

Antimalarial Activity, Mechanism of Action and Drug Interaction Study of Active Constituent Isolated from The Dried Seeds of *Psoralea corylifolia*

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ABSTRACT

Malaria eradication still an enormous global health challenge and getting worse after developing resistance against most of the antimalarial drugs. Plants possess various phytoconstituents that will be a good source for developing novel antimalarial leads. Bakuchiol, a meroterpene was isolated from the seeds of *Psoralea corylifolia* possess many different biological activities. However, its antimalarial potential is unexplored till now. In this study, we first time demonstrated that the hexane extract of *P. corylifolia* seeds showed good anti-plasmodial activity (IC₅₀ 18.3 ± 0.38 µg/ml). The purified product from the hexane extract, bakuchiol showed improved anti-plasmodial activity with IC₅₀ 3.1 ± 0.35 µg/ml (12.1 ± 0.92 µM) in dose dependent manner. Bakuchiol did not shows hemolytic activity at higher concentration (3900 µM). The blood stage specific study showed that late trophozoite and early schizont parasite stages were found to be most sensitive to bakuchiol treatment. The mode of action study revealed that bakuchiol induced oxidative stress *via* generation of reactive oxygen and nitrogen species. Bakuchiol prompts depolarization of mitochondrial membrane potential ($\Delta\psi_m$) and causes DNA fragmentation after the treatment in concentration dependent manner. Bakuchiol showed synergistic interaction with the two most clinically used antimalarial drugs (chloroquine and quinine). The results of this study indicated that bakuchiol inhibits *P. falciparum* growth, inducing oxidative stress lead to mitochondrial membrane depolarization, DNA fragmentation and also act synergistically. Considering the need of new therapeutic alternative options, results showed that bakuchiol can be a potential lead phytomolecule for combination therapy against multidrug resistant malaria.

Key Words: Bakuchiol, Reactive oxygen species, *P. falciparum*, Oxidative Stress, mitochondrial membrane potential ($\Delta\psi_m$), DNA fragmentation.

INTRODUCTION

Plants are practically unlimited sources of novel substances with many therapeutic applications. About half of all drugs in clinical use today are plant based¹. Therefore, the exploration of plants for their possible medicinal uses needs to be continued. In our continuing search for antimalarial compounds from plant sources, we have isolated bakuchiol from the seed of *P. corylifolia* Linn, a tree native to China. The seeds of *P. corylifolia* L. (Fabaceae) have been used traditionally in Chinese and Indian folk medicine for leucoderma and vitiligo². Bakuchiol is a principal constituent of *P. corylifolia* seeds, possesses multiple biological activities such as antimicrobial, anticancer, anti-inflammation and antifungal etc.^{3,4}. Chemically, bakuchiol [4-(1E, 3S)-3-ethenyl-3, 7-dimethyl-1, 6-octadienyl] is made up of styryl moiety in conjunction with a monoterpene and together named as meroterpene⁵.

Malaria is one of the most prevalent and pernicious disease of humans. *Plasmodium sp.* has already acquired resistance against most of the clinically used antimalarial drugs, including artemisinin, which is considered to be the last resort. Artemisinin's in combination remain the only regimens for which clinical failures have not been identified. However, most of these regimens are administered in low doses and are currently having difficulty in implementation, especially in rural areas of developing countries due to affordability and approachability limits⁶. The foci of artemisinin resistance have been documented in various parts of the world, and if it is continued, the malaria control programs could be dire, as no alternative antimalarial drug available for treatment. Hence, a continue efforts are needed to develop new antimalarial agents for combating resistance malaria.

Malaria parasites are highly susceptible to variation in redox equilibrium. Oxidative stress is the result of

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imbalances in cellular redox equilibrium and inability of antioxidant defense system to regulate stress causing agents like reactive oxygen species (ROS) or reactive nitrogen species (RNS)⁷. Free radicals have a useful role in physiological adaptation and in the regulation of intracellular signal transduction. However, it is harmful some time because oxygen free radicals attack biological molecules such as lipids, proteins and DNA⁸. Free radicals are oxygen containing molecules that have one or more unpaired electrons, making it highly reactive with other cellular molecules. Hydrogen peroxide (H₂O₂), Hydroxyl radical (HO[•]), Singlet oxygen (¹O₂), Superoxide radical (O₂^{•-}), Hydroperoxyl radical (HOO[•]), alkylhydroperoxide (LOOH), Alkylperoxyl radical (LOO[•]), Ferryl ion (Fe⁴⁺O), Periferryl ion (Fe⁵⁺O), Nitric oxide (NO[•]) are major active oxygen species⁸. Interestingly, not all reactive oxygen species are harmful to the body, some of them are useful in killing invading pathogens or microbes⁹. H₂O₂, NO and their reaction products reduce parasite viability and susceptibility of *Plasmodium* spp. to oxidant mediated killing has been well established^{10, 11}. In fact, currently used antimalarial therapy constitutes a source of oxidation, as many drugs such as chloroquine, Primaquine and artemisinins are inducers of free radical production¹².

The emergence of resistance to chloroquine and quinine, the most widely used, safest and cheapest antimalarial drugs adversely affect the malaria control programs with the increasing cost of alternative drugs, especially in developing countries have necessitated the need to optimize antimalarial actions of plant phytomolecules and restore efficacy of these old arsenals. Remarkably, chloroquine remains the regimen of choice against malaria in pregnancy due to its enormous safety and cheapness, which remains undefeatable by other antimalarial alternatives¹³. Quinine also a drug of choice currently in areas where quinine resistance is not widespread¹⁴. Taken together, the above findings have provided a justifiable evidences and need to develop strategies of reestablishing chloroquine/quinine efficacy and prolong its clinical utility as an antimalarial drug. The aim of the present study was to examine the anti-plasmodial activity of bakuchiol and explore mechanism of action. Further, *In vitro* combination study was performed to assess potential interactions between bakuchiol with two widely used antimalarial drugs (Chloroquine, Quinine).

MATERIAL AND METHODS

Extraction and Isolation of bakuchiol

The extraction and isolation of bakuchiol were done using methods described previously¹⁵. Briefly, air-dried seeds (400g) of *P. corylifolia* were crushed and powdered which was percolated by *n*-hexane (1L x 3). Removal of solvent under vacuum provided viscous extract (32g). *n*-hexane extract (20g) was subjected to vacuum liquid chromatography [VLC], using silica gel and the column was eluted with hexane, hexane: ethyl acetate (99:1, 98:2, 95:5, 90:10, 75:25). Purity analysis of bakuchiol was performed by HPLC using a Shimadzu LC-10AD liquid

chromatograph equipped with two LC-10A pumps controlled by a CBM-10 interface module, SPD-M10A VP diode array detector, and SIL-10ADVP auto injector. Data were collected and analyzed using a class LC-10 Work Station. The samples were analyzed by using reverse phase chromatography on waters spherisorb ODS2 (250 x 4.6mm i.d., 10 mm) column, mobile phase, acetonitrile: ethyl acetate: iso propyl alcohol (propane-2-ol) 80:10:10 at a flow rate of 0.8ml/min, with column temperature of 25° and UV detection at λ 260 nm.

In-vitro culture of parasite

The chloroquine sensitive *P. falciparum* strain NF-54 was cultivated in human B⁺ red blood cells using RPMI-1640 medium supplemented with 25 mM HEPES, 0.2% NaHCO₃, 370 μM hypoxanthine, 40 μg/mL gentamycin, 0.25 μg/mL fungizone, and 0.5% albumax II at 37 °C as described previously with minor modification¹⁶. Culture was maintained in a standard gas mixture consisting of 5% CO₂. The culture was synchronized by 5% D-sorbitol treatment to obtain ring-stage parasites¹⁷.

