Preparative HPLC fractionation of *Cinnamomum cassia* Water Extract and their *in-vitro* Antimalarial Activities

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**ABSTRACT**

Due to the development of antimalarial resistance strains of *Plasmodium falciparum*, the search for new antimalarial drugs is immediately needed. Such drugs might work by obstructing the heme detoxification pathway in the malaria parasite, a vital requirement for parasite existence in host erythrocytes. The present study is aimed to investigate the antimalarial activity of four preparative HPLC fractions from *Cinnamomum cassia* water extract using semi-quantitative *in vitro* micro-assay that is based on the inhibition of ferriprotoporphyrin IX (FP) biomineralization. A reversed phase C18 inch preparative column and water-acetonitrile binary solvent mixture mobile phase were used to collect the fractions at 275 nm. UHPLC-MS revealed that the major compounds in water crude *Cinnamomum cassia* extract are coumarin and cinnamic acid. Containing coumarin as a major compound, fraction 2 showed superior efficacy compared to chloroquine and 2-mercaptopyrimidine positive controls. Fraction 3 which contains cinnamic acid showed moderate activity, while fraction 4 was the least potent.

**INTRODUCTION**

Malaria is still considered to be a serious public health concern in tropical and subtropical areas (WHO, 2015). It is caused by the transmission of the plasmodium parasite through the bite of infected anopheles mosquitoes. Humans can be infected by five species of plasmodium genera, namely, *P. falciparum*, *P. vivax*, *P. ovale*, *P. malariae* and *P. knowlesi*. However, infections caused by *P. falciparum* species are the most fatal (Rathore, 2006). In 2015, 95 countries and territories had ongoing malaria transmission (WHO Fact sheet, 2016). Despite the progress toward minimizing malaria cases and deaths, it remains a cause of severe morbidity and mortality (WHO, 2015).

The situation has been further deteriorated particularly in Asia, Africa and South America due to the emergence of resistant Plasmodium strains to almost all available drugs, specifically chloroquine, amodiaquine, sulphadoxine-pyrimethamine, artemisinin and its derivatives. Consequently, searching for new alternative, effective leads and eventually affordable novel new drugs from medicinal plants origin is highly demanded. Cinnamon is one of the well-known and widely used spices which has gained recent attention due to its important medicinal traits. Among the numerous increased health benefits reported on cinnamon are antimicrobial (Matan et al., 2006), antifungal (Wang et al., 2005), antitermitic (Tung et al., 2006), antifungal (Wang et al., 2005), insecticidal (Cheng et al., 2009), anti-inflammatory (Tung et al., 2008), antioxidant (Mathew and Abraham, 2006), antiulcerogenic (Shiraga et al., 1988), anti-diabetic (Kim et al., 2006) and anticancer activities (Lu et al., 2010). Moreover, it has been reported to have an influence on neurological disorders such as Parkinson’s and Alzheimer’s diseases (Petersona et al., 2009).

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Further studies showed that it reduces the risk of colon cancer (Wondrak et al., 2010), increases blood circulation in the uterus and improve tissue regeneration (Minich and Msom, 2008), treats toothaches and removes foul breath (Gupta et al., 2011; Jakheta et al., 2010). In our previous work on Cinnamomum cassia, it was found that powdered bark water infusion exhibit effective inhibitory effects on heme bio-crystallization, obstructing hemozoin (malaria pigment) formation (Attieh et al., 2015). The current work however is aimed to collect preparative HPLC fractions using reversed phase inch column and to explore their effect on hemozoin formation.

MATERIALS AND METHODS

Materials

Dimethyl sulfoxide (DMSO), chloroquine diphosphate salt, sodium acetate, coumarin, cinnamic acid, glacial acetic acid and hemin chloride were procured from Sigma-Aldrich, Israel. Cinnamon (Cinnamomum cassia) used was from a local Palestinian market. All the research has been conducted at the Faculty of Science & Technology and the Faculty of Pharmacy in Al-Quds University, 2016.

