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The Potential of Green as well as Roasted Coffee Water Extracts as *in-vitro* Inhibitors of Beta-Hematin Formation

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Abstract. This study investigates the antimalarial potential of coffee extracts exploring their relationship with roasting, absorption, effectiveness, and hemozoin production, while also identifying flavonoids and phenolic compounds. Research on the antimalarial properties of coffee extracts is crucial for developing new therapies. Coffee extracts could provide a natural and accessible source and understanding the effects of the roasting process can optimize their efficacy. Water extracts were obtained from both green and roasted coffee beans subjected to varying roasting times.

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The effectiveness of the extracts was measured by the absorption of dissolved β- hematin at a wavelength of 405 nm. Chromatographic analysis using high-performance liquid chromatography (HPLC) was employed to separate and detect flavonoids and phenolic compounds within the extracts. Specific compound identification was achieved by comparing retention times and UV spectrum wavelengths of standards and samples. The study found that the absorption of coffee extracts was inversely correlated with their effectiveness, indicating that lower absorption corresponds to higher effectiveness. Green coffee water extracts exhibited limited efficacy, while roasted extracts demonstrated the highest efficacy. Chromatographic analysis identified flavonoids and phenolic compounds. Overall, the study reveals the antimalarial potential of coffee extracts, with extract effectiveness inversely related to absorption and inhibitory effects on hemozoin production. Chrysin as well as Galangin were identified as key constituents, highlighting their potential in antimalarial therapies. Further research is needed to understand their mechanisms of action.

Resumen. Este estudio se investigó el potencial antipalúdico de los extractos de café, explorando su relación con el tostado, la absorción, la eficacia y la producción de hemozoína, a la vez que se identificaron flavonoides y compuestos fenólicos. La investigación sobre las propiedades antipalúdicas de los extractos de café es crucial para el desarrollo de nuevas terapias. Los extractos de café podrían proporcionar una fuente natural y accesible, y comprender los efectos del proceso de tostado puede optimizar su eficacia. Se obtuvieron extractos acuosos de granos de café verde y tostado, sometidos a diferentes tiempos de tostado. La eficacia de los extractos se midió mediante la absorción de β-hematina disuelta a una longitud de onda de 405 nm. Se empleó un análisis cromatográfico mediante cromatografía líquida de alta resolución (HPLC) para separar y detectar flavonoides y compuestos fenólicos en los extractos. La identificación de compuestos específicos se logró comparando los tiempos de retención y las longitudes de onda del espectro UV de los estándares y las muestras. Se determinó que la absorción de los extractos de café está inversamente correlacionada con su eficacia, lo que indica que una menor absorción corresponde a una mayor eficacia. Los extractos de agua de café verde mostraron una eficacia limitada, mientras que los extractos tostados demostraron la mayor eficacia. Mediante el análisis cromatográfico se identificaron flavonoides y compuestos fenólicos. En general, el estudio revela el potencial antipalúdico de los extractos de café, cuya eficacia está inversamente relacionada con la absorción y los efectos inhibidores sobre la producción de hemozoína. La crisina y la galangina se identificaron como componentes clave, lo que destaca su potencial en terapias antipalúdicas. Futuras investigaciones deberán enfocarse en la comprensión de sus mecanismos de acción.

Introduction

Beta-hematin, commonly referred to as hemozoin, is a crystalline compound formed by malaria parasites to neutralize free heme, which is released during hemoglobin breakdown. Since free heme is toxic to the parasite, its conversion into the stable crystalline structure of beta-hematin is essential for survival. This crystallization process has been widely investigated to gain insights into malaria pathogenesis and to explore potential targets for antimalarial treatment [1]. Inhibiting beta-hematin formation has been a key strategy in antimalarial drug development. Quinoline-based drugs, including chloroquine and amodiaquine, disrupt the conversion of heme into beta-hematin, causing toxic free heme to accumulate inside the parasite. Similarly, hematinadducts formed from artemisinin-class drugs through parasite metabolism exert their lethal effects by preventing beta-hematin crystallization, thereby increasing the levels of toxic free hematin [2,3]. This interference induces oxidative stress, ultimately leading to the parasite's death. Recent research has utilized high-throughput screening techniques to discover new inhibitors of beta-hematin formation, broadening the range of potential antimalarial drugs. For example, one study analyzed 144,330 compounds and identified 530 with strong inhibitory effects on beta-hematin crystallization. Nevertheless, there remains a lack of sufficient information on the mechanisms of action of medicinal plants with antimalarial properties [4,5]. Studies on the mechanisms of these inhibitors have shown that they bind to specific beta-hematin crystal surfaces, providing valuable insights for the strategic development of more potent antimalarial drugs.

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The prevalence of malaria is concentrated in developing countries, primarily in Africa, where children under the age of five and pregnant women are especially at risk. In 84 malaria-endemic nations, there were an estimated 247 million cases of malaria in 2021, up from 245 million in 2020, with most of this increase coming from countries in the WHO African Region. The WHO African Region was responsible for about 95% of all cases worldwide, with an estimated 234 million cases in 2021 [5]. Malaria is a deadly disease caused by the bites of infected female *Anopheles mosquitoes*. Generally, it is caused by one of four protozoan parasites of the *Plasmodium genus: P. malaria*, *P. ovale*, *P. vivax* as well as *P. falciparum* [6, 7].

