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Extraction, isolation, characterization, semi-synthesis and antiplasmodial activity of *Justicia adathoda* leaves

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Abstract

There is a need to investigate the new sources of antimalarial drugs which are more effective against *Plasmodium falciparum*. The present study was undertaken to evaluate the *in vitro* antiplasmodial activity of vasicinone, vasicine and 9-oxo-1, 2, 3, 9-tetrahydropyrrolo [2,1-b]quinazolin-3-yl acetate (VA-1). Vasicinone and vasicine were extracted from the leaves of *Justicia adhatoda*. The novel compound VA-1 was synthesized from alkaloid the alkaloid vasicine, which was isolated from the ethanol extract of *J. adhatoda* leaves. Vasicine (IC_{50} = 89.8 µg/mL) and vasicinone (IC_{50} = 38.9 µg/mL) showed moderate antiplasmodial activity whereas the compound VA-1 (IC_{50} = 06.0 µg/mL) showed excellent antiplasmodial activity when compared with standard drug chloroquine (IC_{50} = 12.6 µg/mL). The results achieved suggest that both isolated and semi-synthetic compounds may serve as a lead compound to antiplasmodial activity. Further, the compound VA-1 is for the first time reported for antiplasmodial activity with IC_{50} value.

Introduction

Malaria still kills nearly a million people worldwide each year and most malarial deaths are due to *Plasmodium falciparum*. To overcome such disputes of malaria the rapid emergence of antiplasmodial activity is required.

Plant and animal materials have been used successfully for the treatment of human diseases since ancient times. The extracts of *Justicia adhatoda* (*Adhatoda vasica*) were shown to comprise a good antimicrobial (Kavitha et al., 2012; Ignacimuthus and Shanmugam, 2010; Balachandran et al., 2014; Balachandran et al., 2012), antitussive activity (Dhuley, 1999), abortifacient (Atal, 1980), tuberculosis (Karthikeyan et al., 2009), anti-inflammatory (John and Snell, 1996), hypoglycemic activity (Chakrabarty and Brantner, 2001), hepatoprotective effect (Modak and Rao, 1966; Dhar et al., 1968), anti-oxidant effect (Bhattacharyya et al., 2005) and anti-ulcer activity (Jahangir et al., 2006).

The *J. adhatoda* leaves have been used in the treatment of different diseases. The literature survey revealed that no work has been done for the antiplasmodial activity of reported vasicine derivative. The objective of the present study is to identify the antiplasmodial activity of vasicinone, vasicine and reported semi-synthetic derivatives.

Materials and Methods

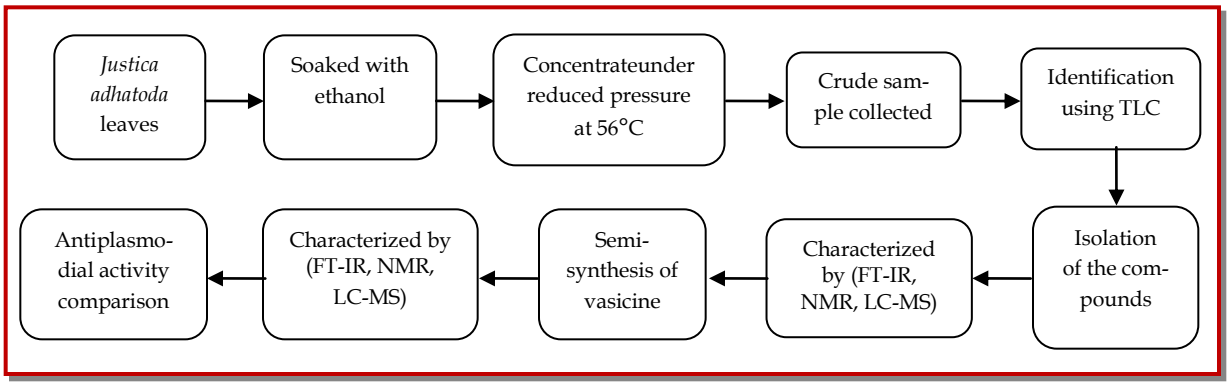
Methods

All the chemicals and reagents used in the present study were of analytical grade. The pure isolated and semi-synthesis compounds were subjected to FT-IR spectra were recorded on KBr medium on a Perkin Elmer Rx, spectrophotometer in wave number region 400-4000 cm^{-1}. The ^{1}H and ^{13}C-NMR spectra were recorded on a Bruker instrument operating at 300 MHz. Mass spectrum was obtained using Agilent compact

