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Piperine of *Piper retrofractum* Antagonizes H_1 Receptor: In vitro and In silico Study on Isolated-Guinea Pig Illeum Smooth Muscle

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Abstract

Piperine is a major Alkaloid that found in *Piper retrofractum.* Empirically, This herbs used as an antispasmodic. In the previous study. Piperin was reported may inhibit the release of histamine from mast cells by inhibiting the signal pathway mediated by IgE. Based on the fact, piperin was expected to has the antagonizm effect on the histamine receptor. The aim of this study was to investigate its activity on H_1 receptor. This research was conducted by investigated the effect of piperin on the guinea pig ileum smooth muscle (in vitro model). Piperin was administered at the doses of 1 mM and 5 mM. The result showed that piperin could inhibit the contraction of isolated guinea pig ileum smooth muscle that induced by histamine. The pD_2 values of H_1 receptor shifted significantly at the dose of 5 mM ($p < 0.05$). According to the contraction response data, piperin showed non-competitive antagonists activity. Its can observed from the shape of the contraction response curve that was not reach 100% E_{max}. Reversibility assay showed that by replaced buffer tyrode every 5 minutes for 30 minutes , the bond of alkaloid to the receptor was able to dissociate. In the in silico study (Autodock), piperine was observed can be bind to H1 receptors (docking score :-5.70). Piperine bound at Lys179 which is one of the important proteins in the histaminergic activity. The conclusion of this research is piperin has the activity as a non-competitive antagonist at the H_1 receptor.

Keywords

Piperine, *Piper retroractum,* H_1 receptor, Antagonism

1. Introduction

Indonesia is known to have a vast biodiversity herbal. Not only used as a spice in cooking, the herb is also sometimes used people as a traditional medicine for various diseases. The availability of various types of spices have the potential to study its contents and used as a guide compounds (lead compound) or the discovery of new drugs.One of the spices that has great potential of medicinal plants is pepper (*Piper nigrum* Linn.) family Piperaceae. This plant comes from India and is growing well in some Southeast Asian countries[1]. Pepper was traditionally used as an analgesic, antipyretic, depressant of central nervous system, anti-inflammatory, antioxidant, anticonvulsant, anti-bacterial, anti-tumor, and has hepatoprotective activity[2].The major constituent of *Piper nigrum* Linn. is piperine alkaloid (5-9%), volatile oil (1-2.5%), resin (6.0%), piperidine and starch (about 30%)[1].

Some of pharmacologial studies of *Piper nigrum* Linn. Have been reported. Alkaloid compound from *Piper nigrum* Linn. Was known has promising activity. The total of 5-9% of alkaloids contained in the pepper was a piperin[3]. Piperine from *Piper nigrum* Linn. Was reported has anti-inflammatory effects in rats that induced by karagenin[4]. From this in-vivo study, indicate that piperine has anti-inflammatory effects, antinociception and antiatritis by inhibiting multiple inflamatory mediators[5]. On the other hand, the mixture of herbal extract (polyherbal) that containing *Piper nigrum* L. showed bronchodilation effects in mice that induced by ovalbumin[6]. From the In-vitro study showed that piperine inhibits degranulation of the mast cell culture (RBL-2H3) through inhibition of phosphatidylinositol 4-kinase(s)[7]. Its also found the decreasement of intracellular Ca^{2+} levels that play a role in the inhibition of mast cell degranulation[8].

The aim of this study is to determine piperine activity on the ileum smooth muscle contraction. The effect was observed by the selectivity occupation of the histamine receptors. The study was conducted by in vitro methods(organbath). This research is expected to yield data that can be used as a reference for future research.

2. Material and Methods

2.1. Materials

Piperine alkaloid, Male guinea pigs with a body weight ranging between 400 and 500 grams were obtained from the School of Pharmacy, UniversitasMuhammadiyah Yogyakarta. All animal handling protocols were performed in accordance with the guidelines of laboratory animal care of the department. The chemicals used in the study were Tyrode's buffer solution, carbogen gas (containing 95% oxygen and 5% carbon dioxide, obtained from PT. Aneka Gas and Industrial Semarang), histamine (obtained from Sigma, USA), distilled water (obtained from pharmacology laboratory of Universitasmuhammadiyahyogyakarta).