Plasmodium falciparum is considered the deadliest of the human malaria parasites and represents 90% of overall deaths [8]. The parasites enter the human host's liver cells, where they mature further before being released into the bloodstream [9]. During the intra-erythrocytic stage, hemoglobin is digested inside the parasite's food vacuole as a source of amino acids, producing oxygen and an oxidized ferric form of protoporphyrin IX. The eventual result of this consumption is the release of highly reactive ferriprotoporphyrin IX, which can kill the parasites through cellular oxidative stress [8]. This stage is responsible for the disease's clinical symptoms [10]. The parasite avoids heme toxicity by polymerizing heme within the food vacuole at a pH of 4.5 to 5.0, generating hemozoin, or "malaria pigment," a non-toxic, unreactive, insoluble crystalline compound [11]. Hemozoin is a polymer made of dimers of hematin molecules. These dimers are formed through an iron-oxygen coordinate bond that links the central ferric iron of one hematin to the oxygen of the carboxylate side chain of the adjacent hematin, arranged in a crystalline ordered structure through intermolecular hydrogen bonding [7, 8, 12]. The synthetic version of hemozoin is called β-hematin, which has the same linkage between the heme groups as purified hemozoin and is structurally, morphologically, and spectroscopically identical [6-8], making it an excellent target for biochemistry studies [9]. Hemozoin formation is a specialized process utilized by *Plasmodium* species to neutralize free heme toxicity. This process serves as a validated target for many well-established antimalarial drugs and is widely regarded as a promising avenue for developing new antimalarial therapies [13].

Chloroquine, a well-known quinoline derivative, inhibits this process by forming a complex with free hematin molecules and interfering with the formation of β-hematin. Several additional quinolones also prevent free hematin from polymerizing [14]. The emergence of *Plasmodium falciparum* strains resistant to chloroquine and various other medications has sparked efforts to find new antimalarial treatments [15].

In ancient therapies, natural products were utilized. Because they have fewer side effects and complications, researchers have focused more on them in drug development in recent decades. As a result, there has been an increase in medicinal and pharmacological research around the world [16-25].

Coffee is one of the world's most well-liked and commonly consumed beverages, attributed to its enticing flavor and aroma as well as its stimulating effect [26]. Many studies have focused on the benefits of the compounds found in coffee, which are primarily alkaloids, phenolic acids, flavonoids, terpenoids, sterols, and volatile components. These chemicals have a variety of pharmacological effects, including insulin sensitization, improved sugar metabolism, anti-diabetes effects, and liver protection [27]. Besides caffeine, which is known worldwide, coffee contains many other chemical compounds such as trigonelline, chlorogenic acids, vitamin B-3, amino acids, biogenic amines, and diterpenes, totaling about 2000 compounds [28]. These compounds are responsible for their biological activities, including anti-inflammatory, sunscreen, antiviral, anti-aging, anti-cancer, and anti-cellulite effects, as well as strong antioxidants and promising antimicrobial activities [29,30,31]. On the other hand, Arabic coffee may increase the risk of cardiovascular diseases [32]. Roasting is a very important process to ensure good quality coffee brew. Chemistry-wise, roasting is a complex process, given that numerous chemical reactions occur simultaneously [33]. The precise quantities of chemical composition depend on many factors, one of them being how the coffee was roasted. The degree of roasting is influenced by the roasting temperature and time, and these two factors determine whether the coffee is light, medium, or dark roasted in terms of color [32]. Additionally, most chemicals related to coffee's aroma are created during roasting. After roasting, more than 950 different chemicals have been discovered [34]. While natural phenolic components may be lost during the roasting process, other antioxidant chemicals are created, and the biological activity of the coffee is altered [35].

In this study, we aim to investigate the potential antimalarial activity of different water extracts from green and roasted coffee of Palestinian origin using a semi-quantitative in vitro assay that detects the inhibition of β-hematin formation. HPLC analysis of these extracts will also be performed.

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Materials and methods

Collection and extraction

Materials

Chloroquine diphosphate salt, sodium acetate (99 % purity), hemin chloride, as well as dimethyl sulfoxide (DMSO) with 99.5 % purity were all acquired from Sigma-Aldrich. Glacial acetic acid was sourced from Fluka. Solvents containing ethanol (EtOH) were procured from Merck.

Coffee samples

A market in a neighborhood of Jerusalem provided eleven samples of Addani coffee beans. These Yemeni-grown beans, which are sold under the Eden coffee brand, were prepared for investigation. They were ground into a fine powder and then roasted for varying durations, from 0 to 17 hours, at 300 °C.

Extraction of coffee

To make coffee, steep 2 grams of beans in 150 mL of distilled hot water. This combination was held at 90 °C for a 20-minute infusion before cooling to room temperature. After cooling, the solution was filtered through 110 mm MN 615 filter paper to remove any particles. A 50% extract was made by diluting the original extract (1:1 dilution; for example, combining 5.0 mL of extract with 5.0 mL of distilled water to make a total of 10.0 mL), whereas the 100% extract was utilized undiluted. The water was evaporated using a rotary evaporator (IKA WEREKRV06-ML) at 60 to 70°C under reduced pressure to produce dried, concentrated extracts from the infusions. The material was then lyophilized (Labconco freeze dryer) until it reached a constant weight. After being stored in a desiccator, the final dry extract was transferred to opaque vials for HPLC analysis.

Semi-quantitative *in vitro* test to check anti-malarial activity

The experimental procedure entails the following steps, as per the protocol described by Deharo *et al.* [27]: The experimental procedure entails the following steps, as per the protocol described by Deharo *et al.* [27]: 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 potential anti-malarial drug solution or solvent was incubated in a non-sterile 96-well flat-bottom plate at 37 °C for 18-24 hours. The solutions were added to the plate in the above order. The plate was then centrifuged for 10 min at 4000 rpm. The supernatant was removed, and the pH of the reaction was measured. The final pH of the mixture was 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, and the supernatant was discarded. The remaining β-hematin was then dissolved in 200 µL of 0.1 M NaOH to form an alkaline hematin that could be measured spectrophotometrically at 405 nm using an ELISA reader. Ultra-pure water was used as a negative control, while positive controls and the tested extracts were dissolved in ultra-pure water.

Chromatographic evaluation

Experimental

HPLC – PDA detection of phytochemicals

A PDA-detection HPLC system was employed to examine 27 phytochemical compounds, comprising flavonoids as well as phenolic compounds. The investigation was carried out using a gradient elution technique with an RP BDS Hypersil C18 column (Thermo Scientific, 150 x 4.6 mm, 3 µm) at a flow rate of 0.7 mL/minute. Table 1 displays the gradient elution program and the composition of the mobile phase used to identify the primary components. The mobile phase consisted of 1.0% acetic acid (A, HAc) and acetonitrile (B, ACN). The column temperature was maintained at 25 °C, and the PDA detector was set to scan wavelengths from 210 to 400 nm. All samples were filtered using a disposable 0.45 µm filter, and a 20 µL injection volume was employed. To prepare the standards, they were dissolved at a concentration of 25 mg/100 mL in a solvent containing 20 % ethanol. The standard mixture was prepared by adding 1.0 mL of each standard solution to a 25 mL volumetric flask and diluting to volume with the same solvent.