In-vitro anti-plasmodial activity assay

In vitro anti-plasmodial activity was determined using SYBER green-I based assay as described previously¹⁸. Briefly, a synchronous ring stage cultures with 1.5% parasitaemia and 3% hematocrit were incubated under standard culture condition in the sterilized black flat bottom 96-well tissue culture plate with different concentrations of bakuchiol. Artemisinin (Sigma) and Chloroquine (Sigma) were used as positive control. After 48 h of incubation, 100μl of SYBER Green I solution was added to each well, mixed gently and incubated in dark at 37°C for 1 hour (h). Fluorescence intensity was measured using a microplate reader (BMG Labteck) with excitation and emission wavelength bands centered at 480 nm and 520 nm, respectively. The fluorescence counts were plotted against the drug concentration, and IC₅₀ (50% inhibitory concentration) was determined by analysis of dose response curves with nonlinear regression analysis. The experiments were performed thrice (n=3) and data expressed as mean values ±SEM. **P<0.01 vs control.

Hemolytic activity

The hemolytic assay was performed as described previously¹⁹. Briefly, 250 μl of 10% (v/v) RBCs suspension were incubated under agitation at room temperature for 1 h containing 5 μl of bakuchiol at 3900 μM concentration. Triton X-100 1% (v/v) was used as a positive control (100% lysis) and PBS (pH 7.4) as a negative control (0% lysis). The mixtures were centrifuged at room temperature for 5 min at 8,000 × g and absorbance of the supernatants were measured at 550 nm with a microplate reader (BMG LabTech). The red blood cell lysis percentage was determined as follows:

$$\text{Percent RBCs lysis} = \frac{(\text{OD}_{550\text{nm sample}} - \text{OD}_{550\text{nm PBS}})}{(\text{OD}_{550\text{nm Triton X-100 1\%}} - \text{OD}_{550\text{nm PBS}})}$$

The experiments were performed thrice (n=3) and data expressed as mean values ±SEM. **P<0.01 vs control.

Blood stage specific study

The effect of bakuchiol on different blood stages of parasite was studied using the method described previously²⁰. In brief, a highly synchronized ring stage *P.*

falciparum culture at 2.5% parasitaemia and 4% hematocrit was incubated in the presence of bakuchiol at $3 \times \text{IC}_{50}$ concentration for 80 h. To study the blood stage specific effect and reversibility of drug action, a highly synchronized ring stage culture at the same parasitaemia level was divided in three separate wells of 24-well tissue culture plate. Each well was treated with $3 \times \text{IC}_{50}$ concentration of bakuchiol, after 1h (ring stage), 12h (trophozoite stage), 24h (schizont stage) and incubated for 12h followed by removal of the bakuchiol through washing with complete medium by centrifugation ($500 \times g$ for 2 min). Finally, cultures were re-suspended in drug free complete medium. Thin blood smears were prepared at 12 h intervals up to 80 h and stained with Giemsa. Parasite morphology was evaluated by light microscopy (Nikon, Japan).

Measurement of reactive oxygen species

The intracellular ROS level was determined by spectrofluorometric and flow cytometric assay as described previously²¹. Briefly, *P. falciparum* (15% parasitaemia) culture was incubated with different concentrations of bakuchiol for 36 h at 37°C in the CO₂ incubator. After incubation, the cultures were washed twice with culture medium and further incubated for 30 minutes in complete culture medium containing CM-H₂DCFDA (10 µM). After that, culture washed twice with ice cold PBS and parasites were isolated and lysed by mild sonication. The ROS level was determined in lysate by measuring the formation of fluorescent dichlorofluorescein (DCF) using a microplate reader (BMG Labteck) at wavelengths 480 nm and 520 nm for excitation and emission, respectively. The experiments were performed thrice (n=3) and data expressed as mean values \pm SEM. **P<0.01 vs control. Simultaneously, Intracellular ROS level was also measured by flow-cytometry²². In brief, *P. falciparum* culture (15% parasitaemia) was treated and stained as above. Stained parasite cultures were analyzed using LSRII flow-cytometer (BD Biosciences) equipped with 488 nm argon laser as light source. The experiment was performed three times, a representative data are shown.

Measurement of nitric oxide level

Measurement of nitric oxide (NO) level was determined using the Griess reagent kit (Invitrogen). Briefly, *P. falciparum* (15% parasitaemia) cultures were incubated with different concentrations of bakuchiol for 36 h at 37°C. After incubation, 150 µl of culture supernatant containing nitrite, 130 µl of deionized water and 25 µl of Griess reagent were mixed in sterilized flat bottom 96-well plate and incubated for 30 minutes at room temperature. A NO producer, Sodium-nitroprusside (Sigma) was used as a positive control. The absorbance was measured using a microplate reader (BMG Labteck) at 548 nm. The experiments were performed thrice (n=3) and data expressed as mean values \pm SEM. **P<0.01 vs control.

Measurement of mitochondrial membrane potential ($\Delta\psi_m$)

Mitochondrial membrane potential was investigated using BD MitoScreen Flow Cytometry kit. Briefly, *P.*

falciparum cultures (15% parasitaemia) were treated with different concentrations of bakuchiol and incubated for 3 h at 37°C. CCCP (2.5 µM) was used as positive control. After incubation, the cultures were washed twice with PBS and pelleted at $500 \times g$ for 2 minutes. The 100 µl of packed cells was suspended in 1x assay buffer and stained with JC-1 dye and analyzed in Flow-cytometer (LSRII BD Biosciences) equipped with 488 nm argon laser beam as light source. The experiment was performed three times, a representative data are shown. Simultaneously, spectrofluorometric measurement of $\Delta\psi_m$ was also determined²³. In brief, *P. falciparum* culture (15% parasitaemia) was treated and stained as above. Absorbance of stained cultures were recorded using a microplate reader (BMG Labteck) at 520 nm and 590 nm wavelengths for excitation and emission, respectively. The experiments were performed thrice (n=3) and data expressed as mean values \pm SEM. **P<0.01 vs control.

DNA fragmentation assay

DNA Fragmentation was measured using APO-BrdU apoptosis detection kit (BD Biosciences). Briefly, *P. falciparum* cultures (15% parasitaemia) were treated with the different concentrations of bakuchiol for 36 h at 37°C. Camptothecin (CPT) was used as positive control. After incubation, parasite cultures were washed twice with PBS (pH 7.4) and re-suspended in 4% (w/v) para-formaldehyde in PBS for fixing the cells. After fixation cultures were stained as per kit protocol and analyzed using flow-cytometer (BD Biosciences) equipped with 488 nm argon laser beam as the light source. The experiment was performed three times, a representative data are shown. Simultaneously, after staining, spectrophotometric measurement of fluorescence intensity was recorded using microplate reader (BMG Labteck) at wavelengths 485-10 nm and 520 nm for excitation and emission, respectively. The experiments were performed thrice (n=3) and data expressed as mean values \pm SEM. **P<0.01 vs control.

