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An experimental *in vitro* study to evaluate the antimalarial activity of select homeopathy preparations

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Abstract
Malaria has become the public health concern worldwide. Although it is preventable and curable; *P. falciparum* and *P. vivax* have developed resistance to nearly all anti-malarial in current use.
New drug discovery and novel drug leads are required to control the current malaria disease burden. The inventor has developed malaria nosode and has subjected it for evaluation of antimalarial activity in vitro assay along with few other homeopathy preparations.
The potential antimalarial activity of the Malaria nosode, Malaria officinalis and China officinalis was evaluated by β-Hematin Formation Assay. The hemozoin content was determined by measuring the absorbance at 400 nm. The results were recorded as % inhibition of heme crystallization compared to negative control (DMSO)
Malaria nosode, Malaria officinalis and China officinalis exhibited inhibition of hemozoin and the inhibition was greater than the positive control Chloroquine diphosphate used in the study.
The study has shown anti-disease activity of an ultra-dilute (potentized) homeopathic preparation. The Malaria nosode prepared by potentizing *Plasmodium falciparum* organisms has demonstrated antimalarial activity, which supports the basic principle behind homeopathy, the law of similar.

Keywords: malaria, P. falciparum, nosode, in vitro activity, hematin, hemozoin, homeopathy

Introduction
Malaria is caused by Plasmodium parasites that are transmitted to people through the bites of infected female Anopheles mosquitoes and is a life-threatening disease [1]. In 2015, nearly half of the world's population was at risk of malaria. Most malaria cases and deaths occur in sub-Saharan Africa. However, South-East Asia, Latin America and the Middle East, are also at risk. According to the latest WHO estimates, released in December 2016, there were 212 million cases of malaria in 2015 (49% India)) and 429 000 deaths [2]. Malaria has become the public health concern worldwide. Although it is preventable and curable; *P. falciparum* and *P. vivax* have developed resistance to nearly all anti-malarial in current use [3].
New drug discovery and novel drug leads are required to control the current malaria disease burden. Homeopathy is an alternative system of medicine based on the principle of treating diseases using ultra-dilute (potentized) medicinal substances having potential capacity to produce similar disease condition in physiological or crude dose. The nosodes are the homeopathic preparations sourced from organisms (or biological materials), and used against the same infections as well as other disease conditions. Some of the recently developed nosodes from HIV virus, Hepatitis C virus and cancer tissues have demonstrated specific anti-HIV [4] anti-Hepatitis C [5] and anti-cancer activities [6] in clinical trials and in vitro studies.
China officinalis, a potentized extract of the plant is in use in homeopathy against Malaria. Certain *in vitro* studies have shown antimalarial efficacy of homeopathic medicines like China officinalis, Chelidonium and Arsenic album [7] Anti-plasmodia potential of a homeopathic medicines have been reported in an in-vivo model (BALB/c mice) against P. berghei infection [8, 9].
Based on this background, the inventor has developed Malaria nosode and has subjected it for evaluation of anti-malarial activity using an *in vitro* assay along with few other homeopathy preparations.
Digestion of hemoglobin in the food vacuole of the malaria parasite produces very high quantities of redox active toxic free heme. Hemozoin (beta-hematin) formation is a unique process adopted by Plasmodium species to detoxify free heme. Hemozoin formation is a validated target for most of the well-known existing antimalarial drugs and considered to be a suitable target to develop new anti-malarial. The chemical nature of new antimalarial compounds showing antimalarial activity through the inhibition of hemozoin formation has also been incorporated, which may help to design future anti-malarial with therapeutic potential against multi-drug resistant malaria [10]. A spectrophotometric assay for *in vitro* beta-hematin formation is widely used for screening of antimalarial activity [11].

2. Materials and Methods
2.1 Chemicals
Hematin porcine, chloroquine diphosphate, Oleic acid, sodium dodecyl sulfate (SDS), sodium acetate, magnesium sulfate, sodium hydrogen phosphate, sodium chloride, potassium chloride and sodiumhydroxide were purchased from Sigma-

Aldrich Chemical Company while Hydrochloric acid was purchased from Merck.

