FORMULATION AND EVALUATION OF COMBINED HERBAL EXTRACTS LOADED CAPSULES AGAINST CHLOROQUINE-RESISTANT PLASMODIUM FALCIPARUM WITH *IN VITRO* CYTOTOXICITY ASSESSMENT

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ABSTRACT

The present study aimed to develop a herbal capsule formulation using Acalypha indica and Citrullus lanatus extracts for antimalarial application against the chloroquine-resistant Plasmodium falciparum 3D7 strain. The formulation underwent preformulation studies, in vitro antimalarial testing, cytotoxicity evaluation on Vero cells, and dissolution testing. The developed formulation demonstrated a sustained release profile with high therapeutic indices, exhibiting potent antimalarial activity without cytotoxic effects, suggesting its potential in therapeutic applications.

KEYWORDS

Acalypha indica, Citrullus lanatus, antimalarial activity, capsule formulation, Plasmodium falciparum, cytotoxicity, phytochemicals

1. Introduction

INTRODUCTION

Medicinal plants are recognized as potential source of bioactive compounds. More than 80% of modern drugs are derived directly from sources of plants and microbes. Natural products derived from medicinal plants have wide range of pharmacological significance. Bioactive compounds as they contain therapeutic and their complex nature will able to interact with mammalian cell targets. Phytochemicals naturally isolated from the medicinal plants (MAPs) are used specifically in drug industries. However, these Phytochemicals' have

certain limitations of low absorption, high toxicity, and other side effects, bioavailability and efficacy. Irrespective of the advantages of synthetic, combinatorial chemistry and molecular modelling, they remain an important source or new drugs discovery. [1]

Malaria remains a major global health challenge, particularly due to the emergence of drug- resistant P. falciparum strains. Traditional medicinal plants offer a rich source of bioactive compounds with potential antimalarial properties. Combining herbal extracts may enhance therapeutic efficacy and reduce the likelihood of resistance development. This study investigates the antimalarial activity of capsules loaded with a combined herbal formulation against the chloroquine-resistant P. falciparum 3D7 strain and assesses their cytotoxicity in Vero cells. [23]

Acalypha indica also known as Indian nettle, exhibits anti-malarial activity by inhibiting plasmodium falciparum growth and reducing parasitemia, due to the presence of flavonoids, terpenoids, and phenolic acids, and has been traditionally used to treat fever and malaria in India and Africa. [6]

FIG 1.0 ACALYPHA INDICA

Citrullus lanatus commonly known as watermelon exhibits anti- malarial activity due to its rich content of citrulline, lycopene and other bioactive compounds which have been shown to inhibit plasmodium parasite growth and reduce malaria symptoms. [5

FIG 1.2 CITRULLUS LANATUS

MALARIA:

The Plasmodium group of single-celled bacteria is responsible for human malaria. Only female Anopheles mosquitoes carrying the infection can transmit it through bites. The parasites from the mosquito's saliva enter a person's bloodstream through a mosquito bite. After reaching the liver, the parasites develop and procreate there. There are five Plasmodium species that usually infect people. Three species—P. falciparum, P. vivax, and P. knowlesi—are linked to more severe instances. A milder form of malaria is typically caused by P. ovale

and P. malariae. Usually, antigen-based fast diagnostic tests or microscopic analysis of blood utilizing blood films are used to diagnose malaria. Although techniques for detecting the parasite's DNA using the polymerase chain reaction have been developed, their expense and complexity prevent them from being extensively employed in regions where malaria is prevalent.

The parasites have become resistant to a number of antimalarial drugs; for instance, P. falciparum, which is resistant to chloroquine, has spread across the majority of malarial regions, and artemisinin resistance has become an issue in certain Southeast Asian countries.[25]

