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Formulation and evaluation of *Morinda lucida* based-phytosome complexes for malaria treatment.

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Abstract

Morinda lucida plant is known to possess potent antimalarial activity. However, its low *in vivo* bioavailability has become a major concern to formulation scientists. Therefore, this study seeks to address this problem by delivery *Morinda lucida* extract as phytosomes.

Morinda lucida leaves were dried and extraction was done via maceration using methanol. Phytochemical analysis was done on the extract. Phytosomes were prepared using the extract and phospholipon 90 H at various ratios with / without surfactants. *In vitro* and *in vivo* evaluations were done on all the formulations.

The phytochemical analysis showed high amount of tannins, flavonoids and terpenoids. The highest anti-plasmodium suppressive effects (97 % and 93%) were seen with the artesunate (standard antimalaria) and the 1:1 extract complex respectively. *In vivo* anti-plasmodium studies confirmed higher antimalarial effect of 1:1 complex at dose of 800 mg/kg comparable/similar with the standard drug.

Keywords: *Phytosomes, Morinda lucida, phospholipon 90 H, anti-plasmodium*

Introduction

Novel drug delivery system has been useful to improve solubility and bioavailability of chemical entities/drugs. They can be designed to deliver drugs to the site of action and achieve controlled drug release [1]. A number of novel drug delivery systems have emerged; some examples are the lipid based drug delivery, polymeric based drug delivery and the vesicular drug delivery system [2,3]. The vesicular delivery systems are well ordered assemblies of lipid bilayers which are formed when the amphiphilic building blocks of these systems are in contact with the aqueous environment. These novel delivery systems can confer various advantages to the drugs including prolonged existence of the drug in systemic circulation and reduced toxicity [4, 5]. Various vesicular drug delivery systems exists and they include liposomes, niosomes, ethosomes, aquasomes, crytosomes, discomes, transferosomes, pharmacosome, virosomes and phytosomes [6, 7].

Phytosomes is an innovative drug delivery system with the purpose of safe, effective and proper delivery of active drug substances particularly phyto-constituents that are absorbed less efficiently. Phytosomes consists of two words – "Phyto" (plant) and "somes" (cell-like). Phytosomes can be defined as microscopic cell like structures which can trap standardized plant extract or phytoconsituents into phospholipids typically the phosphatidylcholine to produce lipid compatible molecular complexes [8]. Phosphatidylcholine consists of the phosphatidyl moiety (lipophilic in nature) and the choline head which is hydrophilic in nature. The choline head binds to the compound while the phosphatidyl portions envelopes the choline bound materials forming a phyto-phospholipid complex [9, 10]. Thus absorption and bioavailability of plant extract is improved. Many herbal extract like milk thistle, ginkgo biloba, grape seed, green tea ginseng and *Musa paradisiaca* has been effectively delivered via the phytosome technology [6, 9].

Morinda lucida (L.) (Rubiaceae) is a tropical West Africa rainforest whose English name is "Brimstone tree". The leaves are used traditionally as anti-malaria, analgesic, laxative and anti-infections. The leaves have also been reported to possess strong trypanocidal and aortic vasorelaxant activities. Other studies have shown that the leaf and stem bark of *M. lucida* possess anticancer, hepatoprotective, cytotoxic, antispermatogenic, hypoglycemia and antidiabetic activity [11, 12]. The alkaloids, anthraquinones and anthraquinols are the major active constituents of *M. lucida* extract. Despite the profound therapeutic activity of this plant, challenges still exist in delivery/formulation of this plant extract to achieve good therapeutic outcome. Therefore, the aim of this study is to extract and deliver the methanol extract of *Morinda lucida* as phytosome in other to improve the bioavailability of the extract.

Methods

Collection of plant

The plant used in this study was collected at Onuiyi in Nsukka, Enugu state, Nigeria. The specimen was authenticated by Felix Nwafor, a taxonomist at the herbarium section of the Department of Pharmacognosy and Environmental Medicine, Faculty of Pharmaceutical Sciences, University of Nigeria Nsukka, Enugu State. The leaves of *M. lucida* were air dried under shade for 2 weeks.