2.2. In Vitro Study

The first step of the assay was to test the effect of 100 mL DMSO on ileal smooth muscle contraction induced by histamine. The purpose of the assay was to ensure that the DMSO used as a piperin solvent would not affect the response of the ileal smooth muscle contraction induced by histamine. Piperin activity as a H_1 receptor antagonist was evaluated by observing changes and shifts in the curve of ileal smooth muscle contraction. The contraction was induced by cumulative concentrations of histamine, ranging from $2x10^{-8}$ to $2x10^{-3}$M.

An organ bath was filled with 20.0 mL of Tyrode's buffer solution, then the organ was placed in the organ bath until a steady state equilibrium was reached (30 min). Subsequently, the single concentration of agonist was introduced to the organ bath and the contraction response was recorded (iwx software). After the contraction reached a plateau, the organ was washed by Tyrode's buffer for 60 min with replacement of the tyrode solution every 15 min. Subsequently, cumulative concentrations of the agonist ranging from $2x10^{-8}$ to $2x10^{-3}$ M were added to the organ bath. After maximum contraction, the organ was washed. After a washing period of 60 min,1 and 5 mM piperine was added to the organ bath at 10 min prior to administration of cumulative concentrations of agonist. After rewashing the organ, this procedure was repeated for each concentration.

A reversibility assay was performed to observe the ability of the organ tissue to return to basalt condition after the piperinetreatment. The assay was performed to evaluate the reversibility of the interaction between the receptor and its agonist. The

assays were performed before and after the piperineactivity assay. The ileum was washed briefly for 30 minutes with Tyrode's buffer solution and with replacement every five minutes. After reaching a stable condition of ileum, the organ was contracted by cumulative concentrations of histamine, after which the contraction response was recorded. The receptor agonist concentration curves before and after treatment with piperine were compared.

2.3. In Vitro Data Analysis

In the in vitro study, the research data concerned ileal smooth muscle contraction. The data were transformed into a percentage of the maximum response achieved by the agonist. Subsequently, the response percentages were plotted against the logarithm of the agonist concentrations.

The EC50 values (concentration of agonist that can produce a response of 50% of the maximum response) of receptor agonist in presence and absence of piperine were calculated based on the curve of the response percentages vs. the logarithm of the agonist concentrations. The EC50 was calculated based on Equation 1 and then transformed into a pD2 value (Equation 2). The data were then represented as mean of pD2 agonist ± standard error (pD2 ± SE). The pD2 values were statistically analyzed using the ANOVA test.

Piperinewas designated as AChM3 receptor antagonist if there was a decrease of the pD2 value of histamine due to piperine. The data distribution of the pD2 values of histamine was analyzed using a normality test (Kolmogorov-Smirnov method). Subsequently, the shift in pD2 value was analyzed with parametric statistical methods (ANOVA test followed by LSD test at 95% confidence level).

Determination of antagonist type was performed using a Schild-plot analysis in the form of a regression analysis. The Y axis is the ratio of the EC50 of agonist in presence of antagonist to EC50 of agonist in absence of antagonist, and then minus one. The X-axis is the logarithm of the concentration of antagonist. The antagonist type is determined based on the value of the slope generated by the Schild-plot equation. If the slope value is close to one, the receptor antagonist is competitive, whereas if the value of the slope is not close to one, it is non-competitive. The pA2 value (antagonist affinity of piperineto the receptor) is the intercept value of the Schild-plot [7].