1120 and Thermo surveyor instrument. The thin layer chromatography (TLC) analysis was carried out on 5 x 20 cm plate coated with silica gel GF254.

Collection and identification of plant material

The leaves of *J. adhatoda* were selected for the present study based upon their medicinal uses and biological activities. The material was collected from Mecheri, Salem District, Tamil Nadu. An expert taxonomist identified plants at the Botanical Survey of India, Tamil Nadu, Agriculture University Campus, Coimbatore, India. Voucher specimens (BSI/SRC/5/23/2016/TECH/433) are stored in the Department of Chemistry laboratory and available for further reference. The flowchart is given below:



Preparation of plant extracts

The leaves (500 g) were shade dried for 10-14 days under room temperature. Commercial electrical stainless steel blender was used to make the material as a fine powder. The material was soaked with 99% ethanol (500 mL) and constantly stirred for 24 hours. The resulting extract was filtered and the filtrate was concentrated under reduced pressure at 56°C to afford a green mass of 80 g (20% w/w).

Isolation of the compounds

To the above 70 g of crude mass, 700 mL of 6% glacial acetic acid was added and the residue solution was warmed at 45°C. The solution was filtered, cooled and the filter was basified with liquor ammonia to pH 8-9. Then the solution was extracted with chloroform (3 x 250 mL). The resulting chloroform layer was washed with water (2 x 250 mL) and saturated sodium chloride (1 x 400 mL), then dried over sodium sulfate and concentrated under reduced pressure to afforded 12 g (12% w/w), (Joshi et al., 1994).

Isolation and characterization of vasicinone and vasicine from the above crude

The resulting above crude was purified by silica gel (60-120) mesh column chromatography. The vasicinone appear first at 3.5% chloroform : methanol as light brown solid (1.2 g, 12% w/w), whereas vasicine arrives at 35% chloroform : methanol as white solid (2.8 g, 28% w/w).

Characterization of compound vasicinone

The compound vasicinone obtained as a pale yellow crystal having $C_{11}H_{10}N_2O_2$, m.p. 199-201°C and the positive test was obtained for alkaloid (Figure 1). FT-IR (KBr) υ: 3150 (Ar-C-H stretching), 2985 (C-H stretching), 1669 (C=O stretching), 1211 (C-N stretching), 1133 cm^{-1} (C-O stretching). ^{1}H-NMR ($CDCl_3$): The chemical shift value δ 5.2-5.2 (H-1) δ 2.2-2.3 and 2.6 -2.7 (H-2), δ 3.9 - 4.1 and 4.3 -4.4 (H-3), δ 7.4 - 8.3 (H-4, H-5, H-6, H-7). 13C-NMR ($CDCl_3$): Experimental values (δ - ppm) 29.2 , 43.8, 71.7, 118.0, 125.9, 126.7, 127.2, 134.6, 147.2, 156.0, 160.7. LC-MS showed (M) + peak at m/z 203.

