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Full Length Research Paper

Cinnamon bark water-infusion as an *in-vitro* inhibitor of β-hematin formation

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Malaria remains one of the prominent public health problems that lead to severe morbidity and mortality in many developing countries around the globe. New antimalarial drugs are urgently needed due to the emergence of antimalarial-resistant strains of *Plasmodium falciparum.* In previous studies, we tested several plants extracts that are capable of inhibiting β-hematin formation, with efficiency similar to chloroquine. In the current study, the effect of cinnamon ethanol and water extracts on inhibiting β-hematin formation was studied. Powdered cinnamon extracts and bark in a stick form were investigated using various extraction methods. A semi-quantitative *in vitro* method, based on the inhibition of ferriprotoporphyrin IX (FP) bio-crystallization developed by Deharo et al. (2002) was utilized. Water extracts of cinnamon revealed potential activity even at low concentration of infusions, which was manifested by a high capability to inhibit β-hematin formation *in vitro*.

Key words: Cinnamon, ferriprotoporphyrin (IX), β-hematin, chloroquine, hemozoin, malaria.

INTRODUCTION

Malaria is a mosquito-borne infectious disease, caused by a plasmodium parasite that is transmitted to humans through the bites of infected anopheles mosquitoes. Five species of plasmodium parasites are responsible for malarial infection. However, *Plasmodium falciparum* species are the most fatal among the four other species (Rathore, 2006).

According to recent World Health Organization (WHO) Malaria Report, about 198 million cases of malaria occurred globally in 2013 and the disease led to an estimated 584,000 deaths. The burden is the heaviest in the WHO African Region where an estimated 90% of all malaria deaths occur, mostly in children under five years of age. Therefore, malaria is considered to be one of the major life-threatening diseases (WHO, 2014).

In the human host, the parasite develops through two main stages: exo-erythrocytic stage (in liver) and intra-erythrocytic stage (in red blood cells, RBCs). During the intra-erythrocytic cycle, the trophozoite ingests the host cytoplasm forming a large food vacuole where the digestion of host hemoglobin takes place giving it the appearance of a ring of cytoplasm (Roberts et al., 1996).

The parasite consumes up to 80% of the host cell hemoglobin in its vacuole (Moore et al., 2006). The

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digestion of hemoglobin releases hematin (oxidized heme), which is toxic to the parasite. Therefore, to avoid destruction by hematin, the parasite detoxifies the hematin through bio-crystallization (Pagola et al., 2000; Kumar et al., 2008) converting it into insoluble and chemically inert β-hematin crystals known as hemozoin or malaria pigment.

Thus, considering the importance of hemozoin for parasite survival encouraged researchers to discover new antimalarial drugs that inhibit hemozoin biocrystallization leading to the release of high quantities of free heme, which is fatal to the parasite (Pandey and Chauhan, 1998).

The emergence of antimalarial-resistant plasmodium strains instigates research endeavors to discover new antimalarial drugs. The process of hematin polymerization is essential for the survival of the malarial parasite. Chloroquine, a well-known quinoline derivative inhibits this process by interfering with β-hematin formation, through the formation of a complex with free hematin molecules (Slater et al., 1991; Kumar et al., 2007). Several other quinolones also inhibit the polymerization process of free hematin (Slater, 1993).

Throughout history, plants have been used for medical purposes. They are effective, cheap, available, have fewer side effects and can be used as an alternative to mainstream medicines (Ghosh, 2003). Numerous natural plants that are known in folk medicine were tested for β-hematin formation inhibition to develop effective antimalarial drugs. We had previously investigated active extracts from *Salvia officinalis* (Akkawi et al., 2012), wild *Salvia palaestina* leaves (Jaber et al., 2013), *Artemisia annua* herb in comparison to Palestinian *Artemisia sieberi* (Akkawi et al., 2014).

Cinnamon is one of the oldest herbal medicines that have been mentioned in Chinese texts as early as 4000 years ago (Torizuka, 1998), and has been traditionally harvested in Asian countries. It is an ever green tree, which belongs to the family Lauraceae. It is available as Cinnamon chips (like wood chips), rolled into Cinnamon sticks or ground into cinnamon powder. Numerous health benefits of Cinnamon were reported, such as anti-bacterial, anti-microbial (Singh et al., 2007), anti-oxidant, anti-fungal (Tabassum and vidyasagar, 2013) and anti-diabetic (Kannappan et al., 2006).

It contains a hepatotoxic molecule called coumarin (~1%), which is unsafe when taken in high-doses and for long-term use. It is safe within a dosage range of 1 to 6 g daily (Medagama and Bandara, 2014). No major reactions have been reported with doses of less than 6 g per day. In the current investigation, a screening of the *in vitro* potential inhibitory effect of different water extracts of cinnamon on β-hematin formation was established.