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Table 1. Mobile phase composition

Time	A (1.0 % HAc)	B (ACN)
0	93	07
40	80	20
50	65	35
70	40	60
75	10	90
78	93	07
80	93	07

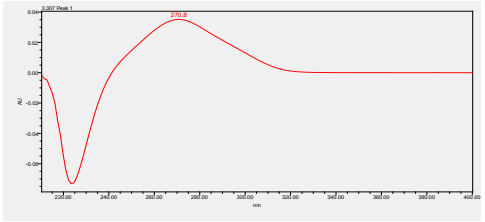
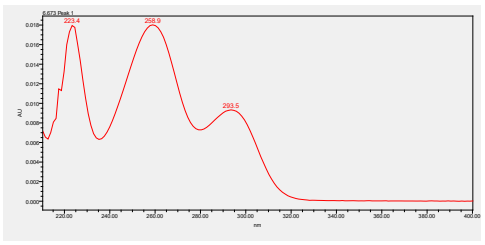
Data analysis and statistical methods

The findings of this investigation are presented as mean values accompanied by their respective standard deviations (SD). Statistical evaluations were conducted using the Statistical Package for the Social Sciences (SPSS), version 16. The threshold for statistical significance was established at a P-value of less than 0.05.

Results

The results of the analysis of polyphenolic compounds are summarized in Table 2. The retention time and UV spectrum for each compound were determined. These results provide valuable information about the elution times and the absorbance characteristics of the polyphenolic standards.

Table 2. Retention time and UV spectrum of the polyphenolic standards separated using the method.

	Name	R.T. (min)	UV scan
1	**Gallic acid**	**3.3**	
2	**3,4-Dihydroxybenzoic acid**	**6.67**	

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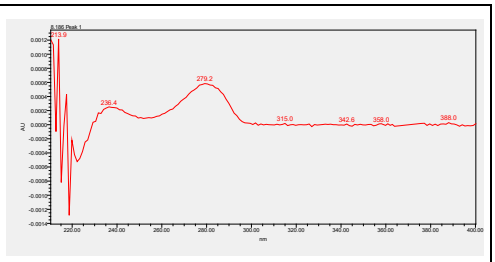
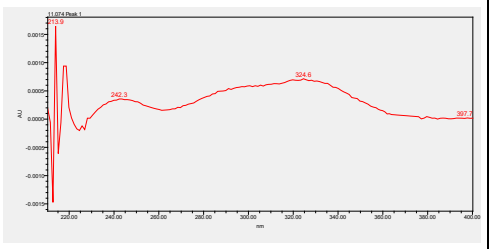
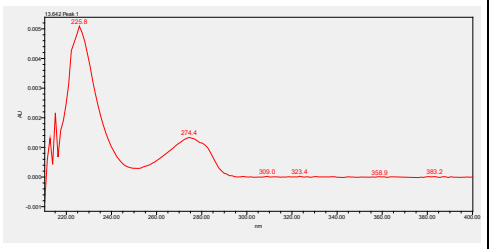
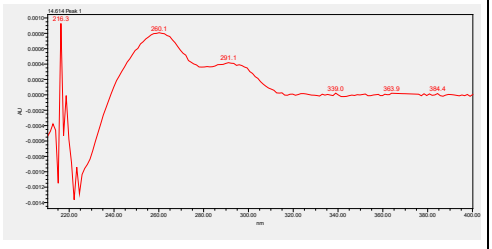
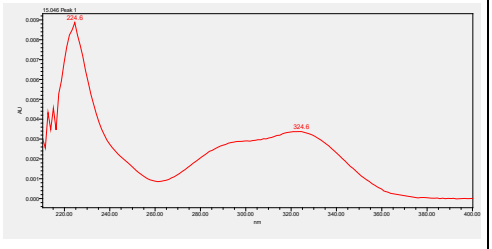
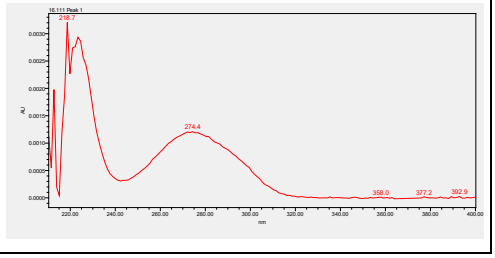
3	**3,4-Dihydroxyphenyl acetic acid**	**8.19**	
4	**Chlorogenic acid**	**11.07**	
5	**4-Hydroxyphenyl acetic acid**	**13.64**	
6	**Vanillic acid**	**14.61**	
7	**Caffeic acid**	**15.05**	
8	**Syringic acid**	**16.11**	