Characterization of the compound vasicine

The compound vasicine obtained as a yellow crystal having $C_{11}H_{12}N_2O$, m.p. 205-208°C and positive test was obtained for alkaloid (Figure 1). FT-IR (KBr) υ: 3390 (O-H stretching), 3063 (Ar C-H stretching), 2848 cm^{-1} (C-H stretching), 1631 cm^{-1} (C=N stretching), 1595 cm^{-1} (C=C stretching), 1230 cm^{-1} (C-N stretching), 1179 cm^{-1} (C-O stretching). ^{1}H-NMR ($CDCl_3$): Shift value δ 4.4 (H-1), δ 1.7 -1.8 (H-2), δ 2.1-2.2 (H-2), δ 3.1-3.1 and 3.2-3.3 (H-3), δ 4.5 (H-4), δ 6.8-6.9 (H-5, H-6, H-7) and 7.07-7.1 (H-8). All the above ^{1}H-NMR spectral data clearly confirmed the structure of vasicine. ^{13}C-NMR ($CDCl_3$): Experimental values (δ - ppm) 27.9, 44.7, 45.7, 69.9, 118.1, 126.3, 122.0, 129.4, 124.4, 141.4, 161.5. LC-MS showed (M)+ peak at m/z 188. The two quinazoline alkaloids of vasicinone and vasicine were structurally isolated from

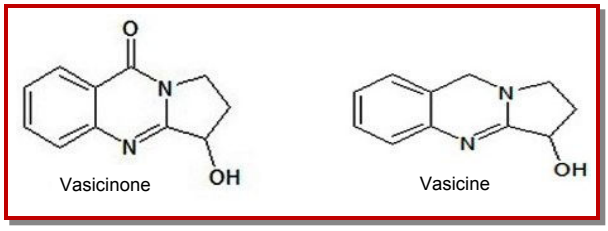


Figure 1: Chemical structure of vasicinone and vasicine

Box I: *In vitro* antiplasmodial assay

Principle

In vitro assessment of drug resistance in *P. falciparum* is usually done using WHO micro-test (Mark III test). The schizont maturation test is developed to monitor drug resistance in malaria parasites.

All cases of severe malaria should be presumed to have *P. falciparum* malaria. It is safer to treat these cases as chloroquine -resistant malaria with drugs like quinine.

Requirement

Isolated and semi-synthetic compound, human O Rh+ red blood cells using RPM1 1640 medium, 10% O Rh+ serum, 5% sodium bicarbonate, 96-well tissue culture plates, 200 µL of *P. falciparum* culture, fresh red blood cells diluted to 20% hematocrit, 2% parasitized *P. falciparum* diluted to 2% hematocrit, parasitized blood cells culture treated with chloroquine and artemether, 40 µg/mL of gentamicin sulfate.

Procedure

Step 1: The isolated and semi-synthetic compound with different concentrations of (3.15, 6.25, 12.5, 25, 50 and 100) µg/mL into 96-well tissue culture plates containing 200 µL of *P. falciparum* culture with fresh red blood cells diluted to 20% hematocrit.

Step 2: Negative control was maintained with fresh red blood cells and 2% parasitized *P. falciparum* diluted to 2% hematocrit, positive control was maintained with parasitized blood cells culture treated with chloroquine and artemether.

Step 3: Parasitemia was evaluated after 24 and 48 hours by Giemsa stain and the average percentage suppression of parasitemia was calculated by the following formula:

Average %parasitaemia =
Average %parasitaemia in control – Average %parasitaemia in test X 100 / Average %parasitemia in control

Video clip: Antiplasmodia activity procedure

Precaution

The entire culturing process was conducted in a validated biosafety level II cabinet.

The culture is very sensitive to contamination, therefore, all reagents weremust be sterile.

References

Ravikumar et al., 2011

J. adhatoda leaves extract. The structures of both compounds were confirmed by FT-IR, ^{1}H-NMR, ^{13}C-NMR and LC-MS. All the resulting data are exactly matching with reported values (Rashmi et al., 2012; Joshi et al., 1994).

Synthesis of VA-1 from vasicine (semi-synthesis)

To the above compound vasicine (0.3 g, 1.5 mmol), acetic anhydride (4 mL) and pyridine (0.5 mL) was added at 25°C. The reaction mass was heated at 90°C for 8 hours. The progress of the reaction was monitored by TLC. The reaction mass was poured into water (50 mL) and extracted with ethyl acetate (3 x 40 mL). The ethyl acetate layer was separated, washed with water (2 x 50 mL) and saturated sodium chloride (1 x 100 mL) then dried on sodium sulfate and concentrated under reduced pressure. The resulting crude product was purified by 60-120 mesh silica column; the product emerges at 2% chloroform : methanol as brown semi-solid (150 mg, 15% w/w).