Extraction

Approximately, 1 kg of *Morinda* dried leaves were pulverised to powdered form, 250 g of the powdered leaf was soaked in 2.5 L of methanol for 72 hours with periodic stirring. The mixture was filtered using whatman No.1 filter paper; filtrate was allowed to evaporate under room temperature. Then the residue was de-fattened using diethylether and collected as the extract, which was further dried in a desiccator [12]. The extract was stored in the refrigerator for further use.

Determination of extract yield

The extract yield and its percentage were determined using the equation below:

$$\%\, Extract\ yield = \frac{Amount\ of\ extract\ produced}{Amount\ of\ powdered\ leave\ used\ for\ extraction} \times 100 \quad \text{...Eqn1}$$

Loss on drying

A 0.5 g of the extract was weighed out in a porcelain evaporating dish of known weight. The crucible with the extract was placed in an oven, at temperature of 105˚C until constant weight was obtained. The % loss on drying was calculated as in equation 2:

$$\%\ Loss\ on\ drying = \frac{Wet\ weight\ of\ the\ sample - weight\ of\ the\ sample\ after\ drying}{Wet\ weight\ of\ the\ sample}$$

Eqn2

The beer's calibration plot

A 10 mg of the extract was dissolved in 2 ml of methanol and made up to 10 ml with SIF to give the stock solution. Several dilutions were made to obtain these concentrations 0.01, 0.02, 0.03, 0.04 and 0.05 mg/ml. The solution with minimum concentration was used to obtain the wavelength of maximum absorbance and the absorbance values of the dilutions were measured at this wavelength using UV-Visible spectrometer (Spectrulab, United Kingdom). The concentrations versus absorbance values were plotted to obtain the calibration plot.

Formulation of lipid complex

The method of Ugwu *et al*., was adopted [6]. An amount of extract equivalent to each ratio was placed in a 50-ml beaker as presented in Table 1, and 10 ml of methanol added, the beaker was placed on a magnetic stirrer to facilitate dissolution. In another 50-ml beaker the required amount of phospholipon 90 H was added and dissolved with 5 ml of ethanol, and the temperature was increased to 32 ± 2˚C to increase dissolution but not to induce evaporation. The phospholipon solution was then mixed with dissolved extract and allowed to mix for about 10 min. The mixture was then poured into a clean petri-dish and the solvent was allowed to evaporate. This batch was coded as batch A. The above procedure was repeated for another preparation with the incorporation of a 0.5 ml of surfactant. The resultant phytosomes was then encapsulated and kept for further analysis.

Phytochemical analysis of extract

Test for Tannins: 0.5 g of the extract was stirred with about 10 ml of distilled water and then filtered. Few drops of 1% ferric chloride solution were added to 2 ml of the filtrate a blue-black, green or blue-green precipitate indicates the presence of tannins [13].

Fehling's test for free reducing sugar: 0.5 g of the extract was dissolved in distilled water and filtered. The filtrate was heated with 5 ml of equal volumes of Fehling's solution A and B. A red precipitate indicates the presence of reducing sugars [14].

Test for Saponins: One gram of the extract was boiled with 5 ml of distilled water, filtered using a filter paper. To the filtrate, about 3 ml of distilled water was further added and shaken vigorously for about 5 minutes. Frothing which persisted on warming showed the presence of saponins [14].

Ferric chloride test for flavonoids: Distilled water was added to 0.5 g of extract, boiled and filter. Few drops of 10% ferric chloride solution were added to 2 ml of the filtrate. A green-blue or violet colouration indicated the presence of a phenolic hydroxyl group [13].

Test for terpenoids: 0.5 g of the extract was dissolved in ethanol. Then 1 ml of acetic anhydride was added followed by the addition of concentrated sulphuric acid. A change in colour from pink to violet showed the presence of terpenoids [14].

Morphology of complexes

A smear of each formulated complex was prepared on a slide and viewed under the microscope at magnification of X 400 (Weltzlar, Germany). The morphology was captured using a digital camera.

