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**Research Article** 

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# Epi-croomine and croomine from Stemona tuberosa antimalarial drug for inhibiting dihydrofolate reductase (DHFR) activity and their molecular modeling

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# **ABSTRACT**

One of the mechanism actions of antimalarial drugsis by an inhibiting on the enzyme dihydrofolate reductase (DHFR), an enzyme target antifolate drug. Epi-croomine and croomine, are alkaloids isolated from Stemonatuberosa showed DHFR inhibition with  $K_i$  of 61.14 and 100.59  $\mu$ M and  $K_M$  values of 30.68 and 27.06  $\mu$ M at 10 ppm. The  $IC_{50}$  to the DHFR of croomine and pyrimethamine were 5.29 and 7.71  $\mu$ M, respectively. Tuberostemonine is not active to the enzyme. The kinetic analysis showed that both epi-croomine and croomine competitively inhibited to the human DHFR recombinant. The molecular modeling of the compounds to the human DHFR was estimate depi-croomine and croomine's binding free energy of -6.66 and -7.60 kcal/mol. The docking showed that both epi-croomine and croomine could possibly form hydrogen bonds with the amino acid residue of theAla9, which residues on the active site of the enzyme.

Keywords: malaria, epi-croomine, croomine, DHFR, Stemonatuberosa

# INTRODUCTION

Malaria, is major tropical disease, and public health problem. In 2012, WHO reported 104 countries being endemic of malaria. In Asia, India recorded the highest malarial infection cases (1,765,371) followed by Indonesia (347,197) and Myanmar (200,679). In 2010, almost half of the total Indonesian population (234 million) lives at risk infection transmitted by *Plasmodium falciparum* and the threat still remain now days. High cases of malaria were recorded in the East part of Indonesia, such as Maluku Islands, East of Nusa Tenggara, and West Papua contributed to more than 80% of the nation's 450,000 confirmed malaria cases in 2011<sup>[5]</sup>. In 2013, WHO estimated that the number of deaths due to malaria in Indonesia is approximately 3,000 per year, And new antimalarial drugs are necessary to explore here.

The first anti-malarial drug, quinine, is derived from Chincona bark, and was extensively used during the First World War. Growing upon resistance of quinine, chloroquine (CQ, quinine derivative) was synthesized and introduced in 1934. Twenty years later, CQ resistant cases were reported in South-East Asia. Artemisinine a new generation malarial drug is resistance also in South-East Asia, such as Thailand, Cambodia, Myanmar and Vietnam [1].

Pyrimethamine resistance was first recorded during the Vietnam War in the 1970's. The drug remained in use apotent malarial medicine in Indonesia until 1990,<sup>[7]</sup> when resistance started was emergency. Resistance has also been recorded on combination therapy drugs sulfadoxin-pyrimethamine (SP) in Asia, Africa, and South of America<sup>[8]</sup>. Different antimalarial drug classes with different bearing mechanismof action have been identified including, pyrimethamine, asdihydrofolate reductase (DHFR) inhibitor.

Resistance to pyrimethamine appeared as a result of spontaneous mutations of DHFR. The first mutation was at 108 amino acid residue, and followed by mutations atamino acid residues at50, 59, 51, 164 resulting in decrease if binding of the pyrimethamine binding to DHFR<sup>[7,9,10]</sup>. Single codon mutation was reported in Africa (108 codon)<sup>[11]</sup>, Vietnam (140)<sup>[12]</sup>, Indonesia (50,59)<sup>[13]</sup>, Bolivia (30, 50, 164)<sup>[12]</sup>, Sudan (108, 59, 51)<sup>[14]</sup>; double mutation occurred in South-East of Asia (59/108)<sup>[11]</sup>; triple mutation was reported in China (51/59/108)<sup>[11]</sup>, Oceania (59/108/164)<sup>[11]</sup>, Peru  $(108/51/164)^{[9]}$ , Malawi  $(51/59/108)^{[15]}$  and quadruple mutation was recorded in South of America  $(51/59/108/164)^{[11,16]}$ .

In Papua-Indonesia, SP is a second line antimalarial agent<sup>[17]</sup> and currently, has become ineffective, therefore the need anew class DHFR inhibitors is urgently required. In our easierresearch was able to isolate two prospective antimalarial alkaloids, *epi*-croomineand croominefrom the root extracts of *Stemonatuberosa* collected from Maluku, Indonesia<sup>[18]</sup>. Pyrimethamineis an alkaloid synthesis with 2,4-pyrimidinediamine skeleton, while *epi*-croomine, crominesand tuberostemonineare alkaloids derived from a octahydro-pyrolo[1,2-a]-azepine backbone<sup>[19]</sup>. In this paper we report the activity of *epi*-croomine, croomineand tuberostemonine as class of inhibitors of DHFR *in vitro*. Molecular docking studies were also performed to study the possible binding modes of these alkaloids to the DHFR active site.