Parasite cultivation

The antiplasmodial activity of isolated bacterial extracts was assessed against *P. falciparum* obtained from the Jawaharlal Nehru Centre for Advanced Scientific Research, Bangalore, India. *P. falciparum* is cultivated in human O Rh+ red blood cells using RPM1 1640 medium (HiMedia Laboratories Private LTD, India) (Ravikumar et al., 2011a). Sublimated with 10% O Rh + serum, 5% sodium bicarbonate (HiMedia Laboratories Private LTD, India) and 40 µg/mL of gentamicin sulfate (HiMedia Laboratories Private LTD, India). Hematocrits was adjusted at 5% and parasite culture was used when they exhibited 2% parasitemia (Ravikumar et al., 2011b).

Antiplasmodial activity cultivation and analysis

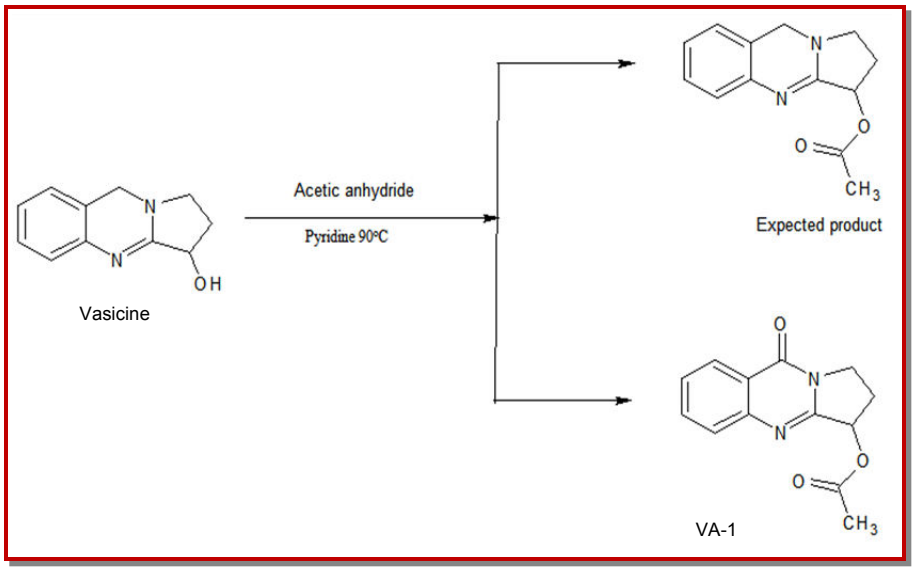
The antiplasmodial activities of isolated bacteria were expressed by the inhibitory concentrations (IC_{50}) of the drug that induced a 50% reduction in parasitemia compared to the control (100% parasitemia). The IC_{50} values were calculated (concentration of extract in x-axis and percentage of inhibition in y-axis) using Office XP (SDAS) software with linear regression equation (Ravikumar et al., 2011c). This activity was analyzed by the norms of antiplasmodial activity.

Chemical injury to erythrocytes

To assess any chemical injury to erythrocytes that might be attributed to the extract, 200 µL of erythrocytes were incubated with 100 µg/mL of the extract at a dose equal to the highest used in the antiplasmodial assay. The conditions of the experiment were maintained as in the case of the antiplasmodial assay. After 48 hours of incubation, thin blood smears were stained with Giemsa stain and observed for morphological changes under high-power light microscopy. The morphological findings were compared with those in erythrocytes findings that were uninfected and not exposed to the extract.

Results

The chemical components derived from plants used in traditional medicine for the treatment of malaria are



Scheme 1: Vasicine acetylation reaction

investigated. The semi-synthetic derivative VA-1 was derived from vasicine by performing acetylation reaction. Initially, the acetylation of vasicine, OH was tried by using the reagents acetic anhydride and pyridine. But unexpectedly there was the formation of vasicinone –OH acetylated product. The vasicine acetylation reaction is given below in Scheme 1.