MATERIALS AND METHODS

Dimethyl sulfoxide (DMSO) of 99.5% purity was purchased from Sigma Aldrich. Chloroquine diphosphate salt was purchased from Sigma. Glacial acetic acid was purchased from Fluka. Sodium acetate of 99% purity was purchased from Aldrich. Hemin chloride was purchased from Sigma. Cinnamon was provided from a local market in Palestine.

Extraction of plant component

Infusion of cinnamon powdered bark [Method-A]

Two grams of cinnamon fine-powdered bark was soaked in 150 ml of hot distilled water (at 90°C), and left for 30 min at room temperature, then filtered using MN 615.Ø110 mm filter paper. The extract then was evaporated at 70°C under reduced pressure using (IKA WEREK RV06-ML) rotary evaporator, and a crude water extract was obtained. Then, it was lyophilized using Labconco freeze drier, until constant weight was achieved. The final dried extract was stored in opaque bottles and kept in a desiccator until use.

Preparation of cinnamon bark (sticks) extract [Method-B]

Cinnamon bark water decoction [Method-B1]

Four grams (one stick) of cinnamon bark was boiled in 300 ml of distilled water for 20 min, and left for 30 min at room temperature, and then filtered using MN 615.Ø110 mm filter paper.

Cinnamon bark water infusion [Method-B2]

Four grams (one stick) of cinnamon bark was soaked in 300 ml of hot distilled water (at 90°C), and left for 30 min at room temperature, then filtered using MN 615.Ø110 mm filter paper. The results of Figure 3 correspond to this extraction method.

Preparation of cinnamon powdered bark alcoholic extract [Method-C]

Two grams of cinnamon powdered bark was soaked in 150 ml of 20% ethanol, and left for about 20 min at room temperature, and then filtered using MN 615.Ø110 mm filter paper. Same method was prepared using 35% ethanol. The results of Figure 4 correspond to this extraction method.

In vitro semi-quantitative test for screening the inhibition of β-hematin formation

As per Deharo et al. (2002) method, a mixture containing 50 µl of 0.5 mg/ml hemin chloride freshly dissolved in DMSO, 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°C for 18 to 24 h. It is important that the solutions be added to the plate in this order. The plate was then centrifuged for 10 min 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 and 5.2. 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 FP that can be measured spectrophotometrically.

Finally, the absorbance was read at 405 nm using ELISA reader. Ultra-pure water was used as negative control. Chloroquine (CQ) and 2-mercaptopyrimidine (2-MP) were dissolved in ultra-pure