Drug combination assay

Fixed ratio combinations of bakuchiol with chloroquine and quinine were tested as previously described²⁴. Briefly, seven drug combination solutions were prepared of which only five were combinations of the two drugs in a fixed ratio of 4:1, 3:2, 1:1, 2:3, 1:4 and the first and last of these seven preparations had the drug alone at a concentration approximately 5 times higher than IC_{50s} of the drugs. The IC_{50s} were calculated for each drug alone and for their respective fixed ratio concentrations using Sybergreen I assay. The individual 50% fractional inhibitory concentrations (FIC₅₀), sum 50% fractional inhibitory concentrations (Σ FIC₅₀) was calculated and isobologram was prepared. Σ FIC < 0.5 represents substantial synergism, Σ FIC < 1 represents synergism, Σ FIC \geq 1 and < 2 represents additive interaction, Σ FIC \geq 2 and < 4 represents slight antagonism where as Σ FIC \geq 4 represents marked antagonism.

RESULTS

Isolation of bakuchiol

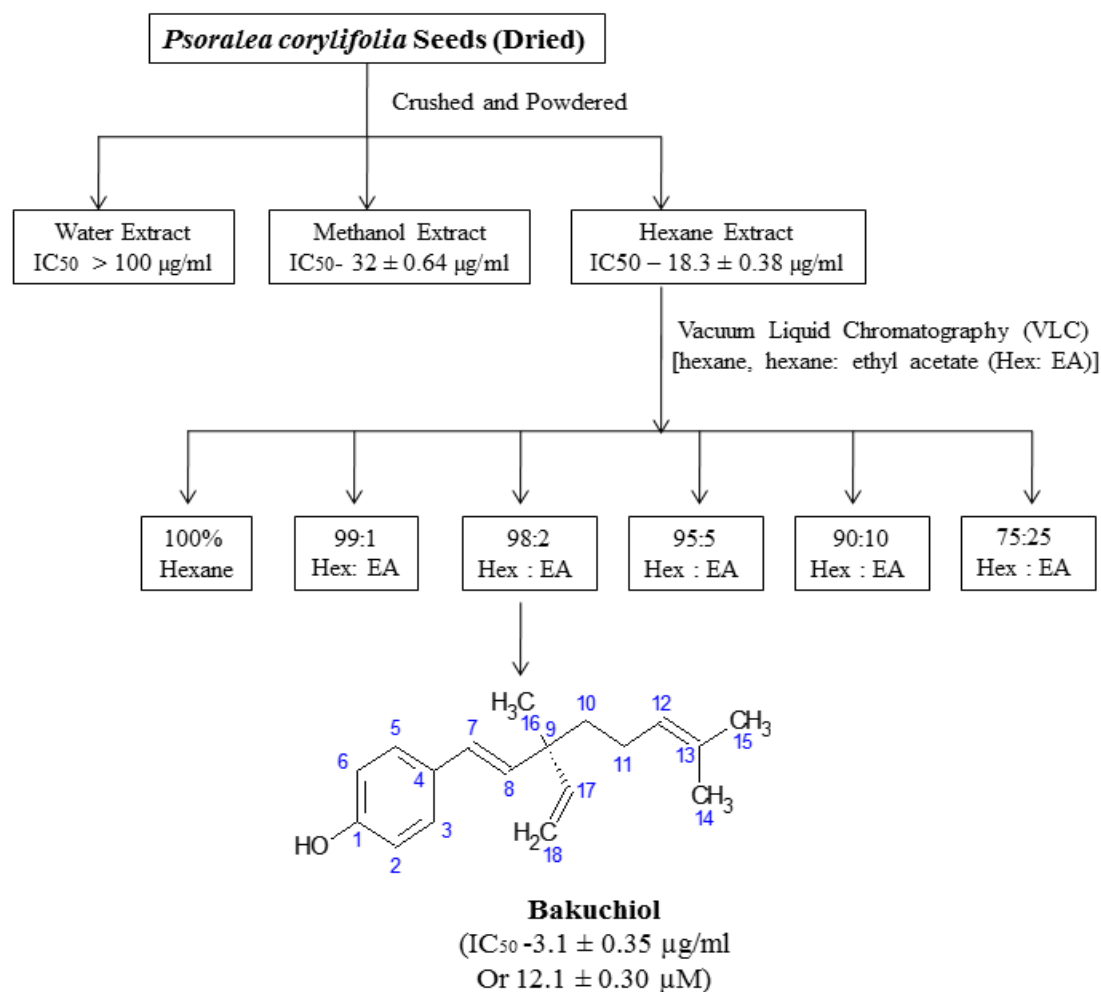
Figure 1: Scheme of extraction and isolation of bakuchiol from dried seeds of *P. Corylifolia*.

Table 1: Interaction between Chloroquine and Bakuchiol.

Combination	Ratio of Drugs		Chloroquine mean FIC_{50}	Bakuchiol mean FIC_{50}	$\Sigma FICs$	Interaction
	Chloroquine	Bakuchiol				
1	5	0	1.0	0.0	1.0	Additive
2	4	1	0.6	0.9	1.5	Additive
3	3	2	0.3	0.6	0.9	Synergistic
4	1	1	0.6	0.8	1.4	Additive
5	2	3	0.5	0.7	1.2	Additive
6	1	4	0.6	0.7	1.3	Additive
7	0	5	0.0	1.0	1.0	Additive

After fractionation of hexane extract, a viscous compound (ASPC-2) was found in *n*-Hexane:ethyl acetate (98:2) fractions (17-22). IR ν^{max} (KBr): 3365(phenolic OH), 2860, 1622 (double bond), 1405, 1243, 981 (aromatics) cm^{-1} ; ^1H NMR (300 MHz, Acetone- d_6): δ 1.22 (3H, s, H_3 -16), 1.41 (2H, m, H_2 -10), 1.49 (3H, s, H_3 -15), 1.57 (3H, s, H_3 -14), 1.91 (2H, m, H_2 -11), 4.91 (1H, m, H_a -18), 4.96 (1H, br s, H_b -18), 5.04 (1H, m, H_2 -12), 5.89 (1H, dd, $J=18.0, 10.2\text{Hz}$, H_2 -17), 6.02 (1H, d, $J=16.2\text{Hz}$, H_2 -8), 6.22 (1H, d, $J=16.2\text{Hz}$, H_2 -7), 6.70 (2H, d, $J=8.4\text{Hz}$, H_2 -3, H_2 -5), 7.18 (2H, d, $J=8.4\text{Hz}$, H_2 -2, H_2 -4), 8.25 (1H, br s, OH); ^{13}C and DEPT NMR (75MHz, Acetone- d_6): δ 17.20 (C-15), 23.30 (C-16), 23.50 (C-11), 25.35 (C-14), 41.69 (C-10), 42.65 (C-9), 111.58 (C-18), 115.72 (C-3, C-5),

125.25 (C-12), 127.35 (C-7), 127.62 (C-2, C-6), 129.93 (C-1), 130.99 (C-13), 134.89 (C-8), 146.58 (C-17), 157.1 (C-4); GC-MS (m/z): 256 [M^+], 241, 213, 173, 158, 145, 107. Comparison of these spectral data with reported literature revealed that the compound ASPC-2 was bakuchiol (Fig. 1), a meroterpenoid phenol¹⁵.