Reaction scheme

The resulting unexpected product VA-1 was confirmed by performing FT-IR, ^{1}H-NMR, ^{13}C -NMR, and LC-MS. In FT-IR spectrum characteristic absorption peak at 1595 cm^{-1} for C=O stretch and 1281 cm^{-1} due to C-N stretch (amide) confirmed the resulting product. The acetylation of vasicine OH confirmed by the disappearance of C-O stretch (C-OH) at 1179 cm^{-1} which was not present in VA-1. Furthermore, there was the disappearance of a C-N stretch (amine) at 1108 cm^{-1} and formation of new absorption frequency at 1281 cm^{-1} (C-N, amide) confirmed the unexpected compound VA-1. The FT-IR spectrum of VA-1 is shown in Figure 2.

In ^{1}H-NMR the characteristic peak for the acetylated compound was observed at δ 2.1 (s, 3H, CH3) and disappearance of methylene proton of vasicine δ 4.63 confirmed the above unexpected compound of VA-3. The ^{1}H-NMR of VA-1 is shown in Figure 2.

In ^{13}C-NMR there was the presence of new C=O group at δ 155.4 and observed acetyl methyl carbon at δ 20.9 confirmed the resulting unexpected compound of VA-3. The ^{13}C- NMR of VA-1 is shown in Figure 2 .

The molecule mass of the unexpected compound recorded by LC-MS which showed mass 245.2 (M+1) + exactly matching for VA-1. The LC-MS spectrum of VA-1 is shown in Figure 2 .

In vitro antiplasmodial activity

The antiplasmodial activity of vasicinone, vasicine and VA-1 were tested at a different concentration from 3.15 µg/mL to 100 µg/mL and chloroquine used as a positive control. The IC_{50} values of all three compounds against *P. falciparum* strains at 24 and 48 hours of parasitemia suppression are listed in Table I and II.

It is understood that the semi-synthetic derivative VA-1 showed more potent than chloroquine at 24 hours. The microscopic observation of antiplasmodial treatment with vasicinone, vasicine and VA-1 against *P. falciparum* is shown in Figure 3.

Table I

% Suppression of parasitemia at 24 hours							
Samples code	3.125	6.25	12.5	25	50	100	IC_{50}
Vasicinone	29.1 ± 0.1	36.6 ± 0.2	43.0 ± 0.1	49.8 ± 0.1	58.3 ± 0.1	69.3 ± 0.1	38.9
Vasicine	13.2 ± 0.1	19.8 ± 0.4	27.5 ± 0.2	32.2 ± 0.1	41.7 ± 0.1	49.8 ± 0.1	89.8
VA-1	42.6 ± 0.1	49.9 ± 0.5	54.0 ± 0.1	62.9 ± 0.5	70.0 ± 0.0	80.4 ± 0.1	06.0
Chloroquine	30.4 ± 0.2	42.2 ± 0.8	49.8 ± 0.1	65.2 ± 0.1	72.9 ± 0.1	80.5 ± 0.2	17.6