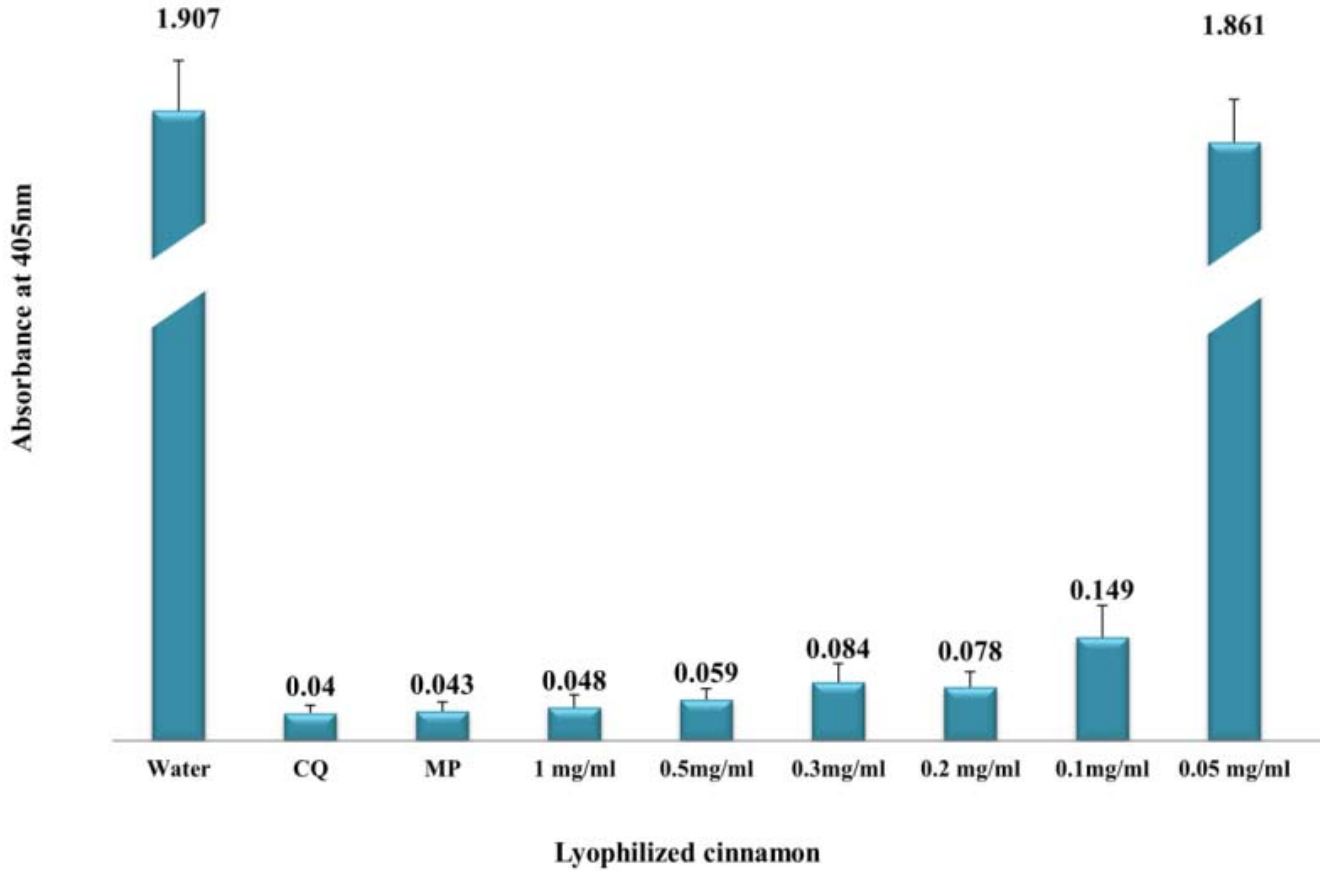

Figure 1. Column diagram representing the efficacy of cinnamon powdered bark water infusion using [method A], compared to the negative and positive controls: ultra-pure H_2O and CQ 0.1 mg/ml, respectively, showing the absorption values of dissolved β-hematin (alkaline hematin) at 405 nm using ELISA reader.

water and both used as a positive control.

Chromatographic analysis

HPLC instrumentation system

The analytical high pressure liquid chromatography (HPLC) system consists of Waters e2695 Alliance Module 2998-Photodiode Array detector (PDA). Data acquisition and control were carried out using Empower 3 software (Waters, Germany).

Chromatographic conditions

The HPLC analytical experiments were run on C18 column (Waters × Bridge, 4.6 × 150 mm, 5 µm). The flow rate was 1 ml/min. The injection volume was 10 µl of 1 mg/ml of the dried bark extract. Analysis was performed at room temperature. The mobile phase consisted of water and acetonitrile solvents in the gradient mode. The linear gradient used was started with 98% water for 2 min and then raised linearly to 100% acetonitrile in 20 min. Before analysis, the column was equilibrated with the starting mixture for 15 min. The PDA scans a range of wavelengths from 210 to 400 nm. Wavelengths of 220 and 275 nm were extracted for peaks comparison.

RESULTS

Results of infusions and dilutions made in distilled water (method-A) are shown in Figure 1. Each absorption value is the average of 32 experiments. Figure 2 shows the antimalarial activity of lyophilized cinnamon water infusion (Method-A). Each absorption value is the average of 24 experiments. A comparison between cinnamon bark water decoction traditional method (Method-B1) and cinnamon bark water infusion (Method-B2) is shown in Figure 3. Each result represents the average of 8 individual experiments. Figure 4 represents a comparison between 20 and 35% ethanol extract of cinnamon powdered bark.Each result represents the average of 8 individual experiments.

At this stage of the study, we tried to learn more about the crude water-soluble peaks profile, numbers, retention times as well as their corresponding UV-Vis spectra using HPLC-PDA. Infusion of cinnamon powdered bark (extraction using method-A) was dissolved in pure water, filtered and injected freshly to the HPLC-PDA. Figure 5 shows the peaks at 220 nm and 275 nm, respectively.

