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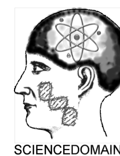

Screening and Characterization of Antimalarial Heme Polymerase Inhibitors from Garlic Cloves

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Authors' contributions

This work was carried out in collaboration between all authors. Authors SM, RD, KMNP Performed Experiments; authors MS, RD, VT Analyzed the Results; Authors RD and VT wrote the manuscript. All authors read and approved the final manuscript.

Research Article

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ABSTRACT

Aim: Garlic (*Allium sativum* L) aqueous extract was investigated to identify antimalarial compounds inhibiting heme polymerization.
Methods: Solvent fraction of aqueous garlic extract was tested in heme polymerization assay and antimalarial assay to identify active factor. Mass spectroscopy, TLC and optical spectroscopy was used to characterize the active factor and mechanism of inhibiton.
Results: Solvent fractionation and silica chromatography of aqueous garlic extract yields partially purified active constituent. The crude garlic extract has a high level of heme polymerization inhibition activity. Mass spectroscopy analysis of the high activity fraction indicates quercetin as a promising hit with an acceptable limit of error. Pure quercetin was found to inhibit heme polymerization and inhibit parasite growth in a dose dependent manner with an activity comparable to the activity present in the purified garlic aqueous fractions. Quercetin forms two distinct complexes with hemin as evident by TLC Chromatogram of hemin and quercetin mixture. ESI-MS analysis of quercetin-hemin reaction mixture gives two prominent peaks; 1st peak with m/z 929 (Hemin+Q+Li+3H) and 2nd peak with m/z 1244.7 (H+2Q+Na) with a clear indication of the formation of quercetin: hemin (1:1) and 2:1 complexes. The dissociation constant (Kd) of quercetin-hemin is 9.35 μM.
Conclusions: In summary, aqueous garlic extract has heme polymerization inhibitor with high antimalarial activity. Quercetin is the main active constitution responsible for the

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activity and it inhibits heme polymerization by chelating free available hemin for polymerization.

Keywords: Malaria; garlic; quercetin; heme polymerization.

1. INTRODUCTION

Malaria caused by Plasmodium Sp is a life-threatening disease causes more than 225 million cases with an annual death toll of 781,000 [1]. During intraerythrocytic stages, parasite depends on the RBC hemoglobin as main source of food for growth and multiplication [2]. Hemoglobin digestion occurs in parasite food vacuole and involves an ordered activity of different proteases [3]. The globin part cleaved into amino acids is used for protein synthesis and free heme is being polymerized into haemozoin to avoid toxic effects of free hemin [4,5]. Conversion of heme to haemozoin through a polymerization reaction is an exclusive metabolic to parasite [6,7]. Considering the paramount importance of heme polymerization in parasite life cycle, number of antimalarial drugs target heme polymerization for their activity [6,8,9]. An additional advantage of exploiting heme polymerization as an axis for drug development is that parasite cannot develop resistance against it. But parasite has devise other mechanisms to over-come drug induced death to acquire resistance [5]. Hence, searching novel heme polymerization inhibitors is in great demand for designing novel drugs against malaria.

From ancient times medicinal plants are always being first choice of source to develop drugs against pathogenic organisms especially malaria [10,11]. In a recent study our laboratory has identified potential inhibitors from a phytochemical reservoir of northeastern india plants against the PfRIO-2 kinase [12]. A Phytochemical reservoir in a plant represents a large collection of naturally occurring bioactive molecules which can be screened in a heme polymerization assay to develop potent antimalarials. *Allium sativum* L or commonly known as “garlic” is an integral part of human diet [13,14]. Presently medicinal and dietary preparations such as garlic oil, powder, water and ethanol extracts is in use as health supplements [15]. Over the past few decade garlic was studied extensively for health benefits and is considered to be one of the excellent disease-preventive plant [16]. Garlic is known to show a wide spectrum of therapeutic effects, such as antibacterial, antiatherosclerotic, antihypertensive, hypolipidemic, hepetoprotective, hypoglycemic, and antidote to control mercury toxicity [17-21]. In addition, it modulates the immune system and prevents from cold and flu [22-24]. The antioxidant activity of garlic is due to a variety of sulfur-containing compounds and their precursors [25]. It contains sulfur-containing compounds such as alliin, γ-glutamylcysteine which are mainly responsible for flavor and health promoting functions. Additional constituents include steroidal glycosides, flavonoids, essential oil, anthocyanins, lectins, nicotinic acid, pectin, adenosine, prostaglandins, phospholipids, biotin, fructan, vitamins B1, B2, B6, C and E, fatty acids, glycolipids, and essential amino acids [26,27].