Loading efficiency

Appropriate quantity of phytosomes equivalent to 50 mg of the extract were weighed out and transferred to a beaker. Simulated intestinal fluid (SIF) pH 6.8 (10 ml) was added to the beaker and stirred well to give a concentration of 5 mg/ml. A 1 ml was withdrawn from the solution, made up to 100 ml with SIF (pH, 6.8) to give a concentration 0.05 mg/ml. Several dilutions (0.005 mg /ml, 0.01 mg /ml, 0.015 mg /ml, 0.020 mg /ml and 0.025 mg /ml) were done and absorbance was measured at 220 nm using UV-Visible spectrometer. The percentage drug content was calculated using equation 3:

$$loading\ efficiency = \frac{Amount\ of\ drug\ obtained}{Amount\ of\ drug\ loaded}\ x\ 100$$
…… Eqn (3)

***In vitro* drug release studies**

The basket (Apparatus II) method was adopted to study the dissolution behaviour of the formulations. This apparatus consists of a beaker and magnetic stirrer setup, with a basket suspended in the medium. A capsule containing suitable quantity of phytosome equivalent to 50 mg of the extract was put in a basket and suspended with a thread into a 500 ml beaker containing 500 ml of SGF (simulated gastric fluid) pH 1.2. (The SGF consists of 2 g of sodium chloride and 7 ml concentrated HCl made up to 1 L with distilled water). The temperature and speed was maintained at 37 ± 0.5° C and 50 RPM respectively. At predetermined interval, 10 ml aliquots of dissolution medium was sampled and replaced with 10 ml of corresponding fresh medium. This experiment was also repeated with simulated intestinal fluid pH 6.8, which contained adequate amount of sodium hydroxide and sodium hydrogen biphosphate dissolved in distilled water). The experiment was done in triplicates and the withdrawn samples were assayed at a wavelength of 220 nm. The amount of drug release and percentage drug release was calculated using equation 4.

$$\%\ Drug\ release = \frac{Amount\ of\ drug\ released\ (mg)}{Loaded\ dose\ (mg)}\ x\ 100$$
…… Eqn (4)

FTIR of phytosome complexes

Fourier transform infrared spectrum (FTIR) was recorded for the phytosome complexes using infrared spectrophotometer (Shimadzu coporation, Japan). The FTIR is a device which generate spectrum representing the molecular fingerprints of a sample, when the infra red light passes through a sample. The complexes and the excipients were prepared in KBr disk (2 mg sample / 200 mg KBr) with a hydrostatic press at a force of 275790.292 Pascal's for 4 minutes and the spectrum was produced within the wavelength number of 4000 to 400 cm^{-1} [8].

***In-vivo* antimalaria study**

Chloroquine sensitive strain of *Plasmodium berghei* NK-65 was obtained from the Department of Veterinary Medicine, University of Nigeria Nsukka. A 4 day suppressive test was adopted for the animal studies [15]. The study was conducted in accordance with Ethical Guidelines of Animal Care and Use Committee (Research Ethics Committee) of the University of Nigeria, Nsukka, following the Federation of European Laboratory Animal Science Association and the European Community Council Directive of November 24, 1986 (86/609/EEC) [16, 17]. A 0.2 mL quantity of donor mouse blood diluted with Phosphate buffer saline (PBS), containing parasitized erythrocytes was intraperitoneally inoculated into 8 groups of 5 Swiss albino mice per group which weighed between 22 – 34 g. The groups include: Group A- treated with 800mg/kg batch A complex (1:1) with surfactant, Group B – treated with 400mg / kg batch A complex (1:1) with surfactant, Group C- treated with 800 mg /kg batch B complex (1:1) without surfactant, Group D – treated with 400 mg /kg batch B complex (1:1) without surfactant, Group E –treated with 800 mg /kg of extract, Group F- treated with 400 mg / kg of extract, Group G – treated with 5mg/kg artesunate (standard drug) and Group H – treated with distilled water (control). The extract and standard drug were administered to the mice per oral at a single dose per day. On the fourth day, blood was taken from the mice and thinly smeared on a microscope slide. The blood films were fixed on the slide using methanol and stained with Giemsa, pH 1.2. Percent parastaemia and activity was calculated using equation 5, 6 and 7.

$$\%\ Parasitaemia = \frac{Number\ of\ infected\ RBCs}{Total\ number\ of\ RBC} \times 100$$
…… Eqn (5)

$$\%\ Difference\ (day\ 1\ \&\ 4) = \frac{\%\ Parasitaemia\ (day\ 1-4)}{\%\ Parasitaemia\ day\ 1} \times 100$$
…… Eqn (6)

$$Activity\ (\%) = 100 - \frac{MPTG}{MPCG} \times 100$$
…… Eqn (7)

* MPTG stands for mean parasitemia of treated group; MPCG is mean parasitemia of controlled group.