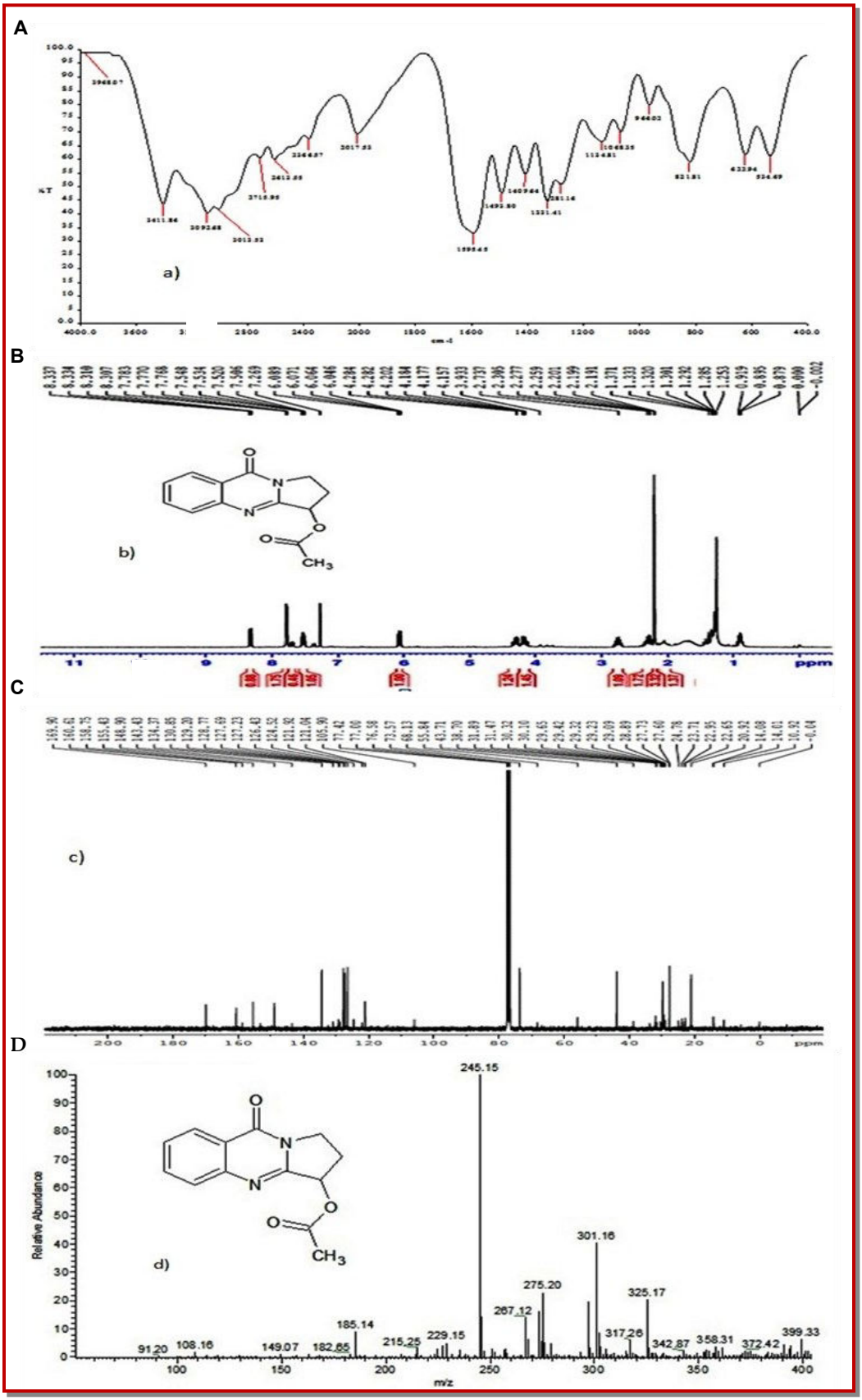


Figure 2: A) FT-IR Spectrum, B) ^{1}H NMR, C) ^{13}C NMR and D) LC-MS of VA-1 compound

Table II

% Suppression of parasitemia at 48 hours							
Samples code	3.125	6.25	12.5	25	50	100	IC_{50}
Vasicinone	36.0 ± 0.1	41.2 ± 0.1	49.8 ± 0.1	59.0 ± 0.1	67.1 ± 0.1	73.9 ± 0.1	20.5
Vasicine	19.6 ± 0.1	24.4 ± 0.1	33.1 ± 0.1	41.0 ± 0.1	49.8 ± 0.2	51.2 ± 0.1	67.5
VA-1	42.7 ± 0.1	49.8 ± 0.1	59.0 ± 0.5	67.3 ± 0.1	78.2 ± 0.1	85.0 ± 0.1	< 50
Chloroquine	33.5 ± 0.1	44.7 ± 0.1	51.3 ± 0.1	67.0 ± 0.1	76.6 ± 0.1	83.2 ± 0.1	12.6