Medical and pharmacological properties of garlic are known to hindus for centuries and documented in Ayurveda [13,28]. Unfiltered garlic juice (aqueous extract) appears to be the best form of garlic preparation for medicinal usage against TB [19,29]. Garlic has biologically active compounds to control malaria by directly targeting parasite invasion, hemoglobin digestion and indirectly stimulating immune system or working as insect repellent [30-32]. In the present study, we have performed a systematic extraction and fractionation of aqueous garlic extract and evaluated the fractions for their antimalarial potential by monitoring

inhibition of heme polymer formation. Quercetin was identified as an active chemical constituent present in garlic responsible for antimalarial activity through inhibition of heme polymerization. It prevents heme polymerization by forming a complex with free heme. In ancient india, garlic is used as an antiseptic agent and it was believed that consuming garlic reduces the chances of getting infectious diseases. Our work supports increased intake of dietary garlic to enhance resistance against malaria and other infectious diseases.

2. MATERIALS AND METHODS

2.1 Material

Hemin chloride, Quercetin, was purchased from Sigma, St. Louis, MO, USA. Silica gel, Tween 20, Sodium dodecyl sulphate (SDS), iodine crystals were purchased from merck. Other reagents and solvents were of analytical grade purity.

2.2 Collection of Plant Material

Fresh garlic (*Allium sativum* L.) was purchased from local vegetable market (guwahati, Assam) in April and was identified by botanists. The bulbs (50g) in good physical shape were peeled, washed with 1% SDS and dried in hot air oven at 37º C for overnight and made into fine powder. The powder was stored at -20ºC until further use. The yield of this process was 43.25% (w/w).

2.3 Solvent Extraction and Fractionation of Garlic

100mg of garlic powder was weighed and homogenized in 10ml of cold and sterile double distilled water in a blender at high speed for 15 min. It was followed by ultrasonication (10 sec on-pulse and 30 sec off-pulse at 33% energy) for 2 mins and filtered three times through a 0.45µm filter. The obtained aqueous extract of garlic was acidified by adding HCl (final concentration 1.2 N). Homogenate was sequentially fractionated with ethyl acetate (80% v/v) and hexane (60% v/v), allowed to stand for 6 hours at room temperature for optimal extraction and phase separation. Aqueous and organic phases were separated and tested for antimalarial activity in in-vitro schizonticidal inhibition assay and heme polymerization inhibition assay.

2.4 In-vitro Schizonticidal Inhibition Assay

The in vitro antimalarial assay was carried out in 96-well microtitre plates as described previously [33,34]. In brief, test compound or plant extract was incubated with ring stage synchronized *P.falciparum* (3D7) parasitised cell. After 40 h incubation, the blood smears from each well were prepared to record maturation of ring stage parasites into trophozoites and schizonts. The test concentration, which inhibits the complete maturation into schizont was recorded as the MIC.

2.5 Heme Polymerization Inhibition Activity Assay

Heme polymerization assay was done as described(35). Briefly, In a total assay volume of 200µl of sodium acetate buffer pH 5.2, 90 µl of heme solution (100 µM) prepared in dimethyl sulfoxide was added into the plate. Finally, tween-20 was added into each well at 6.25 µg/ml to start the polymerization reaction. The plate was incubated at 37º C for 250 minutes and

absorbance was taken at 415/630 nm in infinite M 200 microtiter plate reader (Tecan). To test the activity of plant extract or quercetin, 10µl of garlic extract/quercetin was added before initiation of polymerization reaction with tween-20.