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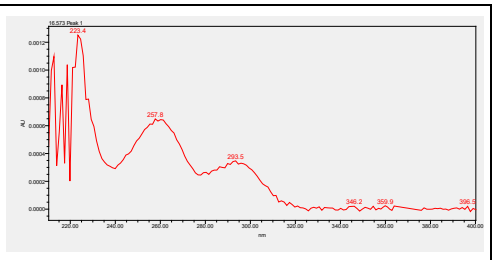
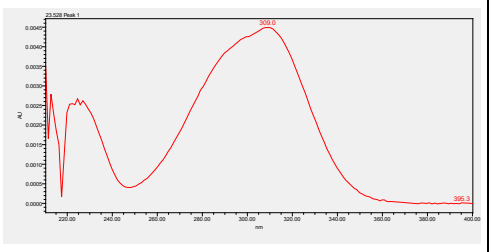
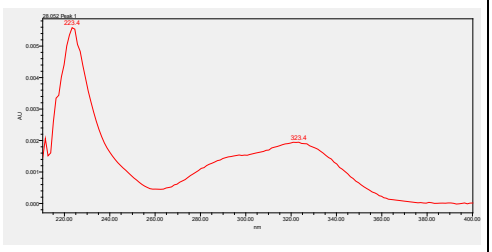
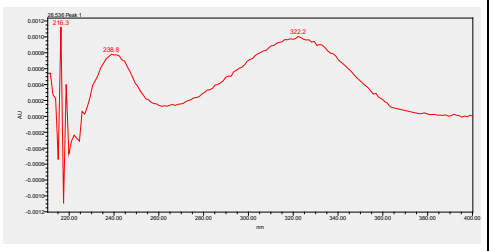
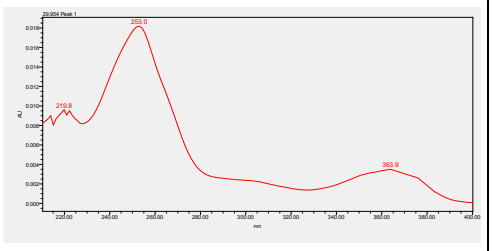
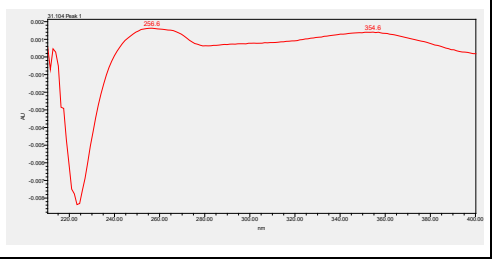
9	**Isovanillic acid**	**16.58**	
10	**p-Coumaric acid**	**23.53**	
11	**Ferulic acid**	**28.05**	
12	**Sinapic acid**	**28.54**	
13	**Ellagic acid**	**29.95**	
14	**Rutin**	**31.10**	

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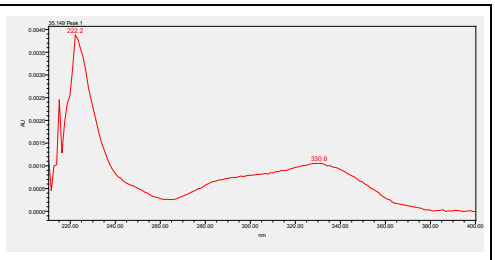
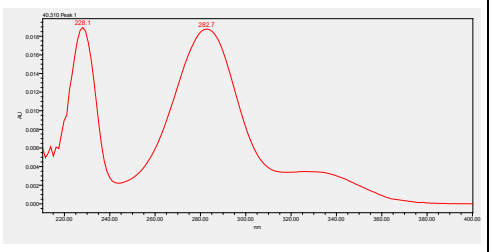
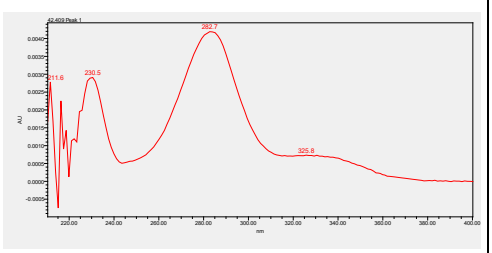
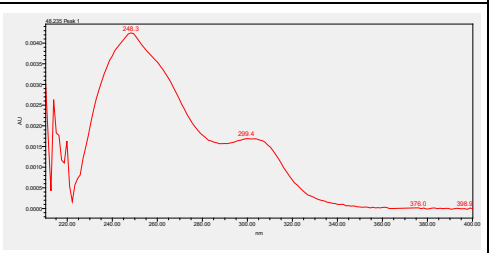
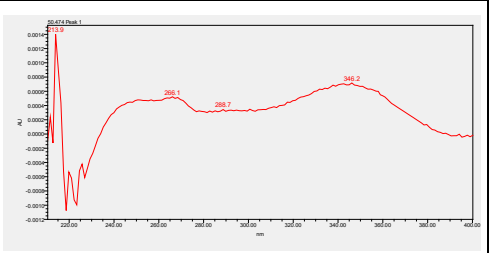
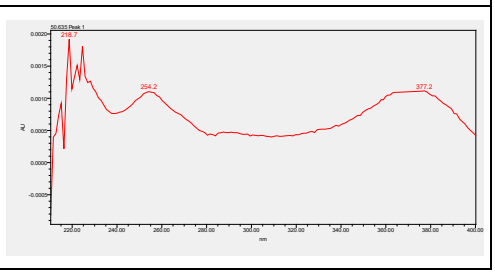
15	**Verbascoside**	**35.15**	
16	**Naringin**	**40.31**	
17	**Hesparidin**	**42.41**	
18	**Daidzein**	**48.23**	
19	**Luteolin**	**50.48**	
20	**Quercetin**	**50.64**	

