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Evaluation for Efficiency of Drug Delivery Systems *In vitro* and *In vivo* Plasmodium Culture using Crude Extract of *Artemisia annua* and artesunate

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ABSTRACT

Parasitic diseases such as malaria, leishmaniasis, and trypanosomiasis represent a significant global burden and pose a great challenge to drug discovery and delivery to scientists due to their intracellular nature and disseminated locations. Moreover, poor rate of discovery in the anti-parasitic segment seen in last few decades has necessitated effective management of existing drugs by modulating their delivery. Nanoparticles (NPs) were proposed as drug carriers over 30 years ago and have received growing attention since, mainly due to their stability, enhanced loading capabilities and control over physicochemical properties. This research work emphasized on the biological and biopharmaceutical issues to be considered in the design of delivery strategy for treating *plasmodium* infection and role of nanoparticles in optimizing the delivery of anti-malarial drugs of natural and synthetic origin. In present study nanoparticles were prepared by using ionotropic gelation with Tpp anions ,liposomes were prepared by using thin film hydration,and phytosomes were prepared using complexing polyphenolic phytoconstituents with phosphatidylcholine.This study through *in vitro* and *in vivo* assays indicates that drug delivery system increases therapeutic index of traditional and novel drugs is enhanced via the increase of specificity due to targeting of drugs to a particular tissue, cell or intracellular compartment, the control over release kinetics, the protection of the active agent or a combination of the above.

Keywords: Drug delivery system, nanoparticles , liposomes, malaria, Plasmodium, phytoconstituents

1. INTRODUCTION

In Asia, *P. falciparum* parasites have become increasingly resistant to quinine, and therefore artemisinin derivatives have been evaluated in pregnant women. Parasites reappeared after treatment in 6.8% of women receiving artesunate alone as primary treatment, 15.9% in women receiving artesunate as a re-treatment, and 37 % in women receiving quinine as a re-treatment . The rate of abortions, stillbirths and congenital abnormalities was similar among women who received artemisinins and the general population drug delivery system holds the key to these problems, by targeting the molecules and slow release of these molecules.

2. MATERIAL AND METHODS

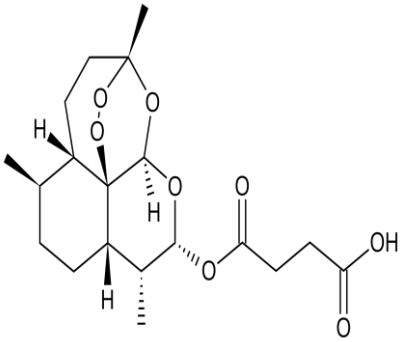
2.1 Plant selected for study

Plant selected for antiplasmodium activity: *Artemisia annua*, Family: Asteraceae, Compositae , Synonym: Sage Brush and Quinghaosu in china. Vernacular name: Maderwood, Wormwood. Aerial parts were used in this study.

2.2 Preparation and Extraction procedure for plant material

Aerial part of *Artemisia annua* were dried in shade, grounded to coursed powder stored in air tight amber colored bottle. Artemisinin, a sesquiterpene lactone is an antimalarial constituent of *Artimisia annua*. Extraction process which was followed in this study.Dried leaves of *Artimisia annua* were coarsely powdered and extracted with the help of petroleum ether (over night). Petroleum ether was then filtered then concentrated to dryness with the help soxhlet then concentrated extract found was then dissolved in chloroform. Acetonitrile was added to the chloroformic solution which is responsible for the sepration of impurities and pricipetation of waxes. Filtration was done to separate out the impurities. The filtrate was again concentrated and was subjected to cool down. Crystals of artimisinin were deposited which were further purified by washing and recrystallization with alcohol.[1,2]

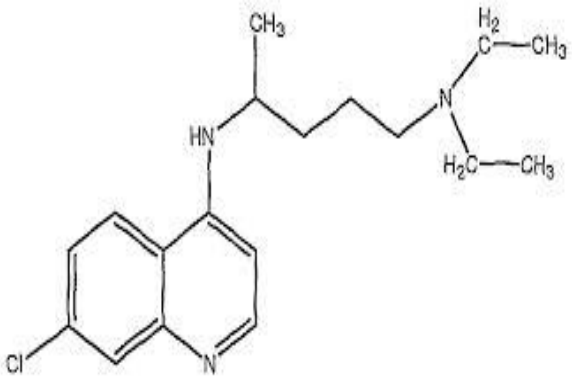
Figure 1: Plant of Artemisia annua



(a)

2.3 Synthetic drug selected for present study

Artesunate with IUPAC name (3R,5aS,6R,8aS,9R,10S,12R,12aR)-Decahydro-3,6,9-trimethyl-3,12-epoxy-12H-pyrano(4,3-j)-1,2-benzodioxepin-10-ol hydrogen succinate was used for study. Chloroqunine was used as standard drug.