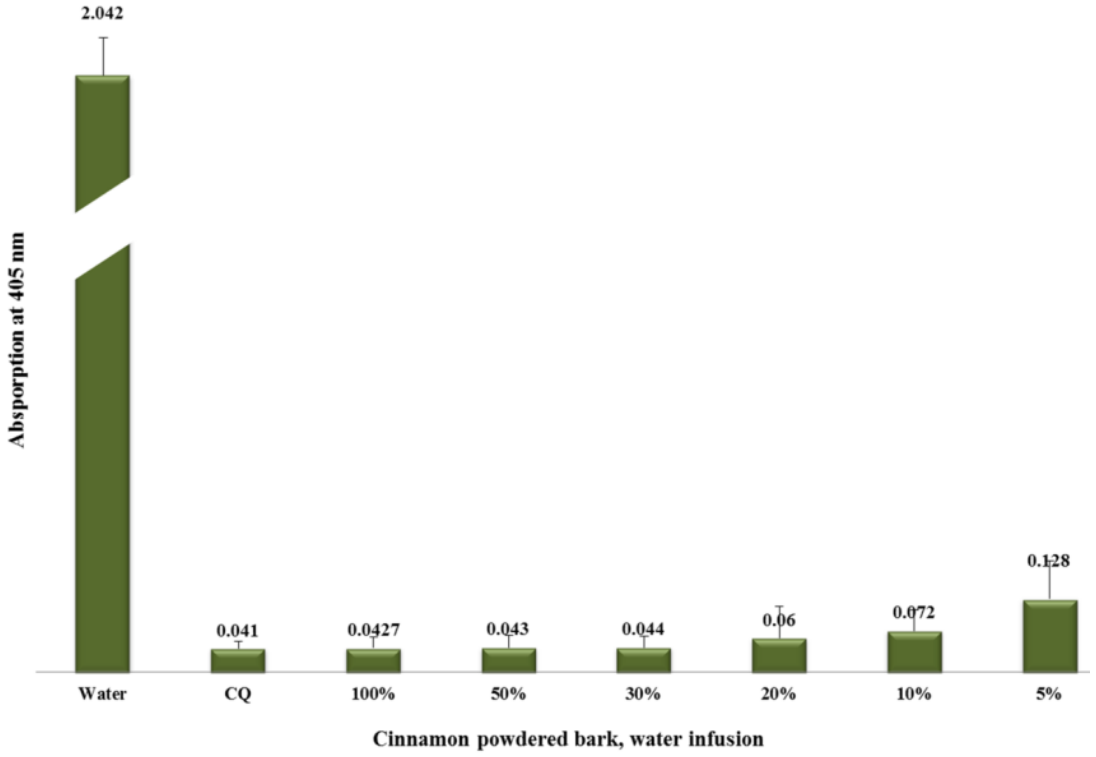

Figure 2. Column diagram representing the efficacy of lyophilized cinnamon powdered bark water infusion using method A, compared to the negative control; ultra-pure H_2O and positive controls: CQ 0.1 mg/ml and 2-MP (2-mercaptopyrimidine) 1 mg/ml, showing the absorption values of dissolved β-hematin (alkaline hematin) at 405 nm using ELISA reader.