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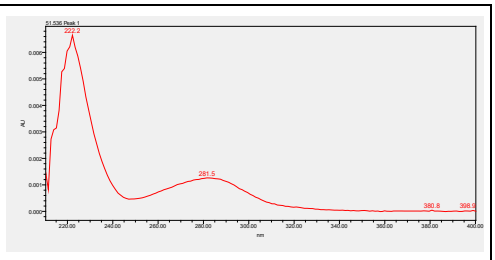
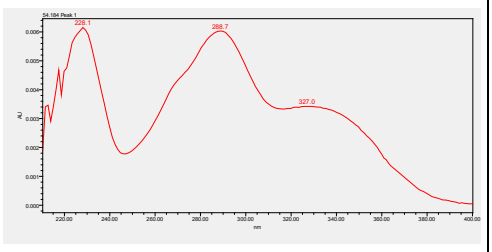
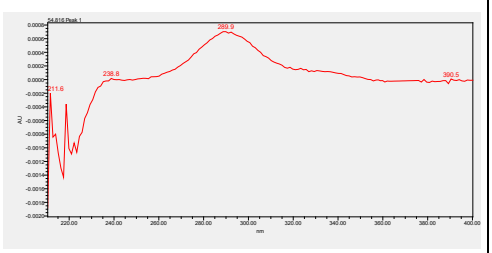
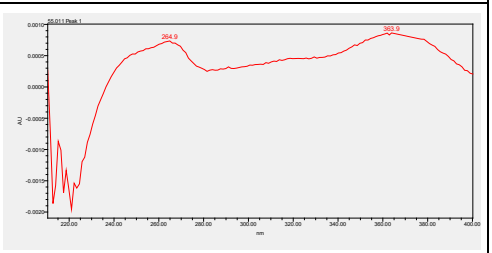
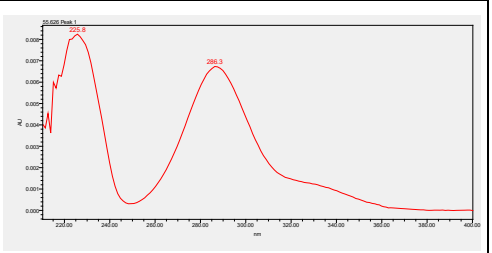
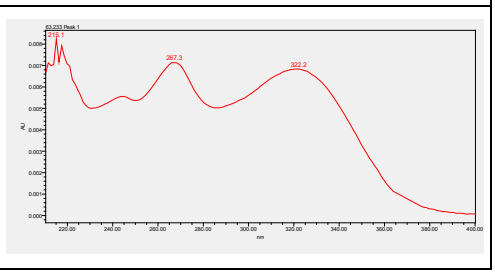
21	**Trans- Cinnamic acid**	**51.53**	
22	**Naringenin**	**54.18**	
23	**Apigenin**	**54.81**	
24	**Kaempferol**	**55.0**	
25	**Hesperitin**	**55.62**	
26	**Chrysin**	**63.23**	

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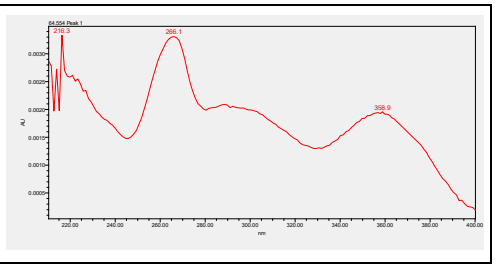
27	**Galagnin**	**64.55**	

Potential anti-malarial activity

Figures 1-3 show the outcomes of semi-quantitative in vitro experiments carried on water extracts of various coffee bean varieties, including both roasted and green (unroasted) beans. The antimalarial efficacy of these extracts is demonstrated in Figure 1 through the measurement of 405 nm absorption of dissolved alkaline β- hematin. The figure compares the absorption values of the positive control (CQ-0.1 mg/mL) as well as the negative control (water) with the extracts of both green as well as roasted coffee. This analysis offers insights into the potential antimalarial properties of the coffee extracts' by comparing them with the control samples.

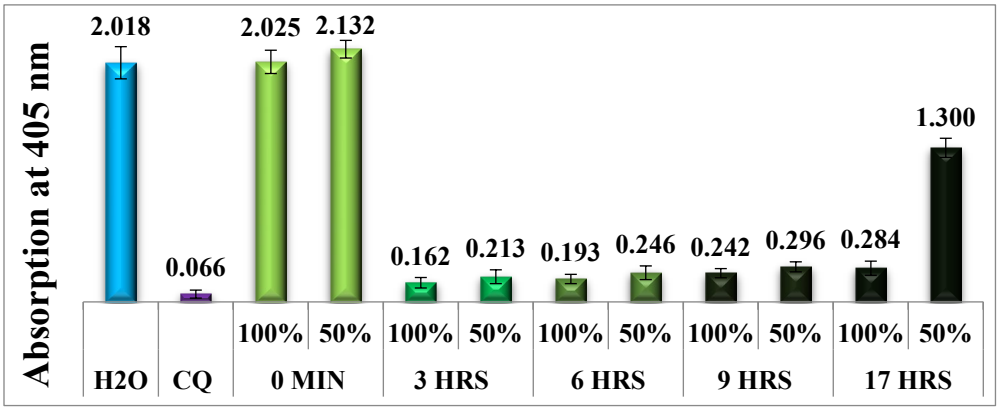


Fig. 1. Column diagrams representing the potential antimalarial efficiency of water extracts of green and roasted coffee beans for different time periods, each result is the average of 16 experiments.

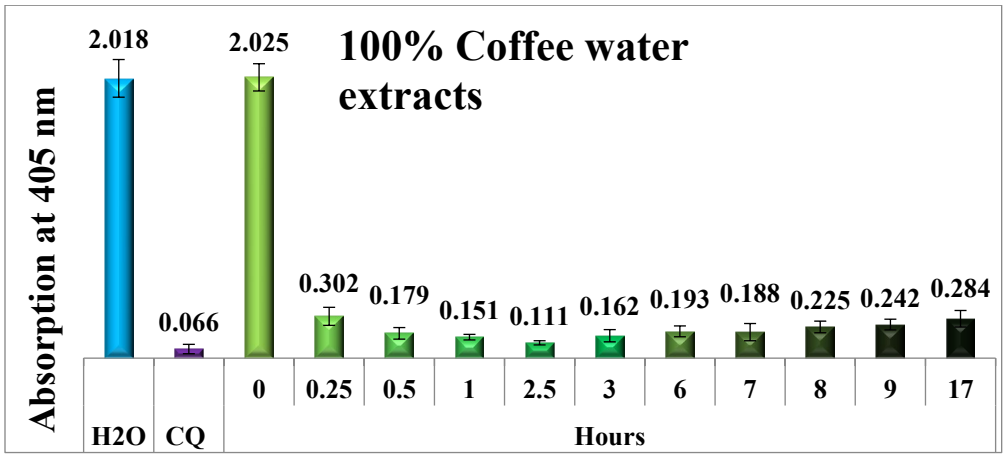


Fig. 2. Column diagrams representing the potential antimalarial efficiency of undiluted (100 %) water extracts of green and roasted coffee beans for different time periods, each result is the average of 16 experiments.

