Inhibition of plasmepsin II, a malarial aspartic hemoglobinasefrom *Plasmodium falciparum*by novel peptidomimeticsmall molecules

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Abstract

Ongoing efforts to develop new treatments for malaria, the study of hemoglobin-degradation in the food vacuole of the parasite generates substantial interest because it is a major metabolic pathway of the parasite and is believed to be essential for the survival of the parasite. The *Plasmodium* aspartic proteinases (plasmepsins) involved in hemoglobin-degradation pathway have been recently recognized by World Health Organization as suitable target for new anti-malarial intervention. It is believed that inhibition of plasmepsins could lead to kill parasites in human red blood cells in culture. Our goal of present studies incorporating synthesis and role of new peptidomimeticsmall molecule inhibitors of plasmepsin II to address the problem of malaria, highlight the importance of our effort.In the present study, a series of novel peptidomimeticchalcones, 1-[4-(N-phthaloyl-L-leucinyl)-amino phenyl]-(3-substitued aryl)-2-propen-1-ones3(i-x)were synthesized by Claisen-Schmidt condensation and characterized by elemental analyses, and Infra red, Mass and ^{1}H nuclear magnetic resonance spectroscopy. All the new products were exploited for their *in vitro* anti-malarial activity against a chloroquine-sensitive isolate of *Plasmodium falciparum* (MRC 2) and recombinant plasmepsin II (Plm II). The catalytic and inhibitory activities of plasmepsin II against its natural substrate (hemoglobin) were also demonstrated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE).The 50% inhibition concentration (IC_{50}) values for cell growth inhibition calculated were found between 2 to 232μg/ml. The interactions between these chalcones and plasmepsin II, a hemoglobin degrading malarial aspartic proteasewere assayed by UV-Vis spectroscopy. The IC_{50} values for inhibition of plasmepsin II by these chalcones were found from 2 to 57 μM. The selectivity of peptidomimeticchalcones towards aspartic protease of malaria parasite, plasmepsin II and human aspartic proteasescathepsin D is evaluated. The pivotal mechanism of inhibition of plasmepsin II in the *Plasmodium falciparum* malaria parasite were also demonstrated by docking study.Our results clearly depict the advantage of these chalcones as they kill *P. falciparum* malaria parasite in culture via inhibition of plasmepsin II activity.

Keywords: Peptidomimetic, chalcones,Claisen-Schmidt condensation, anti-malarial activity, *Plasmodium falciparum,* plasmepsin II.

1. Introduction

Malaria remained uncontrolled to date as its parasite rapidly develops resistance to existing antimalarial drugs.Although new drugs are consistently introduced into wide spread clinical and field use but resistance appears often within a few years. The emergence of resistance to anti-malarial drugs highlights the need of the discovery and development of novel anti-malarial agents. To achieve this goal, anti-malarial drug research needs to identify new drug candidates and new drug targets. World Health Organization estimated 3.3 billion people were at risk of malaria in 2010, causing nearly 655 000 deaths, mostly of children under 5 years in the African Region [1].Among the four species of *Plasmodium* parasite responsible for disease in human beings, *Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium ovale* and *Plasmodium malariae*[2], *P. falciparum* is the most wide spread and hazardous as it can lead to the fatal cerebral malaria, which often results in death[3].

The *Plasmodium*plasmepsins known as aspartic proteases are endopeptidases and believed to play essential role in hemoglobin degradation in acidic food vacuole of intra-erythrocytic stage of malaria parasite[4]. Plasmepsins have been recognized by World Health Organization as suitable target for the design of novel chemotherapeutic compound for the treatment of malaria[5]. Three different classes of enzymes have been found in the acidic food vacuole of the parasite that digest hemoglobin; cysteine proteases (falcipains 1, 2 and 3)[6], aspartic proteases [plasmepsin I (Plm I), plasmepsin II (Plm II), plasmepsin IV (Plm IV), and histo-aspartic protease (HAP)][7]and metalloprotease (falcilysin)[8]. During asexual development of malaria parasite (trophozoites) in human erythrocyte (RBC), *Plasmodium falciparum* invades human erythrocytes and consumed 80% of hemoglobin present[9]and is transported into acidic food vacuole of parasite where it is cleaved by aspartic proteases (Plm I, II, IV and HAP), cysteine proteases (falcipains 2 and 3) and metalloprotease (falcilysin) as a source of amino acids for protein synthesis in an ordered pathway[10].

Plm I and Plm II have a high degree of sequence homology to one another (73% identical)[4]and are closely related in structure to human cathepsin D. Malaria parasites both in culture and in animal models, are killed by inhibitors of plasmepsins, suggesting reliable proof of concept that these proteases are viable drug targets[11]. A number of chemical functionalities and structural units viz, hydroxyethylamine[12],dihydroxyethylene[13], hydroxypropylene[14], reduced amide [15], statine[16],reversed statine[17] and norstatine[18] have been frequently employed as non-cleavable transition state isosteres in the design and synthesis of Plm I and II inhibitors. Moreover, copper (II) synthesized nanohybrid solids have been reported as plasmepsin II inhibitors [19].

Chalcones or 1,3-diaryl-2-propen-1-ones belonging to the flavonoid family are natural products with wide spread distribution in fruits, vegetables, spices and tea.Chemically, they consist of open-chain flavonoid in which two aromatic rings are joined by a three carbon α, β-unsaturated carbonyl framework and possess a diverse array of substituents on the two aromatic rings of 1,3-diaryl-2-propen-1-one which is derived by the cleavage of the C-ring in flavonoid[Fig.1]. Recently, the compounds with chalcone (1,3-diaryl-2-propen-1-ones) moiety have been reported to exhibit anti-malarial activity [20]. A number of chalcone derivatives have also been found to inhibit several important enzymes in cellular systems, includingmalarial aspartic protease (plasmepsin II)[21]and malarial cysteine protease (falcipain-2)[22]. Interest in

development of chalcones as anti-malarial drugs began after the isolation of licochalcone A from the roots of the Chinese licorice (Gan Cao) which inhibited the growth of both chloroquine-sensitive (3D7) and resistant (Dd2) strains of *P. falciparumin vitro*[23].

Owing to multifarious pharmacological spectrum of chalcones and their ease to synthesize from substituted benzaldehydes and acetophenones as attractive drug scaffold led us to design novel L-leucine linked chalcones for the inhibition of cell growth of *Plasmodium falciparum* parasiteand its aspartic proteaseactivity in this communication.

2. Materials and Methods

2.01. Chemicals used

Hemin chloride and human liver cathepsin D, licochalcone A and chromogenic substrate (Lys-Glu-Phe-Val-Phe-NPh-Ala-Leu-Lys) of plasmepsin II were purchased from Sigma (USA), Calbiochem (Merck) and TechnoConcept Pvt. Ltd. (India) respectively. Pro-plasmepsin II expression plasmid was generous gift from Ben M Dunn's laboratory, and isopropyl-*β*-D-thiogalactopyranoside (IPTG) was purchased from Fermentas (USA). All other chemicals and solvents were purchased locally and were of analytical grade. Milli-Q water was used to prepare the solutions.

2.02. Test of homogeneity/purity

Homogeneity/purity of all the products was tested by conducting their thin-layer chromatography with silica gel "G". Sample solutions of last step products in MeOH were loaded on silica gel layers and plates were developed in $CHCl_3$-MeOH (9.5:0.5,v/v) solvent. Chromatograms with multispots visualized in iodine fumes, indicating impurity of samples were purified by column chromatography on silica gel (mesh size 60-120, CDH) columns using $CHCl_3$ as eluent with increasing percentage of MeOH (1-4%). The purity of the final compounds was checked again by thin-layer chromatography with silica gel "G".

2.03. Analyses and physical measurements

Melting points determined in the open glass capillaries were uncorrected. Microanalyses for carbon, hydrogen and nitrogen contents of samples were done on Vario-el-III, Element-R at I.I.T., Roorkee, India. Infrared spectra were recorded at I.I.T., Roorkee, India in KBr medium on Thermo Nicolet Nexus FT-IR spectrometer whereas ^{1}H NMR spectra were recorded in DMSO-d_6 medium on Brucker-400 MHz spectrometer at JamiaHamdard University, Delhi, India. Mass spectra (MS-ES$^+$) of compounds were recorded on LC-MS spectrometer (Water) at University of Delhi, Delhi, India.

2.04. Synthesis of 4-(N-phthaloyl-L-leucinyl)-amino acetophenone(2) (Scheme 1)

To a well stirred solution of N-phthaloyl L-leucinyl chloride [24](**1**) (0.08 mol) and 4-amino acetophenone (0.08 mol) in 100 mL dichloromethane at room temperature triethyl amine (4 mL) was added slowly and further stirred for 24h. Solvent was driven off from the reaction mixture and crude residues dissolved in 30 mL dichloromethane, washed with water, dried on Na_2SO_4 and concentrated under reduced pressure upto dryness. The residues were used for next step without further purification.

Pale yellow solid, 89% yield; IR (KBr): ν 3334, 3260 (N-H, amide), 2935, 2833 (C-H), 1775, 1720 (C=O, phthalimide), 1655 (C=O,ketone), 1580, 1495 (C=C,benzene), 837 (*p*-substitution)

cm^{-1}; ^{1}H NMR (DMSO-d_6,400 MHz): δ_H 0.94 (t, 3H, *J* = 6.6 Hz, -CHMe$_2$), 0.96 (t, 3H, *J* = 6.6 Hz, -CHMe$_2$), 1.54 (m, 1H, -CHMe$_2$), 1.62 (m, 1H, -CH$_2$), 2.53 (m, 1H, -CH$_2$), 2.56 (s, 3H, CH$_3$), 5.13 (dd, 1H, *J* = 11.0 Hz, 4.4 Hz, -CHN), 7.45 (m, 4H, phenyl), 7.78 (m, 2H, Phth), 7.98 (m, 2H, Phth) ppm; Anal. Cacld. for $C_{22}H_{22}N_2O_4$: C, 69.84; H, 5.82; N, 7.40; found: C, 69.45; H, 5.34, N, 7.23 %.

2.05. General method for the synthesis of 1-[4-(N-phthaloyl L-leucinyl)-amino phenyl]-3-(substituted phenyl)-2-propen-1-ones, 3(i-x)(Scheme 1)

To a well stirred solution of 4-(N-phthaloyl L-leucinyl)-amino acetophenone (**2**) (1 mmol) and substituted aromatic aldehydes (i-x) (1 mmol) in 8 mL dry MeOH, solid NaOH (100 mg) was added at room temperature and stirred for 24h. There after reaction mixture was poured into crushed ice. The solids were washed with water, dried and purified by column chromatography.

