Research Article

The Effect of Aqueous Extract of Cinnamon on the Metabolome of Plasmodium falciparum Using $^1$HNMR Spectroscopy

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Malaria is responsible for estimated 584,000 deaths in 2013. Researchers are working on new drugs and medicinal herbs due to drug resistance that is a major problem facing them; the search is on for new medicinal herbs. Cinnamon is the bark of a tree with reported antiparasitic effects. Metabonomics is the simultaneous study of all the metabolites in biological fluids, cells, and tissues detected by high throughput technology. It was decided to determine the mechanism of the effect of aqueous extract of cinnamon on the metabolome of Plasmodium falciparum in vitro using $^1$HNMR spectroscopy. Prepared aqueous extract of cinnamon was added to a culture of Plasmodium falciparum 3D7 and its 50% inhibitory concentration determined, and, after collection, their metabolites were extracted and $^1$HNMR spectroscopy by NOESY method was done. The spectra were analyzed by chemometric methods. The differentiating metabolites were identified using Human Metabolome Database and the metabolic cycles identified by Metaboanalyst. 50% inhibitory concentration of cinnamon on Plasmodium falciparum was 1.25mg/mL with $p < 0.001$. The metabolites were identified as succinic acid, glutathione, L-aspartic acid, beta-alanine, and 2-methylbutyryl glycine. The main metabolic cycles detected were alanine and aspartame and glutamate pathway and pantothenate and coenzyme A biosynthesis and lysine biosynthesis and glutathione metabolism, which are all important as drug targets.

1. Introduction

Malaria is one of the major infectious diseases particularly in tropical countries. It is caused by the protozoan parasite of genus Plasmodium and transmitted by the female Anopheles mosquito [1].

So far, only 5 of the 170 species of this parasite have been found which are the cause of disease in humans. They consist of Plasmodium falciparum, Plasmodium knowlesi, Plasmidium ovale, Plasmodium vivax, and Plasmodium malariae [2]. Plasmodium falciparum is the most dangerous of all and can even lead to death. The latest released statistics by December 2014 showed 198 million cases of malaria in 2013 comprised of estimated 584,000 deaths. Malaria mortality rates have fallen by 47% globally since 2000 and by 54% in the WHO African Region [3].

Since the 17th century, the bark of the Cinchona tree which was the source of quinine had been the first effective western treatment for malaria [4]. However, chloroquine replaced it from the 1940s, although quinine is still used under certain circumstances [5]. The resistance against anti-malaria drugs is a drawback of standard drugs like chloroquine, sulphadoxine-pyrimethamine, and even artemisinin. The search for new herbal drugs is of prime importance [6].

Cinnamon consists of cinnamonaldehyde compounds, volatile oils, tannins, mucilage, limonene, and safrole that possesses antibacterial, antiseptic, antiviral, and antifungal properties [7]. Senhaji et al. in 2005 tested different extracts of cinnamon like the aqueous, hexane, methanol, and ethanol on gram positive and negative bacteria as well as yeast, Leishmania, and Toxoplasma with positive results [8].

More recently, Nkanwen and colleagues in 2013 tested the bark of cinnamon for antiplasmodial activity and found that it had an inhibitory effect on Plasmodium falciparum enoyl-ACP reductase enzyme [9].
Metabonomics is the recent omics that studies simultaneously all the metabolites and small molecules in biological fluids, cells, and tissues. It uses high throughput technology like 1H Nuclear Magnetic Resonance (1H NMR) and Liquid Chromatography Mass Spectrometry (LC-MS). It plays the most important role in direct observation of the physiological status of an organism or the cell and is a faster and more affordable way of testing drugs and their mechanism of action [10].

Earlier studies have reported antiplasmodial effect of cinnamon extract and its result on one of its enzymes. It was decided to study the metabolome of *Plasmodium falciparum* after exposure to cinnamon extract by 1H NMR spectroscopy.

2. Materials and Methods

2.1. Preparation of Cinnamon Extract. Cinnamomum cassia obtained from Mumbai, India, were ground into a fine powder. 50 grams of cinnamon powder was dissolved in 500 mL of distilled water and boiled for 3 hours and then filtered through a gauze. The obtained extract was concentrated into an oily extract using a rotary machine and then lyophilized to 12.48 grams of cinnamon powder.