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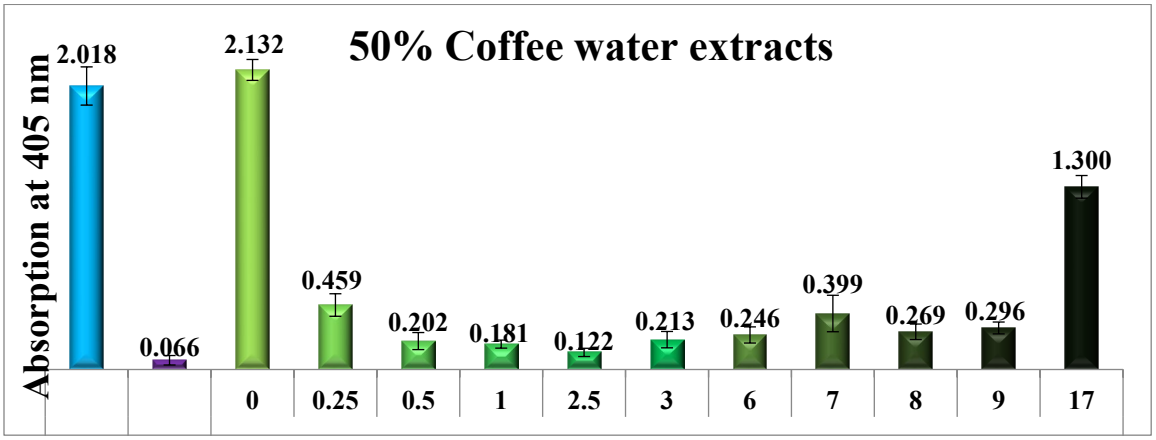

Fig. 3. Column diagrams representing the potential Antimalarial efficiency of diluted (50%) water extracts of green and roasted coffee beans at different time periods, each result is the average of 16 experiments.

HPLC analysis

HPLC chromatogram of standards

The compounds' elution profiles are clearly visible in Figure 4, which displays the chromatogram obtained at 330 nm. The individual standards corresponding to each peak in the chromatogram facilitate the identification and measurement of flavonoids and phenolic compounds present in the sample. The chromatogram helps to better understand and explore the distribution and concentration of these compounds within the tested samples.

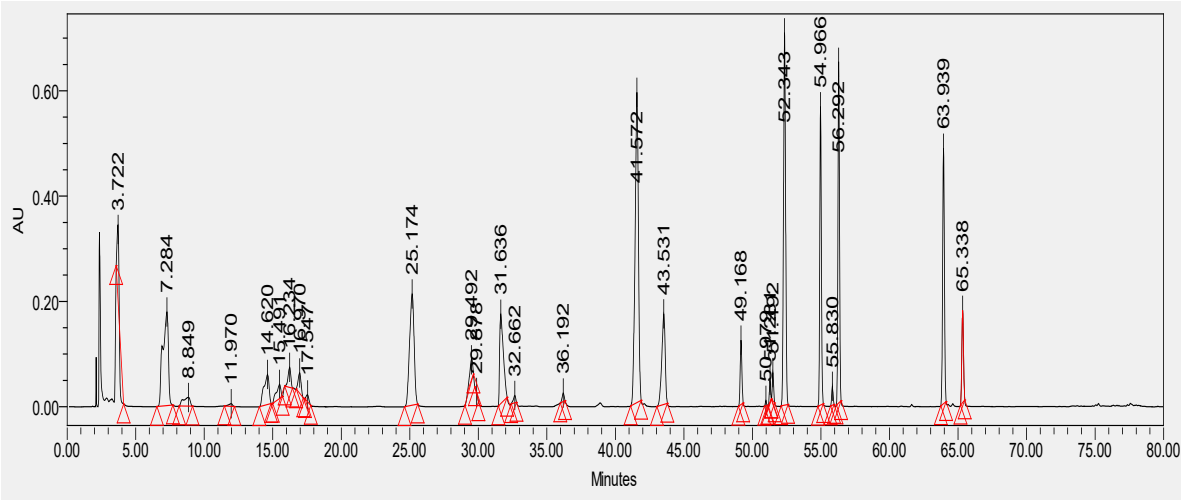

Fig. 4. Chromatogram of 27 standards of phenolic compounds and flavonoids at 330 nm using the developed method.

HPLC chromatogram of coffee sample extracts

Table 3 presents the number of peaks detected in coffee samples roasted for different durations.

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Table 3. Number of peaks detected in coffee samples roasted for different times (6, 5, 4, 3, 2, 1, and 0 hours).

Coffee extract (time of roasting)	No. of peaks detected	Peaks detected	Compounds identified
C6	13	62.84, **63.24**, 63.91, 64.26, **64.60**, 65.18, 66.15, 67.85, 68.14, 68.68, 69.25, 69.66, 70.00	Chrysin and Galagnin
C5	9	59.51, 60.08, **63.26**, 63.91, **64.60**, 65.13, 67.86, 68.67, 73.17	Chrysin and Galagnin
C4	7	59.52, **63.27**, **64.60,** 65.14, 67.86,68.68, 73.18	Chrysin and Galagnin
C3	7	59.52, **63.39, 64.60,** 65.31, 67.86, 68.67, 73.19	Chrysin and Galagnin
C2	9	28.03, 45.81, 59.52, **63.26, 64.60,** 65.13, 68.04, 68.67, 73.18	Chrysin and Galagnin
C1	13	6.54, 11.01, 12.58, 14.99, 23.45, 28.00, 45.79, 59.50, **63.24**, **64.60,** 65.11, 67.98, 68.80	Chrysin and Galagnin
C0	12	6.58, 11.22, 12.63, 14.99, 23.44, 28.02, 59.49, **63.24**, 63.88, **64.60**, 68.44, 71.68	Chrysin and Galagnin

Figures 5 and 6 illustrate the chromatographic analysis of the 27 standards of phenolic compounds and flavonoids, as well as C5, C4, C3, C2, C1, and C0, using the developed method at a wavelength of 330 nm.