2.05.1. 1-[4-(N-phthaloyl-L-leucinyl)-amino phenyl]-3-phenyl-2-propen-1-one,**3(i)**

Yellow solid, m.p. 55-58˚C, 89% yield; IR (KBr): ν 3469, 3345 (N-H, amide), 2913 (C-H), 1781, 1716 (C=O, phthalimide), 1646 (C=O, ketone), 1603, 1524, 1492 (C=C, benzene), 980, 894 (trans olifinic C-H), 831(*p*-substitution) cm^{-1}; ^{1}H NMR (DMSO-d_6, 400 MHz): δ_H 0.92 (t, 3H, *J* = 6.6 Hz, -CHMe$_2$), 0.94 (t, 3H, *J* = 6.6 Hz, -CHMe$_2$), 1.59 (m, 1H, -CHMe$_2$), 1.69 (m, 1H, -CH$_2$), 2.59 (m, 1H, -CH$_2$), 5.12 (dd, 1H, *J* = 11.0 Hz, 4.4 Hz, -CHN), 7.32 (d, 1CHα, *J* = 15.6 Hz), 7.38 (m, 9H, phenyl), 7.46 (d, 1CHβ, *J* = 15.6 Hz), 7.71 (m, 2H, Phth), 7.84 (m, 2H, Phth) ppm; Anal. Cacld. for $C_{29}H_{26}N_2O_4$: C, 74.67; H, 5.57; N, 6.00; found: C, 74.67; H, 5.27; N, 6.25 %.

2.05.2.1-[4-(N-phthaloyl-L-leucinyl)-amino phenyl]-3-(4-chlorophenyl)-2-propen-1-one,**3(ii)**

Yellow solid, m.p. 108-110˚C, 78% yield; IR (KBr): ν 3457, 3426 (N-H, amide), 2917 (C-H), 1789, 1720 (C=O, phthalimide), 1640 (C=O, ketone), 1605, 1489 (C=C, benzene), 987 (trans olifinic C-H), 816(*p*-substitution), 674 (C-Cl) cm^{-1}; ^{1}H NMR (DMSO-d_6, 400 MHz): δ_H 0.96 (t, 3H, *J* = 6.6 Hz, -CHMe$_2$), 0.98 (t, 3H, *J* = 6.6 Hz, -CHMe$_2$), 1.57 (m, 1H, -CHMe$_2$), 1.65 (m, 1H, -CH$_2$), 2.53 (m, 1H, -CH$_2$), 5.18 (dd, 1H, *J* = 11.0 Hz, 4.4 Hz, -CHN), 7.36 (d, 1CHα, *J* = 15.6 Hz), 7.40 (m, 8H, phenyl), 7.48 (d, 1CHβ, *J* = 15.6 Hz), 7.72 (m, 2H, Phth), 7.86 (m, 2H, Phth) ppm; Anal. Cacld. for $C_{29}H_{25}ClN_2O_4$: C, 69.46; H, 4.99; N, 5.38; found: C, 69.56; H, 4.43; N, 5.47 %.

2.05.3. 1-[4-(N-phthaloyl-L-leucinyl)-amino phenyl]-3-(4-bromophenyl)-2-propen-1-one, **3(iii)**

Pale yellow solid, m.p. 142-145˚C, 66% yield; IR (KBr): ν 3457, 3341 (N-H, amide), 2925 (C-H), 1775, 1712 (C=O, phthalimide), 1656 (C=O, ketone), 1604, 1486 (C=C, benzene), 981 (trans olifinic C-H), 815(*p*-substitution), 592 (C-Br) cm^{-1}; ^{1}H NMR (DMSO-d_6, 400 MHz): δ_H 0.95 (t, 3H, *J* = 6.5 Hz, -CHMe$_2$), 0.97 (t, 3H, *J* = 6.5 Hz, -CHMe$_2$), 1.56 (m, 1H, -CHMe$_2$), 1.69 (m, 1H, -CH$_2$), 2.55 (m, 1H, -CH$_2$), 5.23 (dd, 1H, *J* = 11.0 Hz, 4.4 Hz, -CHN), 7.33 (d, 1CHα, *J* = 15.6 Hz), 7.37 (m, 8H, phenyl) 7.44 (d, 1CHβ, *J* = 15.6 Hz), 7.75 (m, 2H, Phth), 7.90 (m, 2H, Phth) ppm; MS: *m/z* 545 [M+1]; Anal. Cacld. for $C_{29}H_{25}BrN_2O_4$: C, 63.85; H, 4.58; N, 5.13; found: C, 64.11; H, 4.76; N, 4.88 %.

2.05.4. 1-[4-(N-phthaloyl-L-leucinyl)-amino phenyl]-3-(4-fluorophenyl)-2-propen-1-one, **3(iv)**

Yellow solid, m.p. 98-102˚C, 69% yield; IR (KBr): ν 3416, 3334 (N-H, amide), 2921 (C-H), 1782, 1712 (C=O, phthalimide), 1655 (C=O, ketone), 1587, 1556 (C=C, benzene), 971 (trans olifinic C-H), 821 (*p*-substitution), 1025 (C-F) cm^{-1}; ^{1}H NMR (DMSO-d_6, 400 MHz): δ_H 0.94 (t,

3H, *J* = 6.5 Hz, -CHMe$_2$), 0.96 (t, 3H, *J* = 6.5 Hz, -CHMe$_2$), 1.55 (m, 1H, -CHMe$_2$), 1.63 (m, 1H, -CH$_2$), 2.49 (m, 1H, -CH$_2$), 5.10 (dd, 1H, *J* = 11.0 Hz, 4.4 Hz, -CHN), 7.34 (d, 1CHα, *J* = 15.6 Hz), 7.39 (m, 8H, phenyl),7.47 (d, 1CHβ, *J* = 15.6 Hz), 7.77 (m, 2H, Phth), 7.89 (m, 2H, Phth) ppm; MS: *m/z* 483 [M+1]; Anal. Cacld. for $C_{29}H_{25}FN_2O_4$: C, 72.04; H, 5.17; N, 5.78; found: C, 71.68; H, 4.87; N, 5.97 %.

2.05.5. 1-[4-(N-phthaloyl-L-leucinyl)-amino phenyl]-3-(4-nitrophenyl)-2-propen-1-one, **3(v)**

Yellow solid, m.p. 155-157˚C, 75% yield; IR (KBr): ν 3484, 3388 (N-H, amide), 2921 (C-H), 1750, 1722 (C=O, phthalimide), 1656 (C=O, ketone), 1588, 1509 (C=C, benzene), 978 (trans olifinic C-H), 827(*p*-substitution), 1509, 1342 (C-NO$_2$) cm^{-1}; ^{1}H NMR (DMSO-d$_6$, 400 MHz): δ_H 0.95 (t, 3H, *J* = 6.6 Hz, -CHMe$_2$), 0.97 (t, 3H, *J* = 6.6 Hz, -CHMe$_2$), 1.58 (m, 1H, -CHMe$_2$), 1.65 (m, 1H, -CH$_2$), 2.57 (m, 1H, -CH$_2$), 5.15 (dd, 1H, *J* = 11.0 Hz, 4.4 Hz, -CHN), 7.39 (d, 1CHα, *J* = 15.5 Hz), 7.44 (m, 8H, phenyl), 7.51 (d, 1CHβ, *J* = 15.5 Hz), 7.73 (m, 2H, Phth), 7.88 (m, 2H, Phth) ppm; MS: *m/z* 511 [M+1]; Anal. Cacld. for $C_{29}H_{25}N_3O_6$: C, 68.10; H, 4.89; N, 8.21; found: C, 68.65; H, 4.85; N, 8.07 %.

2.05.6. 1-[4-(N-phthaloyl-L-leucinyl)-aminophenyl]-3-(4-methoxyphenyl)-2-propen-1-one, **3(vi)**

Yellow solid, m.p. 105-109˚C, 68% yield; IR (KBr): ν 3467, 3332 (N-H, amide), 2959 (C-H), 1765, 1720 (C=O, phthalimide), 1648 (C=O, ketone), 1599, 1509 (C=C, benzene), 984 (trans olifinic C-H), 820 (*p*-substitution), 1168, 1027 (C-O-C) cm^{-1}; ^{1}H NMR (DMSO-d$_6$, 400 MHz): δ_H 0.94 (t, 3H, *J* = 6.6 Hz, -CHMe$_2$), 0.96 (t, 3H, *J* = 6.6 Hz, -CHMe$_2$), 1.53 (m, 1H, -CHMe$_2$), 1.65 (m, 1H, -CH$_2$), 2.54 (m, 1H, -CH$_2$), 3.52 (s, 3H), 5.10 (dd, 1H, *J* = 11.0 Hz, 4.4 Hz, -CHN), 7.35 (d, 1CHα, *J* = 15.6 Hz), 7.38 (m, 8H, phenyl), 7.43 (d, 1CHβ, *J* = 15.6 Hz), 7.74 (m, 2H, Phth), 7.83 (m, 2H, Phth) ppm; MS: *m/z* 496 [M+1]; Anal. Cacld. for $C_{30}H_{28}N_2O_5$: C, 72.58; H, 5.64; N, 5.61; found: C, 72.25; H, 6.03; N, 5.26 %.

2.05.7. 1-[4-(N-phthaloyl-L-leucinyl)-aminophenyl]-3-(3,4-dimethoxyphenyl)-2-propen-1-one, **3(vii)**

Pale yellow solid, m.p. 85-88˚C, 67% yield; IR (KBr): ν 3432, 3355 (N-H, amide), 2842 (C-H), 1778, 1711 (C=O, phthalimide), 1650 (C=O, ketone), 1596, 1512 (C=C, benzene), 978 (trans olifinic C-H), 835 (*p*-substitution), 1159, 1022 (C-O-C) cm^{-1}; ^{1}H NMR (DMSO-d$_6$, 400 MHz): δ_H 0.96 (t, 3H, *J* = 6.5 Hz, -CHMe$_2$), 0.98 (t, 3H, *J* = 6.5 Hz, -CHMe$_2$), 1.57 (m, 1H, -CHMe$_2$), 1.62 (m, 1H, -CH$_2$), 2.57 (m, 1H, -CH$_2$), 3.56 (s, 6H), 5.10 (dd, 1H, *J* = 11.0 Hz, 4.4 Hz, -CHN), 7.37 (d, 1CHα, *J* = 15.6 Hz), 7.43 (m, 7H, phenyl), 7.45 (d, 1CHβ, *J* = 15.6 Hz), 7.80 (m, 2H, Phth), 7.89 (m, 2H, Phth) ppm; MS: *m/z* 528 [M+1]; Anal. Cacld. for $C_{31}H_{30}N_2O_6$: C, 70.72; H, 5.70; N, 5.32; found: C, 70.91; H, 5.81; N, 5.42 %.