(b)

Figure 2 Chemical structure (a) Artesunate (b) Chloroquine

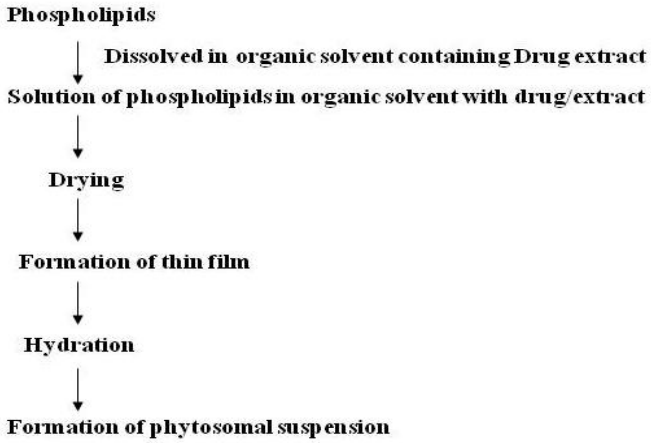


Fig. 3: Common stages for preparation of phytosomes

2.4 *In Vitro P.falciparum* study

Fresh infected blood samples were obtained for clinical isolation of Plasmodium falciparum from Malaria unmulan kendra Bhopal (M.P.) and from People's hospital, Bhopal. Enquiry was made on drug intake of the patients to select those who had taken any antimalarial drug. Giemsa stained thin smear were examined for Plasmodium species identification. Samples having co infection were discarded. The parasite density was examined by counting the number of infected erythrocytes among 20,000 erythrocytes.

2.4.1 Preparation of host erythrocytes

Human erythrocytes for parasite culture are prepared by drawing blood into heparin-treated tubes and washing several times in RPMI 1640 medium to separate the erythrocytes from the plasma and buffy coat. Separation can be achieved by centrifuging the blood at 3500 rpm for 8 minutes at 4º C in a swing-out rotor (centrifuged machine). Leukocyte-free erythrocytes are typically stored at 50% hematocrit (i.e. 1 volume of malaria culture media for 1 volume of packed erythrocytes, corresponding to approximately 5×10^9 cells ml^{-1}).[3]

2.4.2 Malaria Culture Media

RPMI 1640 medium containing L-glutamine (High Media), 25mM HEPES (CDH), 10 µg ml-1 gentamicin (CDH), 0.225% NaHCO3 (CDH). Medium was adjusted to a pH of 7.3 to 7.4. Once media was prepared media was filtered through 0 .22 µm syringe filters then stored in air tight plastic bottles, at 4-8º C till further use. During culture maintenance media was supplemented with 5% fresh human serum (O^+blood group).[3]

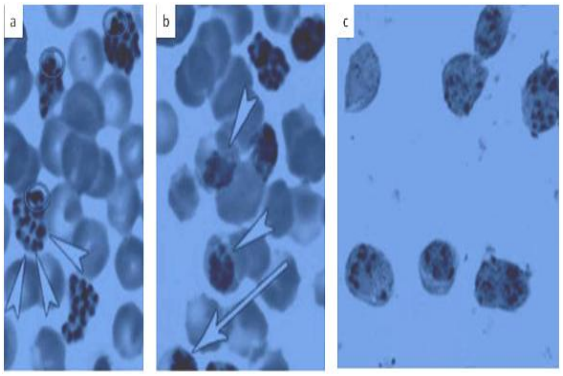


Figure 4. Slides of thin blood film showing *P.berghei*, gametocytes stage (a,b), tropozoites stage (c).(at 100x)