The fraction 'f ' of the heme converted to β-hematin was calculated as follows:

$$f = (A_{control} - A_{sample}) / (A_{control} - A_{min})$$

Where $A_{control}$ is the absorbance of the heme without tween-20 or a plant extract at 415/630 nm, while A_{sample} represents the absorbance of the heme in the presence of both Tween 20 and test compound and A_{min} is the absorbance of the heme with tween-20 in the absence of the test compound at 415/630 nm. The % inhibition of β-hematin formation by plant extract/quercetin was calculated as follows:

$$\% \text{ inhibition} = (1 - f) \times 100$$

2.6 Characterization of Active Fraction from Aqueous Garlic Extract

Active purified fraction was mixed with 0.1% formic acid [v/v]). A mass analysis in Q-tof micromass (waters) with an electrospray source was used to perform electrospray ionization/mass Spectrometry (ESI-MS). The mass spectrometer was operated in the positive ion mode. Samples were directly infused at a rate of 10µl min^{-1} into the ion source. mass-lynx was used to analyze spectrum.

2.7 Characterization of Quercetin-Hemin Complex Formation

Hemin (100µM) was incubated with 100-500µM quercetin (dissolved in 50% methanol) for 250 mins at 37ºC in 100 mM Tris-HCl buffer, pH 7.4. Hemin was precipitated by pouring the reaction mixture into the 10ml sodium acetate pH 5.2 for 30mins at 37 ºC. The precipitate was recovered by centrifugation at 10,000rpm and wash thrice with distilled water. It is analyzed on thin layer chromatography (TLC) using butanol: acetic acid: water (4:1:5) as the solvent system. Chromatogram was developed by I_2 vapor. For ESI-MS analysis of quercetin-hemin complex, total hemin was isolated from quercetin-hemin reaction mixture and precipitate processed directly into mass spectrometer Q-tof Premier & Aquity UPLC (Waters).

2.8 Determination of Binding Constant of Quercetin for Hemin

Quercetin interaction with hemin was studied in optical difference spectroscopy to calculate dissociation constant (K_D) of quercetin-hemin complex. Optical difference spectroscopy was performed as described previously [33]. The equilibrium dissociation constant (K_D) for quercetin-hemin complex was calculated from the following expression= $\frac{1}{\Delta A} = \frac{KD}{\Delta A\alpha}\frac{1}{S} + \frac{1}{\Delta A\alpha}$, where K_D is the dissociation constant of the Quercetin-hemin complex, S is the concentration of Quercetin, ΔA is the observed absorption change at a particular wavelength, and ΔA_α is the absorption change at a saturating concentration of the ligand.

2.9 Data and Statistical Analysis

Statistical analysis was carried out using Student's paired *t*-test and one-way analysis of variance (ANOVA) test. Statistical *P* value > 0.001 was considered significant.

3. RESULTS

The crude garlic extract has a high level of heme polymerization inhibition activity (Table 1). Using heme polymerization inhibition as a screening tool, crude garlic extract was fractionated with different solvent system. Solvent fractionation of crude extract gives 3 fractions, aqueous phase with a more than 2.5 fold enrichment of the activity and completely inactive ethyl-acetate or hexane phase as evident by the heme polymerization assay (Table 1). Testing the crude extract and samples from different fractions in an in-vitro schizonticidal inhibition assay indicate very low levels of antimalarial activity in crude extract which get enhanced several fold after solvent fractionation and major activity was present in the aqueous phase (Table1). The aqueous fraction on repeated chromatography on silica gel yielded partially purified compounds. Mass spectrometry was performed to characterize and identify the molecules present in active fraction. The ESI-mass analysis gives single prominent peaks with m/z 303.13 (Fig. 1). As the compound was protonated, antimalarial phytochemicals with molecular mass 302 were searched in *Allium sativum* L phytochemical database (http://www.ars-grin.gov/duke/). After calculating the theoretical masses of all retrieved compounds, quercetin was found promising hit with an acceptable limit of error. It was further validated by comparing with the authentic pure quercetin on thin layer plates as well as their spectral data. Pure Quercetin was found to inhibit heme polymerization and inhibit parasite growth in a dose dependent manner with an activity comparable to the activity present in the purified garlic aqueous fractions (Table 1). The crude aqueous garlic extract and other fractions at 100µg/ml gives no toxic effects in MCF-7 cells but aqueous fractions containing predominantly quercetin as major constituents give toxic effects to MCF-7 cells. Pure quercetin also gives toxic effects at 100µg/ml in MCF-7 cells.