$$\log EC_{50} = \left[\frac{50 - Y_1}{Y_2 - Y_1}\, X\, (X_2 - X_1)\right] + X_1 \qquad \text{(equation. 1)}$$

where:
X1 : log of concentration with response below 50%
X2 : log of concentration with response above 50%
Y1 : % response below 50%
Y2 : % response above 50%

$$pD2 = -\log EC50 \qquad \text{(equation. 2)}$$

2.4. In Silico Study

Docking process was done by using the Auto Grid 4.2 and 4.2 Autodock via Cygwin Terminal. File from previous preparation that includes Target.pdbqt, Ligand.pdbqt, parameter file (* .gpf), and docking parameter file (* .dpf) was stored in one folder on CygwinTerminal. The results of docking was formated by * .dlg file format. This file contains 10 conformation information and complex.pdb file that used for results visualization. After that, Visualization of the results was done by using DS Visualizeraplication. DS Visualizer app will show the bonding form of a compound with a receptor in 3D.

3. Results and Discussion

Piperine is a compounds that are known could inhibit degranulation of the mast cell cultures through inhibition of phosphatidylinositol 4-kinase[7]. In addition, the study of herbal mixtures that contains extract of *Piper nigrum* L, shows the bronchodilation effect in the rats that induced by ovalbumin[6]. There is the possibility of piperine also have spasmolytic mechanism to inhibit the activation of the H_1 receptor. Therefore, this study was conducted to prove the piperine antagonist activity at the H_1 receptor.

The mechanism of ileum contraction by histamine H1 receptor is mediated by G protein. Because of that, the receptor was categorized as G-protein-coupled Resetor (GPCR). The second messenger of the pathway was mediated by phospholipase C (PLC). Furthermore after PLC was activated, it will catalyze the hydrolysis reaction of fosfoinositol 4,5-diphosphate (PIP2) to form inositol 1,4,5-triphosphate (IP3) and diasil glycerol (DAG). IP3 which has been formed binds to IP3 receptor on the surface of the endoplasmic reticulum and open Trancient potencial Receptor Channels (TRPC) and resulted in the release of Ca2 + from calcium-store. After that the level of intracellular Ca^{2+} will increase. Increased levels of intracellular Ca^{2+} can activate the calcium channels in the membrane cell surface [16].

Activation of calcium channels lead to an influx of extracellular Ca^{2+} and overall would increase the levels of Ca^{2+} instaseluler that induce smooth muscle contraction[17]. The increasement levels of intracellular Ca2 + that derived from GPCR activation or ion channels can cause contraction of the smooth muscle. Ca^{2+} ion can bind toCa^{2+} calmodulin receptor (CaM). Calmodulin binding protein is a Ca which does not have the enzyme activity. Calmodulin will work after forming complexes with Ca^{2+} calmodulin. Furthermore, the complex activates myosin light-chain kinase (MLCK) which phosphorylate myosin. Phosphorylated myosin will interacts with actin filaments and produce contraction[18]. piperine can be said to have activity as an H_1 receptor antagonist if it can reduce the potential for histamine to induce smooth muscle contractions. An in vitro study aims to determine the activity of the piperine as H_1 receptor antagonist. In this study we used piperine with level 1 mM and 5 mM.

3.1. Preliminary study the effect of DMSO on ileal smooth muscle contraction.

DMSO was used as piperine solvent in this study, so that DMSO need to be tested first its effect on smooth muscle contraction. DMSO are expected to has no effect on the contraction of illeum smooth muscle. DMSO was used with the volume 100µL. It was accordance to piperine administration to the organ bath.The test results shown a slight shift in the curve (Figure 1).