In vitro anti-plasmodial activity

In vitro anti-plasmodial activity was evaluated against chloroquine sensitive *P. falciparum* NF-54 strain. Water, methanol and hexane extract exhibited $IC_{50} > 100$, $32.0 \pm 0.64 \mu\text{g/ml}$ and $18.3 \pm 0.38 \mu\text{g/ml}$, respectively. Bakuchiol showed improved anti-plasmodial activity with $IC_{50} 3.1 \pm 0.35 \mu\text{g/ml}$ ($12.1 \pm 0.92 \mu\text{M}$) in dose dependent manner. The IC_{50} for chloroquine and artemisinin was

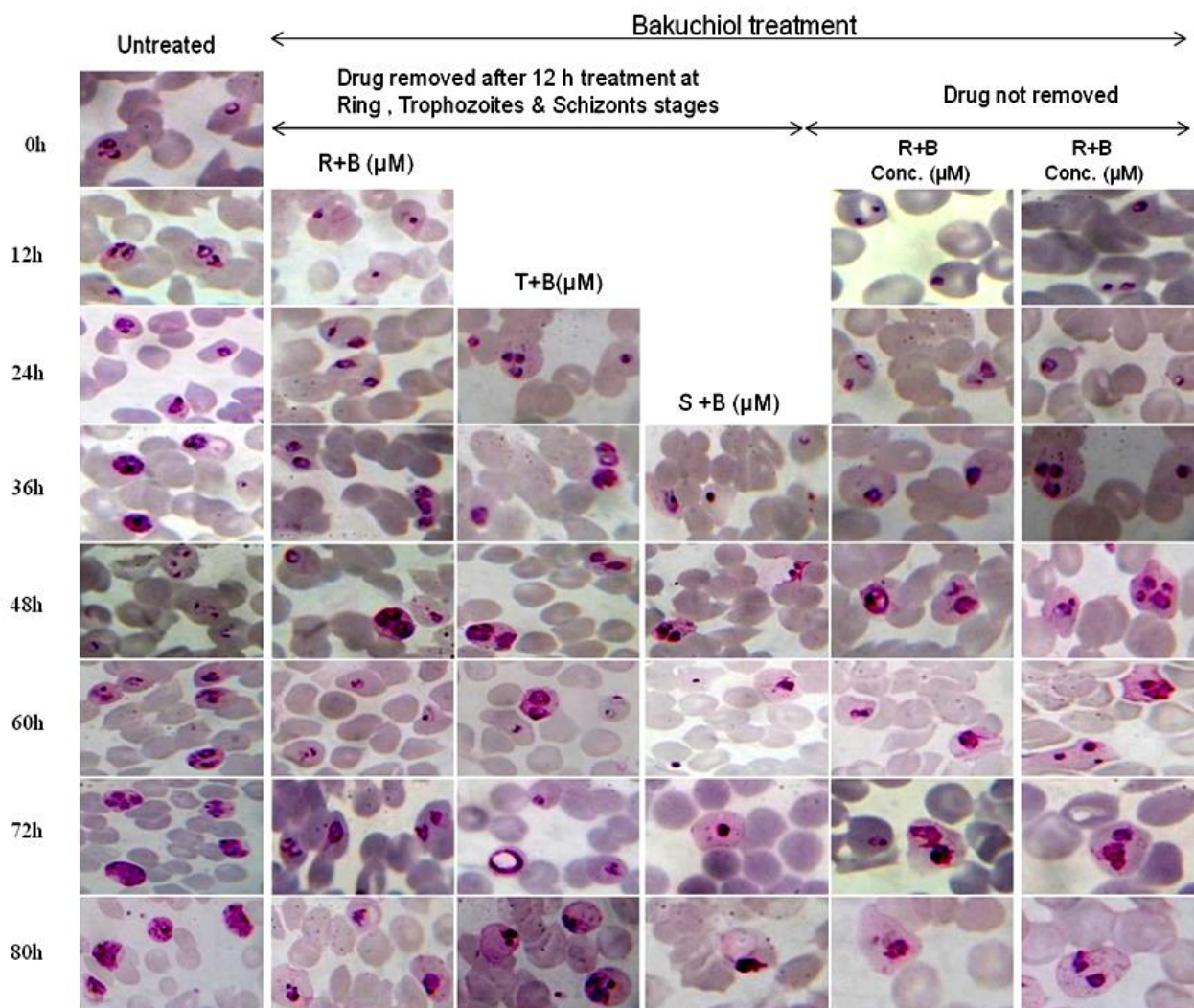


Figure 2: Microscopic observations after bakuchiol treatment at different blood stages of *P. falciparum*. R+B= Ring stage parasites treated with bakuchiol; T+B = Trophozoite stage parasites treated with bakuchiol; S+B= Schizont stage parasites treated with bakuchiol.

$0.046 \pm 0.009 \mu\text{g/ml}$ and $0.026 \pm 0.007 \mu\text{g/ml}$ respectively.

Hemolytic activity of bakuchiol

The hemolytic activity of bakuchiol was tested by measuring the lysis of a 10% (v/v) human red blood cells suspension using spectrophotometric assay. The bakuchiol was tested at a concentration of $3900 \mu\text{M}$ and no significant red blood cell lysis ($5.65 \pm 0.87\%$) was observed.

Blood stage specific study of bakuchiol

To study the effect of bakuchiol on the parasite blood stages, a highly synchronized ring stage culture (2.5% parasitaemia) treated at $3 \times \text{IC}_{50}$ concentration. Parasite growth was significantly arrested at the late trophozoite stage of the erythrocytic cycle in the presence of bakuchiol at $3 \times \text{IC}_{50}$ concentration (Fig. 2). To study the reversibility of drug effect, synchronized ring, trophozoite and schizont stage cultures were treated separately with $3 \times \text{IC}_{50}$ concentration of bakuchiol for 12 h and further incubated in drug free culture medium up to 80 h. It was observed that, the parasite treated at ring stage developed

into trophozoite after 36 h and further to schizont after 48 h and few new rings appeared at 60 h, however parasitaemia was very low as compared to control.

Parasite treated at trophozoite stage did not grow normally. However, some new rings appear at 60 h and develop to trophozoite at 72 h - 80 h with abnormal (condensed) nuclear morphology. Schizont treated parasite stages were most affected by bakuchiol treatment and they did not recover after drug removal up to 80 h (Fig. 2).

Effect of bakuchiol on ROS level

To study the effect of bakuchiol on ROS generation, intracellular level of H_2O_2 was measured spectrofluorometrically and flowcytometrically. In spectrofluorometric assay, the intracellular level of H_2O_2 at different concentrations were observed to be increased significantly (Figure 3A). Percent increase in ROS level was $9.03 \pm 0.54\%$, $26.1 \pm 0.63\%$, $38.4 \pm 0.73\%$ and $40.1 \pm 0.53\%$ at $3.9 \mu\text{M}$, $7.8 \mu\text{M}$, $11.7 \mu\text{M}$ and $15.6 \mu\text{M}$ concentrations respectively (Figure 3B). The flow cytometric analysis was also in agreement with the