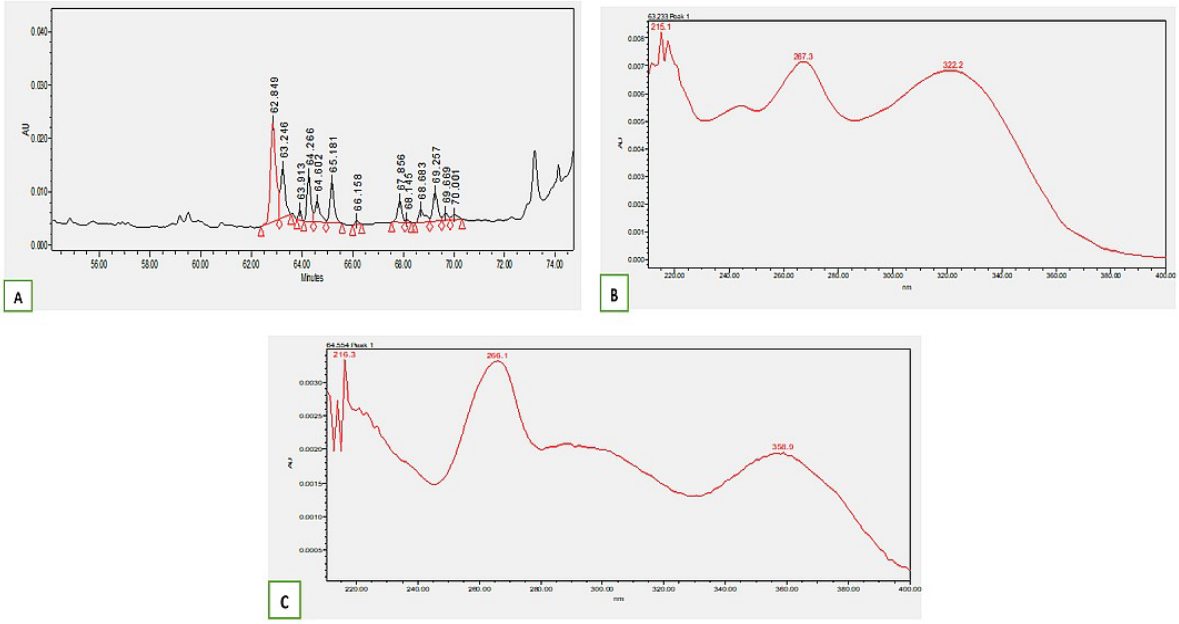

Fig. 5. Chromatogram of C6 at 330 nm **(A)**, and UV spectrum of Chrysin **(B)**.

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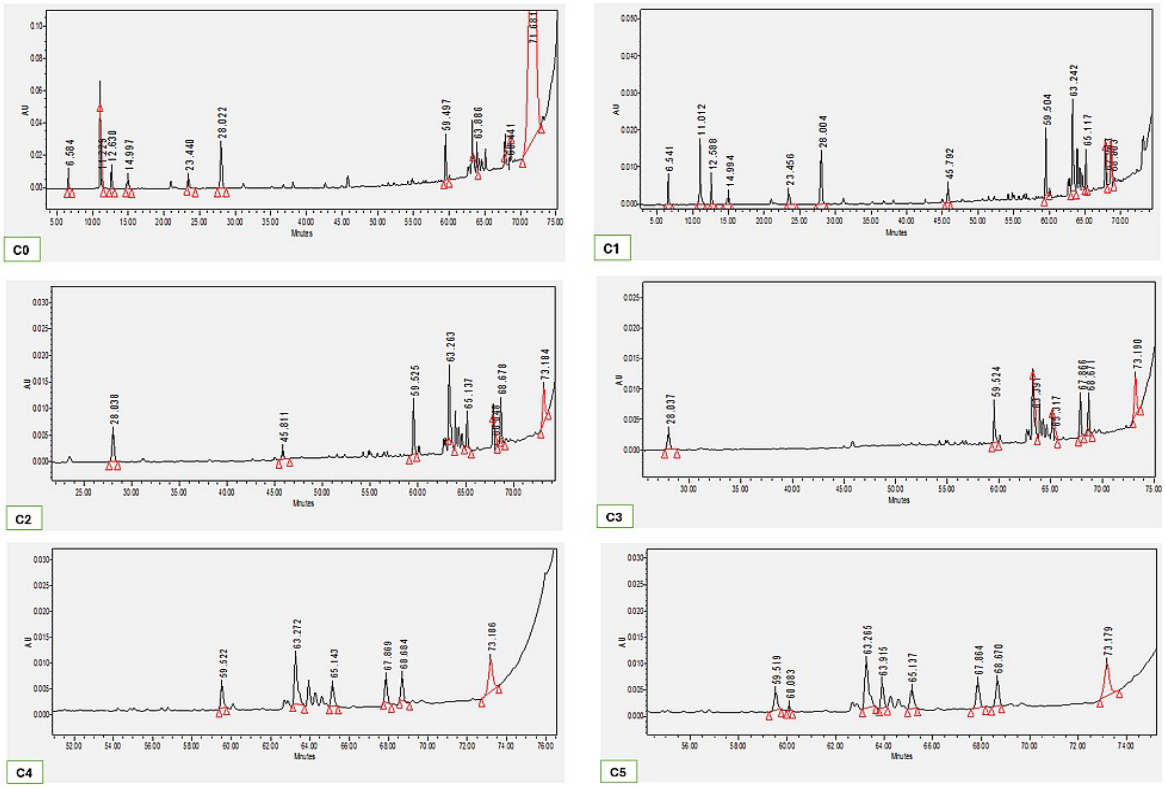

Fig. 6. Chromatogram of C5, C4, C3, C2, C1 and C0.