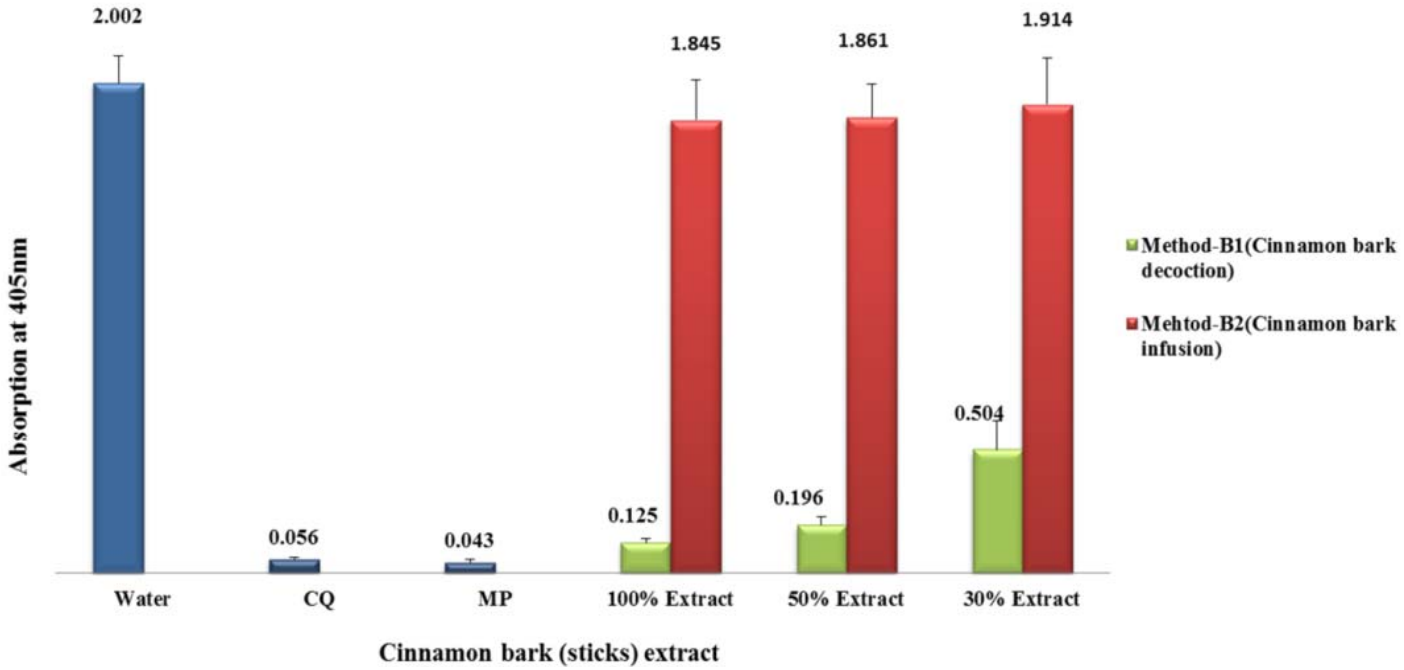

Figure 3. Column diagram representing a comparison of the efficacy between cinnamon water decoction [method-B1] and infusion [method-B2], compared to the negative control; ultra-pure H_2O and positive controls: CQ 0.1 mg/ml and 2-MP (2-mercaptopyrimidine) 1 mg/ml, showing the absorption values of dissolved β-hematin (alkaline hematin) at 405 nm using ELISA reader.

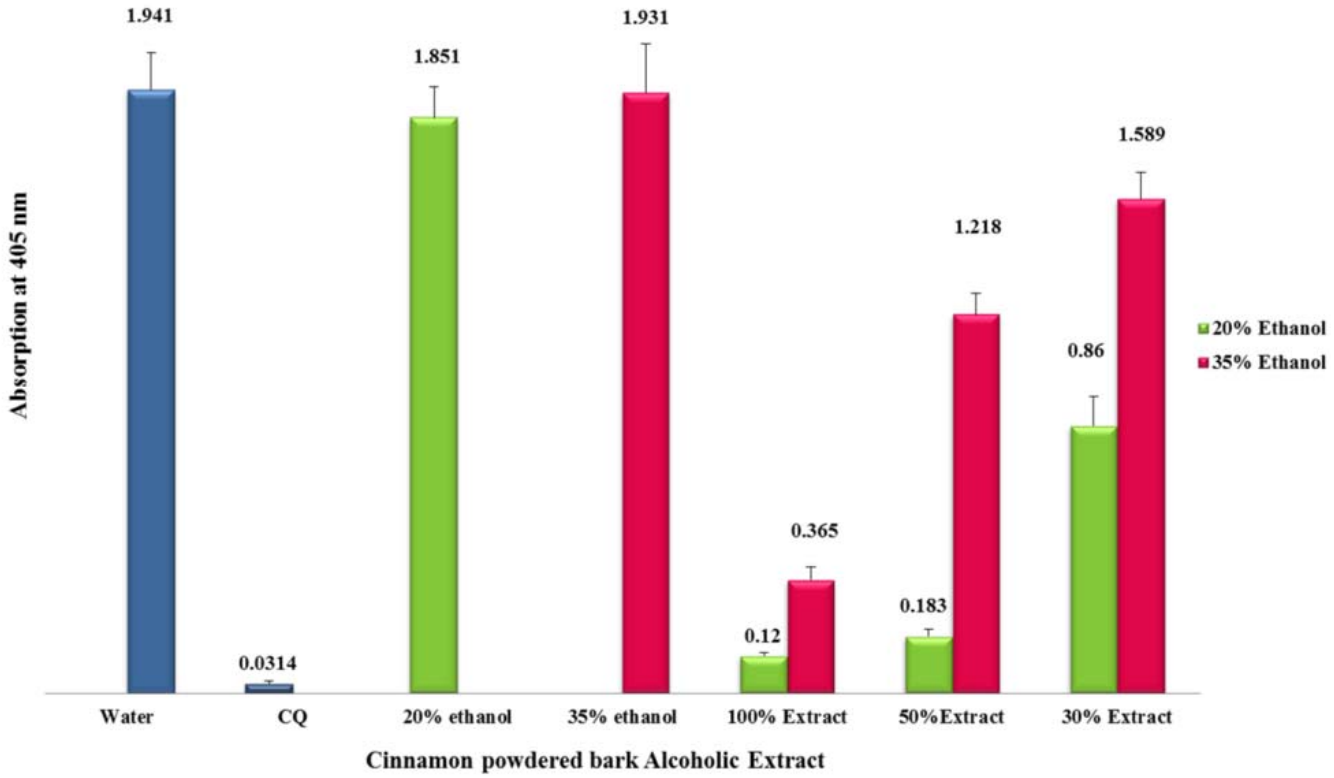

Figure 4. Column diagram representing a comparison of the efficacy between 20 and 35% ethanol extract of cinnamon powdered bark [method-C], compared to the negative control ultra-pure H_2O and positive controls: CQ 0.1 mg/ml, showing the absorption values of dissolved β-hematin (alkaline hematin) at 405 nm using ELISA reader.