# EXPERIMENTAL SECTION

Human DHFR was purchased from Sigma. A 10 mMdihydrofolic acid stock solution (substrate) was prepared by the addition of 2.2 mL assay buffer pH 7.5 to 10 mg dihydrofolic acid. The stock solution 10 mM NADPH was made by the addition of 3 mL suspension buffer to 25 mg NADPH. Four inhibition studies on DHFR to thealkaloids (pyrimethamine, tuberostemonine, croomine and epi-croomine) were made as a 1000 ppm stock solution (in DMSO 0.2% in suspension buffer). The final inhibitors solution concentrations were 0.1, 1, 10 and 100 ppm. The amount of DHFR in each reaction was 1.5 X 10–3 units. The volume of enzyme (5x dilutions) was 10  $\mu$ L. The reactions were measured by the decrease in absorbance at  $\lambda$  340 nm in an UV/Vis spectrophotometer. Alkaloids of croomine, epi-croomine and tuberostemonine were isolated from the roots of S. tuberosa. [18]

**DHFR** NADPH **DHFA** Inhibitor No Compound (unit) (µL)  $(\mu L)$ (ppm) Blank 0.0015 9 Blank 0.0015 9 8 0.0015 9 8 Enzyme Activity Pyrimethamine 0.0015 9 8 Croomine 0.0015 9 8 9 8 Epi-croomine 0.0015 Tuberostemonine 0.0015 9 8

Table 1. The composition of reaction for DHFR assay

Note: Assay buffer was 1500 μL/ sample; \*) alkaloid concentrations were 0.1, 1.0, 10.0; 100.0

The spectrophotometer was adjusted to 340 nm. In the reaction tube was add assay buffer to the test microcentrifuge tube according to the reaction (Table 1) and to the test being performed. Then, the DHFR enzyme or the sample to the appropriate tube was added, and homogenized. For activity assays, without testing an inhibitor, continue to transfer the content of the tube to be tested to a 1 mL quartz cuvette. Then, NADPH solution and dihydrofolic acid were added. For inhibition assay only, add the inhibitor and mix well (pyrimethamine,

tuberostemonine, croomine and epi-croomine) at 0.1; 1; 10 and 100 ppm concentration in the reaction. The absorbance at 340 nm decreases, due to a decrease in NADPH concentration.

Ligand binding mode into human DHFR (hDHFR) was analyzed by docking molecular simulation using Autodock4<sup>[20]</sup>. Re-docking of native ligand (co-crystalization) was used for docking validation. The gridbox that generated complex structure more similar to native structure was used to dock the alkaloids in this paper. The three-dimensional structure of hDHFRwas provided from theProtein Data Bank (PDB) with accession code 1HFP. The gridbox docking was centered on 30.013, 17.759, -2.393 coordination, 19 x 19 x 19 size with spacing of 1.0 Å. Docking preparation was performed using program Autodock Tools 1.5.6 <sup>[21]</sup> and docking results were analyzed using PyMol 1.3 and Discovery Studio Visualizer 2.4.

# RESULTS AND DISCUSSION

The activity of DHFR was recorded before incubating with the test compounds in which dihydrofolic acid (substrate) is converted to tetrahydrofolic acid through a reduction reaction. Three isolated alkaloids, *epi*-croomine, croomine, and tuberostemonine were incubated with DHFR and natural substrate using pyrimethamine as the positive control. Percentage of the decrease of inhibition of DHFR was evaluated as a percent of reduction of DHFR activity (Table 1).

The alkaloids showed no inhibitory activities at low concentrations (0.1 and 1.0 ppm), while the pyrimethamine as a positive control showed 65% inhibition at 1.0 ppm. At increasing concentration (10 ppm) *epi*-croomine and croomine started to show enzyme inhibition. At 100 ppm croomine showed almost complete inhibition (96.32%) of DHFR activity as well as pyrimethamine, but *epi*-croomine is moderate and tuberostemonine is not active. The IC<sub>50</sub> to the DHFR was evaluated using Probit analyze of SPSS 17.0 mode. The values IC<sub>50</sub> to the DHFR of croomine andpyrimethamine were 5.29 and 7.71  $\mu$ M, respectively.

Table 1. The % inhibition activity of DHFR by alkaloids

Compound	The % inhibition activity of DHFR				
	0.1 ppm	1 ppm	10 ppm	100 ppm	
Epi-croomine	0	0	21.17	25.88	
Croomine	0	0.88	36.32	96.32	
Tuberostemonine	0	0	15	15.58	
Pyrimethamine	2.20	65.28	70	100	

An enzyme interacts with anagonist in many ways forming an enzyme-substrate complex. The  $K_i$ s were determined by calculations based on the  $K_M$  values from DHFR with and without inhibitor of alkaloids, from the experimental data, via the Lineweaver-Burk plot using formula:

$$\frac{1}{v} = \left(\frac{K_m}{V_{\text{max}}}\right) \left(\frac{1}{[S]}\right) + \frac{1}{V_{\text{max}}}$$

Based on the Lineweaver-Burk graph, the Vmax and Km values (with and without inhibitors) are summarized in Table 2.