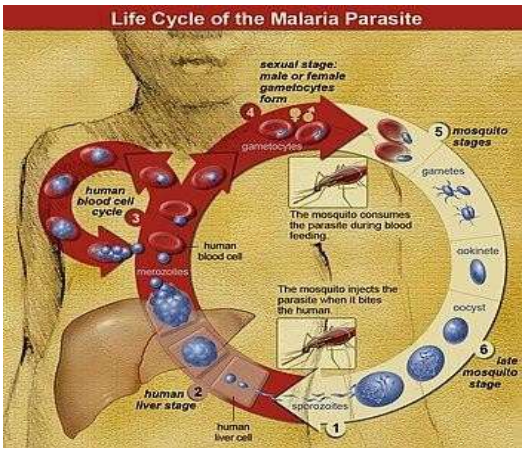


Fig 1.3 Life Cycle of the Malarial Parasite

TREATMENT:

- **Chloroquine and Hydroxychloroquine:** Prevent the parasite from breaking down hemoglobin in red blood cells.
- **Artemisinin-based Combination Therapies (ACTs):** Artemisinin, derived from *Artemisia annua*, is combined with other drugs (e.g., lumefantrine, amodiaquine). First-line treatment for **P. falciparum** malaria, especially in areas with chloroquine resistance.
- **Mefloquine:** Interferes with the parasite's ability to digest hemoglobin. Treatment and prevention in areas with chloroquine resistance.
- **Quinine and Quinidine:** Derived from the bark of the cinchona tree; used to treat severe malaria.
- **Primaquine:** Targets the liver stage of the parasite to prevent relapses, especially in **P. vivax** and **P. ovale**.
- **Atovaquone-Proguanil (Malarone):** Combines two drugs to disrupt the parasite’s mitochondrial function.

3. METHODOLOGY

COLLECTION OF PLANT MATERIAL AND AUTHENTICATION:

Fresh leaves of Acalypha indica and seeds of Citrullus lanatus were collected from Malumichampatti, Coimbatore district, Tamil Nadu during December 2024 and the plant was identified and authenticated by Dr. M. U. SHARIEF, Scientist 'F', Botanical Survey of India (BSI), Tamil Nadu Agricultural University, Coimbatore, India, where a voucher specimen 01,(No. BSI/SRC/5/23/2024-25/TECH-605) and specimen 02,(No. BSI/SRC/5/23/2024-25/TECH-606), on dated 19thDecember2024' of the plant has been kept in Institution herbarium. Fresh healthy and mature leaves of Acalypha indica and seeds of Citrullus lanatus were taken from the plant used for the study.[18]

EXTRACTION

Soxhlet extraction is a method used to extract compounds from solid materials, typically for the purpose of isolating oils, fats, or other soluble substances. In this procedure, a sample is placed in a thimble made of filter paper, which is then placed inside a Soxhlet extractor. The extractor is connected to a condenser, and a solvent is added to a round-bottom flask beneath the extractor. The system is heated, causing the solvent to evaporate and rise into the Soxhlet chamber. As the solvent condenses, it drips onto the solid sample, dissolving the desired compounds. After the solvent has soaked the sample, it is siphoned back into the flask. This process is repeated several times, allowing for the efficient extraction of the target substances. The solvent is then typically evaporated, to remove the impurities and the crude extracts were obtained.[22]

PRE- FORMULATION STUDY

PRELIMINARY PHYTOCHEMICAL SCREENING TEST

Alkaloids: Test of alkaloids (Dragendroffs test) a small amount of sample taken and add a few drops of Dragendroffs reagent. Where reddish brown colour indicates the presence of alkaloids.
Flavonoid: Test for flavonoids (Shinoda's test) sample taken and to this, a small amount of magnesium turnings was added; this was followed by 3-5 drops of the concentrated HCl. The intense cherry red colour indicated the presence of flavonoids.
Phenol: Add a few drops of 1% FeCl3 was added with added sample. Yellow colour confirms the presence of phenolic compound.
Tannins: Ferric chloride test : Take 2ml of sample and a few drops ferric chloride observe for bluish green colour indicates the presence of tannins.[20][13]

ANALYTICAL STUDIES

ABSORBANCE OF DRUG BY UV SPECTROPHOTOMETER:

Ultraviolet spectrophotometric method was carried out using ethanol as solvent media and herbal extracts as standard as per reported method with slight modification. Standard herbal extract solution in ethanol was scanned between UV-Visible range for obtaining

absorbance maxima. Then the absorbance of the different serial diluted samples of standard was measured at the λ max using a UV spectrophotometer and a standard calibration curve was plotted with concentration against absorbance. [26][27]