2.2 Study sample

The samples tested were Malaria nosode 30c, Malaria officinalis 30c and China officinalis 30c. The samples were prepared in alcohol and potentized alcohol (potentized up to 30c potency) which acted as vehicle controls. The standard used was Chloroquine diphosphate (500 µg), a known anti-malarial drug. The given samples were also evaluated in various combinations. The samples provided for testing were coded to conduct blinded assay.

2.3 In vitro β-Hematin Formation Assay

The potential antimalarial activity of the given samples was evaluated by the method described by Afshar *et al*, with some modifications [12]. The given samples were incubated with 3 mM of hematin, 10mM oleic acid, and 1MHCl. The final volume was adjusted to 1mL using sodium acetate buffer, pH 5. Chloroquine diphosphate was used as a positive control. The reaction mixtures were incubated overnight at 37^0C with constant gentle shaking. After incubation, samples were centrifuged (14,000 rpm, 10 min, at 21^0C) and the hemozoin pellet was repeatedly washed with incubation (15 min at 37^0C with regular shaking) in 2.5% (w/v) SDS in phosphate buffered saline followed by a final wash in 0.1M sodium bicarbonate until the supernatant was clear (usually 3–8 washes). After the final wash, the supernatant was removed and the pellets were dissolved in 1mL of 0.1M NaOH before determining the hemozoin content by measuring the absorbance at 400 nm. The results were recorded as % inhibition (*I*%) of heme crystallization compared to vehicle controls using the following equation:

$I\% = [(AN-AS)/AN] \times 100$

Where AN: Absorbance of vehicle control and AS is absorbance of test samples

2.4 Statistical Analysis

The results are presented as mean ± SD of three independent experiments carried out in duplicates. Statistical significance between the groups was assessed by one-way analysis of variance (ANOVA) followed by a post hoc test. The accepted level of significance for the test was $P < 0.05$. All tests were carried out using GraphPad Software (Version 3.06).

3. Results

The results from the *in vitro* β-hematin formation assay of the given samples in single and in various combinations are listed in Table 1 and Table 2, extraction and fractionation yields are listed in Table 1. The inhibition of β-hematin formation expressed as percentage (%) and standard deviations (n= 3) are given for sample.

All the three samples exhibited significant inhibition of hemozoin formation at the concentrations studied as compared to the alcohol and potentized alcohol as vehicle controls and this inhibition was greater than that exhibited by the positive control, Chloroquine diphosphate used in the study (Table 1). Although, the study controls have also exhibited inhibition however the inhibition by the samples was greater than the controls used. In various combinations, all the 3 samples exhibited greater inhibition as compared to the alcohol control and more than that exhibited by the positive control (Table 2). However, the results with various combinations were not statistically significant.

Table 1: Effect of given samples on hemozoin formation (n=6)

Sr. No	Study Groups	% Inhibition
1	Alcohol	44.72 ± 3.01
2	Potentized alcohol	43.64 ± 2.48
3	Malaria nosode 30c	57.96 ± 8.52
4	Malaria officinalis 30c	63.88 ± 11.35@**
5	China officinalis 30c	64.89 ± 15.21@@**
6.	Chloroquine diphosphate (500 µg)	53.83 ± 8.33

All values represent Mean ± SD;
@ p<0.05; @@ p<0.01 as compared to the Untreated Control group;
** p<0.01 as compared to the Treated Control group;
(ANOVA followed by post-hoc tests)