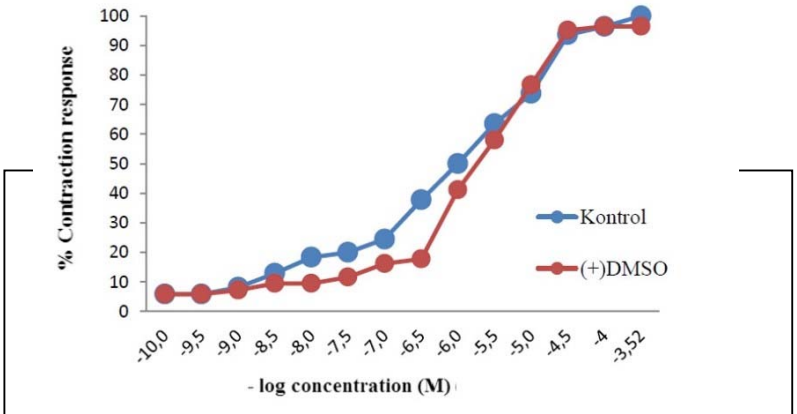

Fig. 1 Concentration-response curves to histamine in the absence or presence of DMSO at volume 1000 uL in guinea pig ileal smooth muscle (data represent n = 5-10, mean ± SEM).

This effect was followed by a decrease in the value of PD_2 of DMSO (Table 1). However, based on statistical tests by using paired t-test, it was not significantly different ($p > 0.005$). Therefore, DMSO does not have the effect of lowering the contraction significantly so that it can be used as a piperine solvent.

Table 1. Effect of DMSO on ileal smooth muscle contraction

Groups	PD_2	E max
Control	5.84 ± 0.12	100 % ± 0.00
DMSO	5.72 ± 0.13	100 % ± 0.00

Effect of DMSO on the response of ileal smooth muscle contraction induced by histamine. The pD2 and Emax values of histamine in absence and presence of 100 mL DMSO (n = 5, mean ± SEM).

3.2. Comparative study using diphenhydramine

H_1 receptor distributed on the surface of the smooth muscle of guinea pig ileum. By histamine H1 receptor activation will result in contraction of smooth muscles both in humans and guinea pig ileum. Diphenhydramine comparative stsudy conducted by using the same method with treatment using piperine. Diphenhydramine is a first-generation H_1 receptor antagonist with has sedative and anti-allergic properties. Diphenhydramine competitively inhibit the H1 receptor. Usually diphenhydramine used for the symptoms caused by endogenous histamine in the bronchi, blood vessels and gastrointestinal smooth muscle. The purpose of this comparison study is to see if there is a same effect between diphenhydramine and piperin as antihistamines. It is also to ensurethe methods that used is valid to prove an H1 receptor antagonist.Test results showed that diphenhydramine could shifts the contraction response curve to the right (Figure 2) and it cause the PD_2impairment (Table 2).

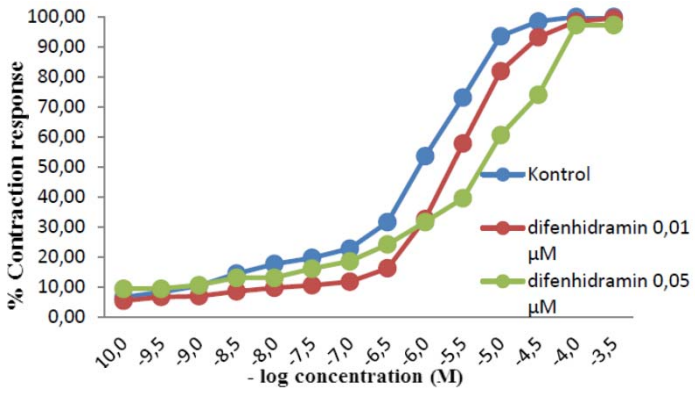

Fig. 2 Concentration-response curves to histamine in the absence or presence of diphenhydramine at concentrations of 1 & 5 mM in guinea pig ileal smooth muscle (data represent n = 5-10, mean ± SEM).

Shape of the curve shows contraction responses (E_{max}) agonist back to 100% after being treated with histamine. The position of competitive antagonist which occupied the same active receptors can be shifted with the addition of the agonist concentration. So that the EC_{50} can be achieved with the addition of the higher agonist concentration. Maximal response (E_{max}) can be returned 100%. It shows that diphenhydramine is a competitive antagonist to the H_1 receptor.