Statistical analysis

All experiments were repeated in triplicates. All statistical calculations were done with the Graph Pad Instat Demo. p values less than 0.05 were considered significant.

Results

Extract yield, phytochemical analysis and loading efficiency

The percentage extract yield obtained was 13.32 %, using methanol as the solvent. The percentage loss on drying for the extract was 22 %. The phytochemical analysis as presented in Table 2 showed the presence of some secondary metabolites such as tannins, flavonoids, trepenoids and reducing sugar. The optical microscopy images showed that the complexes formed were spherical and enclosed by the phospholipids (Fig 2). The loading efficiency ranged from 56.42 ± 1.5 to 91.95 ± 5.30 % as presented in Table 3. Highest loading efficiency was seen in formulation B3 (91.95 %), while formulation A1 had the least drug content (56.42 %).

***In-vitro* drug release studies**

The formulated complex batch A2 gave the highest (68.77 %) drug release in phosphate buffer pH 6.8 (Fig. 4) compared to other complexes. In the acidic medium, the highest drug release (55.33%) was seen in batch A3. The presence of surfactant in the formulations seemed to have enhanced the release of the phytoconstituents.

FTIR of phospholipids and phytosomes

Molecular interaction between the formulation components was shown via FTIR (fig 5). The three spectra appear similar indicating no interaction. The absorption band of the phospholipids is characterized by the C-H stretch of long fatty acid chain seen at wavenumber 2887cm^{-1}, P=O stretch at 1228 cm^{-1} and the O-H stretch between 3170 to 3470 cm^{-1} . The benzene ring stretch was seen at 1612 cm^{-1}.

Anti-plasmodium effects of the extract and complexes

The group taking the standard drug (artesunate) had the highest suppressive effect (97 %), followed by the group that received the 800 mg/kg of batch B (A2) (95 %), and then the group that received 400 mg/kg of formulation A2 (88 %) of batch B. The complexes had significantly higher ($p < 0.05$) malaria suppressive effects compared to the negative control (Table 4). There was gross reduction in parasitaemia level in mice across all test groups (extract, complexes and standard), within and after the experimental period (day 1 to 4), except for the non-treated group where there was an increase in the parasitaemia count throughout the period (Fig. 6).

Effect of the extract and complexes on the animals' weight

There was no significant difference in weight observed across the treated groups compared to the group that received no drug (Table 5 - 8). The total percentage increase in weight of all the animals was in the same range of 17 - 35 %. There was no decrease in weight observed in all the groups (treated and untreated). Also no significant statistical difference in weight was seen when the 400mg /kg and 800mg /kg dose was administered across the groups. The phytosome formulation, extract and the reference drug did not adversely affect the weight of the animals.

Discussion

The test "loss on drying" determines the amount of water and volatile matter in the extract. The presence of microbial contamination as well as instability problems is often linked to the presence of water in the extract. The methanol extract of *Morinda lucida* is hygroscopic; however formulation as phytosomes will confer stability to the extract [1]. These secondary metabolites/compounds are known to be responsible for the medicinal properties of many plant extract. They have curative activity against many pathogens associated with diseases and as such could be developed into herbal medicine [18]. For instance tannin rich plants are associated with healing various diseases such as leucorrhoea and diarrhoea. Researchers have also reported that secondary metabolite such as alkaloids, tannins and flavonoids is linked to the antimalarial activities of *Morinda lucida* plant [12, 19]. In this study, the tannins present in large amount in

the extract could be responsible for its antimalarial activities. Flavonoids and terpinoids present in moderate amount may have potentiated the antimalarial activity of tannins in the extract. On interaction with water, it is observed that hydrophilic part of the phospholipids aligned themselves towards water compartment, while the lipophilic part aligned away from water forming the vesicular system. The planar configuration of the delivery system phytosomes enabled the extract to be entrapped in the organized structure of the phospholipids within the phytosomes [12, 20].

