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 (ΔΨₘ) 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 (ΔΨₘ), 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
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). 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 DNA. 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 (•OH), Singlet oxygen (•O₂), Superoxide radical (O₂⁻), Hydroperoxyl radical (HOO•) alkylhydroperoxide (LOOH), Alkylperoxy radical (LOO•), Ferryl ion (Fe⁴⁺O), Perieryl ion (Fe³⁺O), Nitric oxide (NO) are major active oxygen species. Interestingly, not all reactive oxygen species are harmful to the body, some of them are useful in killing invading pathogens or microbes. H₂O₂, NO and their reaction products reduce parasite viability and susceptibility of Plasmodium spp. to oxidant mediated killing has been well established. 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 production.

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 alternatives. Quinine also a drug of choice currently in areas where quinine resistance is not widespread. 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 antiplasmodial 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 previously. 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-2-01) 80:10:10 at a flow rate of 0.8ml/min, with column temperature of 25°C and UV detection at 260 nm.

**In-vitro culture of parasite**

The chloroquine sensitive *P. falciparum* strain NF-54 was cultivated in human B positive red blood cells using RPMI-1640 medium supplemented with 25 mN 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 modification. Culture was maintained in a standard gas mixture consisting of 5% CO₂. The culture was synchronized by 5% DMSO (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 Labtech) 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 previously. 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:

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\text{Percent RBCs lysis} = \left( \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}}} \right) \times 100
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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 previously. 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×IC50 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×IC50 concentration of bakuchiol, after 1 h (ring stage), 12 h (trophozoite stage), 24 h (schizont stage) and incubated for 12 h followed by removal of the bakuchiol through washing with complete medium by centrifugation (500 x 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 previously. 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 Labtech) at wavelengths 485 nm and 520 nm for excitation and emission, respectively. The experiments were performed thrice (n=3) and data expressed as mean values ±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) paraformaldehyde 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, spectrophotometric measurement of fluorescence intensity was recorded using microplate reader (BMG Labtech) 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 ±SEM. **P<0.01 vs control.**

**Drug combination assay**

Fixed ratio combinations of bakuchiol with chloroquine and quinine were tested as previously described. 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 IC50s of the drugs. The IC50s were calculated for each drug alone and for their respective fixed ratio concentrations using Syb green I assay. The individual 50% fractional inhibitory concentrations (FIC50) were calculated and isobologram was prepared. Σ FIC < 0.5 represents substantial synergism, Σ FIC < 1 represents synergism, Σ FIC ≥ 1 and < 2 represents additive interaction, Σ FIC ≥2 and < 4 represents slight antagonism where as Σ FIC ≥ 4 represents marked antagonism.

**RESULTS**

Isolation of bakuchiol

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IJPPr, Volume 9, Issue 3: March 2017 Page 366
After fractionation of hexane extract, a viscous compound (ASPC-2) was found in n-Hexane:ethyl acetate (98:2) fractions (17-22). IR $\nu_{\text{max}}$ (KBr): 3365 (phenolic OH), 2860, 1622 (double bond), 1405, 1243, 981 (aromatics) cm$^{-1}$; $^1$H NMR (300 MHz, Acetone-d$_6$): $\delta$ 1.2 (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), 191 (2H, m, H$_2$-11), 4.91 (1H, m, H$_2$-18), 4.96 (1H, br s, H$_3$-18), 5.04 (1H, m, H$_2$-12), 5.89 (1H, dd, J=18.0, 10.2Hz, H-17), 6.02 (1H, d, J=16.2Hz, H-8), 6.22 (1H, d, J=16.2Hz, H-7), 6.70 (2H, d, J=8.4Hz, H-3, H-5), 7.18 (2H, d, J=8.4Hz, H-2, H-4), 8.25 (1H, br s, OH); $^{13}$C and DEPT NMR (75MHz, Acetone-d$_6$): $\delta$ 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 meroterpene phenol.

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 ± 0.64 µg/ml and 18.3 ± 0.38 µg/ml, respectively. Bakuchiol showed improved anti-plasmodial activity with IC$_{50}$ 3.1 ± 0.35 µg/ml (12.1 ± 0.92 µM) in dose dependent manner. The IC$_{50}$ for chloroquine and artemisinin was
0.046 ± 0.009 µg/ml and 0.026 ± 0.007 µg/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 µM and no significant red blood cell lysis (5.65 ± 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 x IC₅₀ concentration. Parasite growth was significantly arrested at the late trophozoite stage of the erythrocytic cycle in the presence of bakuchiol at 3xIC₅₀ concentration (Fig. 2). To study the reversibility of drug effect, synchronized ring, trophozoite and schizont stage cultures were treated separately with 3 x IC₅₀ 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 H₂O₂ was measured spectrophurometrically and flowcytometrically. In spectrophluorometric assay, the intracellular level of H₂O₂ at different concentrations were observed to be increased significantly (Figure 3A). Percent increase in ROS level was 9.03 ± 0.54%, 26.1 ± 0.63 %, 38.4 ± 0.73 % and 40.1 ± 0.53 % at 3.9 µM, 7.8 µM, 11.7 µM and 15.6 µM concentrations respectively (Figure 3B). The flow cytometric analysis was also in agreement with the
spectrofluorometric data, wherein the shifting of DCF positive cells towards the FITC channel were 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 (Δψm) was measured. Bakuchiol significantly decreased Δψm 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.
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 (Δψm).

**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 FIC50 values of bakuchiol and CQ or QN, plotted on x and y axis respectively (Figure 7). Mean FIC50 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 (ΣFIC < 1, 0.9) while additive interaction (Σ FIC ≥ 1 and < 2) was observed in other combinations (Table 1). The isobologram also represent synergistic interaction at 3:2 ratio, showing ΣFIC [ΣFIC = 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 [ΣFIC = 0.9 (0.2 &
0.7] (Table 2). The isobologram also represent synergistic interaction at 1:4 ratio, showing ΣFIC 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±0.88µg/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 ± 0.30 µg/ml or 12.1 ± 0.30 µM) than the parent hexane extract (IC_{50} 18.3±0.88 µg/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 µM 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 stage. 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_{2}O_{2}. Therefore, intracellular levels of ROS (H_{2}O_{2}) were determined. The H_{2}O_{2} level at 3.9 µM, 7.8 µM, 11.7 µM concentration was significantly increased while at 15.6 µM, 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.
At 15.6 µM (more than IC₅₀) 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 (NO₂⁻) and nitrate (NO₃⁻) 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 death²⁹,³⁰. In order to investigate whether ROS or NO generate after treatment with bakuchiol can lead to the mitochondrial dysfunction, change in mitochondrial membrane potential (Δψm), 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 cells³⁰,³¹. Henceforth, DNA damage analysis was
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 potential. 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). Artemisinin in combination with curcumin adds a new dimension to malaria therapy. 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$_2$O$_2$ 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), Chlormethyl 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 (TUNEL).

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