Preparation of plant extract

Two grams of Cinnamomum cassia fine-powdered bark was soaked in 150 ml of distilled hot water (at 90°C), left for 30 minutes at room temperature and then filtered using MN 615.0110 mm filter paper. The resultant extract was evaporated using IKA WEREK RV06-ML rotary evaporator at 70°C under reduced pressure. Then, it was lyophilized using Labconco freeze drier (USA), until constant weight was achieved. The final dried extract was stored in an amber bottle and kept in the fridge until fractionated by preparative HPLC (Akkawi et al. 2014).

In-vitro semi-quantitative test for screening of anti-malarial activity

As per the protocol described by Deharo (Deharo et al., 2002), a mixture containing 50 μl of 0.5 mg/ml haemin chloride (freshly dissolved in DMSO), 100 μl of 0.5 M sodium acetate buffer (pH 4.4) and 50 μl of the tested anti-malarial drug solution or control, was incubated in a normal non-sterile 96-well flat bottom plate at 37°C for 18-24 hours. It is imperative 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 and 5.2. The wells were washed with 200 μl DMSO per well to remove free haemin chloride. The plate was centrifuged again, discharging the supernatant afterwards. The β-haematin remaining was then dissolved in 200 μl of 0.1 M NaOH to form an FP that could be measured spectrophotometrically. Finally, the absorbance was determined at 405 nm using an ELISA reader (Stat Fax-2100). Ultrapure water was used as negative control, whereas chloroquine dissolved in ultrapure water was used as positive control.

Chromatographic analysis

Analytical HPLC, Preparative HPLC and UHPLC-MS Instruments

Analytical samples were analyzed using HPLC of Waters Alliance (e2695 separations module), which is equipped with 2998 Photo diode Array (PDA). Data acquisition and control were carried out using Empower 3 chromatography data software (Waters, Germany). The Preparative High Pressure Liquid Chromatography (Prep-HPLC) system consisted of 3535 quaternary gradient module equipped with 996 PDA detector. The UHPLC chromatography was performed under reverse phase conditions using a TSQ Quantum Access MAX (Thermo Scientific, San Jose, CA, USA) which includes a Dionex Pump with a degasser module, an Accela PDA detector and an Accela Autosampler. The chromatographic separations were performed on a Kinetex™ column (C8, 2.6 μm particle size, 100 Å pore size, 100 x 2.1 mm) from Phenomenex, USA. A UHPLC SecurityGuard™ cartridge (C8, for 2.1 mm ID column) was from Phenomenex, USA. The injection volume was 10 μL, the oven temperature was maintained at 35°C (Attieh et al., 2015; Kang et al., 2014).

Chromatographic conditions

Crude Cinnamomum cassia from pure water was run on reversed phase ODS column of Waters (XBridge, 4.6 ID x 150 mm, 5 μm) with a guard column of XBridge ODS (20 mm x 4.6mm ID, 5 μm). The mobile phase consisted of a binary solvent mixture of 0.5% acetic acid solution (A) and acetonitrile (B) in linear gradient mode. A 100% of solvent A was initially used and then descended to 70% A in 40 minutes, then to 40% A in 20 minutes, finally to 10% A in 2 minutes and remained there for 6 minutes and then back to the initial conditions in 2 minutes. The HPLC system was equilibrated for 5 minutes with the initial acidic water mobile phase (100 % A) before injecting the next sample. All the samples were filtered with a 0.45 μm PTFE filter. The PDA wavelengths range was from 210-500 nm. The flow rate was 1 ml/min. Injection volume was 10 μl and the column temperature was at room temperature. The preparative HPLC experiments were run on ODS column (Agilent PrepHT C18, 22.2 x 250 mm, 10 μm).

The gradient program started from 95% water, 5% acetonitrile to 100 % acetonitrile in 17 minutes and back to the starting mobile phase composition in 4 minutes. The flow rate was 12 ml/minutes, the injection volume was 1000 micro l, the column temperature was set at room temperature and the PDA wavelengths ranged from 200-450 nm (Attieh et al., 2015; Kang et al., 2014).