INVITRO ANTI-MALARIAL ACTIVITY

***In vitro* cultivation of P. falciparum:** The CQ-sensitive strain 3D7 of P. falciparum was utilized in in vitro blood stage culture to evaluate the antimalarial activity of several plant extracts. The culture was preserved in the Entomological Research Laboratory, Department of Zoology, Bharathiar University, Coimbatore. The culture of P. falciparum was sustained following the methodology outlined by Trager and Jensen (1976), with slight alterations. P. falciparum 3D7 cultures were sustained in fresh O+ve human erythrocytes at a 4% hematocrit in RPMI 1640 (Sigma), supplemented with 0.2% sodium bicarbonate, 0.5% Albumax, 45 µg/L hypoxanthine, and 50 µg/L gentamicin, and incubated at 37°C in a gas combination of 5% O2, 5% CO2, and 90% N2. Infected erythrocytes were placed daily into fresh complete medium to propagate the culture. For P. falciparum, albumax was substituted with 10% pooled human serum. [28]

Drug dilutions: Stock solutions of CQ were formulated in milli-Q grade water, whereas the test chemicals were dissolved in dimethyl sulfoxide (DMSO). All stocks were subsequently diluted with culture media to attain the specified concentrations; with the exception of CQ, the final solution included 0.4% DMSO, which was determined to be non-toxic to the parasite. Pharmaceuticals and test substances were thereafter positioned in 96-well, flat-bottom, tissue culture-grade plates. [28][29]

Evaluation of Antiplasmodial activity: The formulations were assessed for their antimalarial efficacy against P. falciparum strains 3D7. A SYBR Green I-based fluorescence test for drug screening was established as outlined by Smilkstein et al. (2004). Sorbitol-synchronized parasites were incubated under standard culture conditions at 2% hematocrit and 1% parasitemia, with or without escalating concentrations of formulations. CQ served as the positive control, whereas 0.4% DMSO functioned as the negative control. Following a 48-hour incubation, 100 µl of SYBR Green I solution (0.2 µl of 10,000 X SYBR Green I (Invitrogen) per mL) in lysis buffer {Tris (20 mM; pH 7.5), EDTA (5 mM), saponin (0.008%; w/v), and Triton X-100 (0.08%; v/v)} was introduced to each well, mixed gently twice with a multi-channel pipette, and incubated in the dark at 37°C for 1 hour. Fluorescence was quantified using a Victor fluorescence multi-well plate reader (Perkin Elmer), with excitation and emission wavelengths centered at 485 nm and 530 nm, respectively. The fluorescence counts were graphed against the drug concentration and the 50% inhibitory concentration (IC50) was ascertained through the examination of dose-response curves.[30]

Cytotoxic activity on Vero cells using MTT assay

The cytotoxic effects of formulation on host cells were evaluated by a functional assay as outlined by Mosmann (1983), utilizing Vero cells cultivated in MEM supplemented with 10% fetal bovine serum, 0.21% sodium bicarbonate (Sigma), and 50 µg/mL penicillin-streptomycin (complete medium). Cells (104 cells/200 µl/well) were inoculated into 96-well

flat-bottom tissue culture plates with complete media. Drug solutions were administered 24 hours post-seeding and incubated for 48 hours in a humidified environment at 37°C with 5% CO2. DMSO was included at a concentration of 10%. Twenty microliters of a stock solution of MTT (5 mg/mL in 1X phosphate-buffered saline) were added to each well, mixed gently, and incubated for an additional 4 hours. Following the centrifugation of the plate at 1,500 rpm for 5 minutes, the supernatant was discarded, and 100 µl of DMSO (stop agent) was introduced. The formation of formazon was measured using a microtiter plate reader (Versa Max tunable multi-well plate reader) at a wavelength of 570 nm. The 50% cytotoxic concentration (TC50) of the medication was established through the examination of dose-response curves. The therapeutic index was determined as the ratio of TC50 in Vero cells to IC50 in 3D7. [29]

PREPARATION OF HERBAL EXTRACTS POWDER:

Collect the fresh Acalypha indica leaves and Citrullus lanatus seeds were washed, dried grinded into a coarse powder subjected to extraction using a suitable solvent (ethanol, methanol, water) employing Soxhlet technique. The crude extract was subsequently dried using the lyophilization (BORG Lyophilizer) method to obtain a powdered form. After to store the powdered herbal extracts in an airtight container away from light, heat, and moisture. [2]

FORMULATION OF CAPSULE:

Formulating a capsule involves several simple steps to ensure the active ingredient (API) is effectively delivered in a stable and usable form. First, the active pharmaceutical ingredient (API) is selected and mixed with excipients substances that help in the formulation process, such as fillers (lactose or cellulose), binders (which help hold the ingredients together), disintegrants (to help the capsule break apart in the body), and lubricants (to prevent the ingredients from sticking to the equipment). These ingredients are blended together to form a uniform powder.[13][19]. Very small-scale experimental filling of the hard gelatin capsules can simply be carried out manually, that is, by removing the cap from the body of an empty capsule shell, filling the body with a preweighed amount of API or formulation, and attaching the cap.Small-scale manufacture (several hundred capsules) can be done by using a manual capsule-filling machine.[7]

Ingredients	**Quantity (g)**
Sample 01(*Acalypha indica)*	0.125g
Sample 02(*Citrullus lanatus)*	0.125g
Lactose	0.05g

Sodium Starch Glycolate	0.125g
Talc	0.001g
Magnesium Stearate	0.002g

Table 1: Formulation of Capsule

FIG 1.7 Formulation of Capsule

PRE EVALUATION OF FORMULATED POWDER:

Prepared powders were subjected for determination of bulk density, tapped density, Hausner ratio, Carr's index and angle of repose in order to assess the flow property of powder.

Bulk Density (D0)

10g of accurately weighed powder was poured into a graduated cylinder, powder bed was made uniform without disturbing the cylinder and the volume was measured directly from the graduation mark on the cylinder as ml. The volume measure was called as bulk volume and bulk density is calculated as,

Bulk density (DO) = weight of powder / bulk volume

Tap Density (DF)

After measuring D0 same cylinder was set to measure tap density. The cylinder was tapped with 100 tap drop/minute volume was noted. Tapped density was calculated using the following formula,

Tapped density (DF) = Weight of powder / Tapped volume

Angle of repose

To determine the angle of repose the powder was passed through the walls of a funnel, fixed at a definite position. The powder was poured till the upper tip of the pile surface touched the lower most end of the funnel and the angle of repose was calculated as,

θ = tan−1 (h/r)

Where Ɵ is the angle of repose, h is the height in cm and r is the radius in cm.

Hausner's ratio and Carr's index

Hausner's ratio and carr's index was also calculated with the help of bulk density and tap density using the following formulae, [12]

EVALUATION TEST FOR FORMULATED CAPSULE:

Weight variation test:

Test for uniformity of weight was performed as per Indian pharmacopoeia (IP), 2007 Randomly selected 20 capsules were weighed (individually and together) in a single pan balance. The average weight, variation in the individual capsule and the standard deviation was calculated. IP limit for weight variation in case of capsule weighting more than 400 mg is ± 5%.

Dissolution test:

Dissolution profile of capsule formulation containing lyophilized polyherbal extract was determined according to USP type-2 dissolution tester apparatus (rotating basket) (LABINDIA, DS 8000). An accurately weighed amount of capsule was placed in USP dissolution basket rotated at 100 ± 5 rpm using phosphate-buffer (pH= 6.8) as a dissolution medium and temperature was adjusted to 37 0C. 1 ml aliquot of sample was withdrawn at regular time intervals (0, 10, 20, 30, 40 and 50 min) diluted and assayed spectrophotometrically at 288 nm. Meanwhile an equal volume of PB was added to maintain the constant volume. The cumulative % release was calculated for the formulation from previously constructed calibration curve. [15]

Disintegration time:

Disintegration times for capsules were determined by disintegration apparatus. Six capsules were placed in six tubes of the basket and the apparatus was operated using water as release medium maintained at 37 ± 2°C. The capsules were observed and the times taken for complete disintegration of all capsules were determined. [21]

Stability studies of the capsules:

Stability is defined as the extent to which a product retains, within specified limits and throughout its period of storage and use (i.e., its shelf-life) the same properties and characteristics that it possessed at the time of its manufacture. [18]

Conditions of Stability studies

Accelerated condition of 40 °C ± 2 °C/75% RH ± 5% RH
Long term condition of 25 °C ± 2 °C/60% RH ± 5 % RH
Long term / intermediate condition of 30 °C ± 2 °C/75 % RH ± 5% RH

4. RESULTS AND DISCUSSION

PRELIMINARY PHYTOCHEMICAL SCREENING

S.NO	TEST	OBSERVATION	INFERENCE
1.	Dragendroffs test - extract + dragendroffs reagent	Reddish brown colour	Presence of alkaloids.
2.	Shinoda test –-extract + Mg turnings + con.Hcl	Slightly yellow colour was observed	Presence of Flavonoids.
3.	Ferric chloride test - extract + Fecl3	Yellow colour was observed	Presence of phenols.
4.	chloride test- Extract + a few drops ferric chloride	bluish green colour	Presence of Tannins.

Table 2: Phytochemical Test

The particular test required for detection of phytochemicals present in Acalypha indica and Citrullus lanatus extract were performed and test shows presences of various phytoconstituents, such as phenols, alkaloids, tannins and flavonoids.

ANALYTICAL STUDIES: UV-VISIBLE

CONCENTRATION	ABSORPTION
0.2	0.177
0.4	0.312
0.6	0.367
0.8	0.421
1	0.545

Table 3: citrullus lanatus

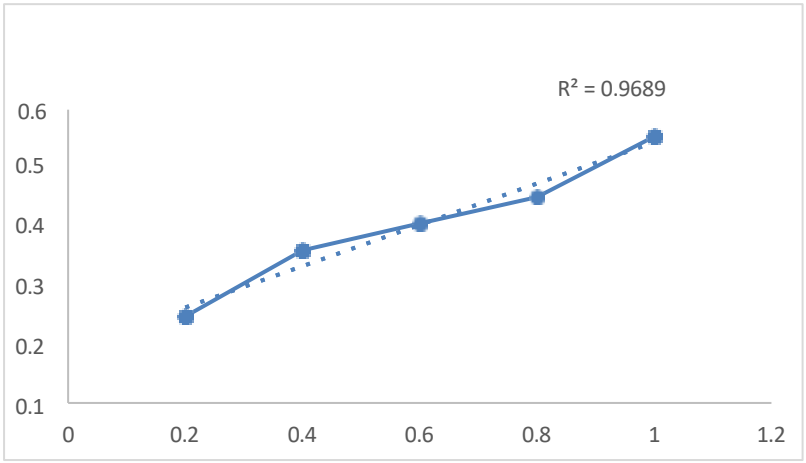


FIG 1.8 Calibration Curve for Citrullus Lanatus

CONCENTRATION	ABSORPTION
0.2	0.195
0.4	0.321
0.6	0.398
0.8	0.496
1	0.587

Table 4: Acalypha indica

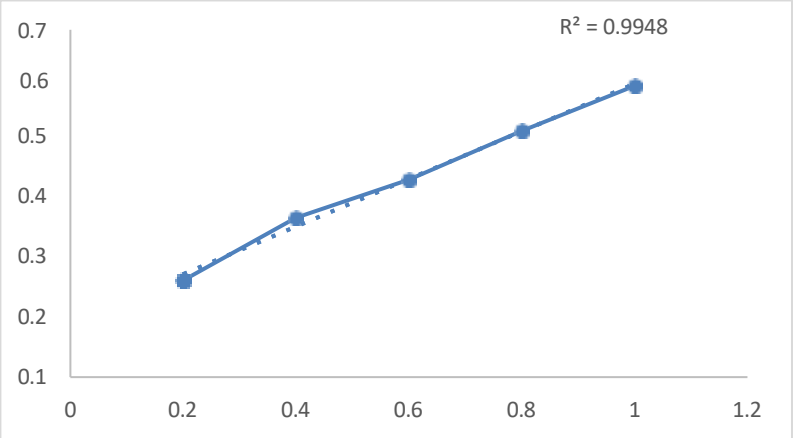


FIG 1.9 Calibration Curve for Acalypha Indica

FORMULATION OF CAPSULE:

S.NO	TEST	OBSERVATION
1.	Bulk density	0.33g
2.	Tapped density	0.52g
3.	Angle of repose	32.10°
4.	Hausner ratio	1.57g
5.	Carrs index	36.53g

Table 5: Pre evaluation of formulated powder

FIG 2.0 Bulk density **FIG 2.1 Tapped density** **FIG 2.2 Angle of repose**



S.NO	**TEST**	**OBSERVATION**
1.	Weight variation test (n= 20)	0.413g
2	Disintegration time	Not more than 30 mins
3	Stability study	No physically instability over 3months

Table 6: Evaluation of Formulated Capsule

DISSOLUTION STUDY:

FIG 2.3 Dissolution test

TIME(MIN)	CUMULATIVE DRUG RELEASE (%)
0	0
10	0.345
20	0.271
30	0.408
40	0.367
50	0.472

Table 7: Dissolution Test

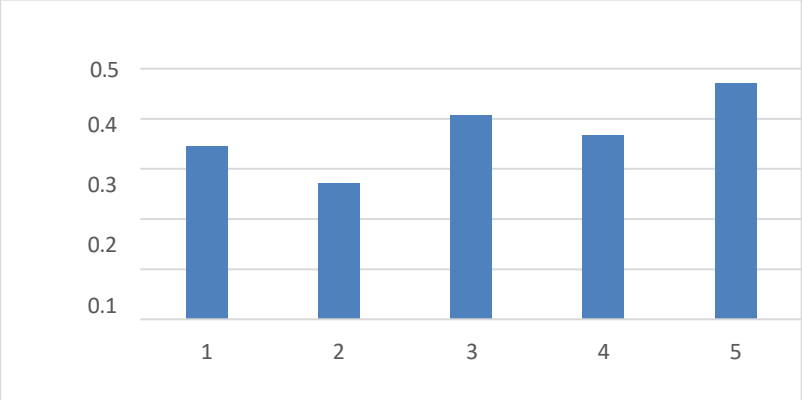


FIG 2.4 DISSOLUTION TEST

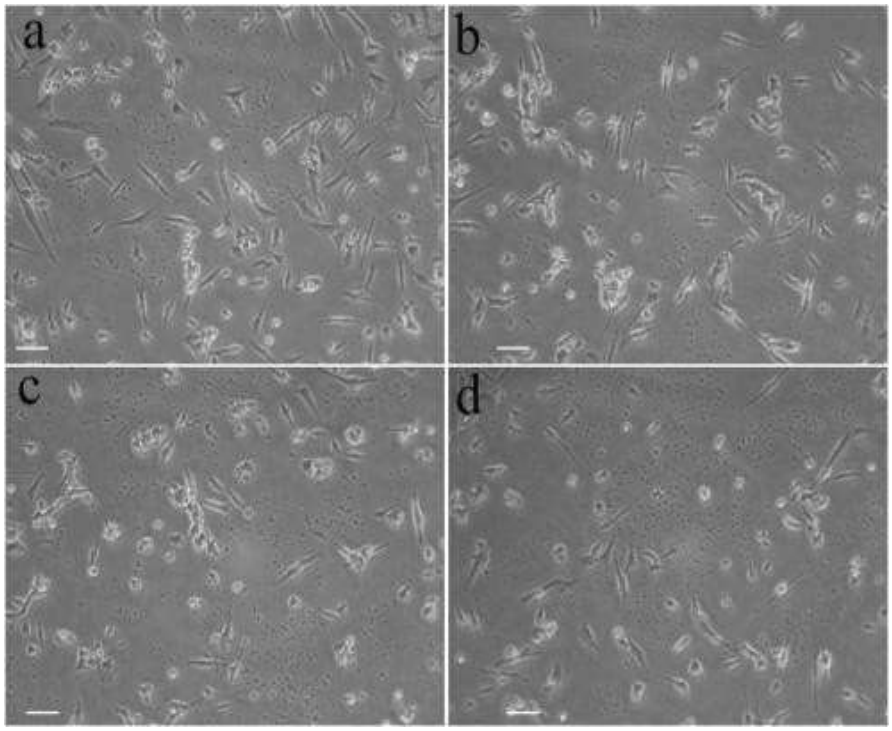
The cumulative drug release data indicates a gradual and steady increase in drug release over time. At 10 minutes, 0.345% of the drug is released, followed by a slight decrease to 0.271% at 20 minutes. However, the release rate picks up again, reaching 0.408% at 30 minutes and 0.367% at 40 minutes. Finally, at 50 minutes, the cumulative drug release reaches 0.472%. This data suggests that the drug release profile is characterized by an initial burst release, followed by a more sustained release phase. The overall release profile appears to be relatively slow and controlled, which may be desirable for certain therapeutic applications.