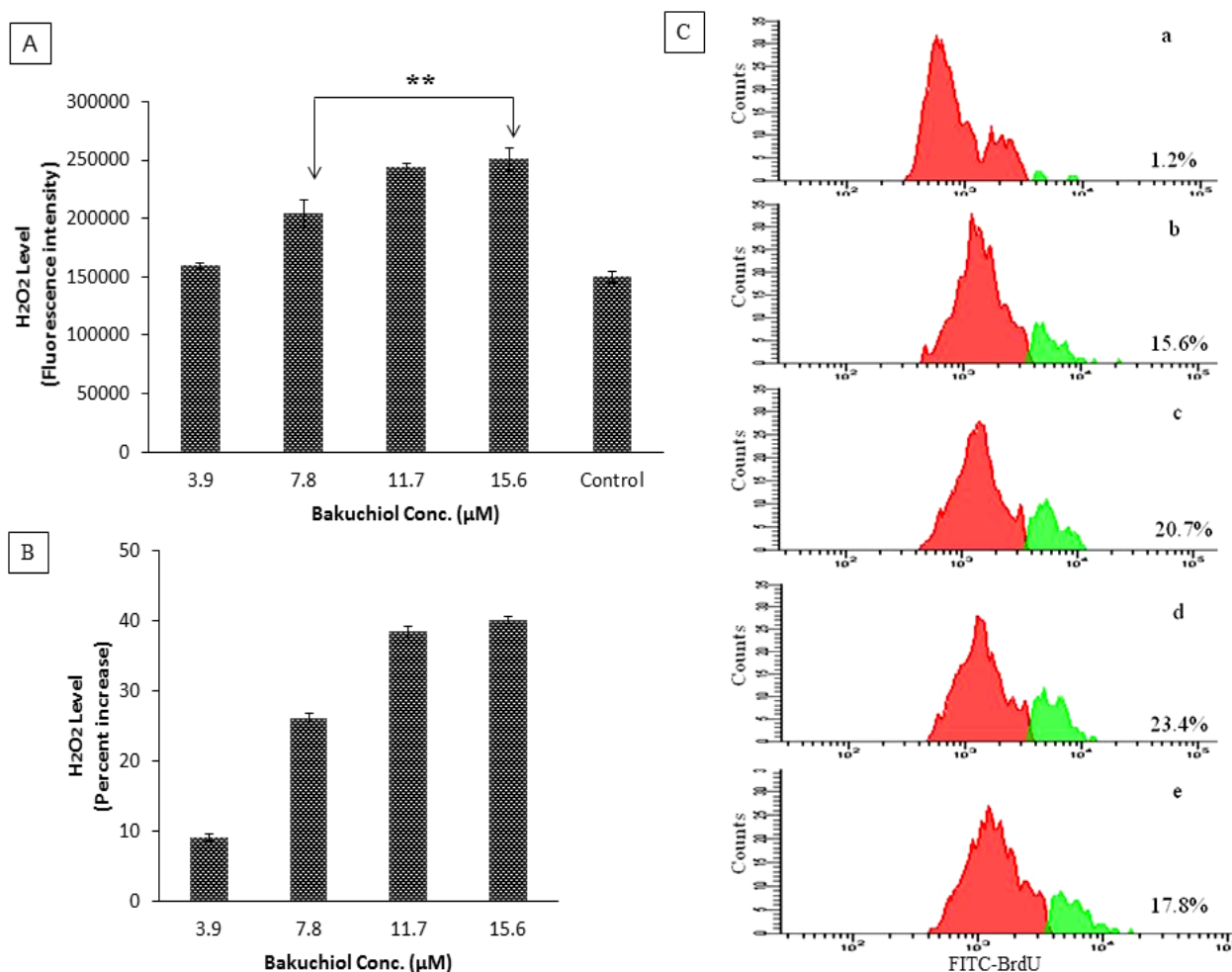


Figure 3: Effect of bakuchiol on H₂O₂ Level. A. Level of intracellular H₂O₂, B. Percent increase in H₂O₂ level. C. Flow cytometric histograms showing the shifting of population towards FITC channel. a. Untreated control, b. 3.9 μM, c. 7.8 μM, d. 11.7 μM, e. 15.6 μM.

Table 2: Interaction between Quinine and Bakuchiol.

Combination	Ratio of Drugs		Quinine mean FIC ₅₀	Bakuchiol mean FIC ₅₀	Σ FICs	Interaction
	Quinine	Bakuchiol				
1	5	0	1.0	0.0	1.0	Additive
2	4	1	0.5	0.9	1.4	Additive
3	3	2	0.4	0.8	1.2	Additive
4	1	1	0.6	0.9	1.5	Additive
5	2	3	0.3	0.7	1.0	Additive
6	1	4	0.2	0.7	0.9	Synergistic
7	0	5	0.0	1.0	1.0	Additive

spectrofluorometric data, wherein the shifting of DCF positive cells towards the FITC channel were also observed and number of DCF positive cells were also increased (15.6%, 17.0%, 20.7%, and 17.8%) as the concentrations increased in comparison to control (Figure 3C).

Effect of bakuchiol nitric oxide level

The nitrite level was observed to be increased (7.73 ± 0.54 %, 16.7 ± 0.93 %, 26.8 ± 0.38 %, 32.3 ± 0.62 %) in a dose dependent manner upon the treatment of bakuchiol at the concentrations of 3.9 μM, 7.8 μM, 11.7 μM and 15.6 μM, respectively. In the case of positive control (SNP), the increased in nitrite level was 42.7 ± 0.54 %.

Effect of bakuchiol on mitochondrial membrane potential
To study the effect of bakuchiol on mitochondrial dysfunction, alteration in mitochondrial membrane potential ($\Delta\psi_m$) was measured. Bakuchiol significantly decreased $\Delta\psi_m$ as indicated by the spectrofluorometric analysis and flow cytometric analysis (Figure 5).

The ratio of fluorescence intensity at 590/520 was observed to be 1.08 ± 0.034 , 0.99 ± 0.021 , 0.93 ± 0.021 , and 0.74 ± 0.008 at the concentrations of 3.9 μM, 7.8 μM, 11.7 μM and 15.6 μM respectively, which was significantly lower (1.24 ± 0.002) than untreated control. The ratio of 590/520 was 0.29 ± 0.002 in case of CCCP