The time taken to release 40 % (T_{40}) of the drug in the alkaline medium ranged from 30 to 40 min for most of the formulations. It was observed that equal amount of extract and phospholipid in batch (A2) and the presence of surfactants in the formulations contributed to the best release profile in the alkaline environment. Generally, drug release came with an outburst, releasing most of its content within 50 minutes. The pytosome complexes showed better drug release profile in the alkaline medium. Researchers have reported good pharmacokinetic profile when herbal extract are complexed with phospholipids. The good drug release observed, explains the compatible molecular complexes associated with phytosomes formulation [9]. The presence of surfactant and the ratio of extract: phospholipids are important factors for good dissolution rate of phytosomes.

The spectra of the formulation shows the presence of the benzene ring stretch at 1616 cm^{-1} and an O-H stretch between 3000 to 3337 cm^{-1} [21]. However, the absorption band at 1228 cm^{-1} seemed to have been altered in the phytosome complex. The shift in the O-H stretch to 3337 cm^{-1} observed in the phytosomes could signify the presence of the weak intermolecular interaction between the phospholipids and the extract as a result of the complex formation [22, 23].

The antimalarial activity observed, seemed to be dependent on the dose given and the duration of treatment. However, the complex formulation (with surfactant) at a dose of 800 mg/kg showed a significant remarkable decrease ($p < 0.05$) in parasite load compared to the extract and other complexes. Consequently, its activity was comparable to the standard reference drug (artesunate). A nanoparticle transport is mediated by the peyer's patches in the small intestine into systemic circulation through the lymphatic transport. Thus, there is a bypassing of first pass metabolism by the liver and consequently enhanced bioavailability [24]. The vesicular delivery system, phytosomes seemed to have improved the solubility of *Morinda lucida* extract, and invariably the *in vivo* absorption via the lipid carrier was enhanced [25]. This was evident in the reduction in parasite load and increased antimalarial activities seen in the formulation as compared to the extract

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Table 1. Formula ratio for phytosome complexes

Batch	Extract	Phospholipon 90 H	Solutol
A_1	1	1	--
A_2	1	1	0.5
A_3	1	2	--
B_1	1	2	0.5
B_2	2	1	--
B_3	2	1	0.5

A_1- 1:1 without surfactant , A_2 - 1:1 with surfactant , A_3 - 1:2 without surfactant , B_1 - 1:2 with surfactant , B_2 - 2:1 without surfactant, B_3 - 2:1 with surfactant

Table 2. Qualitative phytochemical results of methanol extract of *Morinda lucida*

Secondary metabolites / compounds	Results
Tannins	+++
Flavonoids	++
Saponins	–
Trepenoids	++
Reducing sugar	++

- Not detected, + low, ++ moderate, +++ Large

Table 3. Loading efficiency of formulated complexes

Formulation	Loading efficiency (% ± SD)
A1	56.42 ± 1.5
A2	63.96 ± 2.32
A3	63.39 ± 1.42
B1	63.45 ± 1.48
B2	84.08 ± 3.89
B3	91.95 ± 5.30

A_1- 1:1 without surfactant , A_2 - 1:1 with surfactant , A_3 - 1:2 without surfactant , B_1 - 1:2 with surfactant , B_2 - 2:1 without surfactant, B_3 - 2:1 with surfactant