2.05.8. 1-[4-(N-phthaloyl-L-leucinyl)-aminophenyl]-3-(3,4,5-trimethoxyphenyl)-2-propen-1-one, **3(viii)**

Yellow solid, m.p. 95-100˚C, 82% yield; IR (KBr): ν 3440, 3363 (N-H, amide), 2937 (C-H), 1780, 1715 (C=O, phthalimide), 1641 (C=O, ketone), 1588, 1503 (C=C, benzene), 1001 (trans olifinic C-H), 821 (*p*-substitution), 1178, 1028 (C-O-C) cm^{-1}; ^{1}H NMR (DMSO-d$_6$, 400 MHz): δ_H 0.94 (t, 3H, *J* = 6.6 Hz, -CHMe$_2$), 0.96 (t, 3H, *J* = 6.6 Hz, -CHMe$_2$), 1.58 (m, 1H, -CHMe$_2$), 1.68 (m, 1H, -CH$_2$), 2.58 (m, 1H, -CH$_2$), 3.54 (s, 9H), 5.11 (dd, 1H, *J* = 11.0 Hz, 4.4 Hz, -CHN), 7.38 (d, 1CHα, *J* = 15.6 Hz), 7.45 (m, 6H, phenyl), 7.49 (d, 1CHβ, *J* = 15.6 Hz), 7.77 (m, 2H, Phth),

7.86 (m, 2H, Phth) ppm; MS: *m/z* 557 [M+1]; Anal. Cacld. for $C_{32}H_{32}N_2O_7$: C, 69.06; H, 5.75; N, 5.03; found: C, 69.64; H, 6.06; N, 4.87 %.

2.05.9. 1-[4-(N-phthaloyl-L-leucinyl)-aminophenyl]-3-(N,N'-dimethylaminophenyl)-2-propen-1-one, **3(ix)**

Yellow solid, m.p. 115-119˚C, 66% yield; IR (KBr): ν 3407, 3322 (N-H, amide), 2917 (C-H), 1767, 1708 (C=O, phthalimide), 1644 (C=O, ketone), 1597, 1549 (C=C, benzene), 939 (trans olifinic C-H), 821 (*p*-substitution), 1344 (C-N) cm^{-1}; ^{1}H NMR (DMSO-d$_6$, 400 MHz): δ_H 0.96 (t, 3H, J = 6.5 Hz, -CHMe$_2$), 0.98 (t, 3H, J = 6.5 Hz, -CHMe$_2$), 1.53 (m, 1H, -CHMe$_2$), 1.66 (m, 1H, -CH$_2$), 2.59 (m, 1H, -CH$_2$), 2.77 (s, 6H), 5.15 (dd, 1H, J = 11.0 Hz, 4.4 Hz, -CHN), 7.37 (d, 1CHα, J = 15.5 Hz), 7.43 (m, 8H, phenyl), 7.48 (d, 1CHβ, J = 15.5 Hz), 7.70 (m, 2H, Phth), 7.85 (m, 2H, Phth) ppm; MS: *m/z* 509 [M+1]; Anal. Cacld. for $C_{31}H_{31}N_3O_4$: C, 73.08; H, 6.09; N, 8.25; found : C, 73.68; H, 5.85; N, 8.49 %.

2.05.10. 1-[4-(N-phthaloyl-L-leucinyl)-aminophenyl]-3-(2-chloro-quinolin-3-yl)-2-propen-1-one, **3(x)**

Yellow solid, m.p. 120-126˚C, 68% yield; IR (KBr): ν 3422, 3356 (N-H, amide), 2934 (C-H), 1768, 1722 (C=O, phthalimide), 1655 (C=O, ketone), 1597 (C=C, benzene), 969 (trans olifinic C-H), 835 (*p*-substitution), 674 (C-Cl) cm^{-1}; ^{1}H NMR (DMSO-d$_6$, 400 MHz): δ_H 0.98 (t, 3H, J = 6.6 Hz, -CHMe$_2$), 1.02 (t, 3H, J = 6.6 Hz, -CHMe$_2$), 1.56 (m, 1H, -CHMe$_2$), 1.68 (m, 1H, -CH$_2$), 2.59 (m, 1H, -CH$_2$), 5.12 (dd, 1H, J = 11.0 Hz, 4.4 Hz, -CHN), 7.43 (d, 1CHα, J = 15.6 Hz), 7.51 (m, 9H, phenyl), 7.56 (d, 1CHβ, J = 15.6 Hz), 7.85 (m, 2H, Phth), 7.92 (m, 2H, Phth) ppm; MS: *m/z* 553 [M+1]; Anal. Cacld. for $C_{32}H_{26}ClN_3O_4$: C, 69.56; H, 4.71; N, 7.60; found : C, 69.70; H, 5.06; N, 7.97 %.

2.06. In vitroPlasmodium falciparum red blood cell growth inhibition assay

The asynchronous parasites of *Plasmodium falciparum* (MRC 2) were synchronized after 5% D-sorbitol treatment to obtain ring stage parasites. Licochalcone A was used as a drug control. 10 µL parasite in total volume of 100 µL of RPMI-1640 medium was uniformly maintained in 10 wells (1 control and 2-10 test wells) of 96-microtitre plate. 100 µL of test compound (1mg/ml) was added in tenth well. Taking 100 µL aliquot from tenth well (highest concentration is 500 µg/ml), eight serial dilutions of each test compound were carried out from tenth to second well (lowest concentration is 1.95 µg/ml). 96 well-microtitre plates were incubated at 37 ^{o}C in candle jar [25]. After 36-40h of incubation, the blood smear from each well were prepared and stained with JSB-II and JSB-I stains respectively. The slides were microscopically examined to record maturation of schizonts in the presence of different concentrations of compounds. The test concentration, which inhibits the 50% maturation into schizonts was recorded as IC_{50} and calculated by dose response curve plotted between drug concentration and percentage inhibition. The percentage inhibition was calculated by following formula.

$$inhibition = 1 - \frac{Number\ of\ schizonts\ in\ test\ well}{Number\ of\ schizonts\ in\ control\ well} \times 100$$

2.07. In vitroPlasmodium falciparum hemoglobin digestion inhibition assay

For the evaluation of *in vitro* effects of potent peptidomimeticchalcones on the accumulation of hemoglobin in trophozoite stage of parasites, synchronized ring stage of *P. falciparum* (MRC 2) parasites were incubated at 27 ^{o}C for 24 h with 10 μM of pepstatine A, compounds 3ix and 3x and in parallel controls, parasites were treated with drug solvent (DMSO) and 10 μM chloroquine. After 24h of growth, JSB-II and JSB-I stained smears were made from the drug treated parasites and compared with controls by microscopic examination. In order to determine the effect of above mentioned chalcones on hemoglobin digestion, trophozoites infected erythrocytes were collected after 24h, and lysed with saponin. The cell lysate was further homogenized by sonication for 2 min. The obtained extracts were subjected to 12% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). The gel was subsequently stained with Coomassie brilliant blue R-250.

2.08. In vitro heme crystallization inhibition assay

The heme crystallization inhibition assay was performed according to the reported procedure [26]. Briefly, 50 μL solution of hemin chloride (4mM) in dimethyl sulphoxide (DMSO) was distributed in 96-well micro plate. 50 μL solution of each test compound in DMSO (100 μM) was added in triplicate in test wells. Controls contained either water, DMSO or Licochalcone A and chloroquine (50 μL). *β*-hematin formation was initiated by the addition of 100 μL of acetate buffer (0.2M, pH 4.4). The plate was incubated at 37°C for 48 h to allow completion of the reaction and centrifuged (4000 RPM X 15 minutes). After discarding the supernatant, the pellet was washed twice with 200 μL of DMSO and finally dissolved in 200 μL of 0.2 N NaOH. The dissolved aggregates were further diluted 1:2 with 0.1 NaOH and absorbances recorded at 405 nm (Microplate Reader, ECIL MICROSCAN-MS5605A). The results were expressed as a percentage of inhibition of *β*-hematin formation and calculated by following formula.

% inhibition = 1- (Optical density in test well) / (Optical density in control well) X 100

2.09. Expressionofrecombinantpro-plasmepsinII

Pro-plasmepsin II waspreparedaccordingtothe reported procedure [27].Briefly, pET-3a expression plasmid containing PlmII precursorgenewastransformedintoBL21-(DE3) pLysS*Escherichia.coli*.Asinglecolonywas usedtoinoculate100mlofLuria-Bertanimedium.Theculturewasgrownovernightat30°Cand 60ml of itwasusedtoinoculate3L offresh Luria-Bertanimedium.Cellsweregrownuntil the optical densityat600nmreached0.4.Theyweretheninducedwithisopropyl-*β*-D-thiogalactopyranosideatafinalconcentrationof1 mM.After 3hofincubation,cellswereharvestedbycentrifugationandresuspendedin 100mMtris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl), pH8,containing0.2%TritonX-100.Aftertheadditionoflysozyme (1mg/ml),resuspendedcellswereincubatedat37°Cfor30minandsonicated.Then solidureawasaddedtoreachafinalconcentrationof500mMandthesolutionwas stirredatroomtemperaturefor30min.Inclusionbodieswereharvestedbycentrifugation at16,000*g*for10minandthenresuspendedinthesamebuffer,stirredfor30min,and spunasbefore.Inclusionbodiesweresolubilizedin6 Murea,100mMTris-HCl,pH8.5, and32mM*β*-mercaptoethanol.Proteinwasrefoldedbydialysisagainst10L of10mM Tris-HCl, pH 8.5. Protein

concentration wasdetermined according to the methodof Lowry*etal*., 1951[28].

