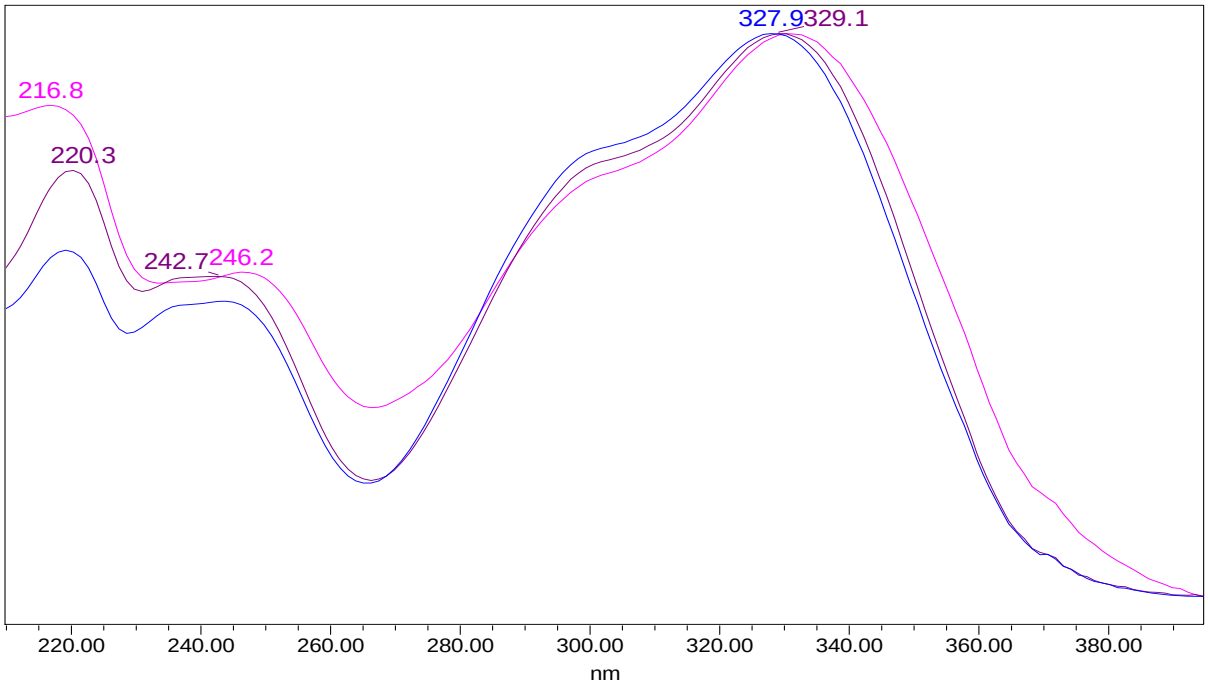


Fig. 6 Overlaid scanned UV-Visible spectra of the major three eluted peaks (4.85, 7.35 and 7.55 minutes) from crude plant extract solution.

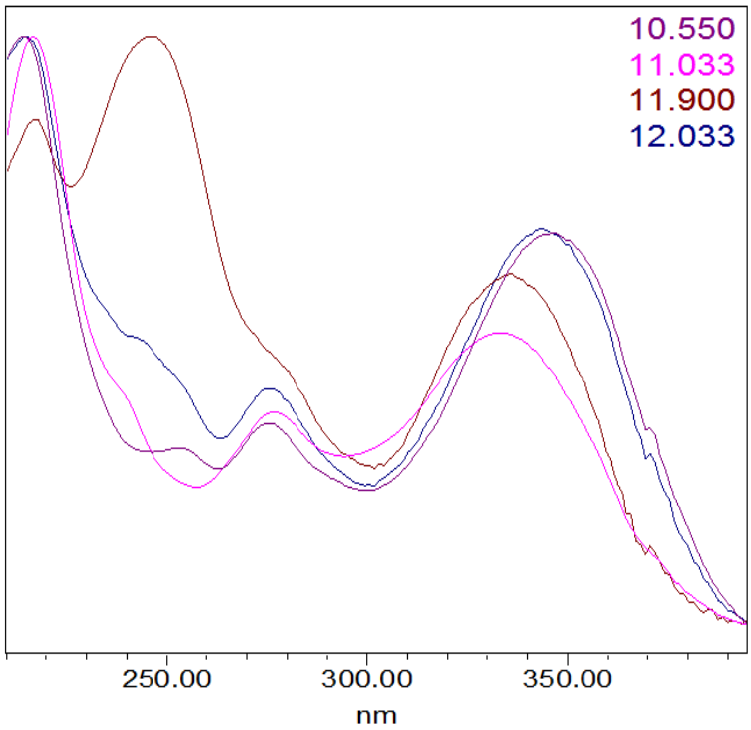


Fig. 7 Overlaid scanned UV-Visible spectra of the major 4 eluted peaks (10.55, 11.03, 11.9 and 12.03 minutes) from crude plant extract solution.

been mentioned that the bicarbonate has the ability to participate in free radical reactions accompanying oxygen reduction to water [25-27]. Recently, it became clear that the so-called reactive oxygen species (ROS) play the basic role in regulation of practically all vital processes, though the particular mechanism of their action remains unclear.

The antimalarial effect increases with bicarbonate. It is important to mention that WHO recommended treatment for severe malaria is injectable artesunate that is dissolved in 5% bicarbonate before being administered to the patients [28-30].

Acidosis due to an increase in blood lactate and decrease in carbonate leads often to a fatal outcome in cerebral malaria. It can be corrected by moderate administration of bicarbonate [31].

The findings of this *in-vitro* research could be a breakthrough in the battle against malaria. Bicarbonate influence may have a significant effect on the way Africans use antimalarial herbs including *A. sieberi* and *A. afra,* which are growing wild in Africa. However, a through *in-vivo* investigations are needed to take place prior advocating this alternative medicine.

4. Conclusions

Malaria is a global disease causing millions of deaths mostly in Africa and currently, there is still an urgent need for antimalarial new candidates due to the increasing spread of resistant strains. When comparing the *in vitro* activity of *A. sieberi* stem water infusion with that extracted with addition of bicarbonate, the activity of bicarbonate extract was always superior. This enhanced antimalarial activity is probably due to synergistic effect, which may be attributed to the presence of different anti-malarial compounds in *A.sieberi.* However, comprehensive *in-vivo* pharmacological studies still have to be investigated with a restricted dose prior any recommendation for treatments.

Acknowledgments

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