INVITRO ANTIMALARIAL ACTIVITY

Therapeutic indices of formulations harboring potent antimalarial activity

S.NO	SAMPLE	ANTIMALARIAL POTENCY (IC_{50} MG/ML) AGAINST *PLASMODIUM FALCIPARUM* 3D7	CYTOTOXICITY (CC_{50} MG/ML) AGAINST VERO CELLS	THERAPEUTIC INDEX TC50/IC50
1	*E1- Acalypha indica*	3.71	>100	>47
2	*E2- Citrullus Lanatus*	6.23	>100	>33
3	Std (CQ)	0.031	32	>64

Table 8: Invitro Antimalarial Activity



Vero cells Scale bars - 50µm Control (a) 25µg/ml (b) 50µg/ml (c) 100µg/ml (d)

Values enclosed in parenthesis represent resistance index CQ-resistant strain 3D7.

In the current investigation, a variety of formulations were tested to determine the effectiveness of their antimalarial properties. Additionally, sample E1- Acalypha indica had very high levels of potency. The formulations demonstrated an astonishingly superior response against CQ-resistant strain 3D7, with resistance indices ranging from >47. In order to determine the therapeutic utility of the potent formulations, the formulations were tested

for their ability to cause cytotoxicity in the African green monkey kidney cell line (Vero). At a dose of 100 μg/mL, it was revealed that these formulations exhibited no toxicity, resulting in a therapeutic index that ranged from more than 33.

DISCUSSION

The evaluation of the physicochemical properties, including bulk density, tapped density, angle of repose, Hausner ratio, and Carr's index, suggests that the formulation has moderate flowability and compressibility, which are crucial for ensuring uniformity in dosage forms. The phytochemical screening of Acalypha indica and Citrullus lanatus extracts confirmed the presence of bioactive compounds such as phenols, alkaloids, tannins, and flavonoids, which are known for their antioxidant, anti-inflammatory, and therapeutic properties. The drug release study revealed a biphasic pattern, with an initial burst release followed by a sustained release phase, which is desirable for prolonged therapeutic effects. The controlled drug release profile indicates that the formulation may help maintain consistent drug levels over time, reducing the need for frequent dosing. Furthermore, the remarkable activity of the formulation against CQ-resistant strain 3D7, with resistance indices exceeding 47, highlights its potential as an effective antimalarial agent. Importantly, the cytotoxicity assay using Vero cells demonstrated that the formulation is non-toxic at 100 μg/mL, with a high therapeutic index (>33), indicating a favorable safety profile. These findings support the potential application of this formulation in therapeutic settings, warranting further in vivo studies to validate its efficacy and safety.

5. CONCLUSION

In conclusion, the formulated system demonstrated favorable physicochemical properties, ensuring good flowability and compressibility for potential pharmaceutical applications. The phytochemical analysis confirmed the presence of bioactive compounds, reinforcing the therapeutic potential of Acalypha indica and Citrullus lanatus extracts. The drug release profile exhibited an initial burst followed by sustained release, indicating controlled and prolonged drug availability. Notably, the formulation showed significant activity against CQ-resistant strain 3D7, with high resistance indices, and exhibited no cytotoxicity in Vero cells at 100 μg/mL, ensuring a high therapeutic index. These findings suggest that the developed formulation holds promise for safe and effective therapeutic applications, warranting further investigation for clinical translation.

6. CONFLICT OF INTEREST

The authors declare no conflict of interest.

7. ACKNOWLEDGEMENTS

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