2.10. Determination of inhibition concentrations (IC_{50}) of chalcones toward Plm II

Pro-plasmepsin IIwasactivatedbyadditionofonetenthvolumeof100mM sodiumacetatebuffer,pH4.7 followedbyincubationat37 °Cfor90min.Theenzyme activityofPlmIIwasevaluatedspectrophotometricallyat 300nm wavelengthonCary50 spectrophotometer(Varian)by followingthecleavageof chromogenic substrate (Lys-Glu-Phe-Val-Phe-NPh*-Ala-Leu-Lys, where NPh* is *p*-nitrophenylalanine) as described [29]. For IC_{50} determinations, six different concentrations of each inhibitor were madeinDMSO (final concentrationof 1%ofthesamplevolume) and allowed to fully equilibrate with enzyme at 37 °C for 5 min. The final substrate concentration in assay was 160 μM. IC_{50} values were obtained from dose response curve of v_i / v_o as a function of inhibitor concentration by the Enzyme Kinetic Module of Sigma Plot 11 (SYSTAT Software Inc.) where, v_i and v_o are initial velocities of the inhibited and uninhibited reactions respectively [30].

2.11. In vitro inhibition of hemoglobin digestion

For the evaluation of the effects of only two potent chalcones,3ix and 3x *in vitro* on the hemoglobin digestion as typical examples were studied. 12 μg human hemoglobin was incubated with pre-activated 250 ng Plm II at 37 ^{o}C for 30 minutes in a final digest volume of 100 μL 100 mM sodium acetate buffer, pH 4.7, in presence and absence of the 10 μM pepstatine A, compounds 3ix and 3x of series. The reaction was stopped by the addition of SDS loading dye. Samples were denatured by boiling for 5 minutes and loaded on 18% SDS-PAGE for analysis. The gel was stained by Coomassie brilliant blue R-250.

2.12. Human cathepsin D assay

The enzyme activity was measured as described for Plm II by incubating 0.800 U of human cathepsin D enzyme with 30 μg of chromogenic substrate in 100 mM sodium acetate buffer, pH 4.7. Final incubation volume of reaction was 0.7 mL. The compounds were tested against cathepsin D at concentrations 10-fold higher than that required to inhibit Plm II.

2.13. Structural docking of chalcones into Plm II

The structure of *P. falciparum*plasmepsin II was retrieved from PDB (protein data bank) (www.rcsb.org/pdb). The 3D coordinates used in this study is 1j8j[31] (crystal structure of plasmepsin II from *P. falciparum* in complex with inhibitor diphenyl urea). To perform computational docking screen, only two chalcones, viz, compounds 3(ix) and 3(x) were selected as typical examples. Scigress 7.7 (Fujitsu) software was used for docking study. Before performing docking, all water molecules were removed from crystal structure of enzyme. The three dimensional structure of selected chalcones were superimposed on diphenyl urea inhibitor and docked into the active sites of plasmepsin II using potentials of mean force (PMF) scoring function [32]. For docking stimulations, protein-ligand complex composed of a rigid protein structure and a flexible ligand was modeled. The docked molecules were allowed to move by 40 degree during the docking. The minimization took place using 40000 iterative steps of genetic algorithm.

3. Results

3.01.Syntheses

Chalcones, 1-[4-(N-phthaloyl-L-leucinyl)-aminophenyl]-3-(substituted aryl)-2-propen-1-ones, **3**(i-x)were prepared by Claisen-Schmidt condensation of 4-(N-phthaloyl-L-leucinyl)-amino acetophenone, **2** with substituted aromatic aldehydes in presence of NaOH in MeOH at room temperature. On the basis of infrared (IR) and proton nuclear magnetic resonance (^{1}H NMR) studies all the condensation products obtained, α, β-unsaturated ketones, have been proposed as trans-alkenes (E-form). In these condensations a water molecule is eliminated and a new moiety, $-\overset{O}{\overset{\|}{C}}-C=C-$, is formed.Mass spectral data (molecular weights) of chalcones further confirm the structure of new products.

3.02. In vitroPlasmodiumfalciparum infected red blood cell assay

The anti-malarial activities of chalcones, 3(i-x) were tested *in vitro* against a chloroquine-sensitive isolate of *Plasmodium falciparum* (MRC 2) parasites. The results are depicted in table 1. Introduction of different substituents on the aromatic rings enabled investigation of the relationships between the substitution pattern and the anti-malarial activity. Ten compounds were tested for their ability to inhibit parasite development by incubating parasites in different concentrations for 48h, beginning at the ring stage. The development of parasites was compared with untreated control. Licochalcone A was used as drug-control [Fig.2a]. Out of ten, only four compounds, viz. 3(iv), 3(v), 3(ix) and 3(x) inhibited parasite development at concentration 10 µg/ml or at lower than 10 µg/ml.

3.03. In vitroPlasmodium falciparum hemoglobin digestion inhibition assay

For better understanding of the effect of new peptidomimeticchalcones on the development of *P. falciparum*, we examined the morphology and hemoglobin digestion pattern of ring stage parasites treated with 10 µM pepstatine A, chloroquine, and compounds 3ix and 3x along with control parasites treated with DMSO for 24h beginning at the ring stage. These compounds affect the morphology of *Plasmodium* parasite in which hemoglobin digestion takes place.

3.04. *In vitroβ*-hematin formation inhibition assay

To evaluatethe potential anti-malarial activity of compounds,3(i-x), their ability to inhibit heme crystallization was tested. The cause of this assay is that most anti-malarial drugs available in market interfere with hemozoin pathways. It is believed that heme crystallizes spontaneously under acidic and low oxygen conditions found in the vacuole of the parasite [33].Results (table 1) showed that compounds 3(ii), 3(iv), 3(ix) and 3(x) inhibit significant heme crystallization. The presence of electron-withdrawing groups such as halogens (chloro and fluoro), and *N,N'*-dimethyl group and quinoline ring showed to be favorable for the potential anti-malarial activity. While electron donating groups like methoxy, dimethoxy and trimethoxy at *p*-position of chalcone A ring show poor effect on inhibition of *β*-hematin formation.Chloroquine [26]shows strong inhibition of *β*-hematin formation [Fig.4], whereas licochalcone A was ineffective.Mishra *et al*., [34]reported that hemozoin formation was remained unaffected in licochalcone A treated malaria parasite.

3.05. Inhibition of plasmepsin II and selectivity toward human cathepsin D

All compounds were spectrophotometrically assayed against purified recombinant Plm II enzyme at 300 nm wavelength by hydrolysis of chromogenic substrate (Lys-Glu-Phe-Val-Phe-NPh-Ala-Leu-Lys). The values of inhibition concentrations (IC_{50}) are depicted in table 1. The compounds 3(ix) and 3(x)showed IC_{50} values lower than 10 μMwhereas others showed IC_{50} values considerably higher than 10 μM.

3.06. In vitro inhibition of hemoglobin digestion

In order to ascertain substrate specificity of purified recombinant *P. falciparum*Plm II towards its natural substrate (hemoglobin), the enzyme was incubated with hemoglobin for 30 min at pH 4.7 in presence and absence of 2 most potent chalcones, 3ix and 3x. SDS-PAGE analysis [Fig. 5] of hemoglobin digestion clearly shows that pepstatine A [35] and peptidomimeticchalcones inhibit Plm II activity at micromolar concentration.

1.1.1.1.1 3.07. Structural docking

1.1.1.1.2 The structural docking experiments on selected compounds, 3(ix) and 3(x) were conducted to understand how test compounds inhibited Plm II activity.The structures of the inhibitors were docked into active sites of Plm II by potentials of mean force (PMF) method. PMF method randomly places the ligand near the active site in a random conformation. The programs (Scigress 7.7) generally have an algorithm that at least allows full flexibility of the ligand while the receptor is kept rigid. The inhibitors were allowed to rotate during docking. The docking study of Plm II reveals the important interactions between the enzyme and inhibitors.

4. Discussion

The effect of nature of substituted groups on *Plasmodium falciparum* growth has been studied on para substituted chalcones as at this position steric hinderence is least. Activities increasing in the order of Br < Cl < F < NO_2 clearly reveal that more electron-withdrawing nature of para-substituents on A ring of chalcones increases anti-malarial activity. Electron-donating methoxy group has adverse effect on anti-malarial activity. With increasing number of methoxy substituents in chalcones, the anti-malarial activity fall in order of 4-OMe > di-OMe(3&4) > tri-OMe(3,4&5).

The results of IC_{50} values of table 1 divulge lowest values of 4-*N,N'*-dimethyl amino (3ix)quinolinyl (3x) substituted chalcones [Fig. 2b and 2c]. High anti-malarial values of these compounds could be accounted for in terms of protonation of quinolinyl and 4-*N,N'*dimethyl amino groups on A ring of chalcones under weakly acidic conditions which may enhance their interaction with protease resulting in inhibition of parasite development. However, the exact mechanism of action of anti-malarial chalcones is not known hitherto.

It has been observed from hemoglobin digestion inhibition assay that the parasite food vacuole becomes abnormally enlarged and more pronounced with the staining characteristics of erythrocyte cytoplasm on treatment with above mentioned chalcones. Similar morphological changes are also observed in parasites incubated with chloroquine. It has been expected that hemoglobin digestion was inhibited in chloroquine-treated

parasites[36] whereas pepstaine A does not alter parasite morphology [37-39]. To verify the food vacuole abnormality caused by aforesaid chalcones owing to blockage in hemoglobin digestion, we isolated parasite proteins after 24h of incubation and analysed by SDS-PAGE [Fig. 3]. A distinct difference was seen. The gel clearly shows that chloroquine and chalcones (3ix and 3x) treated parasites contained more undegraded globin (three vertical arrowheads) than control parasites. The hemoglobin digestion profile for pepstatine A treated parasite being similar to that of DMSO control shows that pepstatine A (specific inhibitor of all aspartic proteases) does not inhibit hemoglobin digestion in *in vitro* parasite culture. Thus it could be inferred from present studies that chalcones investigated are more cell permeable to parasitophorous vacuolar membrane of parasite than pepstatine A and blocked parasite hemoglobin degradation and development at micromolar concentration. Moreover, the chalcones contain peptidomimetic bond, a common feature of protease inhibitor binding and inhibiting hemoglobin degrading enzyme activity in the food vacuole of malaria parasite.

The effect of peptidomimeticchalcones on plasmepsin II activity, was studied on para position as at this position steric hinderance is least. Inhibitory activities of compounds containing electron-withdrawing groups decrease in order of F > Cl > Br > NO_2 in accordance to their electron-withdrawing ability of substituted groups on inhibition of Plm II. While the inhibitory activities of compounds containing electron-donating nature of substituted groups increase in the order of OMe<di-OMe(3&4) < tri-OMe (3,4,5) as the numbers of methoxy groups increased. Moreover, human cathepsin D was inhibited by two most potent compounds 3(ix) and 3(x) at higher concentrations than required for Plm II, indicating good selectivity for the *Plasmodium*enzyme.