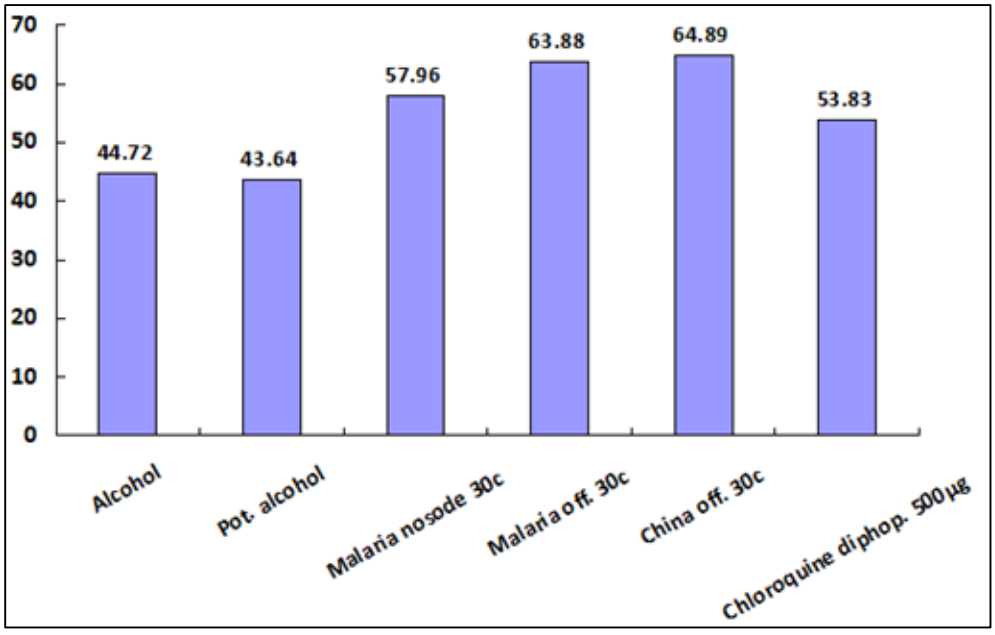

Fig 1: Effect of given samples on hemozoin formation

Table 2: Effect of given samples used in various combinations on hemozoin formation (n=6)

Sr. No	Study Groups	% Inhibition
1	Potentized alcohol	48.57 ± 10.07
2	PFA 30C + Malaria officinalis 30C	60.25 ± 6.42
3	PFA 30C + China officinalis 30C	60.05 ± 11.09
4	Malaria officinalis 30C + China officinalis 30C	62.52 ± 10.78
5	PFA 30C + Malaria officinalis 30C+ China officinalis 30C	62.82 ± 4.66
6.	Chloroquinediphosphate (500 µg)	56.99 ± 9.2