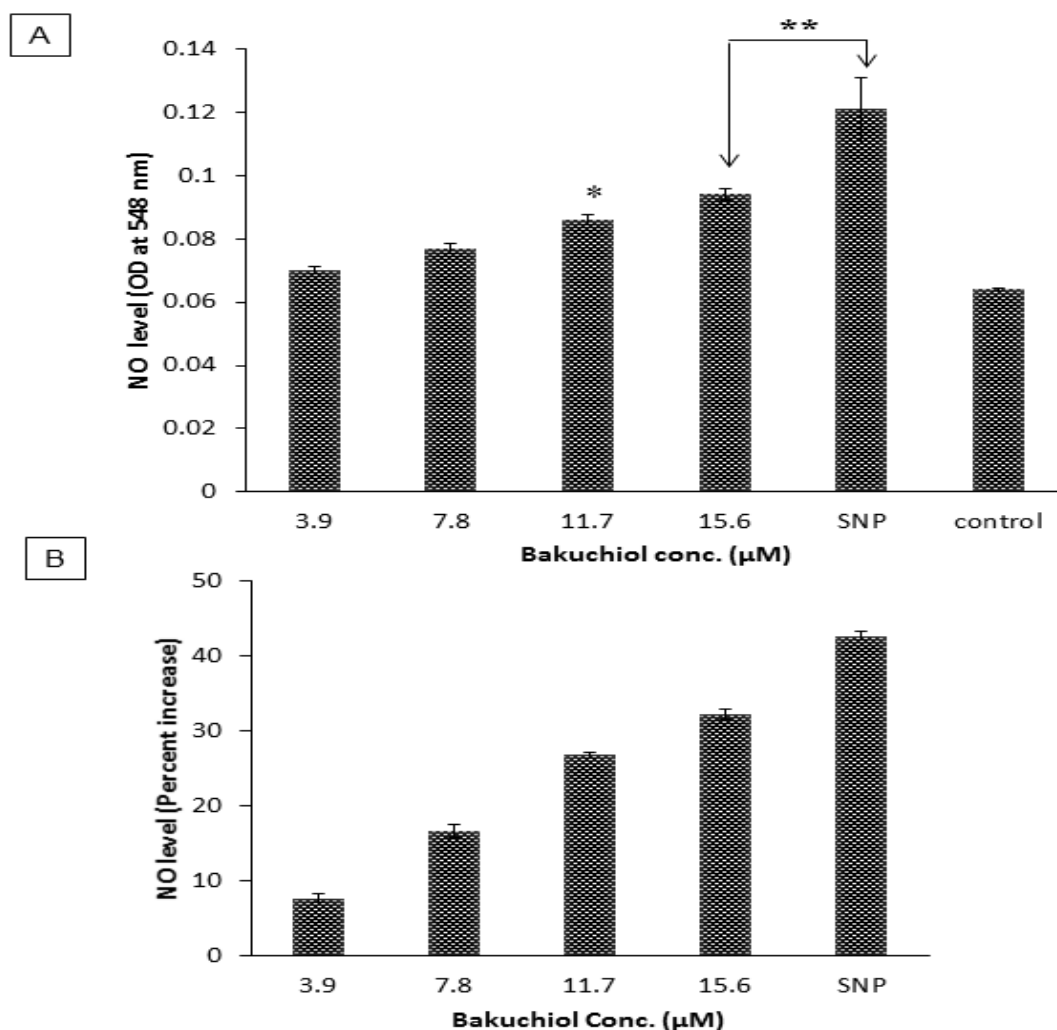


Figure 4: Effect of bakuchiol on NO level. A. Level of nitrite, B. Percent increase in NO level.

(positive control) treated parasites (Figure 5B). The flowcytometry analysis showed that the uptake of the JC-1 dye was decreased as the concentration of bakuchiol increased. The decrease in 590/520 (red/green) ratio of fluorescence intensity and the shift in distribution of cells towards the Q-4 quadrant (or right side in histogram) at different concentration of bakuchiol (Figure 5C), indicates the loss of mitochondrial membrane potential ($\Delta\psi\text{m}$).

Effect of bakuchiol on DNA

To study the DNA fragmentation in bakuchiol treated parasite, spectrofluorometric and flow cytometry based tunnel assay was performed. Flow cytometry based tunnel assay revealed a shift in the distribution of cells towards the right side of the FITC green channel suggesting increase in green fluorescence intensity that is proportional to fragmented DNA (Figure 6C). The tunnel positive cells were increased up to 2.5 %, 2.7 %, 3.4 % and 7.3 % at 3.9 μM , 7.8 μM , 11.7 μM and 15.6 μM concentrations respectively, which was higher than control (1.5 %). In case of positive control Camptothecin, the tunnel positive cells were found to be 5.1 % at 2.8 μM concentration. In spectrofluorometric assay, a significant increase was observed in FITC-BrdU fluorescence

intensity which is associated with the presence of fragmented DNA (Figure 6A). The percent increase in FITC - BrdU positive cells were 5.5 ± 0.80 %, 16.5 ± 0.62 %, 37.0 ± 0.63 %, 40.9 ± 0.46 % and 33.8 ± 0.66 % at 3.9 μM , 7.8 μM , 11.7 μM , 15.6 μM and CPT respectively (Figure 6B).

Interaction between bakuchiol and chloroquine / quinine

The results of *in vitro* drug interaction between bakuchiol and chloroquine or quinine are summarized in Table 1 and Table 2, respectively.

Isobologram was constructed from the Mean $\text{FIC}_{50\text{s}}$ values of bakuchiol and CQ or QN, plotted on x and y axis respectively (Figure 7). Mean $\text{FIC}_{50\text{s}}$ values that were on the straight line showed additive, above the straight line showed antagonistic and below the line showed synergistic interaction. Synergistic interaction was observed between CQ and bakuchiol at 3:2 ratio ($\Sigma\text{FIC} < 1$, 0.9) while additive interaction ($\Sigma\text{FIC} \geq 1$ and < 2) was observed in other combinations (Table 1). The isobologram also represent synergistic interaction at 3:2 ratio, showing ΣFIC [$\Sigma\text{FIC} = 0.9$ (0.3 & 0.6)] values below the straight line (Figure 7A). In the case of QN and bakuchiol combination, synergistic interaction was observed at 1:4 ratio or combination 6 [$\Sigma\text{FIC} = 0.9$ (0.2 &

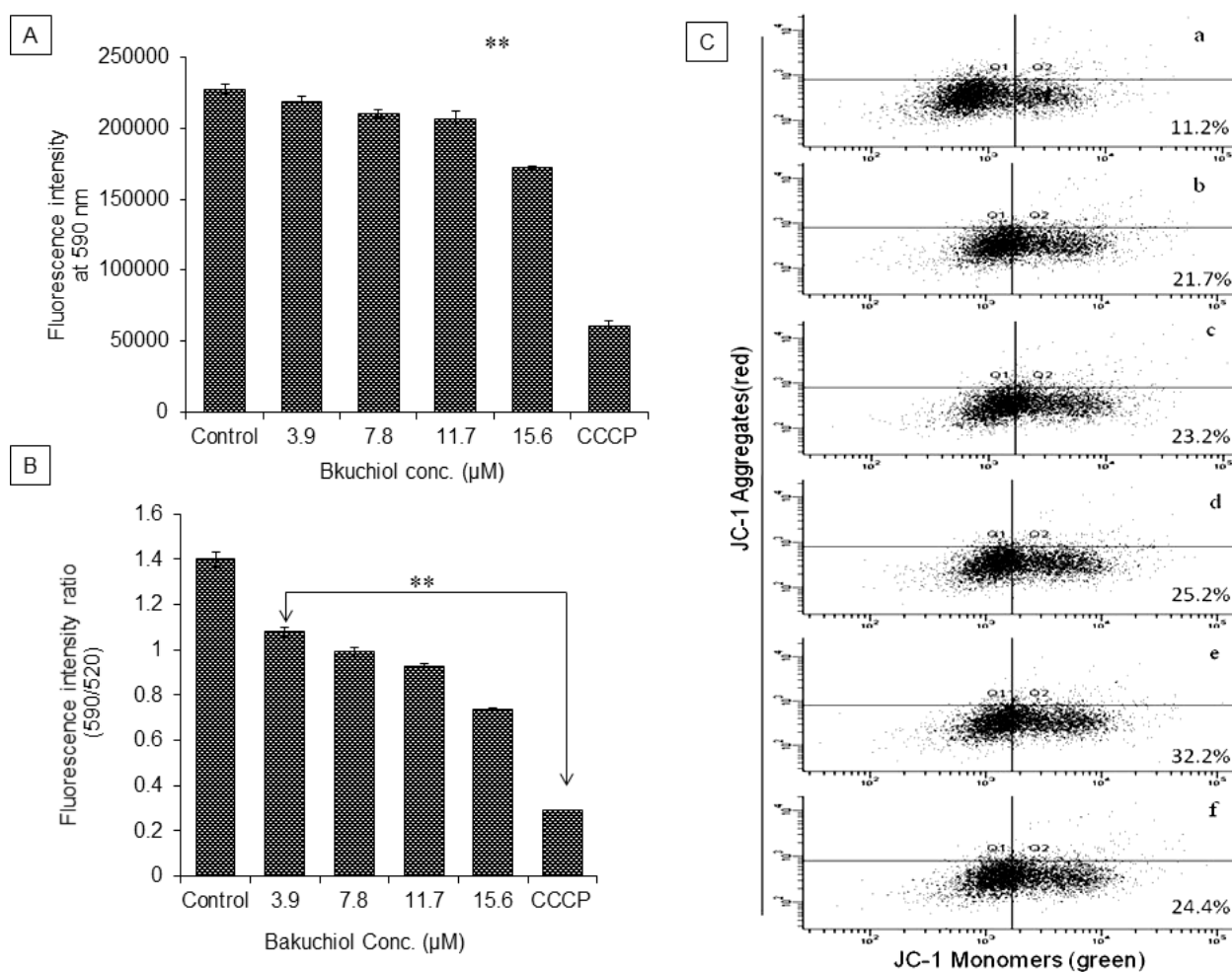


Figure 5: Effect of bakuchiol on mitochondrial membrane potential. A. Change in fluorescence intensity at 590 nm, B.