The UHPLC-MS chromatographic separation was achieved using a linear gradient program at a constant flow rate of 0.4 mL/min over a total run time of 70 min. The mobile phase gradient program was the same as the HPLC except for the use of 0.1% formic acid instead of acetic acid. Samples were detected by a TSQ Quantum Access Max mass spectrometer in positive and
negative ion mode using Electron Spray ionization (ESI) and full scan acquisition. Air was produced (SF 2 FF compressor, Atlas Copco, Belgium). Purified nitrogen was used as source and exhaust gases.

**Cinnamon preparative sample preparation**

When 500 mg of crude *Cinnamomum cassia* was dissolved in 5 ml water, the solution turned turbid but when 5 ml of ethanol was added a clear solution was obtained which was directly filtered through PTFE 0.45 μm membrane filter before injection. One ml of the clear solution was directly injected to the preparative HPLC and four fractions were collected. Fraction 1 was from 0-6.21 minutes, fraction 2 was from 6.21-14.20 minutes, fraction 3 was from 14.20-14.80 minutes and finally fraction 4 was from 14.8-22 minutes, the end of the run.

**RESULTS AND DISCUSSION**

*Cinnamomum cassia* consists of a broad range of compounds such as cinnamaldehyde, cinnamic acid, cinnamyl alcohol, cinnamyl acetate, eugenol and to a large extent of coumarin (Wang et al. 2013). Some of these compounds, particularly the volatile ones, can be separated and determined using GC-MS. High Performance Liquid Chromatography (HPLC) however is better technique to separate the more polar compounds present in *Cinnamomum cassia* which dissolves in water.

Herbal therapies of *Cinnamomum cassia* are usually prepared for diabetic patients by infusion of the powdered bark part of the plant. The dissolved active and inactive ingredients alike are then released into the water solvent. The crude lyophilized *Cinnamomum cassia* extract was injected into an analytical HPLC chromatograph in the reversed phase mode.

Typical chromatogram of the crude extract at 275 nm is shown in Figure 1. Extended gradient conditions were intentionally used in order to separate all the compounds present in *Cinnamomum cassia* extract at a satisfactory resolution.

Two major slightly soluble compounds were eluted at 27.5 and 36.2 minutes while five other minor compounds were eluted earlier (Figure 1). Standard cinnamic acid which is known to present in *Cinnamomum cassia* was injected and eluted at 36.2 minutes. Its UV-Vis spectrum exactly matched the peak eluted at 36.2 minutes from cinnamon (Figure 2). The other peaks present in *Cinnamomum cassia* water extract and their corresponding overlaid UV-Vis profile from 210-500 nm is depicted in Figure 1.

The crude water extract of *Cinnamomum cassia* was also analyzed by ultra-performance liquid chromatography coupled to photodiode array and mass spectrometry (UHPLC-PDA-MS). A full scanned MS in the positive ESI mode revealed the identity of the peak eluted at 27.5 minutes to be coumarin (eluted at 17 minutes using the UHPLC-MS mobile phase conditions). The protonated m/z [M+H]^+ ion shows peak at 147.31 Da, indicating coumarin presence (Figure 3). In general, coumarins are known to have anti-malarial properties (Cubukcu et al., 1990 and Noster et al., 1990).

Preparative HPLC-PDA was utilized in the reversed phase mode using an inch column in order to collect four fractions (Figure 4). Ten successive injections of a total of 500 mg concentration were completed. At 275 nm, fraction 1 mainly showed a major peak at about 2.1 minutes while fraction 2 contained an overlapped mixture of peaks including coumarin, apparently because of the concentration overloading conditions. Fraction 3 however includes almost pure cinnamic acid and fraction 4 only showed a small peak at 16 minutes (Figure 4).