We speculate that peptidomimetic bond (a common feature of protease inhibitor) in above mentioned chalcones bind the active sites of Plm II resulting in inhibition of its action. These results further support the possibility that these molecules are able to inhibit enzyme activity when hemoglobin is the substrate for *P. falciparum*Plm II. A correlation between the potencies of above mentioned chalcones against purified recombinant Plm II and their effectiveness against parasite hemoglobin digestion and development was also seen. These correlations strongly plead that the anti-malarial effects of these chalcones are due to the specific inhibition of Plm II.

Figures 6a and 6b show superimposition of compound 3(ix) and 3(x) with diphenyl urea inhibitor (PDB 1j8j). These compounds are nicely accommodated within the active sites of Plm II and provided several anchoring sites with Plm II as compared with non-peptidyl diphenyl urea inhibitor. The hydrogen and carbonyl oxygen atoms of amide bond in diphenyl urea inhibitor show strong hydrogen bonding with Gly 36 and Val 78 at a distance of around 1.957 A° and 1.987 A° respectively [Fig. 7a]. On the other hand, the predicted polar hydrogen binding conformation in compounds 3(ix) and 3(x) were found to be different as compared with diphenyl urea inhibitor [Fig. 7b and 7c].

The H-bonding pattern in compound 3(ix) [Fig. 8a] can be generalized as: the hydrogen bonding was observed between the carbonyl oxygen atom of amide bond and amide hydrogen atom of Val 78 residue of flap at a distance of around 2.381 A°. The amide hydrogen atom shows interaction with carboxylate oxygen of catalytic aspartic acid, Asp 34 via hydrogen

bonding at a distance of around 2.277 A° and the α,β-unsaturated carbonyl oxygen atom of chalcone moiety shows hydrogen bonding with hydroxyl H-atom of Ser 79 residue of flap at a distance of around 2.114 A°.A strong intramolecular hydrogen bonding was also found between carbonyl oxygen of phthalimidering and amide hydrogen atom at a distance around 1.827A°.The carbonyl oxygen atoms of phthalimide ring and amide bond incompound 3(x) [Fig.8b]show hydrogen bonding with amide hydrogen atom of Val 78 residue of flap at a distance of around 2.236 and 2.247 A° respectively. The other binding modes are same with slight difference in hydrogen bonding distances as seen in compound 3(ix). No intramolecular hydrogen bonding was observed in compound 3(x).

Conclusions

We prepared novelpeptidomimeticchalconesand applied*invitro* anti-malarialscreeningagainst*P. falciparum* isolate (MRC 2). The compounds viz, 3ix and 3x were found to be most promising candidatea s aspartic protease inhibitors and show good selectivity against Plm II vs human cathpsin D.Differentsubstitutionsoffunctionalgroups on aromatic ring of chalconesmay haveimprovedthepotencyofthese compoundsandprovideselectiveaspartic protease inhibitors. Reported big peptidomimetic molecules suchas pepstatin A,SC-50083 and Ro40–4388, however displaypotentinhibitionofplasmepsins butthey do not inhibit parasitegrowth,owing to their large molecular size having difficult access to the parasitic food vacuoles in which hemoglobin degradation takes place.On the basis of our findings it could be inferred thatthesecompoundshavegreateraccessto the parasiticfoodvacuolesinwhich hemoglobindegradationtakesplaceand starve the malaria parasite via inhibition of aspartic protease(plasmepsinII).

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Legends to figures:

Figure 1. Chemical structures of flavonoid **(a)** and Chalcone**(b)** skeleton.

Figure 2. *In vitro Plasmodium falciparum* growth inhibition curves for licochalcone A (**a**), peptidomimeticchalcones, 3ix (**b**) and 3x (**c**).

Figure 3. Inhibition of hemoglobin digestion by peptidomimeticchalcones. Cultured parasites were incubated with inhibitors for 24 h beginning of ring stage.Trophozoite-infected erythrocytes were collected and lysed with saponin. The protein were electrophoresed on 12% SDS-PAGE and then stained with Coomassie brilliant blue R-250. Each lane contains approximately 10^5 parasites. Parasites incubated with pepstatine A (Lane 2 ; 10 μM), chloroquine (Lane 3 ; 10 μM), compound 3ix (Lane 4 ; 10 μM), compound 3x (Lane 5 ; 10 μM). The vertical arrowheads show more accumulation of undegraded globin than controls incubated with DMSO (Lane 1; 1%) and pepstatine A (Lane 2).

Figure 4. Inhibition of β-hematin formation by chalcones, 3(i)-3(x).

Figure 5. 18 % SDS-PAGE analysis of human hemoglobin degradation by purified recombinant plasmepsin II in the presence of inhibitors.12 μg human hemoglobin was incubated with 250 ng purified pre-activated Plm II in 100 μL of 100 mM sodium acetate buffer, pH 4.7 in presence and absence of inhibitors. Lane 1-5 show (hemoglobin (Hb) control), (Hb + Plm II), (Hb + Plm II +10 μM pepstatine A), (Hb + Plm II + 10 μM compound, 3ix), (Hb + Plm II + 10 μM compound, 3x) respectively. The gel was stained by Coomassie brilliant blue R-250.

Figure 6. Active site pocket of plasmepsin II is shown with superimposed molecules (magenta coloured), **(a)** 3(ix) and **(b)** 3(x) with diphenyl urea inhibitor (blue coloured). The blue and red marks of enzyme pocket show a portion of the surface where the ligands need H-bonds and H-bond donors. The yellow marks of enzyme pocket show a portion of surface where ligands need hydrophobic interactions to achieve good binding to the protein.

Figure 7. Active sites of Plm II are shown with inhibitors (magenta colour), **(a)** diphenyl urea (PDB ID1j8j), **(b)** 3(ix)and **(c)**3(x). The activesitesofPlmIIarecolored byelement type(carbon isgray, hydrogen is colorless, oxygen isred,nitrogen isblueandsulphurisyellow).Thebluelinesrepresent hydrogen bonding between receptor andinhibitor.

Figure 8. Schematic representation of hydrogen bonding network for inhibitors **(a)** 3(ix) and **(b)** 3(x).

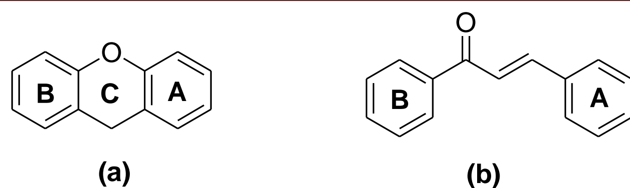
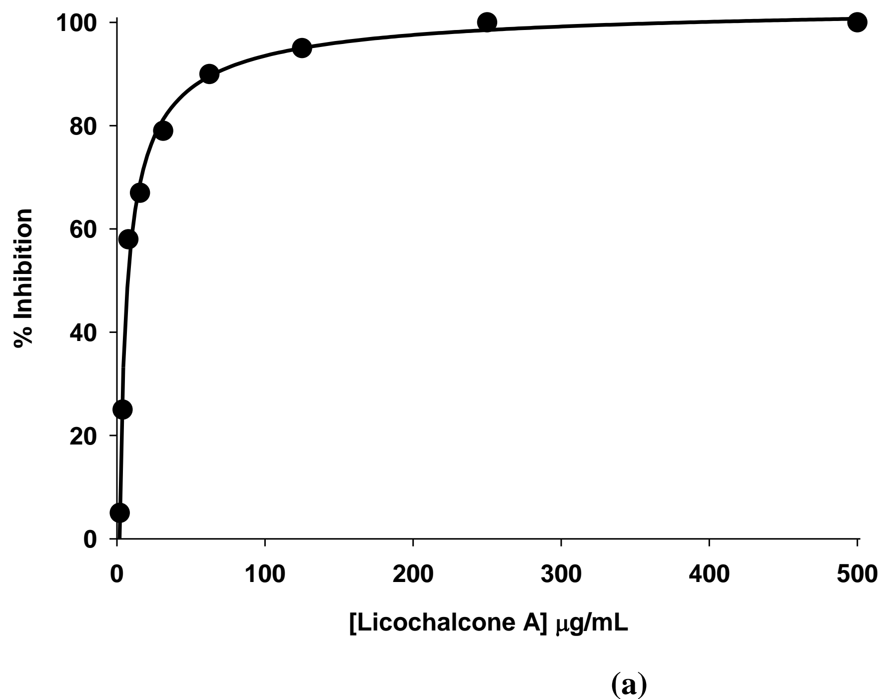
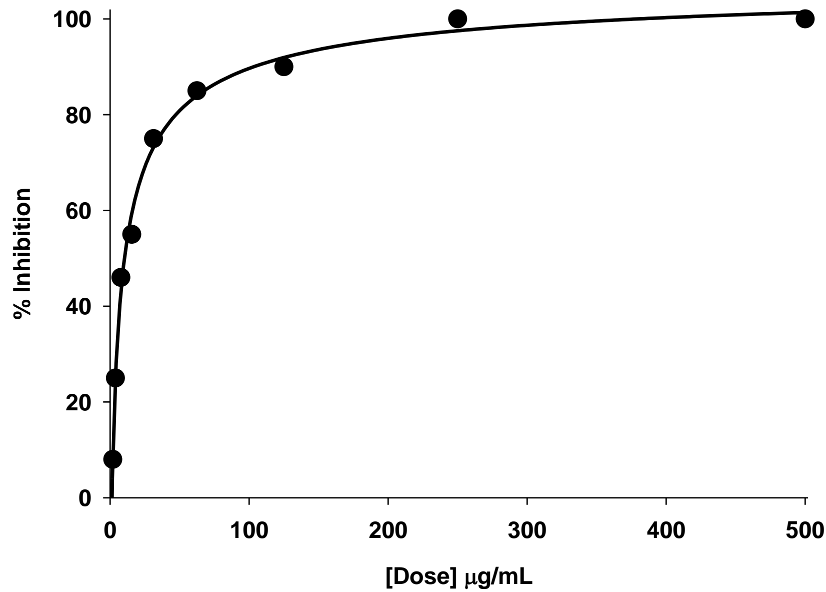


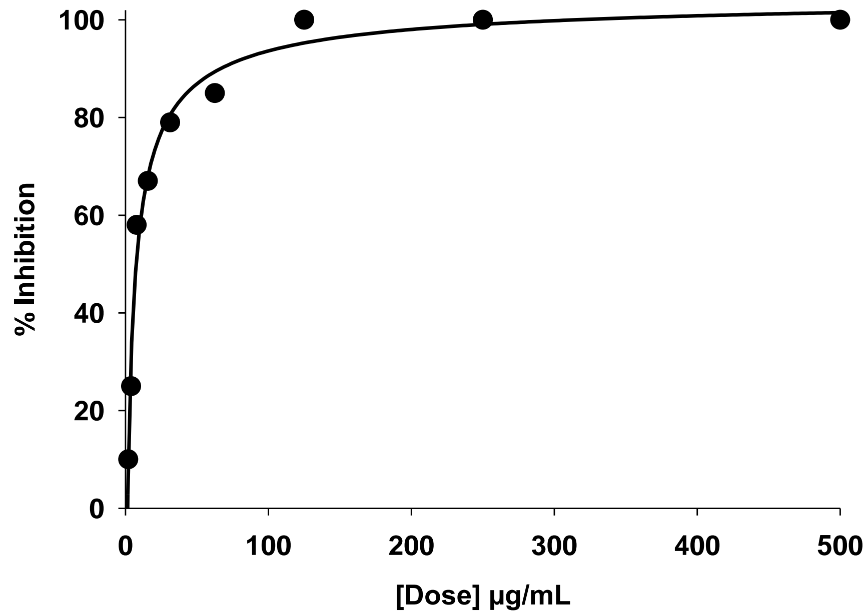
Figure 1. Chemical structures of flavonoid **(a)** and Chalcone**(b)** skeleton.