Ratio of fluorescence intensity at 590 nm / 520 nm. C. Flow cytometric dot plot showing the shifting of population towards JC-1 green channel (Q4 quadrant) after the treatment of bakuchiol. a. untreated control, b. 3.9 μ M, c. 7.8 μ M, d. 11.7 μ M, e. 15.6 μ M and f. CCCP (2.5 μ M).

0.7)] (Table 2). The isobologram also represent synergistic interaction at 1:4 ratio, showing Σ FIC values below the straight line (Figure 7B).

DISCUSSION

Bakuchiol has been widely used in various herbal preparations and in traditional medicine in China. Several therapeutic uses have been reported till now, except, its antimalarial potential. The aim of the current study was to evaluate anti-plasmodial activity of bakuchiol, explore the mechanism of action and test its potential in using combination therapy. Our results indicate that out of three extracts (water, methanol & hexane); the hexane extract of *P. corylifolia* seeds inhibited *P. falciparum* growth in a dose dependent manner with an IC_{50} $18.3 \pm 0.88 \mu$ g/ml. Interestingly, from the 6 fraction (hexane: ethyl acetate) from hexane extract; n-Hexane : ethyl acetate (98:2) fractions (17-22) furnished a major viscous compound bakuchiol. The purified bakuchiol showed improved anti-plasmodial activity (IC_{50} $3.1 \pm 0.30 \mu$ g/ml or $12.1 \pm 0.30 \mu$ M) than the parent hexane extract (IC_{50} $18.3 \pm 0.88 \mu$ g/ml) indicating that it is the major component responsible for the anti-plasmodial action. The bakuchiol

was tested for hemolytic activity at a concentration of 3900 μ M and no significant red blood cell lysis was observed. Next we test the effect of bakuchiol on different blood stage of *P. falciparum* to study their stage specific action. Interestingly, late trophozoite and early schizont stages were most affected by bakuchiol treatment and they did not recover after drug removal (Figure 2).

As per previous reports, the intra-erythrocytic malaria parasite, specifically trophozoites are more susceptible to oxidative stress due to increased energy requirement at this stage²⁵. In addition, trophozoite-schizont stage intra-erythrocytic parasites were killed or inhibited in their development and appeared to be degenerating, a characteristic of crisis forms (condensed DNA) on exposure to small amounts of H_2O_2 ¹⁰. Therefore, intracellular levels of ROS (H_2O_2) were determined. The H_2O_2 level at 3.9 μ M, 7.8 μ M, 11.7 μ M concentration was significantly increased while at 15.6 μ M, it was decreased (Figure 3B). The flow cytometric analysis was also in agreement with the spectrofluorometric data, wherein the number of DCF positive cells increased with the concentration of bakuchiol as compared to control

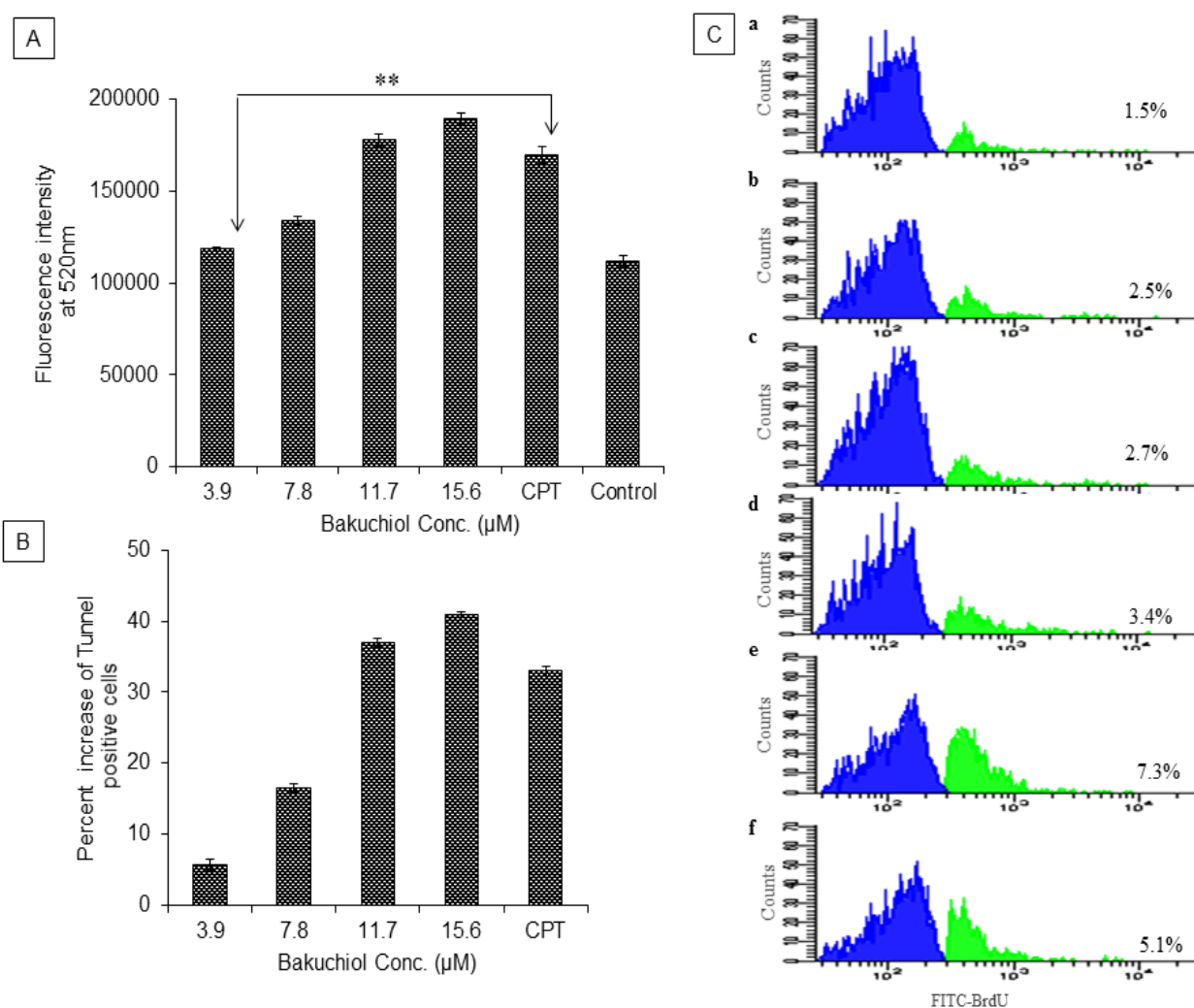


Figure 6: Effect of bakuchiol on DNA. A. Spectrofluorometric measurement of FITC-BrdU fluorescence intensity at 520 nm. B. Percent increase in FITC-BrdU fluorescence intensity. C. Flow cytometric histograms showing the shifting of population towards FITC green channel. a. untreated control, b. 3.9 μM , c. 7.8 μM , d. 11.7 μM , e. 15.6 μM and f. CPT (2.5 μM).