Table 2. Effect of diphenhydramine on ileal smooth muscle contraction

Groups	PD_2	E max
Histamine control	6.10 ± 0.16	100 % ± 0.00
Diphenhydramine 0.01 uM	5.67 ± 0.09	100 % ± 0.00
Diphenhydramine 0.05 uM	5.15 ± 0.23*	100 % ± 0.00

* Significant difference (P < 0.05) compared to control.
Effect of diphenhydramine on the response of ileal smooth muscle contraction induced by histamine. The pD2 and Emax values of histamine in absence and presence of diphenhydramine (n = 5, mean ± SEM).

In addition, the type of antagonist can also be determined by Schild-plot analysis. From this Schild-plot analysis we obtained equation y = 0,7542x + 1.7281. Schild slope value equation-Plot is at 0.7542 (approaching 1.00) and intercept (PA_2 value) of 1.7281. PA_2 value (parameter affinity) showed levels of antagonists which may cause the levels agonist folded into 2 times to get the same effect as before given the antagonistic effect. From thisassay it can be concluded that diphenhydramine acts as a competitive antagonist to the H_1 receptor.

3.3. Antagonism effect of piperine to the H_1receptor.

Antagonism effect of piperineto the H_1 receptor was done by observe the changes in the curve of contraction profiles. Piperine thought to have potential as an H_1 receptor antagonist. This potential can be measured by comparing the value of PD_2 histamine with and without piperine pretreatment. Pretreatment oflleal smooth muscle with piperinshould be able to make value of PD2 histamine lower. Histamine can induce contractions after binding with H_1 receptors on smooth muscle of ileum. increasement concentration of exogenous histamine also resulted in the increasement of percetage contraction response. Response of isolated ileum smooth muscle contraction will achieve 100% on administration of exogenous histamine at the level 3 x 10^{-4} M. The results showed that ileal smooth muscle pretreatment with piperine 1mM and 5mM for 5 minutes, able to reduce the response of isolated ileum smooth muscle contraction

induced by exogenous histamine in a concentration-dependent pattern. Reduction of contraction response occurs primarily in low concentrations of histamine administration. The profile curves (Figure 3) shows the shift of declining curve of histamine concentration series. Curve Shifting shows a decrease in the ability of histamine to induce contraction due to the effect of pretreatment using 1mM and 5 mM piperine.

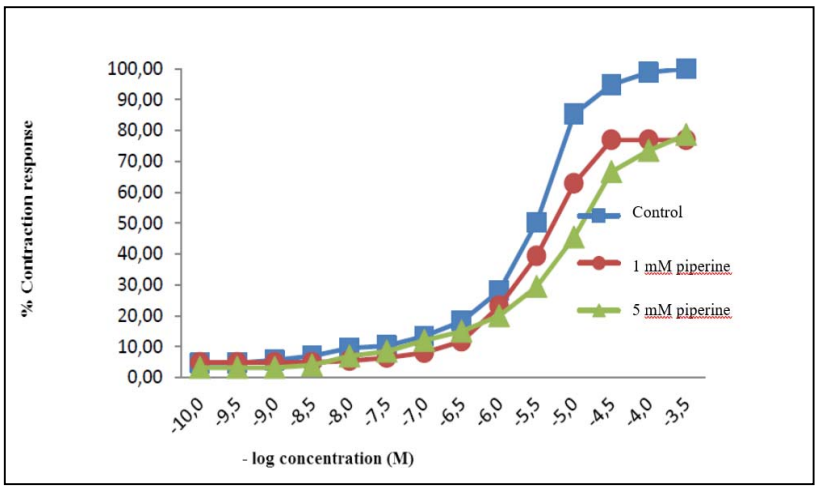

Fig. 3 Concentration-response curves to histamine in the absence or presence of piperine at concentrations of 1 & 5 mM in guinea pig ileal smooth muscle (data represent n = 5-10, mean ± SEM).

This effect is also characterized by impairment PD_2 histamine (Table 3). The PD_2value of histamine with piperine pretreatment 1 mM and 5 mM respectively amounted to 5.61, 5.24 and 4.94. This PD_2 Impairment was statistically significant ($p < 0.05$).PD_2 histamine decline because of the influence of piperine pretreatment. piperine prove have antagonistic effects on the smooth muscle of ileum H1 receptor. To know the type of piperine antagonists, can be seen in the shape of contraction. Ileal smooth muscle can not restore the contraction response (E_{max}) to 100%.