Discussion

The absorption is inversely related to the effectiveness of the extract and the amount of hemozoin, meaning that lower absorption indicates higher effectiveness and a stronger inhibitory impact on hemozoin production [36]. Although the precise mechanism of action of coffee as an antimalarial extract is not fully understood, it is evident that certain extracts have a significant inhibitory effect on the production of β- hematin [37]. Figure 1 demonstrates that green coffee water extracts are ineffective in inhibiting β- hematin, while the effectiveness increases during the first 2.5 hours of roasting and gradually decreases to zero after 17 hours. This trend is further supported by the results in Figures 2 (undiluted) and 3 (diluted), where water extracts from roasted coffee beans are used. These figures illustrate the antimalarial efficiency of water extracts obtained from both green and roasted coffee beans subjected to a temperature of 330 °C for varying time periods. The effectiveness is measured by the absorption of dissolved β-hematin at a wavelength of 405 nm. The comparison of the results with the negative control (water) and positive control (CQ-0.1 mg/mL) provides a reliable representation of the antimalarial effectiveness of the coffee bean extracts under the specified conditions.

The developed method successfully separates and detects 27 standards of flavonoids and phenolic compounds. Fig. 4 displays the chromatogram obtained at 330 nm, clearly showing the elution profiles of the individual standards. This facilitates the identification and measurement of the flavonoids and phenolic compounds present in the sample, enabling a better understanding of their distribution and concentration within the tested samples.

The selective chromatograms presented in Fig. 5**(A)** represent the coffee extract obtained from green coffee roasted for 6 hours (C6). The identification of specific compounds was achieved by comparing the retention times and UV spectrum wavelengths of both standards and samples. Furthermore, thirteen peaks were detected, with one peak at 63.23 minutes matching the retention time and UV spectrum of Chrysin in the standards mixture chromatogram (Fig. 5**(B)**) and another peak at 64.55 minutes matching the retention time and

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UV spectrum of Galangin (Fig. 5**(C)**). Similar analyses were conducted for the extracts from other roasting times, such as C5, C4, and so on, where Chrysin and Galangin were consistently detected (Fig. 6). Table 3 provides information on the number of peaks detected in coffee samples roasted for different times, with variations observed in the number of peaks. However, Chrysin and Galangin were consistently present in all samples.

The chromatographic analysis also revealed the presence of polar peaks in non-roasted samples and those roasted for one hour, while nonpolar compounds were detected in samples roasted for longer durations. Additionally, samples roasted for two hours showed the presence of compounds with medium polarity. These findings indicate that the roasting process influences the composition and polarity of the compounds present in the coffee extracts. Notably, Chrysin and Galangin were consistently detected across all samples, highlighting their stability and potential contribution to the observed antimalarial effects of coffee extracts.

Chrysin and Galangin, both flavonoids with polyphenolic structures, possess the capacity to interact with free heme through hydrophobic stacking and hydrogen bonding with the porphyrin ring, potentially disrupting β-hematin crystallization. These interactions can inhibit the polymerization of toxic ferriprotoporphyrin IX into inert hemozoin, thereby exerting antimalarial effects similar to quinoline-based drugs such as chloroquine. Galangin has been shown to bind heme-containing enzymes and interfere with oxidative processes, suggesting it could stabilize free heme or alter redox potential within the parasite vacuole [38-41]. Chrysin, with its planar flavone backbone, may intercalate into crystal growth sites or chelate iron ions, further contributing to hemozoin inhibition [42]. While their precise targets in Plasmodium species remain to be confirmed, their physicochemical properties and interaction potentials suggest they may act as heme-binding agents or crystal surface blockers, warranting deeper in vivo investigation.

Galangin, a naturally occurring flavonoid, has demonstrated various biological activities, including antimicrobial, anti-inflammatory, and anticancer effects. It has shown bactericidal properties against multiple-resistant bacteria [43,40]. However, its effectiveness against parasitic organisms remains unsubstantiated. Nevertheless, Galangin showed some activity against certain parasites, including *Trypanosoma brucei* and *T. congolense* [44]. While Chrysin has demonstrated antiviral effects against certain viruses, such as the porcine epidemic diarrhea virus [45], its potential against the *Plasmodium* species has not been established. Therefore, further research is necessary to determine whether Chrysin or Galangin possesses any antimalarial properties.

To our knowledge, there are no direct scientific studies or evidence to support the antimalarial efficacy of roasted coffee. While coffee is known for its antioxidant and anti-inflammatory properties, primarily attributed to its polyphenolic compounds, no previous studies have focused on its antimalarial efficacy. For instance, previous studies have reported that coffee extracts possess antioxidant and anti-inflammatory activities, which are inversely related to the degree of roasting. Additionally, some research has indicated that coffee brews exhibit antioxidant capacity and can reduce pro-inflammatory markers after simulated gastrointestinal digestion [46,47].

The consistent presence of Chrysin and Galangin in roasted coffee extracts, coupled with their observed inhibitory activity against β-hematin formation, suggests a promising avenue for the development of novel antimalarial agents derived from natural products. These compounds could serve as lead scaffolds for drug discovery or be evaluated in combination with existing antimalarial drugs to enhance efficacy, reduce resistance development, and potentially lower therapeutic doses. Their integration into multimodal therapeutic strategies represents a valuable opportunity to bridge phytochemical research with clinical application in malaria-endemic regions.

Conclusions

In conclusion, the findings discussed provide valuable insights into the antimalarial potential of coffee extracts and their relationship with roasting time. The results indicate an inverse relationship between absorption and extract effectiveness, as well as the inhibitory effects on hemozoin production. The chromatographic analysis allows for the identification and measurement of flavonoids and phenolic compounds, revealing their distribution and concentration within the tested samples. The presence of Chrysin and Galangin in all samples suggests their significance as potential bioactive constituents responsible for the antimalarial

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activity of coffee extracts. To enhance reproducibility and facilitate comparative analysis, future studies should consider standardizing coffee extracts based on key bioactive markers such as caffeine and chlorogenic acid. These compounds significantly influence the pharmacological profile of coffee and may vary with roast degree and origin. Quantifying these constituents via HPLC and adjusting extract concentrations accordingly would allow for more consistent assessment of biological activity and better correlation between chemical composition and antimalarial efficacy as well as could elucidate the precise mechanisms of action and explore the synergistic effects of different compounds to develop effective antimalarial therapies.

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