(Figure 3C). At 15.6 μM (more than IC_{50}) concentration, it is imperative that the parasite growth would be inhibited leading to less parasitaemia as compared to lower concentrations, which in turn would lead to less ROS generation, but it is still high as compared to control. These observations are in accordance with earlier reports of natural products inducing ROS generation, such as Quercetin, dietary flavonoid, Withaferin A etc^{26, 27}. NO is another important signaling molecule in the cell and play important role in oxidative balance. As per previous reports, a saturated solution of nitric oxide did not inhibit parasite growth, but its oxidation products; nitrite (NO_2^-) and nitrate (NO_3^-) were toxic to the parasite²⁸. When parasite was treated with bakuchiol, NO level was found to be increase in a dose dependent manner after 36 h of incubation (Figure 4). These observations clearly indicated that bakuchiol is responsible for inducing oxidative stress via generation of free radicals (ROS & RNS) in treated parasite. Accumulation of ROS/RNS induces oxidative damage of membrane lipids, nucleic acid, proteins⁷ and also

contributes to the mitochondrial dysfunction, which is often considered as the “point of no return” in the cascade of events leading to cell death^{29, 23}. In order to investigate whether ROS or NO generate after treatment with bakuchiol can lead to the mitochondrial dysfunction, change in mitochondrial membrane potential ($\Delta\psi\text{m}$), was measured using JC-1 dye. In the functionally active mitochondria, JC-1 dye accumulated and formed aggregate inside the mitochondria, which emits orange red fluorescence at 590 nm, but in depolarized state dye remains in the cytoplasm which emits green fluorescence at 520 nm. The ratio of 590 nm / 520 nm and shifting of fluorescence intensity from red to green is the measure of loss of mitochondrial membrane potential²³. The decrease in 590 nm / 520 nm ratio of fluorescence intensity and the shift in distribution of cells towards the Q-4 quadrant at different concentration of bakuchiol, indicates the loss of mitochondrial membrane potential (Figure 5). Previous reports suggest that free radicals (ROS & RNS) are responsible for oxidative DNA damage in various cells^{30, 31}. Henceforth, DNA damage analysis was

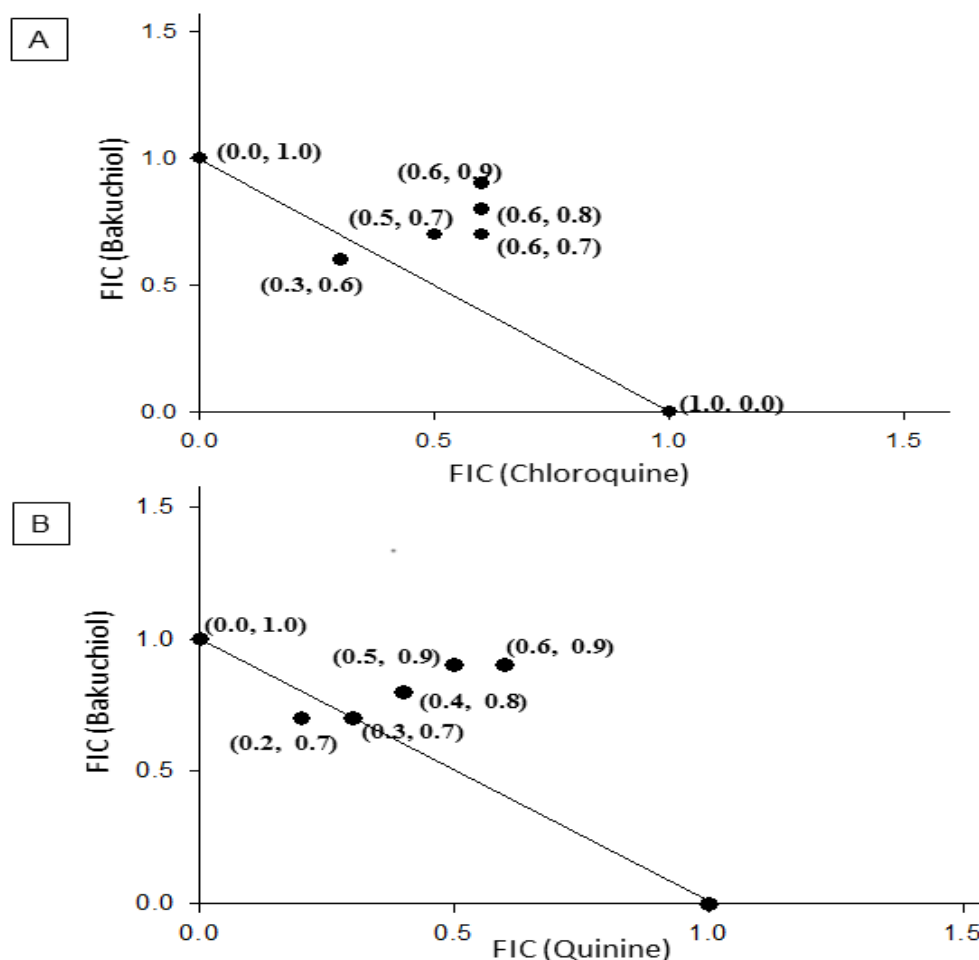


Figure 7: (A) Isobologram showing interaction between chloroquine and bakuchiol. (B) Isobologram showing interaction between quinine and bakuchiol. Mean FICs of test drugs were taken on y and x axis, respectively, along with error bars. The straight line joins both axes at combination 1 and 6.

performed using TUNNEL assay. Remarkably, DNA fragmentation was observed upon bakuchiol treatment in concentration dependent manner (Figure 6). These results are in agreement with earlier reports that cell death in *Plasmodia* sp. has been documented, as featured by the DNA fragmentation and loss of mitochondrial potential³². The use of old drugs in combination with new antimalarial leads is a novel strategy that enhances the therapeutic efficacy and delays the emergence of multidrug-resistance *P. falciparum*. The plant extracts or plant derived molecules are well known to improve the efficacy and slowing down the emergence of resistance by synergistic interaction between test drugs. The major synergistic combination antimalarials recently produced are Malarone (atovaquone-proguanil) and quinimax (quinine-quinidine-cinchonine)³³. Artemisinin in combination with curcumin adds a new dimension to malaria therapy³⁴. *In vitro* drug combination study was performed to assess interactions between bakuchiol with two widely used antimalarial drugs (chloroquine, quinine). Interestingly, synergistic interaction was observed between CQ and bakuchiol at 3:2 ratio, while additive interactions were observed in other combinations. Synergistic interaction among QN and

bakuchiol was observed at 1:4 ratio, while additive interaction was observed in other combinations.

CONCLUSION

It is concluded that the bakuchiol being a natural compound and traditionally used in different preparations for treatment of various diseases, is a suitable candidate for antimalarial drug development. The present study, first time demonstrates that it inhibits malaria parasite growth. Bakuchiol did not show hemolytic activity. It acts as oxidative stress inducer *via* generation of H_2O_2 and NO, causes mitochondrial membrane depolarization and DNA fragmentation leading to parasite cell death. Further, bakuchiol shows synergistic and additive interaction with chloroquine and quinine, that is may be helpful for controlling drug resistance. Considering the need of non-artemisinin based therapeutic alternative options, we feel that, bakuchiol can be a potential lead phytomolecule from cheaper and sustainable source and highly recommended for advance study.

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COMPETING INTERESTS

None declared

ABBREVIATIONS

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT), Chloromethyl 2',7'-dichlorodihydrofluorescein diacetate (CM-H₂DCFDA), Carbonyl cyanide 3-chlorophenylhydrazone (CCCP), Fluorescein isothiocyanate (FITC), 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide (JC-1), Terminal Transferase dUTP Nick End Labeling (TUNNEL).

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