2.2. *Plasmodium falciparum* Culture. Strains of 3D7 provided by the late Dr. Walliker were cultured using the method of Trager and Jensen. Briefly, the parasites were cultured in 7 mL RPMI 1640 medium with 5% serum, 10% hematocrit, hypoxanthine, and gentamicin (complete medium) in 75 mL flasks. The medium was changed every 48 h and flasks were incubated at 37°C; the cells were washed three times with saline (PBS) on ice for 30 min and then centrifuged at 4,000 g for 20 min at 4°C. The cells were washed three times with 1XPBS and pellet collected by centrifugation in the above conditions and final centrifugation was carried out at 14,000 g for 5 min at 4°C; the cells were counted in a hemocytometer and stored at −20°C [14].

2.3. IC50 Determination. Parasites reaching 5% were then diluted to reduce the parasitemia to 0.5%, and the hematocrit was adjusted to 1.5%. This suspension was then added (100 μL per well) to microplates predosed with 90 μL of different concentration of cinnamon or artemisinin and incubated for 48 hours at 37°C in mixed gas of 5% CO2, 5% O2, and 90% N2; after that thin smears were prepared from each well, stained with giemsa stain for determination of percentage of parasitemia and IC50 detected by microscopy [13].

2.4. Isolation of Parasites. Parasites were isolated by adding 40 times the volume of 0.02% saponin in phosphate buffered saline (PBS) on ice for 30 min and then centrifuged at 4,000 g for 20 min at 4°C. The cells were washed three times with 1XPBS and pellet collected by centrifugation in the above conditions and final centrifugation was carried out at 14,000 g for 5 min at 4°C; the cells were counted in a hemocytometer and stored at −20°C [14].

2.5. Preparation of Parasite Extract. The samples containing parasites sonicated in a sonifier (Soniprep 150) at 9 KHz for 5 min in pulse were then centrifuged at 10,000 g for 10 min, and the pellet dissolved in 200 μL of 1.8 mM cold perchloric acid and pH adjusted by addition of 5.4 M KOH to 6.8 and kept on ice for 60 min to precipitate the acid. The parasite extract was then centrifuged for 10 min at 10,000 g and the pH once again adjusted to 6.8 and lyophilized [14].

2.6. Preparation of Sample for 1H NMR. 1 mL of D2O and 0.01% TSP was added to the lyophilized powder and spectroscopy was performed using 2-dimensional NOESY (Nuclear Overhauser Spectroscopy) conditions [15].

2.7. Computational Analysis. The spectra from 1H NMR were Fourier transformed using Mestrec software. To obtain regression values, the variables of the signal intensities and chemical shifts were integrated and were inserted into the Excel file. Normal intensities were used for further analysis with MATLAB.

2.8. Partial Linear Square (PLS). PLS is a supervised method to obtain a model using regression in multivariate techniques via linear combination of original variables in which X is the normal intensities from the 1H NMR spectra and the Y matrix comprises 0 for cinnamon treated and 1 for controls. Orthogonal signal correction (OSC) filters removed noise from the spectrum; only one factor was removed and PLS was applied after OSC [16].

2.9. Identification of Metabolites. Metabolites corresponding to these resonances were then identified using chemical shift assignments of spectra of differentiating metabolites of sera based on comparison with chemical shifts of metabolites in Human Metabolite Database Data Bank (HMDB) (http://www.hmdb.ca/metabolites) [17] and in other published data. Analysis of metabolite cycles was carried out using Metaboanalyst software (http://www.metaboanalyst.ca) [18].

3. Results

The lyophilized cinnamon was redissolved in RPMI medium and tested on 5% *Plasmodium falciparum* and IC50 of 1.25 mg/mL was obtained with significance p < 0.001 (Figure 1). Parasite extract obtained from large-scale cultivation of *Plasmodium falciparum* was analyzed by 1H NMR. The spectra of the cinnamon treated *Plasmodium falciparum* and controls were superimposed in Figure 2. The chemical shifts (ppm) of the spectra were converted into figures and then analyzed using OSC-PLS in MATLAB. Figure 3 shows complete separation of the two groups of samples. Figure 4 shows differentiating metabolites between the two groups. Figure 5 depicts the biplot showing both the score plot and loading plot. The outliers indicate the most significant differentiating metabolites which are detected from their numbers. The metabolites were identified from their chemical shifts using HMDB (Table 1). The metabolites were entered into
4. Discussion

Cinnamon has IC50 of 1.25 mg/mL on *Plasmodium falciparum in vitro* with IC50 of 1.25 mg/mL obtained with significance $p < 0.001$. The altered metabolites comprise succinic acid, glutathione, L-aspartic acid, beta-alanine, and 2-methylbutyryl glycine (Table 1). The most significant biochemical pathways which have changed are discussed below (Figure 6).