Table 4. Percent parastaemia of complexes

Batch	Doses (mg/kg)	Day 1	Day 2	Day 3	Day 4	% activity
A2	800	20.25±2.17	8.00±.913**	4.25±.629**	1.50±.646**	95.00
A2	400	21.00±1.685	9.75±.854**	4.25±.25**	2.50±.50**	88.00
A1	800	20.25±1.55	10.0±.707**	5.00±.408**	3.00±.408**	85.00
A1	400	24.00±2.12	10.5±1.32**	5.2500±.75**	4.25±.75**	82.00
Extract	800	21.00±1.87	8.00±2.12**	4.75±1.70**	2.75±1.11**	78.00
Extract	400	23.00±1.96	7.50±1.19**	5.25±.750**	2.50±.866**	68.00
Artesunate	5	24.67±3.93	8.67±1.43**	2.0±.577**	0.667±.667**	97.00
Negative control	-	23.33±2.91	24.0±1.15	29.7±1.45	30.67±3.53	31.00

A_1- 1:1 without surfactant , A_2 - 1:1 with surfactant

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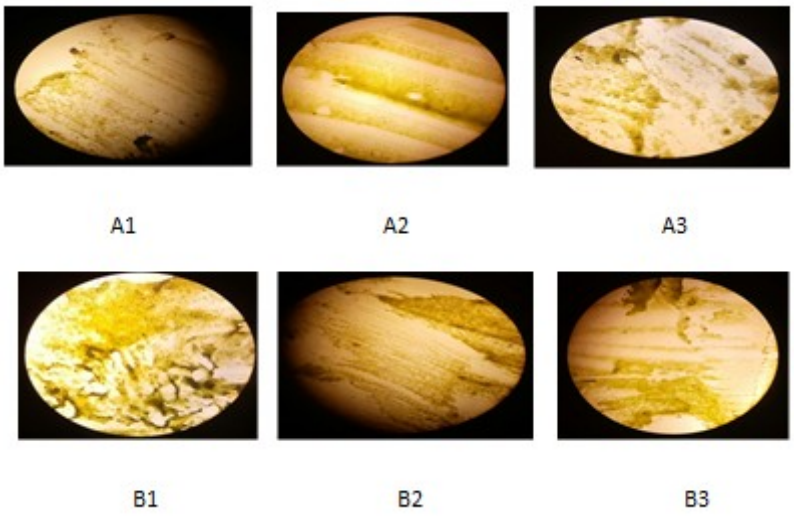
Table 5: Effects of extract and complexes on animal's weight

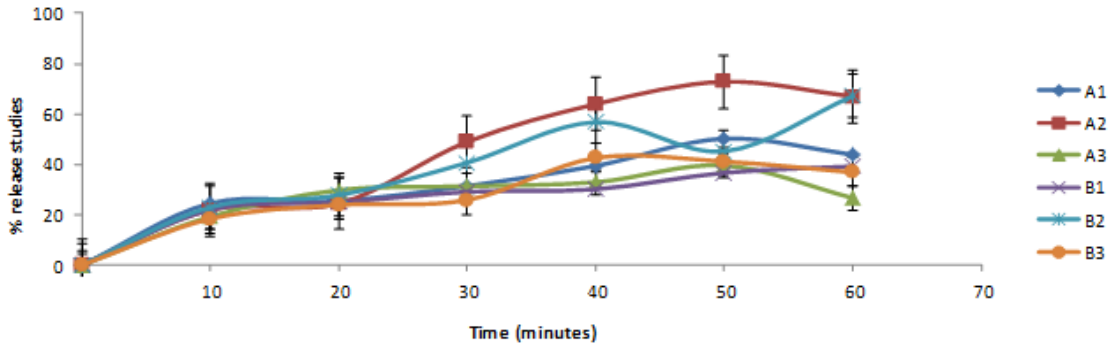
Groups	400 mg/kg		800 mg/kg	
	Weight (g) at Day 0	**Weight (g) at Day 4**	**Weight (g) at Day 0**	**Weight (g) at Day 4**
Group 1- received batch A 2 complex	25.49	32.88	25.84	34.72
	24.78	31.29	28.30	34.86
	25.69	33.10	26.94	34.56
	29.18	39.62	26.20	32.69
Group 2 – received batch A1 complex	32.26	39.66	23.90	31.53
	27.76	37.64	27.18	32.21
	24.59	33.10	27.79	34.90
	32.48	38.27	24.58	33.27
Group 3 - received extract	25.00	31.01	30.06	38.16
	25.38	39.27	25.79	32.02
	30.26	37.17	30.99	40.20
	26.73	36.44	26.46	35.28
	Negative control (water)		**Positive control (artesunate 5 mg/kg)**	
	Weight (g) at Day 0	**Weight (g) at Day 4**	**Weight (g) at day 0**	**Weight (g) at day 4**
Group 4 –received control	21.50	29.13	26.59	32.12
	27.49	34.16	25.06	32.57
	22.83	29.31	26.60	34.16