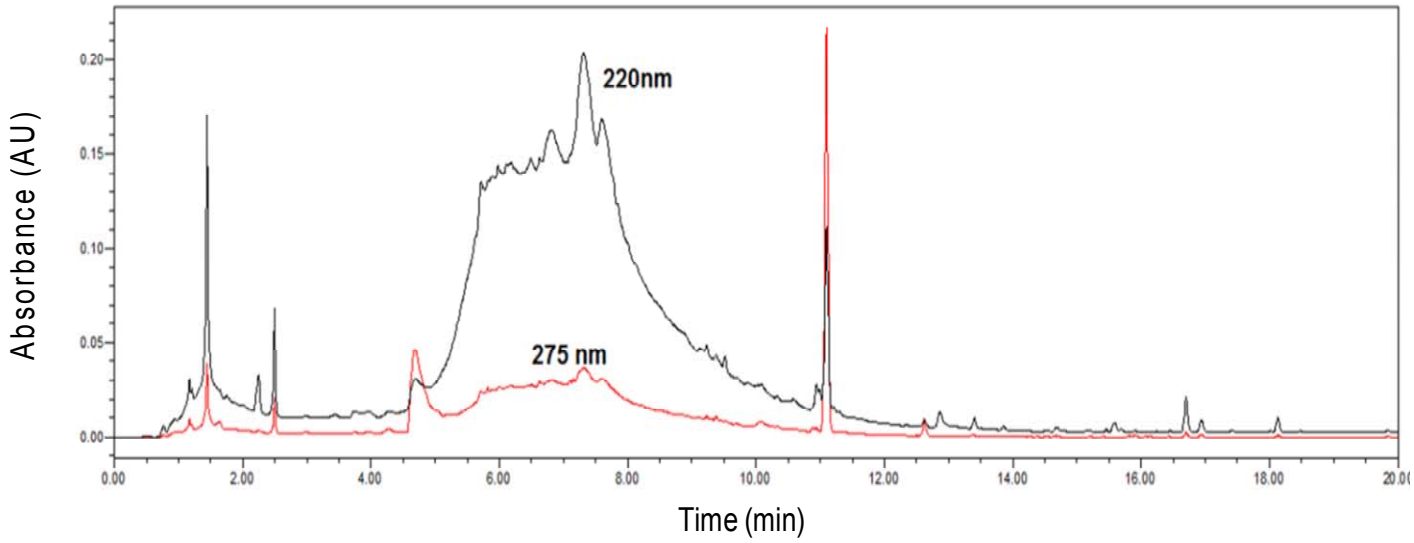

Figure 5. HPLC-PDA peak profiles of crude cinnamon at two wavelengths, 220 and 275 nm, respectively.

Figure 6 shows the UV-Vis absorption spectrum of the major peak that exhibit a maximum wavelength of 275.8 nm and 309. 1 nm, respectively. Using PDA, the scanned UV-Visible spectra from 210-400 nm of the major peaks exist in the crude water extract is shown in Figure 7.