The alanine, aspartame, and glutamate pathway which is one of the amino acid cycles is the first one to be affected. L-Aspartic and succinic acids are the metabolites which take part in it. There are very early reports about the ability of the parasite to fix carbon dioxide and then synthesize alanine, aspartame, and glutamate. But amino acid uptake by the parasite from the infected erythrocytes is confirmed [19]. When culturing *Plasmodium falciparum in vitro* seven amino acids have to be supplied exogenously; they are isoleucine, methionine, cysteine, glutamate, glutamine, proline, and tyrosine [20]. Proteases act on amino acids especially aspartic

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**Table 1:** Differentiating metabolites identified by their chemical shifts using HMDB.

<table>
<thead>
<tr>
<th>Differentiating metabolites</th>
<th>HMDB</th>
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<tbody>
<tr>
<td>Succinic acid</td>
<td>HMDB00254</td>
</tr>
<tr>
<td>Glutathione</td>
<td>HMDB00121</td>
</tr>
<tr>
<td>L-Aspartic acid</td>
<td>HMDB00191</td>
</tr>
<tr>
<td>Beta-alanine</td>
<td>HMDB00056</td>
</tr>
<tr>
<td>2-Methylbutyryl glycine</td>
<td>HMDB00339</td>
</tr>
<tr>
<td>D-Aspartame</td>
<td></td>
</tr>
<tr>
<td>D-Glutamate</td>
<td></td>
</tr>
<tr>
<td>D-L-Aspartic acid</td>
<td></td>
</tr>
<tr>
<td>D-Beta-alanine</td>
<td></td>
</tr>
<tr>
<td>D-2-Methylbutyryl glycine</td>
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</table>
In the asexual erythrocytic cycle, suggesting that they constitute an important epigenetic mechanism of gene regulation in Plasmodium falciparum. Most SET-domain genes and histone methyllysine marks displayed dynamic changes during the parasite asexual erythrocytic cycle, suggesting that they constitute an important epigenetic mechanism of gene regulation in malaria parasites [26]. They are considered as recent targets for designing of antimalarial drugs and cinnamon extract affects them [27].

Glutathione metabolism is the last and most important cycle which has shown a change in the metabolome of Plasmodium falciparum to cinnamon. It is reported that Plasmodium falciparum employs a complex thioredoxin and glutathione system based on the thioredoxin reductase/thioredoxin and glutathione reductase/glutathione couples. Plasmodium falciparum thioredoxin reductase reduces thioredoxin and a range of low molecular weight compounds, while glutathione reductase is highly specific for its substrate glutathione disulfide. Since Plasmodium spp. lack catalase and a classical glutathione peroxidase, their redox balance depends on a complex set of five peroxiredoxins differentially located in the cytosol, apicoplast, mitochondria, and nucleus with partially overlapping substrate preferences. Moreover, P. falciparum employs a set of members belonging to the thioredoxin superfamily such as three thioredoxins, two thioredoxin-like proteins, a dithiol and three monocysteine glutaredoxins, and a redox-active plasmoredoxin with largely redundant functions. It is seen that glutathione metabolism is disturbed by the cinnamon extract.

It can be concluded that cinnamon has an inhibitory effect on Plasmodium falciparum in vitro with IC50 of 1.25 mg/mL with significance of $p < 0.001$. The altered metabolites are succinic acid, glutathione, L-aspartic acid, beta-alanine, and 2-methylbutyryl glycine and the main metabolic cycles
affected were alanine, aspartate, and glutamate pathway, pantothenate and coenzyme A biosynthesis, lysine biosynthesis, and glutathione metabolism, all of which are important as drug targets.

**Conflict of Interests**
The authors declare that there is no conflict of interests regarding the publication of this paper.

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**References**