Figure 1. The morphology of the complexes



A_1- 1:1 without surfactant , A_2 - 1:1 with surfactant , A_3 - 1:2 without surfactant , B_1 - 1:2 with surfactant , B_2 - 2:1 without surfactant, B_3 - 2:1 with surfactant

Figure 2. *In vitro* drug release of *Morinda lucida* phytosomes in phosphate buffer pH 6.8



A_1- 1:1 without surfactant , A_2 - 1:1 with surfactant , A_3 - 1:2 without surfactant , B_1 - 1:2 with surfactant , B_2 - 2:1 without surfactant, B_3 - 2:1 with surfactant.

Figure 3. FTIR spectra of a) Phospholipon 90, b) phytosome complex (batch A1), c) phytosomes complex (batch A2)

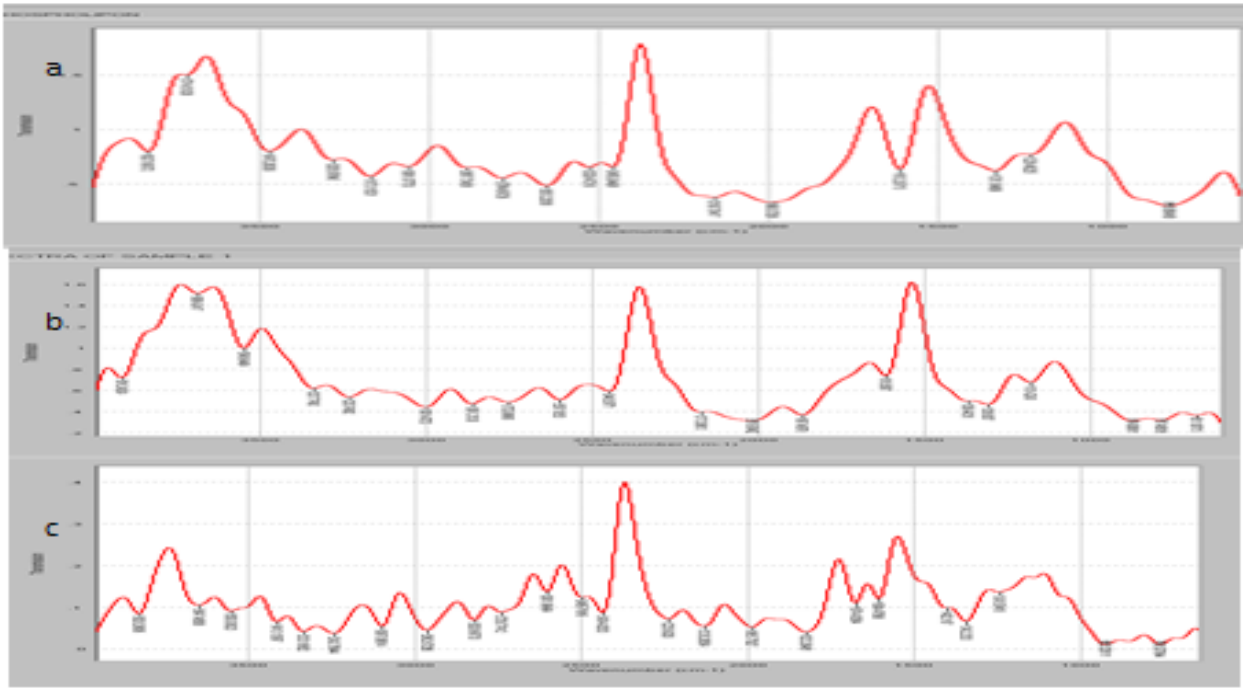


Figure 4: The anti-plasmodium effect of *M. Lucida* extract and its complexes