All values represent Mean ± SD

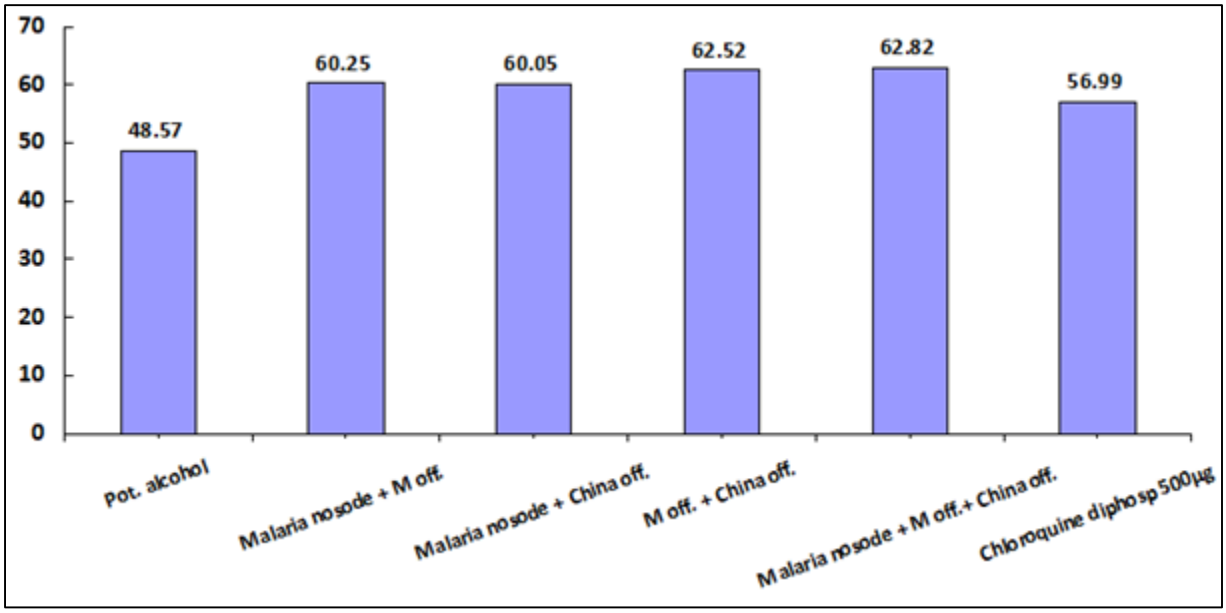

Fig 2: Effect of different drug combination on hemozoin formation

4. Discussion and Conclusion

Digestion of hemoglobin in the food vacuole of the malaria parasite produces very high quantities of redox active toxic free heme. Hemozoin (beta-hematin) formation is a unique process adopted by Plasmodium species to detoxify free heme. Hemozoin formation is a validated target for most of the well-known existing antimalarial drugs and considered to be a suitable target to develop new anti-malarial agents. The possible mechanisms of free heme detoxification in the malaria parasite and the mechanistic details of compounds, which offer antimalarial activity by inhibiting hemozoin formation, is the current area of research [13].

Thus, the inhibition of hemozoin formation is an attractive target for development of several antimalarial drugs such as 4-aminoquinolines (quinine, mefloquine, and chloroquine) and is therefore considered as a suitable target for drug screening programs [13]. Many in vitro assays based on spectral characteristics and differential solubility of monomeric heme and β-hematin (synthetic analogue of hemozoin) have been described and used for screening of novel synthetical [14, 15] and natural [16] antimalarial compounds.

In the given study, all the three samples Malaria nosode 30c (coded as PFA), Malaria officinalis 30c and China officinalis 30c exhibited inhibition of hemozoin and the inhibition was greater than the positive control Chloroquine diphosphate used in the study. All the three samples when used in various combinations also exhibited inhibition greater than the positive control. The vehicle controls alcohol and potentized alcohol had also exhibited inhibition but the inhibition of samples was greater than the controls used.

The study and the findings are of the particular interest due to several reasons:

a) The study has shown anti-disease activity of an ultra-dilute (potentized) homeopathic preparation, in which no detectable residual of the original substances (Plasmodium F, Malaria officinalis source material, and China officinalis herbal extract) is expected to be found. The findings strengthen the therapeutic application of the method of potentization.
b) The Malaria nosode prepared by potentizing Plasmodium falciparum organisms has demonstrated antimalarial activity, which supports the basic principle behind homeopathy, the law of similar. This thought-provoking finding is capable of inviting more research with different nosodes.
c) The result shown with ultra-dilute, potentized dose the homeopathic preparations in in vitro model has demonstrated the effect of homeopathic medicines outside of human body. It has been a common belief that there is (disease producing) primary effect of the ultra-dilute preparation on the human body, while the rebound, secondary action (equal and opposite) is curative in nature. (reference) The basic concept may call for a review.

Way back in 1796, Samuel Hahnemann, MD, introduced homeopathy with an experiment with the first medicine, China officinalis, which has been in use in homeopathy since then. Also, Chloroquine diphosphate, a derivative of quinine is used as antimalarial in the conventional medicine. Interestingly, this experiment has validated antimalarial activity of ultra-dilute China officinalis in laboratory model.

Hence more experiments need to be conducted to understand mode of action, various pathways, and more variants of potentized preparations against malaria; as well as other infectious diseases.

5. Conflict Of Interest

One of the authors has patent pending for Malaria nosode. He had no control over the process of experiment and the results.

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