(a)



(b)



(c)

Figure 2. *In vitro Plasmodium falciparum* growth inhibition curves for licochalcone A (**a**), peptidomimeticchalcones, 3ix (**b**) and 3x (**c**).

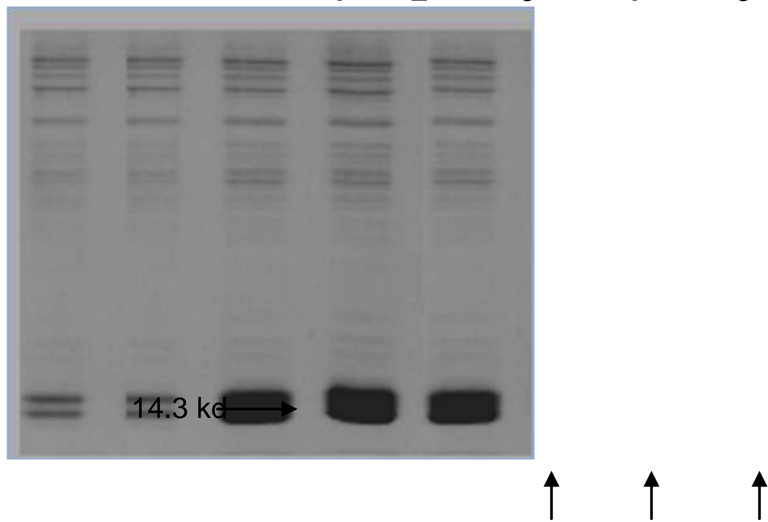


Figure 3. Inhibition of hemoglobin digestion by peptidomimeticchalcones. Cultured parasites were incubated with inhibitors for 24 h beginning of ring stage.Trophozoite-infected erythrocytes were collected and lysed with saponin. The protein were electrophoresed on 12% SDS-PAGE and then stained with Coomassie brilliant blue R-250. Each lane contains approximately 10^5 parasites. Parasites incubated with pepstatine A (Lane 2 ; 10 μM), chloroquine (Lane 3 ; 10 μM), compound 3ix (Lane 4 ; 10 μM), compound 3x (Lane 5 ; 10 μM). The vertical arrowheads show more accumulation of undegraded globin than controls incubated with DMSO (Lane 1; 1%) and pepstatine A (Lane 2).

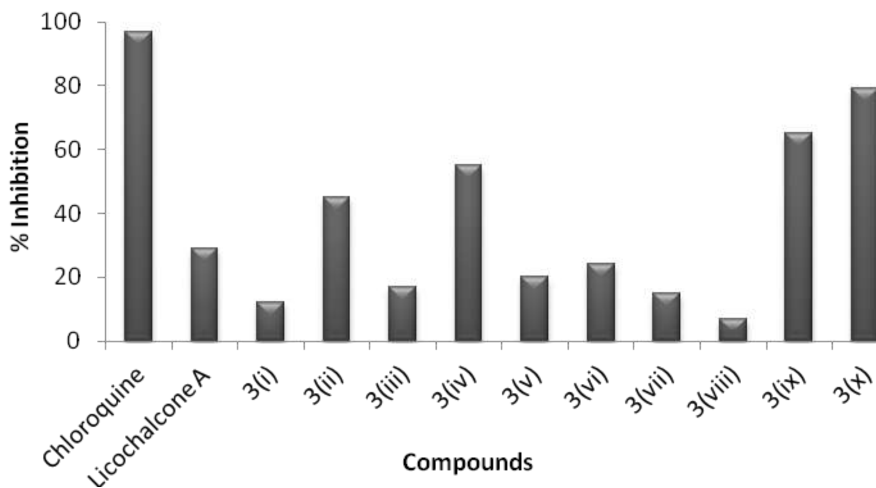


Figure 4. Inhibition of β-hematin formation by chalcones, 3(i)-3(x).

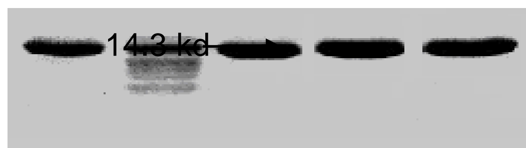
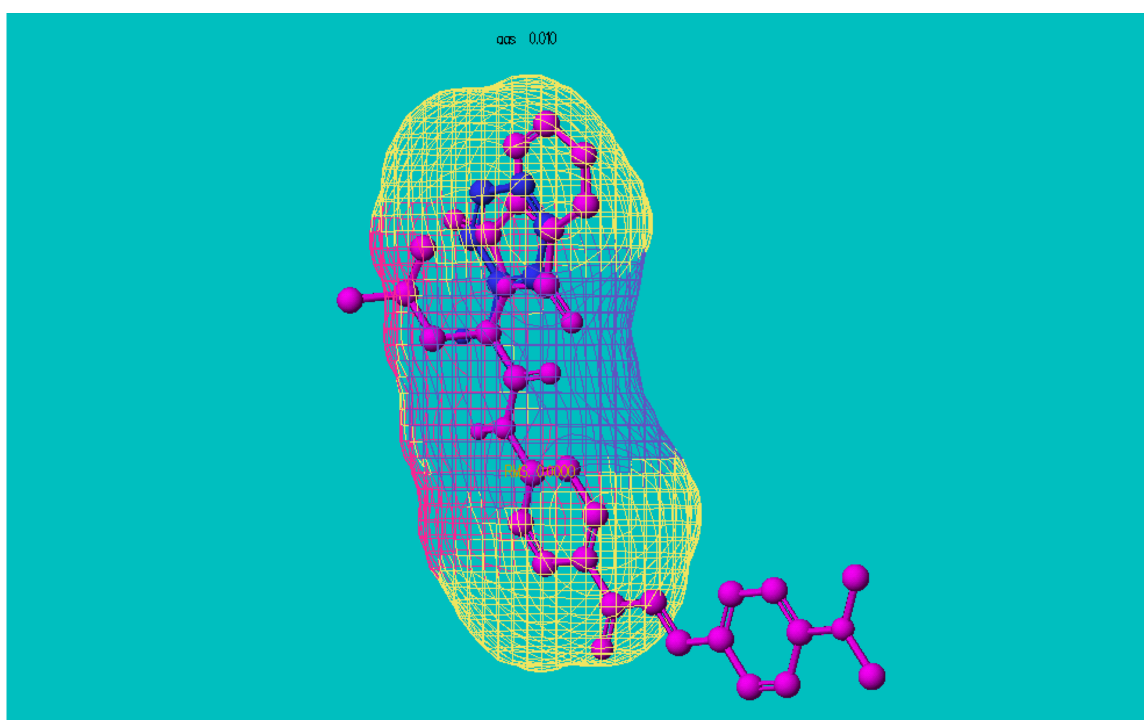
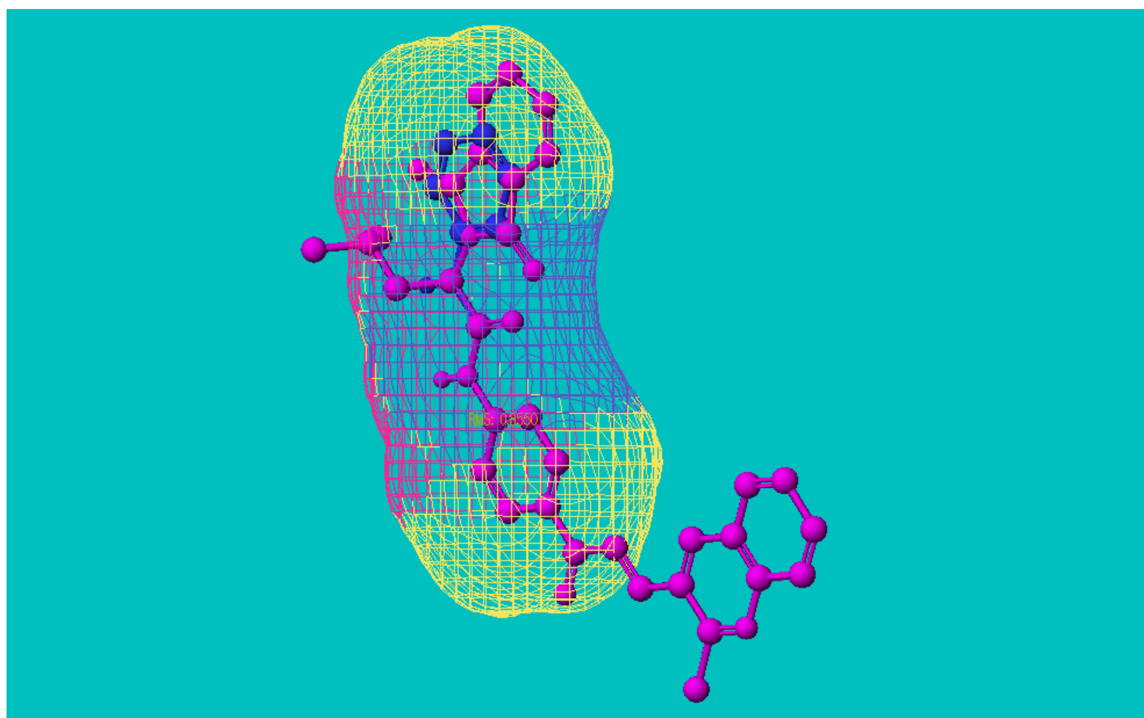


Figure 5. 18 % SDS-PAGE analysis of human hemoglobin degradation by purified recombinant plasmepsin II in the presence of inhibitors.12 µg human hemoglobin was incubated with 250 ng purified pre-activated Plm II in 100 µL of 100 mM sodium acetate buffer, pH 4.7 in presence and absence of inhibitors. Lane 1-5 show (hemoglobin (Hb) control), (Hb + Plm II), (Hb + Plm II +10 µM pepstatine A), (Hb + Plm II + 10 µM compound, 3ix), (Hb + Plm II + 10 µM compound, 3x) respectively. The gel was stained by Coomassie brilliant blue R-250.