Table 1. Antimalarial activity of garlic extracts/fractions

S. No.	Type of the sample	In-vitro Schizonticidal Activity MIC (µg/ml)	Heme Polymerization IC_{50} (µg/ml)
1	**Solvent Control**	NA	NA
2	**Crude extract**	48 ± 4.25	0.5 ± 0.12
3	**Ethyl Acetate fraction**	42 ± 3.23	11 ± 1.34
4	**Hexane fraction**	35 ± 2.12	6 ± 2.45
5	**Aqueous fraction**	12 ± 1.89	0.2 ± 0.031
6	**Silica chromatography**	9.12 ± 3.41	0.18 ±0.017
7	**Quercetin (positive control)**	7.12 ± 2.31	0.15 ±0.021

Aqueous Garlic extract was fractionated using different organic solvent as described in "material and methods" and test for anti-malarial activity in in-vitro schinzonticidal activity against P. falciparum 3D7 or in-vitro heme polymerization assay. Data are the mean ± SD of three independent experiments (n=3) with duplicate measurement for schinzonticidal activity assay or triplicate measurement for in-vitro heme polymerization assay. Pure quercetin from sigma is used for comparison purposes and used as "positive control" where as parasite incubated with solvent as "negative control".

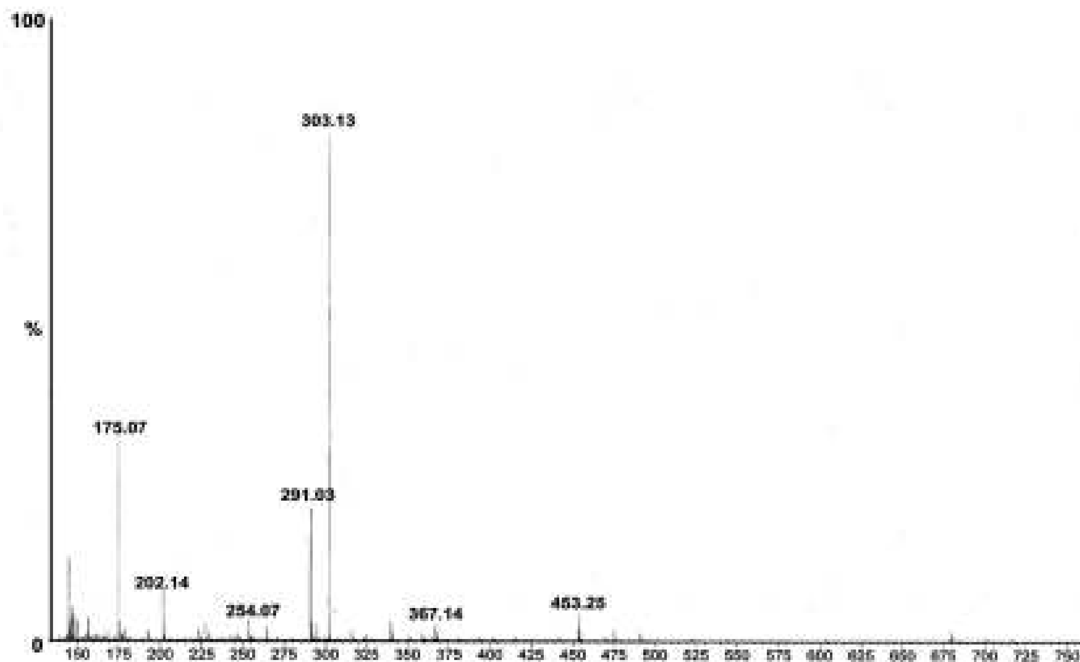

Fig. 1. Characterization of active fraction from aqueous garlic extract. *Electrospray Ionization/Mass Spectrometry (ESI-MS) analysis of the purified fraction after silica chromatography of aqueous fraction after solvent fractionation.*