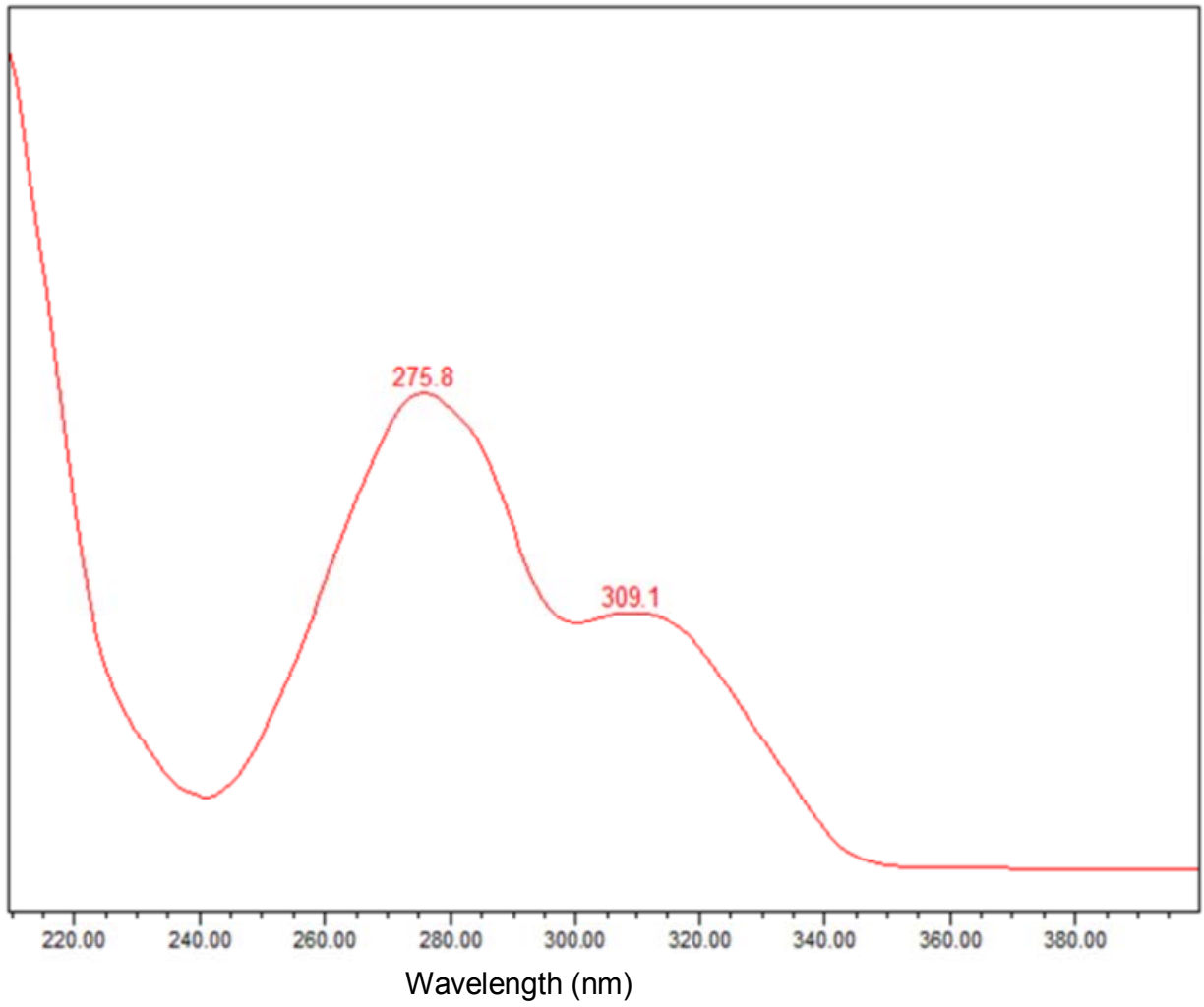

Figure 6. UV-Visible spectrum of main peak cinnamic acid present in crude cinnamon eluted at 11.1 min.

DISCUSSION

The results acquired were compared to negative ultra-pure H_2O and positive chloroquine (CQ) of 0.1 mg/ml and 2-mercaptopyrimidine (2-MP) of 1 mg/ml controls. 2-MP is considered as an internal control to ensure that the reaction environment was ideal, that is, due to its sensitivity to the change in temperature. The absorption is inversely proportional to drug efficiency meaning the lower the absorption, the more efficient the drug is.

The boiled extract of Method-B1 was found to be more effective than that of Method-B2 as shown in Figure 3, because more therapeutic compound is expected to be released when decoction is performed.

Cinnamon powdered bark water infusion provides better results than cinnamon bark sticks, probably due to its higher surface area and small volume that allow its active ingredients to dissolve better.

From Figure 4, it is evident that the extract acts more efficiently in the 20% alcoholic extract rather than the 35% alcoholic extract. This observation probably indicates the capability of water to extract efficient compounds by using this ratio.

Inhibiting heme polymerization “β-hematin formation” of different antimalarial drugs, such as chloroquine through complex formation of with ferriheme monomers has been studied by different spectroscopic methods (Dorn et al., 1995; Blauer et al., 1982).

The activity of cinnamon water infusions is thought to be by inhibition of β-hematin formation which is probably through formation of a complex between ferriheme and the reactive groups of compounds found in the cinnamon infusion. Different non-covalent interactions may be responsible for this inhibition. For example, the possibility of forming hydrogen bonding between propionic acid in ferriheme and corresponding hydrogen-bonding groups found of the active compounds in the cinnamon infusion, carbonyl groups, as an example. In addition, π-π electron