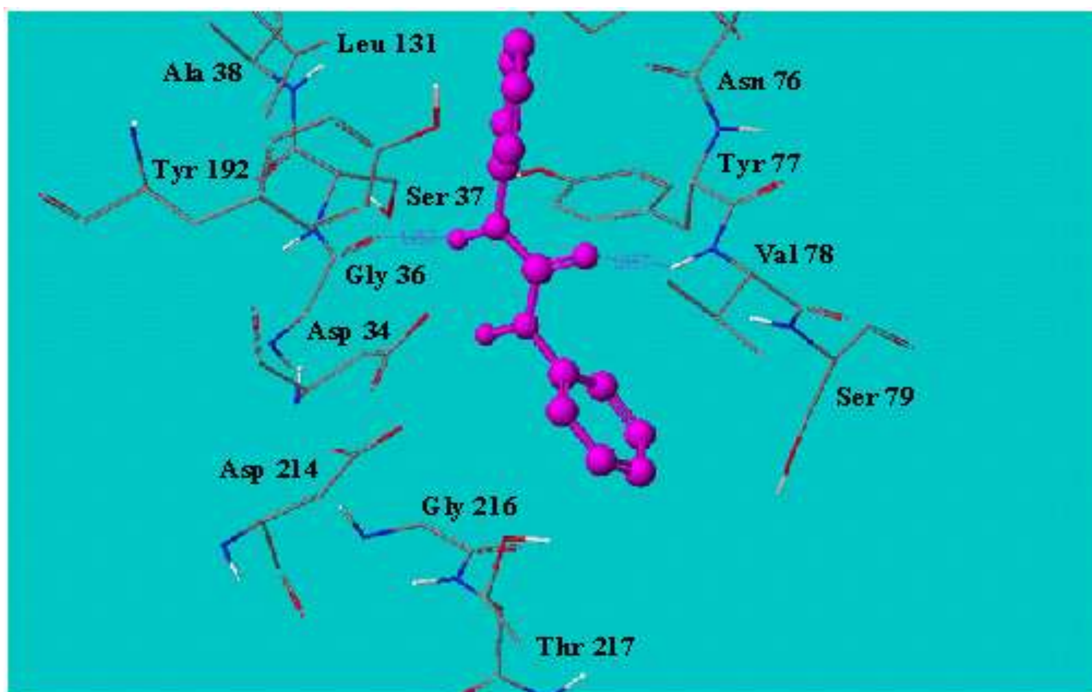


(a)

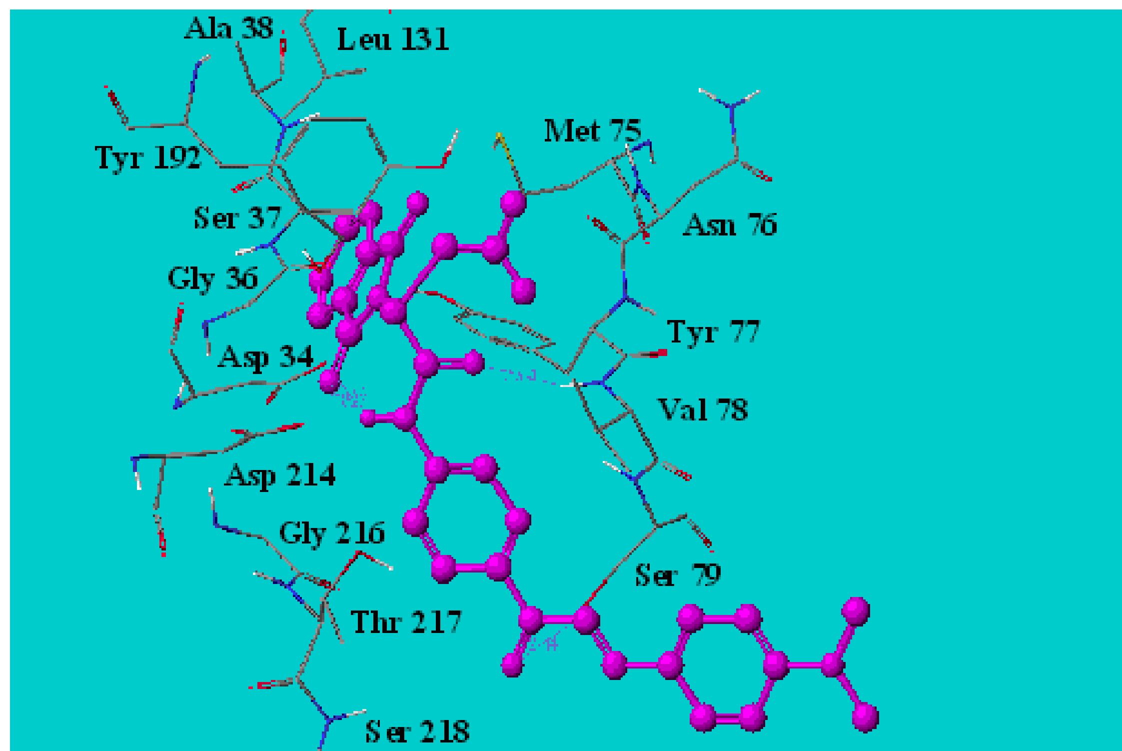


(b)

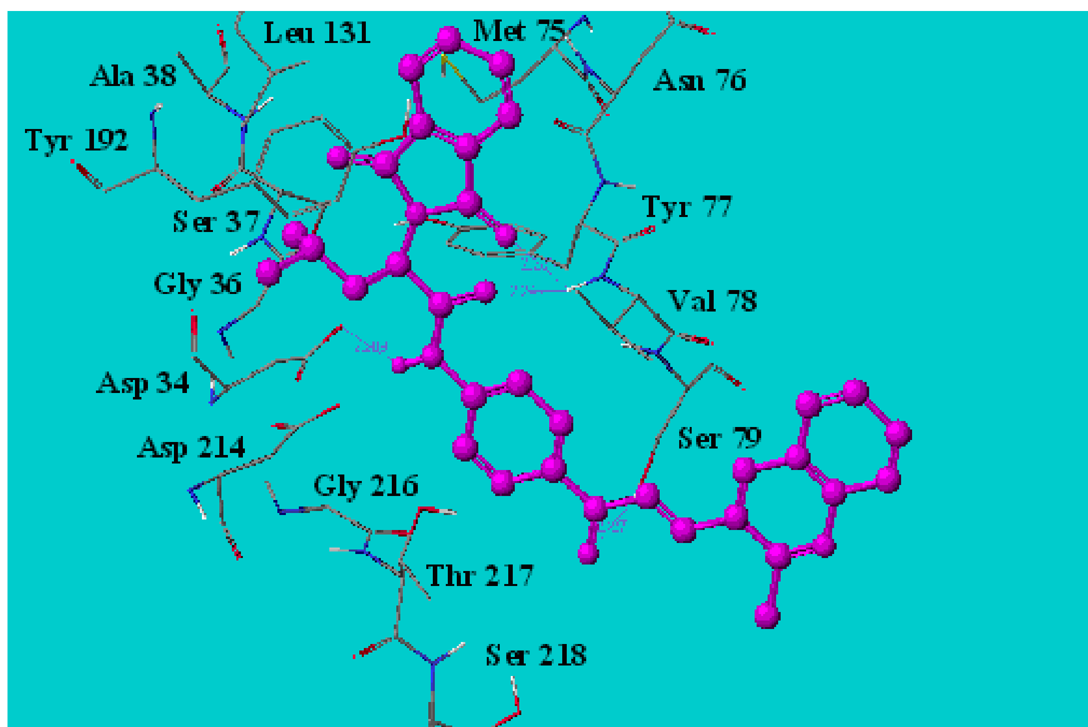
Figure 6. Active site pocket of plasmepsin II is shown with superimposed molecules (magenta coloured), **(a)** 3(ix) and **(b)** 3(x) with diphenyl urea inhibitor (blue coloured). The blue and red marks of enzyme pocket show a portion of the surface where the ligands need H-bonds and H-bond donors. The yellow marks of enzyme pocket show a portion of surface where ligands need hydrophobic interactions to achieve good binding to the protein.