Antimalarial activity of quercetin is known but its mechanism and molecular targets are not known(36). The data in Table 1 clearly indicate a direct role of quercetin in inhibiting heme polymerization. There are two prominent mechanisms known with antimalarial compounds inhibiting heme polymerization; alteration of polymerization conditions or sequestration of hemin to form toxic drug-hemin complexes as in the case of artimisinin(37). Quercetin doesn't have the potentials to change the pH of the reaction mixture (data not shown) but in-vitro incubation of hemin with varying concentration of quercetin gives 2 distinct complexes on TLC Chromatogram (Fig. 2A). Complex 1 is less abundant and complex 2 is much more abundant in the mixture. Further analysis of quercetin-hemin reaction mixture in ESI-MS gives two prominent peaks; 1st peak with m/z 929 and 2nd peak with m/z 1244.7. The 1st peak corresponds to (Hemin+Q+Li+3H) and indicate the formation of quercetin:hemin (1:1) complex where as 2nd peak corresponds to (H+2Q+Na) and indicate the formation of quercetin:hemin (2:1) complex (Fig. 2B). The binding affinity of quercetin towards hemin to form quercetin-hemin complex is further characterized in an optical difference spectroscopy. It indicates that quercetin-hemin dissociation constant (K_D) is 9.35 µM (Fig. 2C). Hence, our study highlight the garlic as a potential source for excellent drug like molecules for malaria.