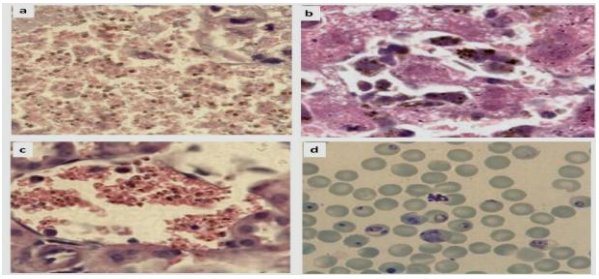


Figure 5. Photomicrograph of *P. berghei* in : placental tissue(a) : spleen (b): kidney (c): peripheral blood of negative control pregnant mice(d) . (200x)

2.4.2.1 Procurement of experimental animal

After taking permission for animal studies from Institutional Animal Ethics Committee (IACE), B.U, Bhopal. (Reg No. CPCSEA/444), Swiss albino mice, weighing 27-35 g and age of 6-8 weeks, were selected for the study , The mice were obtained from the local breeder and maintained in animal house of Department of pharmacy, Barkatullah University, Bhopal. Animals were housed in propylene cages with standard animal house under natural 12h light and 12 dark cycles at 24-28ºC temperature. They were maintained on standard pallet diet (Hindustan lever Ltd. Bangalore) and water ad labium. They have free access to food and water. The animals were acclimatized to the test environment for one week before starting the experiment. Animals were fasted for at least 12 hours before the onset of each activity. Culture and screening were performed.[4]

2.4.2.2 In Vivo Cultivation of Plasmodium berghei

To test the antimalarial activity of the aqueous and alcoholic extracts of each plant, the mouse was infected with, CQ sensitive strains of *P. berghei* (obtained as a kind gift from Dr. S Sharma from Friendicoes, New Delhi) maintained in vivo was used. The parasites were maintained by serial passage of blood from infected mice to the non-infected ones on weekly basis.Donor *Plasmodium berghei* infected mice (parasitemia of 20%) were killed by cervical dislocation. Blood was then collected in slightly heparinized syringe from auxillary vesssels. The collected blood was diluted with PBS in such a way that each 0.2ml contains approximately 1 x 10^7 infected red cells. Mice were then divided in to ten groups of six each; eight groups of mice received the crude extracts or fractions, while the other two groups were used as positive and negative control.[4]

2.4.2.3 In vivo Toxicity Test of the Crude Plant Extracts

The acute toxicity test was carried out according to the CDER guidelines.The crude aqueous and methanol extracts of *A. annua* intended for the antimalarial test against *P. berghei* were evaluated for their toxicity in non-infected male Swiss albino mice aged 6-8 weeks and weighing 27-35g. For each extract test, 20 mice were used by randomly dividing them into four groups of 5 mice per cage.[4,5]

2.4.2.4 In vivo Evaluation of the Antimalarial Activity of natural and synthetic drugs in free and encapsulated for

For an *in vivo* evaluation of each extract the Peter`s 4 day suppressive test against *P. berghei* infection in mice was employed .[4] Male Swiss albino mice weighing 27-32g were infected with *P. berghei* and randomly divided into eight groups of five mice per

cage: eight test groups(extracts ,drugs and drug delivery systems) (G8-CQ a standard drugs as a positive control and G1-vehicles, dH_20 or 3% DMSO, as a negative control). Each mouse received standard inoculums of about 10^6-10^7 parasite infected red cells per gram body weight through the intraperitoneal route in a PBS of 0.2ml on D_0 to produce a steadily rising infection in mice. The stock of aqueous extracts were made dissolved in distilled water (dH_2O) and ethanolic extracts were dissolved in less than 3% DMSO according to the weight of mice in each group. The extracts were given via intragastric route by using gavage in six different doses (100, 200, 400,600,800 and 1000 mg $kg^{-1}day^{-1}$), while the positive control mice received chloroquine (CQ) at $25mg^{-1}kg^{-1}day$ and the negative control received vehicles with in a volume of 0.2ml. Each treatment was administered as a single dose per day and was started after 3 hours of infection on day zero continued daily for four days.[5] On the fourth day drops of blood sample was collected from the tail snip of each mouse. Thin smears were prepared and stained with 10% Giemsa solution. Then, each stained slide was examined under microscope with an oil immersion magnification (100x) power to evaluate the percentage of parasitaemia and suppression of each extract with respect to the control groups.LD_{50} was determined .[6]