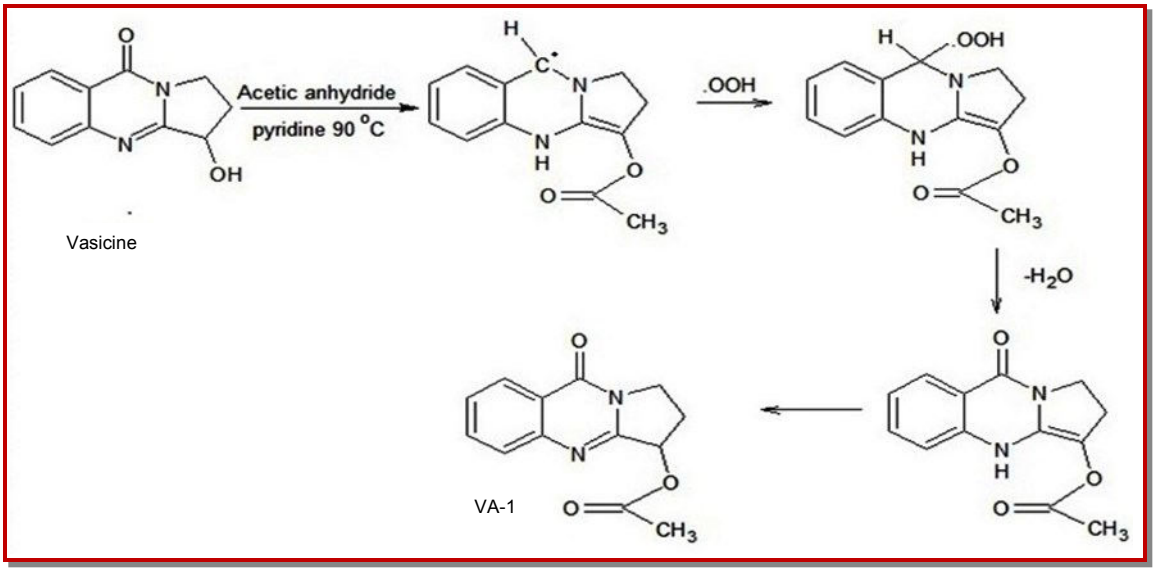


Figure 3: Mechanism of auto-oxidation– vasicine to VA-1 acetylation

The microscopic observation of antiplasmodial treatment with both isolated compounds of (vasicinone and vasicine) and a semi-synthetic compound VA-1 against *P. falciparum* are shown in Figure 4.

Discussion

The present work confirmed antiplasmodial activities of vasicinone, vasicine and semi-synthetic derivatives. The compound VA-1 was most active against *P. falciparum* with IC_{50} value 6.0 µg/mL (24 hours) and >50 µg/mL (48 hours). The compound of vasicine was also active against *P. falciparum* with IC_{50} value 38.9 µg/mL (24 hours) and 20.5 µg/mL (48 hours). The isolated compound of vasicinone showed IC_{50} value 89.8 µg/mL (24 hours) and 67.5 µg/mL (48 hours) which considered as less active or no activity against *P. falciparum*.

The leaves of *J. adhatoda* were taken and extracted in ethanol from crude and isolated compounds of two pure alkaloids vasicinone and vasicine. Furthermore, the attempt was made for the synthesis of vasicine derivative. Since vasicine contain -OH group in its structure, first O-acetylation was tried, but unexpectedly product VA-1 was formed. The isolated two pure alkaloids and semi-synthetic derivatives were subjected to antiplasmodial activity against *P. falciparum*.

Based on traditional medicine, artemisinin has been isolated from the Chinese herb *Artemisia annual* and its semi-synthetic derivatives have been developed (Cumming et al., 1997). Although artemisinin is now effective in the treatment of malaria of both *chloroquine-sensitive* and resistant strains of *P. falciparum*. The *P. falciparum* might rapidly develop resistance to the drugs. Therefore, it is necessary to search for new compounds as backup antimalarials. Hence, there is an urgent need for the discovery of novel and efficient antimalarial drugs to treat malaria and to prevent the emergence of resistance.