(a)

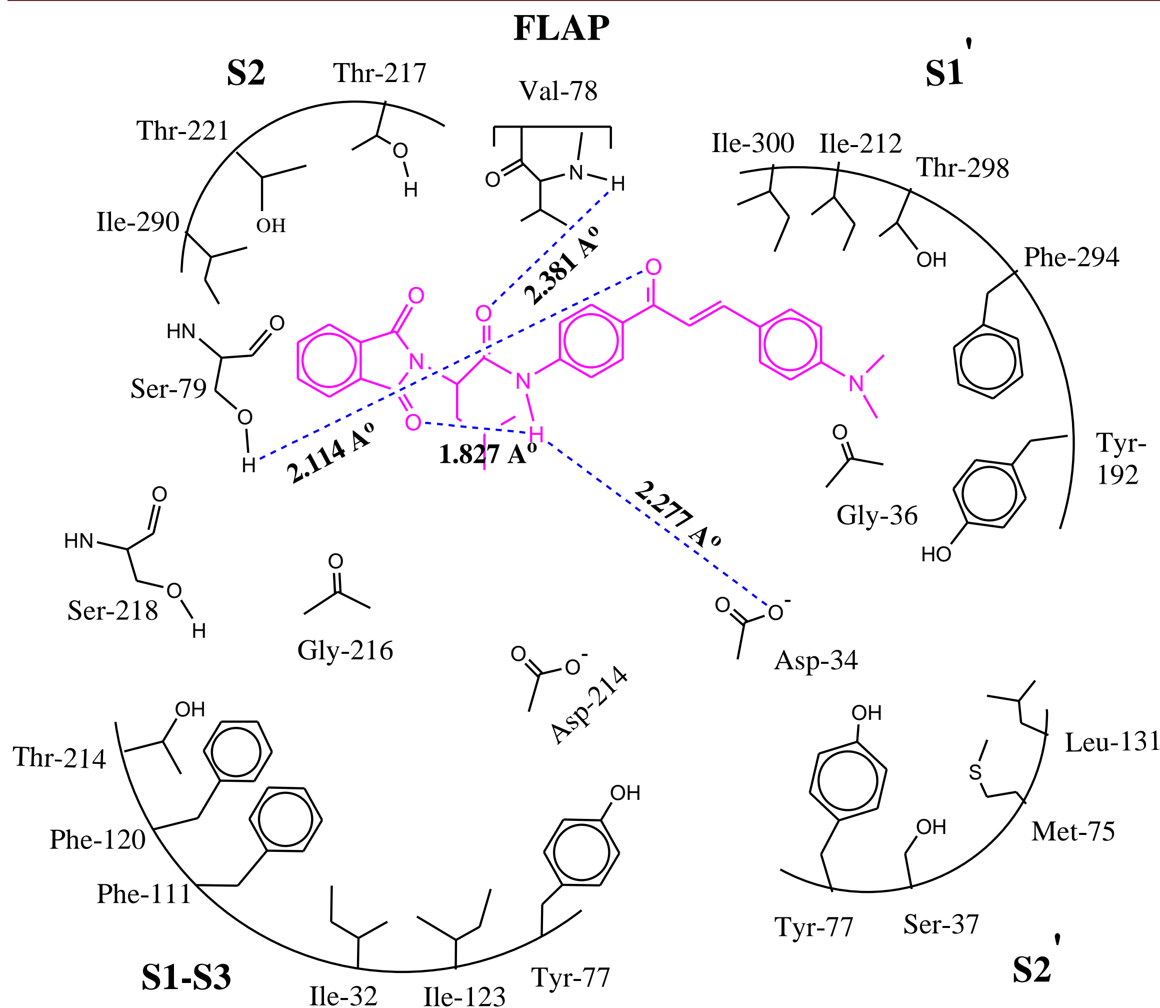


(b)

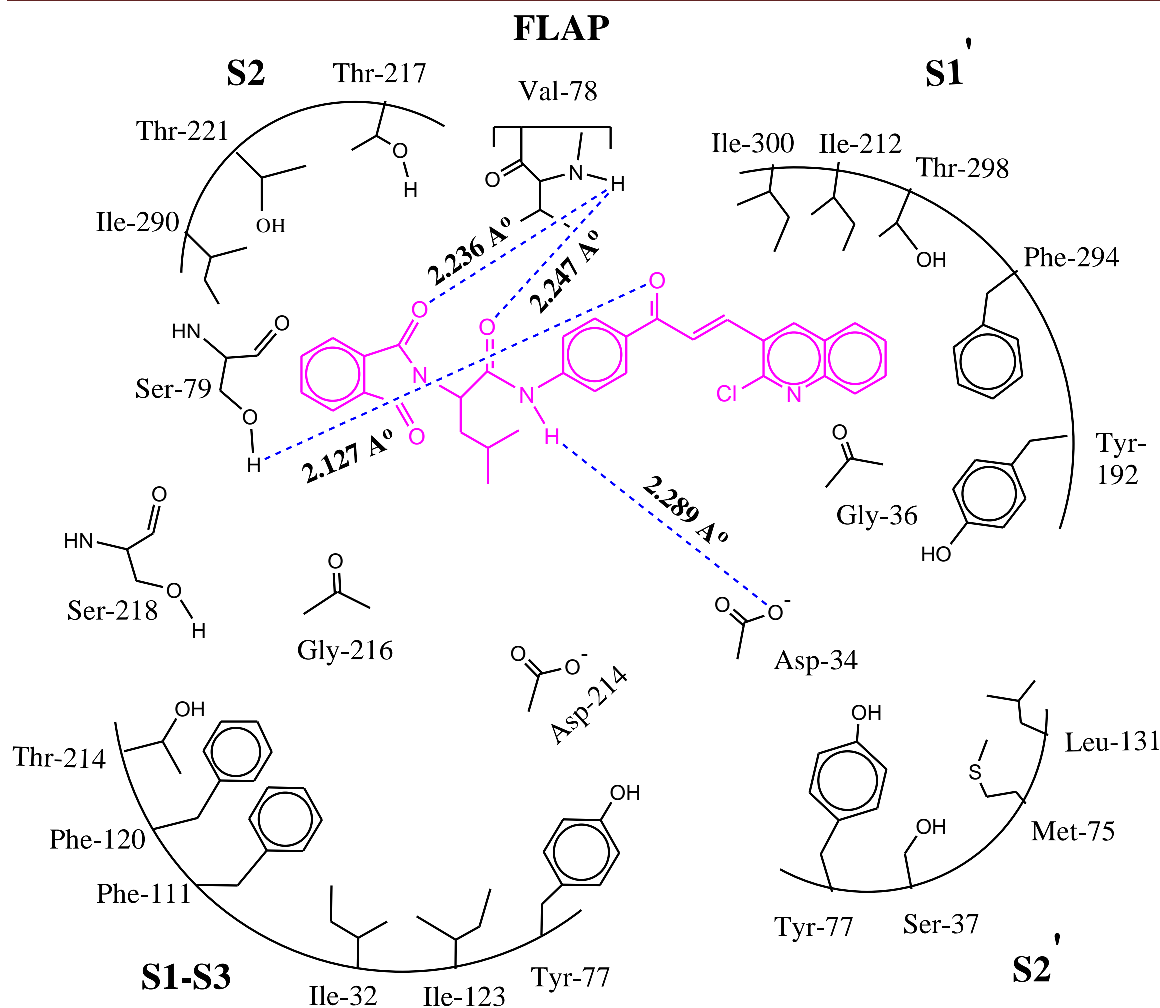


(c)

Figure 7. Active sites of Plm II are shown with inhibitors (magenta colour), **(a)** diphenyl urea (PDB ID1j8j), **(b)** 3(ix)and **(c)**3(x). The activesitesofPlmIIarecolored byelement type(carbon isgray, hydrogen is colorless, oxygen isred,nitrogen isblueandsulphurisyellow).Thebluelinesrepresent hydrogen bonding between receptor andinhibitor.



(a)



(b)

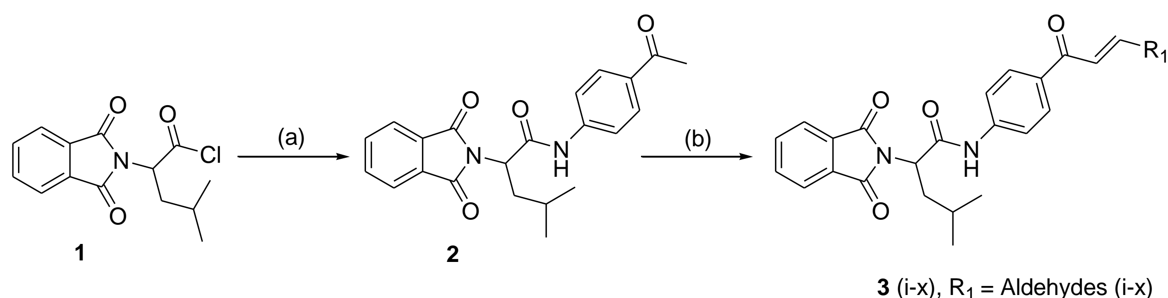
Figure 8. Schematic representation of hydrogen bonding network for inhibitors **(a)** 3(ix) and **(b)** 3(x).

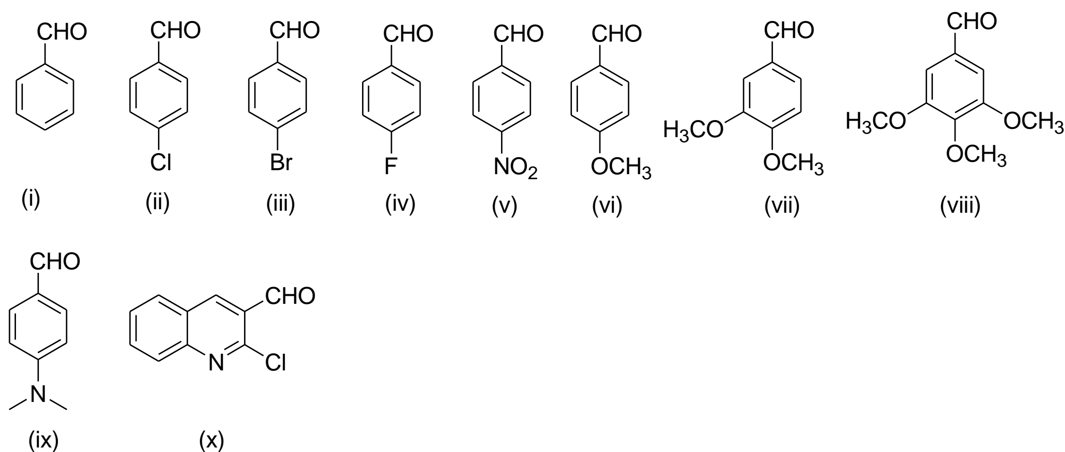
Table 1. Biological results for peptidomimeticchalcones.

S. No.	Compound	*Plasmodium falciparum* IC_{50} (µg/ml)	% inhibition of *β*-hematin formation	Plasmepsin II IC_{50} (µM)	Human cathepsin D IC_{50} (µM)
1	3(i)	44	12	45	24
2	3(ii)	20	45	39	30
3	3(iii)	30	17	48	40
4	3(iv)	10	55	27	19
5	3(v)	5	20	57	38
6	3(vi)	48	24	42	28
7	3(vii)	140	15	29	16
8	3(viii)	232	7	19	8
9	3(ix)	5	65	2	8
10	3(x)	2	79	2	6
11	licochalcone A	2	29	NA	NA
12	Chloroquine	ND	97	NA	NA

NA not applicable, *ND* not determined.

Scheme 1





Scheme 1. Reagents and conditions : (a) 4-amino acetophenone, DCM, Et_3N, rt, 24h; (b) Aldehydes (i-x), MeOH, NaOH, 24h, rt.