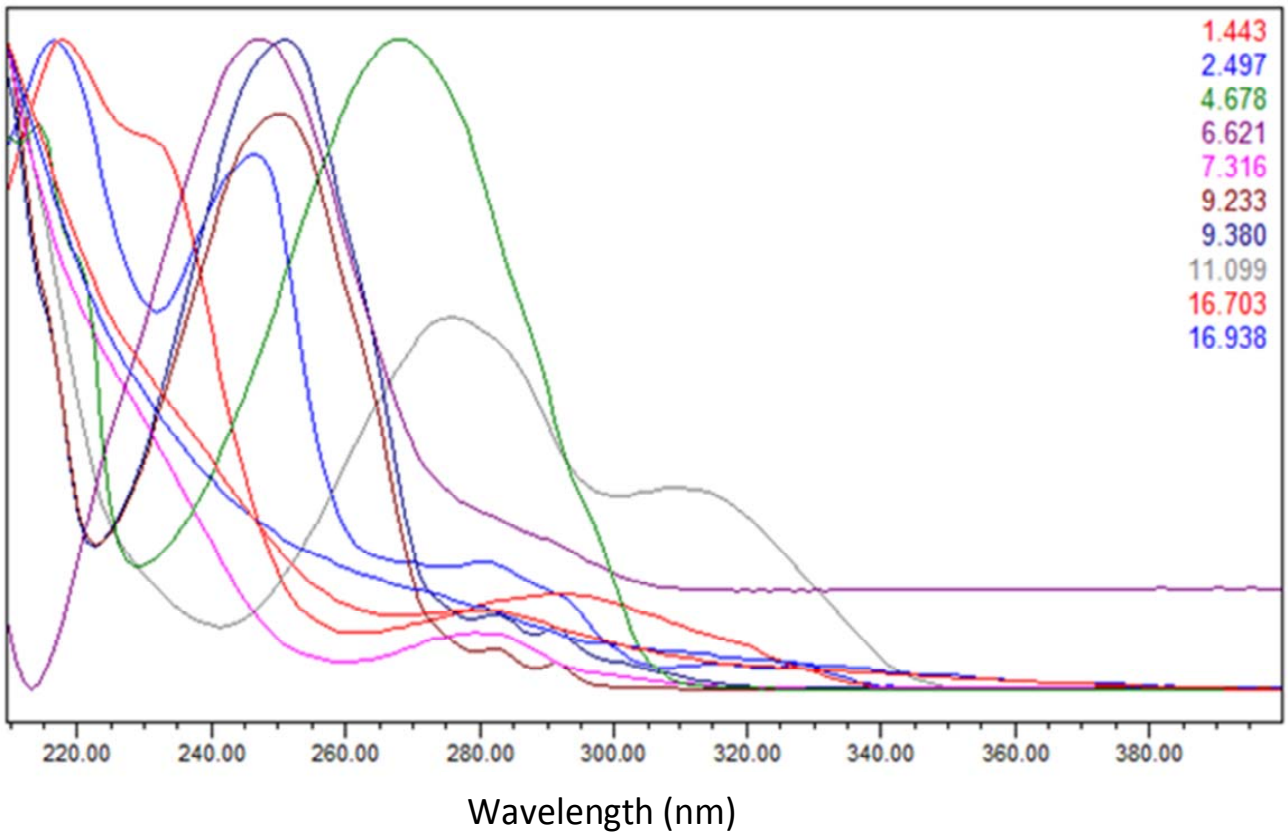

Figure 7. Overlaid other major UV-Visible spectra of the peaks present in crude water cinnamon extract. The colored numbers at the right corner represent the retention time of each eluted peak.

interactions between proximate heterocyclic and aromatic moieties of the complex components could add to the stability of the complex.

In the literature, the bark of various cinnamon species were investigated using GC-MS for the most important constituents of cinnamon essential oil, which contribute to the fragrance and some biological activities observed in cinnamon oil. Among these are *trans*-cinnamaldehyde (65 to 80%), eugenol (5 to 10%) and some other compounds, which present to a lesser extent, such as cinnamic acid, p-methoxycinnamaldehyde, hydroxyl cinnamaldehyde, cinnamyl alcohol, cinnamyl acetate, coumarin, α-muurolene and borneal (Rao and Gan, 2014; Kamaliroosta et al., 2012). The water extracts however, revealed more phenolic glycosides and cinnacassosides constituents (Luo et al., 2013).

From the results obtained after performing HPLC-PDA for cinnamon powdered bark infusion (extraction using method-A), Figure 5, and at 220 nm, the chromatogram manifested more peaks although both indicated a major one at about 11.1 min. While Figure 6 shows the UV-Vis absorption spectrum of the major peak that exhibit a maximum wavelength of 275.8 and 309.1 nm, respectively similar to typical cinnamic acid, a soluble compound in water. Other peaks eluted between 12 and 19 min gave UV-Vis maxima at 287, 307, 309 and 337 nm. Moreover, a bunch of hydrophilic compounds were eluted in close proximity and overlapped between the ranges of 5 to 9 min (Figures 5 and 7). Longer and slower gradient elution in future investigation would show better resolution of this region. Currently, this study is focusing on the preparative HPLC separation of the major peaks to characterize their structures.

Conclusion

Due to the emergence of antimalarial-resistant strains of *P. falciparum*, there is an urgent need to search for new, safe, effective, and cheaply available anti-malarial drugs from natural products. Using the ferriprotoporphyrin bio-crystallization inhibition test, this study revealed cinnamon's crude water soluble extracts ability to inhibit β-hematin formation *in vitro*. Therefore, this endeavor must be given more attention to identify its active β-hematin agent.

Fractionation, purification and identification of the possible potential active ingredients as well as *in vitro*

testing are currently under investigation.

Conflicts of interest

The authors have none to declare.

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