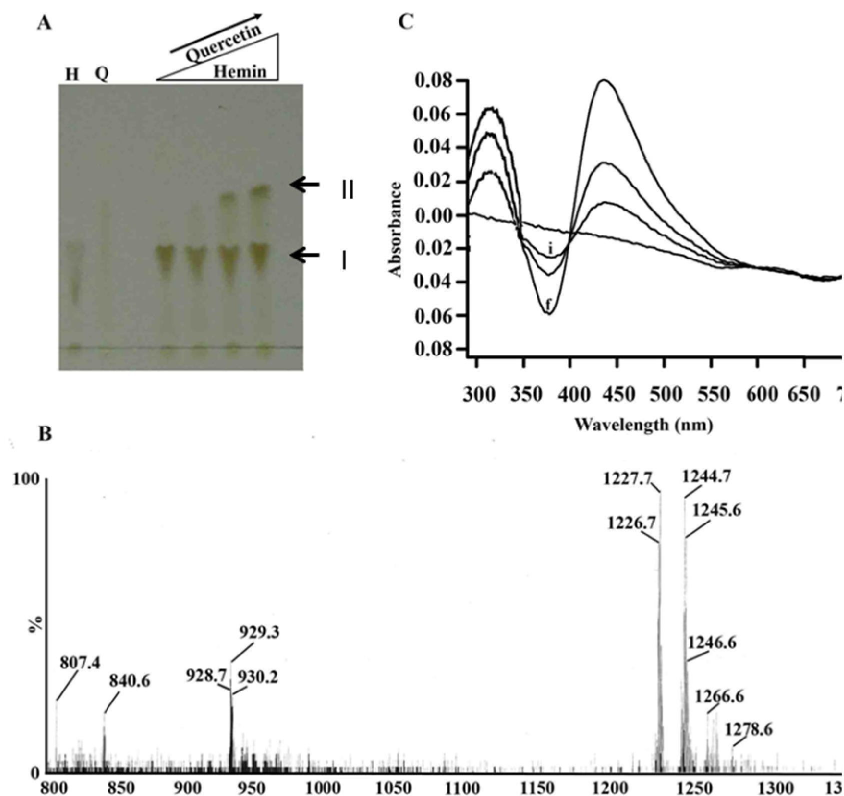

Fig. 2. Quercetin inhibits heme polymerization by forming complex with free hemin *(A)* *hemin (100μg) was incubated with different concentration of quercetin (100, 150, 200, 500 μg) in the heme polymerization assay buffer and after 250mins of incubation, 10μl of hemin recovered from reaction mixture was analyzed on thin layer chromatography in a solvent system containing butanol: acetic acid: water (4:1:5) and chromatogram was developed by I_2 vapors. Complex I with heme/quercetin (1:1) whereas complex II heme/quercetin (1:2) appeared on TLC, both complexes are denoted by arrows. Hemin or quercetin dissolved in the same buffer system was also analyzed under the similar running conditions.* ***(B) Characterization of Heme-Quercetin adduct by ESI-mass spectroscopy.*** *Quercetin and hemin was incubated in reaction buffer at pH 7.2 as described in A, and isolated hemin from reaction mixture was processed directly into liquid chromatography mass spectrometer (LC/MS/MS) in Q-tof Premier & Aquity UPLC (Waters).* ***(C) Quercetin is interacting with hemin.*** *Optical difference spectra of quercetin with hemin (i, initial scan at 1.65 μM; f, final scan at 6.66 μM). The plot of 1/∆ Absorbance (nm) versus 1/[Quercetin] was used to calculate the K_D.*

4. DISCUSSION

Medicinal and pharmacological properties of garlic is documented in Ayurveda [13,28]. The crude garlic extract is found to be the best form of garlic preparation for medicinal usage [19,29]. In addition, antimalarial phytochemicals are documented in garlic and strong evidences are presented from purified phytochemicals present in garlic [30-32,38]. But a schematic fractionation strategy using a heme polymerase assay as a screening tool is not performed. The current study is the first documented effort to isolate bioactive antimalarial compound inhibiting heme polymerization from aqueous garlic extract. It has identified quercetin as the active compound present in the aqueous fraction as indicated by mass

spectroscopic study of purified fraction after solvent fractionation and silica chromatography. NMR or X-ray crystallography might be more robust methods to precisely identify and characterize the active factor from crude extract but mass spectroscopy in conjugation with the exclusion approach is found to be useful to identify quercetin from aqueous extracts of *Caesalpinia pluviosa* [39].

Quercetin has a potent antimalarial activity against *Plasmodium falciparum* 3D7 and inhibits heme polymerization through sequestration of free hemin by forming quercetin-hemin (1:1) and (1:2) complex. A number of hydroxyl group present on quercetin might be potential sites to participate in complex formation. Quercetin is a flavonoid and is responsible for observed therapeutic potentials of medicinal plants [40]. Flavonoids are three ringed molecules with multiple hydroxylation and a structure-function study indicates the crucial role of hydroxylation in the biological activity of the molecule [41]. Quercetin reduces inflammation, haemozoin effects on cytokine production from monocytes and to reduce drug (quinine) toxicity in malaria patients [42-44]. Quercetin is most abundant flavonoid reported in fractionation and purification of antimalarials from different medicinal plants of ethanobotanical potential [10,36,39,45,46]. Quercetin has direct effects on parasite viability through inhibition of permeability pathways, disturbance of hemoglobin metabolism [47] but no report is available on the heme polymerization pathway. Quercetin forms complex with free hemin and reduces available hemin for polymerization. At present exact site on hemin or quercetin involved to form quercetin-hemin complex is not clear but a competition experiment with metal or porphyrin moieties might help us to explore sites on hemin. Hemin-artimisinin complex blocks heme polymerization and drug-hemin complex is more toxic than free hemin inside the parasite [48]. Similar mechanism with quercetin might be operating inside the parasite to exhibit toxicity and death but it is not explored in the current work. The understanding of quercetin-hemin complex formation and its effects towards parasitized RBC is an interesting feature to explore but it is beyond the scope of the current study.

4. CONCLUSION

Solvent fractionation and silica chromatography of aqueous garlic extract yields partially purified active constituent. Aqueous garlic extract has heme polymerization inhibitor with high antimalarial activity. Quercetin is the main active constitution responsible for the activity and it inhibits heme polymerization by chelating free available hemin for polymerization.

CONSENT

Not applicable.

ETHICAL APPROVAL

Not applicable.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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