Significant antimalarial activity of *Z. spectabilis, S. wallichiana, C. pulcherrima* and *Amomum* sp. have been demonstrated with no toxicity to erythrocyte (Chander et al., 2015). On the other hand, 2,6-substituted benzothiazole derivatives have antiplasmodial effect

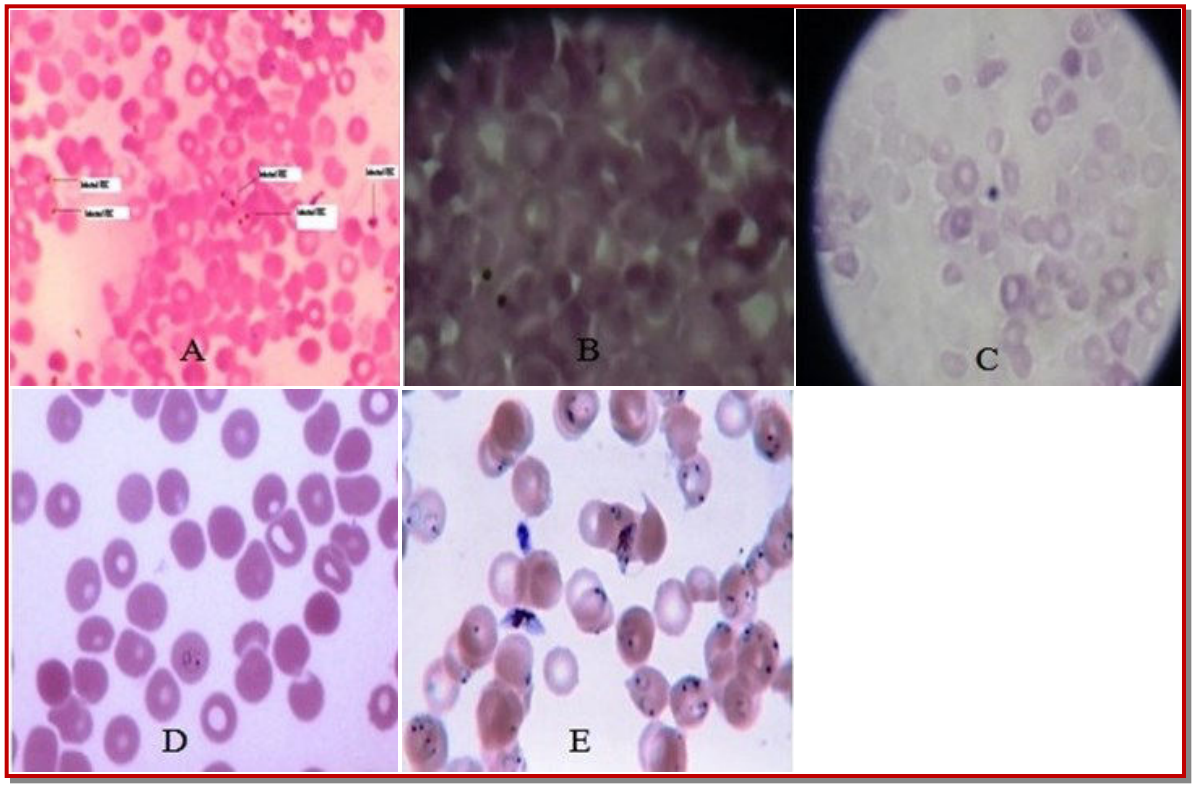


Figure 4: *In vitro* antiplasmodial activity of A) vasicinone, B) vasicine, C) VA-1, D) chloroquine, E) negative control

(Sadhasivam et al., 2016).

Conclusion

Semi-synthesis of VA-1 shows an excellent antimalarial activity compared to vasicine.

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Conflict of Interest

All authors have completed the ICMJE uniform disclosure form and declare no support from any organization for the submitted work.

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