![Fig. 1: HPLC-PDA chromatogram of all the peaks present in crude water extract of Cinnamon at 275 nm & their overlaid UV-Vis spectra between 210-500 nm.](image-url)
Fig. 2: Overlaid HPLC-PDA chromatogram of crude water extract of Cinnamon (blue) and cinnamic acid standard (black) dissolved in 50% ethanol at 275 nm. The overlaid spectra of standard cinnamic acid with the peak from Cinnamon is depicted at the right corner.

Fig. 3: UHPLC-PDA of the Cinnamon water extract at 272 nm (A). The extracted ion chromatogram in the positive ESI mode of the 17.7 minutes peak (B), shows protonated coumarin at m/z of [M+H]^+ of 147.31 Da.
Using the ferriprotoporphyrin bio-mineralisation inhibition test, this study compared the inhibitory effect of four preparative HPLC fractions of water extract on beta-hematin formation in a cell-free system. The mechanism of inhibition is probably through formation of a complex between active ingredients in these extracts and ferriheme; this complex prevents the formation of beta-hematin. Several heme-binding anti-malarial compounds, such as chloroquine, efficiently inhibit this process, and this is believed to be the predominant mechanism by which these drugs work as antimalarials (Akkawi et al. 2014; Attieh et al., 2015). All the fractions were subjected to in-vitro assays. Fraction 2 which contains coumarin as a major compound appeared to be the most effective in inhibiting the formation of beta-hematin. Fraction 2 consists of a mixture of compounds that were clearly seen in the analytical HPLC profile (Figure 1). In the preparative chromatogram however, these compounds were eluted together preceding fraction 3 which is almost pure cinnamic acid compound. Three preparative HPLC fractions out of four from the water extracts of Cinnamomum cassia bark were shown to possess in-vitro antimalarial activities (Figure 5). Results obtained were compared to positive (chloroquine (CQ) of 0.1 mg/ml & MP 1 mg/ml), showing the absorption values of dissolved β-hematin (alkaline hematin) at 405 nm using ELISA reader as per Deharo et al. semi-quantitative method. Each result represents an average of 16 individual experiments.

Fig. 4: Typical preparative RP-HPLC-PDA chromatogram of water extract of Cinnamon. 1 ml was injected at flow rate of 12 ml/min at 275 nm. Fraction 1, from 1-6 minutes, fraction 2 from 6-14 minutes, fraction 3 from 14-15 minutes and fraction 4 was from 15 to the end of the elution 22 minutes.

Fig. 5: Efficiency of the four fractions obtained from preparative HPLC separation of Cinnamon water extract, dissolved in water at concentration of 1 mg/ml, compared to negative control (water) and positive controls (CQ-chloroquine 0.1 mg/ml & MP 1 mg/ml), showing the absorption values of dissolved β-hematin (alkaline hematin) at 405 nm using ELISA reader as per Deharo et al. semi-quantitative method. Each result represents an average of 16 individual experiments.
towards hemozoin formation. Figure 5 showed that the most active fraction is number 2 that contains mainly coumarin and the least is fraction 4. Fraction 3 which contains cinnamic acid is fairly active. Previous studies have shown that cinnamic acid derivatives have an effect on the in vitro growth of Plasmodium falciparum and on the permeability of the membrane of malaria-infected erythrocytes (Kanaani and Ginsburg, 1992). Coumarins also have been found to exhibit anti-malarial activity (Cubukcu et al., 1990 and Noster et al., 1990). The in-vitro activity guided preparative HPLC fractions of Cinnamomum cassia results support these findings. The water extracts of Cinnamomum cassia revealed potential activity even at low concentration of infusions and decocation (Attieh et al., 2015). However, fraction 2 of the current study even showed superior activity in comparison to the crude extract as manifested by a high capability to inhibit β-hematin formation in vitro.

CONCLUSION

Cinnamomum cassia consists of numerous active water soluble compounds which have significant influence in inhibition of hemozoin formation. Water crude extract contains two major compounds, coumarin and cinnamic acid. Coumarin as a major compound in water Cinnamomum cassia extract showed the best antimalarial effect. Cinnamic acid which is also abundant in Cinnamomum cassia presented a moderate antimalarial effect.

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