Table 3. Effect of iphenhydramine on ileal smooth muscle contraction

Groups	PD_2	E max
Histamine control	5.61 ± 0.12	100 % ± 0.00
Piperine 1 mM	5.24 ± 0.16	76.93% ± 0.00
Piperine 5 mM	4.94 ± 0.52*	78.68 % ± 0.00

* Significant difference ($P < 0.05$) compared to control.
Effect of piperine on the response of ileal smooth muscle contraction induced by histamine. The pD2 and Emax values of histamine in absence and presence of piperine (n = 5, mean ± SEM).

In the pretreatment of 1 uM piperine, E_{max} reached 76.93%. And in the treatment with5 mM, E_{max} reached 78.68%. Non-competitive antagonist is an antagonist that reduces the effectiveness of an agonist through a different mechanism (have different binding site). From this result we know that piperine categorized as non competitive antagonist on the H_1 receptor.

The next assay is reversibility study. Reversibility test aims to determine whether the bond alkaloid pepper with H1 receptor can dissociate so that the effect of the contraction of ileal receptor can be returned. The assays were carried through by wash ileum of guinea pigs by replacing the buffer Tyrode every 5 minutes for 30 minutes. Antagonist binding properties of pepper alkaloid said reversible if the value of PD_2 contraction after treatment is not much different from the before. The results can be

seen in the response curve (Figure 4). We can see the shape of the curve is relatively similar.In addition the value of PD_2 (Table 4) are not much different and statistically there was no significant difference between the control and test groups ($p > 0.005$). Based on this, we can conclude piperine bonding can be removed in washing every 5 minutes for 30 minutes. In other words, piperine bonding to H1 receptor was reversible.

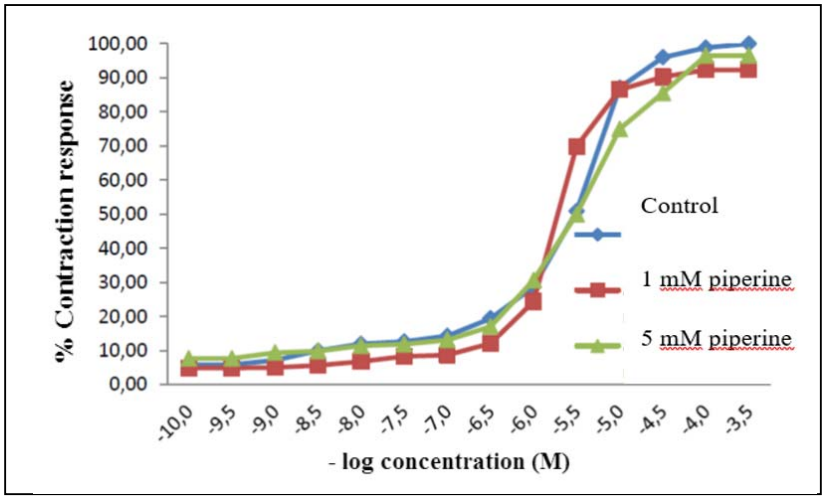

Fig. 4 Reversibility assay: Concentration-response curves to histamine in guinea-pig ileal smooth muscle after treatment with piperine (data represent n = 5-10, mean ± SEM).

Table 4. Reversibility assay of piperin in the H_1 receptor

Groups	PD_2	E max
Histamine control	5.61 ± 0.16	100 % ± 0.00
Piperine 1 mM	5.73 ± 0.09	92.35 % ± 0.00
Piperine 5 mM	4.54 ± 0.23	96.52 % ± 0.00

Reversibility Effect of piperine on the response of ileal smooth muscle contraction induced by histamine. pD2 and Emax values of metacholine (n = 5, mean ± SEM).