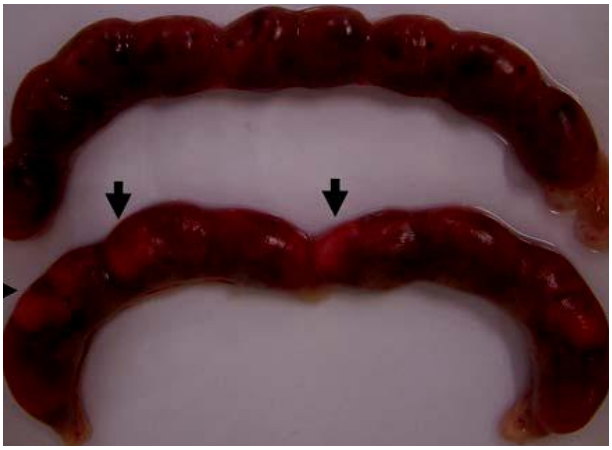


Figure 6. Representative uterus at G18 from pregnant females, treated with encapsulated synthetic drug in nanopartical (upper) and uterus at G13 treated with free synthetic drug (bottom). Arrow indicates the sign of abortion. Both the females were infected with P.berghei by IV injection of 1×10^6

2.5 Drug delivery system preparation

2.5.1 Nanoparticles preparation

Chitosen nanoparticles were prepared with suitable modification based on the ionotropic gelation with Tpp anions. Chitosan (2mg ml^{-1}) was dissolved in aqueous acetic acid (pH 4.0) solution and Tpp was dissolved in distilled water at the concentration of 1mg/ml.Drug (10%) was added to Tpp solution (by dissolving Tpp in 100ml distilled water under contineuous stirring on magnetic stirring).Finally 1.5 ml of drug containing Tpp solution was added to 4 ml of chitosan solution with the help of syringe needle under magnetic stirring at room temperature.The dispersion so formed was sonicated for 15 minutes,then disperse in water and centrifuge the sample at 12000 rpm for 30 minute.the supernatant was discarded and pallet was resupended in distilled water and lyophilized and stored at -20ºC till further use.[7,8]

2.5.2 Liposomes Preparation

Liposomes were prepared using the thin film hydration technique. Briefly, 100 mg ml^{-1} of phospholipids in chloroform taken in a clean moisture-free container was purged with nitrogen gas to remove the solvent. 5 ml of phosphate buffered saline (PBS pH 7.4) were added to the container and the mixture was warmed at 60°C for 30 minutes. The solution was then extruded through polycarbonate membranes of 200 nm pore size using an extruder for ten cycles to obtain extruded liposomes.[9] The liposomes were lyophilized and stored at -20 °C in air-tight vials.

2.5.3 Phytosome Preparation

Phytosomes are prepared by complexing polyphenolic phyto-constituents in 1:2 ratio with phosphatidylcholine. Phospholipid was dissolved in organic solvent and plant extract was also added to the mixture followed by drying and film formation then hydration through phosphate buffer saline .[10]

2.6 *In vitro* drug release

In vitro release of hydrophilic molecule out of drug delivery system was measured spectrophotometrically by incubation of drug delivery systems in 10mM Tris buffer (pH: 7.4) at 37°C in mild shaking (35 rpm) water bath under mild-shaking conditions for determined time intervals. Dialysis bags (Dialysis membrane 110, Hi Media, India) were immersed in water for one hour to remove the preservatives followed by rinsing in phosphate buffered saline (PBS) solution.[11] The drug and extract encapsulated nanoparticles were placed in PBS and loaded in the dialysis bag. The bag was sealed at both the ends and immersed in 4 mL of PBS with 10% methanol (Katrin, 1995). The release of the drug and extract was evaluated at three different pH values (1.2, 7.4 and 9.0). A pH of 1.2 was maintained using 0.1 M HCl -KCl buffer while pH 9.0 was maintained using 0.1 M phosphate buffer. Samples were withdrawn at time intervals of 0.5, 1, 2, 4, 6, 8, 24-h, periodically. *In vitro* release rate results were evaluated kinetically (Zero, first-order and Higuchi models).[11]

2.7 Encapsulation efficiency

The entrapment efficiency of drug and extracts was determined by using following equation.

$$Encapsulation\ efficiency\ (\%) = \frac{Total\ drug - Free\ drug}{Total\ drug} \times 100$$