Table 2. Kinetic constants for human DHFR activities to the epi-croomine and croomine

Compounds	Vmax (µM/ min)	Km (µM)	Ki (µM)
No inhibitor	23.67	20.66	
Epi-croomine (10 ppm)	24.17	30.68	61.14
Croomine (10 ppm)	25.76	27.06	100.59

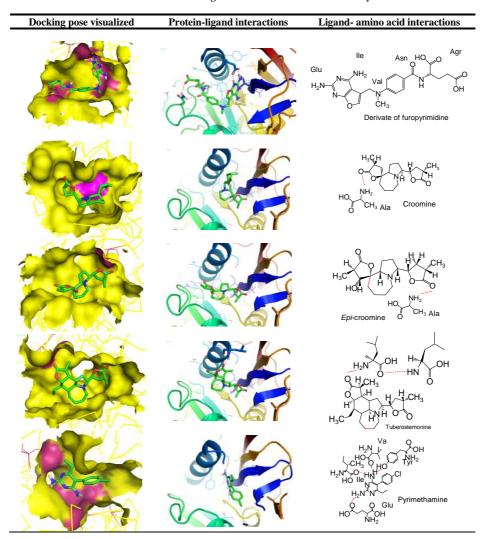
The Lineweaver-Burk double reciprocal plot showed that the inhibited enzyme had similar  $V_{max}$  values while the presence of the inhibitor generated larger  $K_M$  values.  $V_{max}$  of epi-croomine, croomine and without inhibitor are almost the same of 24.17;25.76 and 23.67  $\mu$ M, but  $K_M$  values were different with inhibitor of 30.68 and 27.06  $\mu$ M. From the percent of DHFR enzyme inhibition data in Table 1, the binding affinity  $(K_i)$  was evaluated for epi-croomine and

croomine. The values  $K_i$  of epi-croomineandcroomineare 61.14 dan 100.59 $\mu$ M, it indicated that binding affinities of epi-croomine bigger than croomine. So, the inhibition of epi-croomine is also stronger than croomine. Kinetic analysis of  $K_M$  for a competitive inhibition reaction reflected an effective decrease in the enzyme affinity toward these alkaloids due to their inability to interact with already inhibitor bounded enzyme. For competitive inhibitors, the affinities are derived from  $K_i = K_M I / K_{M(i)}$  -  $K_M$  while for non-competitive inhibitors,  $K_i = V_{max(i)} I / v_{max} V_{max} - V_{max(i)} K_i$  for an inhibitor is analogous to Km for a substrate.

Table 3. Calculated values of binding free energy and H-bonding of alkaloids

Ligand	Binding free energy (kcal/mol)	H-bonding	
Furopyrimidine derivative	-8.43	Ile7, Glu30, Asn64, Arg70, Val115	
Tuberostemonine	-8.06	Leu27	
Epi-croomine	-6.66	Ala9	
Croomine	-7.60	Ala9	
Pyrimethamine	-5.40	Ile7, Glu30, Val115, tyr121	

Table 4. Visual of docking between alkaloids and DHFR enzyme



Alkaloids inhibition activity were evaluated by *in silico*toperform computational analysis of thealkaloid-enzyme complex and to predict the relative binding free energies and to explore the possibility of hydrogen bonding between these compounds and amino acid residues of DHFR wild-type enzyme was obtained from Protein Data Bank (PDB) with code 1HFP (Tab. 3).

The estimation of binding free energy for *epi*-croomine and croomine are almost the same of -6.66 and -7.60 kcal/mol (see Table 3) which indicated that *epi*-cromine and croomine-enzyme complexes are more stable than pyrimethamine-enzyme complex of -5.40 kcal/mol.

The docking studies (Tab. 4) indicated that *epi*-croomineand croominemight interact with Ala9 at the active site of DHFR through hydrogen bonding. Both *epi*-croomineand croomine interact with Ala9, but they have bind at differentpositions, *epi*-croomineand croomine bonded to oxygens of carbonyl at C-17 and C-12, respectively.

# **CONCLUSION**

Konstanta inhibition (K<sub>i</sub>) of *epi*-croomine is bigger than croomineat 10 ppm. Both of *epi*-croomineand croomineare competitively inhibited to the human DHFR recombinant andmight interact with Ala9 amino acid residue at the active site of DHFR through hydrogen bonding.

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