3.4. In silico study of piperin to H_1 receptor.

Before starting the docking process, the first step that must to do is validation of docking protocol. Valid docking method can be indicated by the value of RMSD (Root Mean Square Distance. Valid RMSD valueis ussually below 2,0000. Native ligand that used in this validation stage is doxepin (5EH). RMSD value that obtained was 1.723 (2.0000) with a docking score is -5.6. From this result we can know that the docking protocol docking is valid.

Piperin activity to the H1 receptor can be studied through in silico molecular docking method. Applications that used for in silico study was AutoDockTools. The protein usedas the target is 3RZE which is an H1 receptor protein in humans. Docking process produces 10 conformations that contains information energy of each conformation. Results of 10 conformations are seen on its binding energy to choose the best conformation for binding energy value describes the strength of bonds between ligand and protein. If there is more negative energy value of the bond, it indicate the stronger bonding to the receptor. Score of the bond and the interaction energy between each ligand to the target protein can be seen in Table 5.

Table 5. Binding energy score and interaction of ligand to the amino acid residue of H_1 receptor

Groups	Binding energy	Amino acid residue

	(kkal/mol)	
Piperine	-5.70	Isoleucine 438
		Histidine 450
		Isoleucine 454
		Lysine 179
Doxepine (5EH)	-5.02	Aspartic acid 107
		Histidine 1031
		Leucine 1032
		Phenylalanine 1104
Diphenhydramine	-6.99	Tyrosine 458
		Tryptophan 428
		Tyrosine 431
		Serine 111
		Tyrosine 108
		Phenylalanine 432

Piperine score is slightly higher than the original 5EH as ligands and lower than diphenhydramine as an H_1 receptor antagonist. But in vitro study piperine alkaloid known act as non-competitive antagonist.

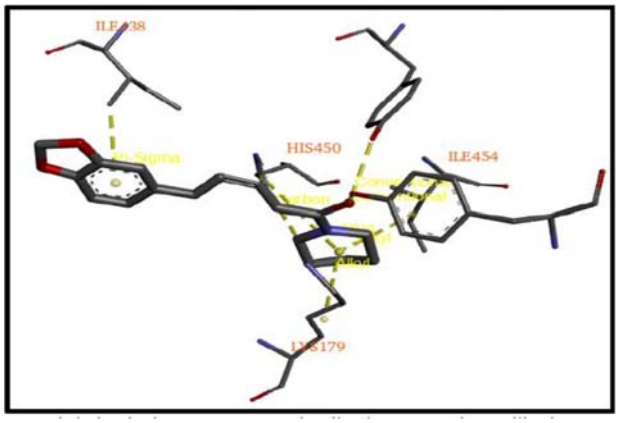

Fig. 5 Docking visualization of piperine on H_1 receptors.

H1 receptor has many amino acids, but only partially that important in histaminergik activity, that is Trp428, Asp107, Asn198, Lys191, and Lys17919. Furthermore Asp107 and Trp428 amino acid is an amino acid that plays an important role in bonding histamine as antagonist [20,21]. In this study, it can be seen conformation 5HE (original ligand) with the highest bond energy value (-5.02) binds to the residue Asp107, while piperine conformation with the highest bond energy value (-5.70) binding to Lys179. Diphenhydramine as a competitive antagonist with an energy value of bonds the highest (-6.99) binds the amino acid Trp428. From the in silico results it can be concluded that diphenhydramine ligand binds to both amino acids Asp107 and Trp428 was the important bonding, in accordance with the histamine antagonist diphenhydramine function that works as a competitive antagonist to the H1 receptor. While piperine in vitro assays pepper alkaloid previously shown to be a non-competitive antagonist that binds to the protein side Lys179 are also important in the histaminergic system.

Conclucion

Piperine have antagonism activity on H_1 receptor, by PD_2 impairment. From schild plot analysis the type antagonism is non-competitive antagonist. From the in

silico study, piperine can bind to the H_1 receptor (docking Score: -5.70). Piperine can bind to Lys179 residue of H_1 receptor, which is one important residue in histaminergic activity.

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