About 10ml of particulate suspension(drug delivery particles) was digested with minimum amount of ethanolic solution (Water: ethanol in 7:3 ratios). The digested homogenates were centrifuged at 12000 rpm for 30 min and supernatant was analysed for drug entrapment. Entrapped drug and extracts in this study were measured at their respective λ maximum.[11]

Table no 1: Effects of drugs/ extract and drug delivery system on pregnant mice weight, number and weight of litters born

S.No	Extract/Drug	Weight: before treatment (gms)	Weight: after treatment (gms)	No:of delivered litters	Weight:of litter (gms)
1	Alcoholic *Artemisia annua* extract	30.86±2.46	33.42±2.08	3.46±1.64	0.95±0.74
2	Liposomes *Artemisia annua* extract	30.82±2.82	33.86±2.94	5.24±2.14	1.22±0.92
3	Phytosomes *Artemisia annua* extract	29.86±2.73	33.92±2.06	2.45±1.42	1.09±0.74
4	Nanoparticles *Artemisia annua* extract	30.84±2.84	33.04±1.92	3.89±0.62	1.19±0.86
5	Chloroquine	32.05±2.74	35.62±1.92	6.82±1.42	1.29±0.69
6	Nanoparticle chloroquine	29.84±2.63	33.94±2.12	7.62±2.24	1.38±0.72
7	Artesunate	28.52±2.84	35.96±1.42	3.32±0.72	0.85±0.62
8	Nanoparticles Artesunate	28.58±2.65	33.62±1.84	4.62±1.41	0.87±0.42
9	Liposomes Artesunate	29.84±2.26	34.84±1.62	6.64±2.42	0.99±0.63
10	Control group	29.86±2.82	35.82±1.46	11.62±1.76	1.94±0.19
11	Negative control	29.97±1.74	30.32±1.92	All females dies *	

3. RESULTS AND DISCUSSION

Extractive value was 29.4% for *Artimisia annua*.LD_{50} for alcoholic *artemisia annua* extract found to be 4.2µg ml^{-1} and LD_{50} for artesunate found to be 32.56 µg ml^{-1} in vitro screening .No sign of toxicity was observed with dose upto 600mg kg^{-1} body weight in mice in toxicity assay performed for the safety analysis of drugs. Encapsulation efficiency of molecules is nearly 80-90% in all delivery system analysed in present study. Results of percentage cumulative release of drug over a time period confirms sustain release. In present study test pregnant mice shows , better response when treated with novel drug delivery systems as compare to free synthetic drugs, even crude extracts when given through these delivery systems shows better response (results are given in table1), in terms of weight gain of mother during gestation period, delivery healthy litters. Efficiency of molecules increased by drug delivery systems. Sign of abortions are high in case of group treated with artesunate, but number of healthy litters delivered increases in group treated through drug delivery. In figure 4 Slides of thin blood film reprents gametocytes stage (a,b), tropozoites stage (c) of *P.berghei*, at 100x magnification .Figure 5 represents *P. berghei* in :placental tissue(a) : spleen (b): kidney (c): peripheral blood of negative control pregnant mice(d) at 200x magnification. Artesunate is known for its side effects and is avoided in pregnancy, this study emphasis that these delivery system reduces the side effect of drugs by sustain release rate ,reducing dosage dependency .Number of viable litters and their boby weight represented in table 1 confirms the reduction of side effects of drug when given with drug delivery system. Figure 6 represents Representative uterus of females infected with *P.berghei* by IV injection of 1×10^6 , upper one from from pregnant females at G18, treated with encapsulated synthetic drug in nanopartical and bottom one from female treated with free synthetic drug at G13. Arrow indicates the sign of abortion, induced as a side effect of artesunte during pregnancy.

4. CONCLUSION

Present study confirms the effectiveness of drug delivery system for selected antimalarial compounds. Out of three drug delivery systems selected phytosomes proves to best for natural compounds, next is liposomes and nanoparticles are least effective. For synthetic molecules nanoparticles shows better results then liposomes. Drug delivery system reduces drug dosage requirement due to control drug releasing property. Parasitic diseases, proves to be a significant global burden drug delivery systems can play a crucial role in effective management of the current and emerging anti-parasitic agents by improving their parasite specificity with concomitant reduction in adverse effects associated with them. In view of this, colloidal carriers such as liposomes and nanoparticles have shown a good potential in improving efficacy and tolerability of anti-parasitic agents.

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