Kinetic and Theoretical Study of the Chalcones as Inhibitors of β-Lactamase Enzyme

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Abstract: *Staphyloccoccus aureus, Escherichia coli, Pseudomona aeruginosa Klebsiella pneumoniae, Acinetobacter baumannii, Enetrobacter cloacae* and more bacterias have shown resistance to antibiotics in Colombia, therefore, resistance to antibiotics is a problem that is on increase in Colombia. The resistance mechanism to penicillin antibiotics in these bacterias is the expression of β-Lactamase enzyme. In order to use the penicillin antibiotics which are still effective against them, we had evaluated 10 chalcones as inhibitors of this enzyme. The most active chalcone showed $K_{m'}$ = 406.7 μM higher than clavulanic acid that showed $K_{m'}$ = 211.9 μM at 37°C during 10 h, and using amoxycillin as substrate. The chalcones were better competitive inhibitors because of they allowed the hydrolysis of smaller quantity of amoxycillin than the clavulanic acid, the most active chalcone showed K_{cat}/K_M = 1.398 $(minM)^{-1}$ and the clavulanic acid showed K_{cat}/K_M = 2.674 $(minM)^{-1}$. The molecular modelling of the Enzyme-chalcones complexes showed that the chalcones with electron-donating groups on ortho, meta position of A ring favour the interaction with the residues Threonine-319, Lysine-67, Serine-64 and Tyrosine-150 of the active site of the enzyme, because of the number and strong of Hydrogen bonds of the chalcones in the active site of the enzyme increase, and therefore the affinity of the chalcones increases too. The electron-donating groups in the chalcones contribute to their inductive effect improving the interaction with the active site of the enzyme because of rising of electrostatic attraction between them.

Key Words: β-Lactamase, chalcones, molecular modelling, inhibitory activity, amoxycillin, clavulanic acid.

INTRODUCTION

Staphyloccoccus aureus, *Escherichia coli*, *Pseudomona aeruginosa Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Enetrobacter cloacae*, etc. have shown resistance to antibiotics in Colombia, and the health system still prescribes Amoxicilina® (Amoxycillin) and Clavulin® (Amoxycillin:Clavulanic acid) as antibiotics to treatment of infection disease, because they are still effective and cheaper than another antibiotics [1-4]. The most widespread resistance mechanism to β-lactam antibiotics is β-Lactamase enzymes expression, which hydrolise this drugs, thereby inactivanting them [5]. Due to the re-emerging resistance of these microorganisms, it is necessary to produce better and specific inhibitors against β-Lactamase in order to extend the life of antibiotics, and because the clavulanic acid was reported as poor inhibitor of class C β-Lactamase and there is already resistant enzymes to this inhibitor [6-8].

The present article describes the kinetic study of the chalcones (1)-(10), and the molecular modelling of the most active chalcones as potential inhibitors of the β-lactamase enzyme (class C *Enterobacter cloacae*, EC 3.5.2.6). The Table **1** shows the chalcones with different substituent on the A and B ring which were evaluated as enzymatic inhibitors. The inhibitory activity of the chalcones was compared with the activity of the clavulanic acid as inhibitor.

The geometry of enzyme-chalcone, enzyme-amoxycillin and enzyme-clavulanic acid complexes was optimized using Gaussian 98 at B3LYP/3-21G level. The electronic density of each compound was calculated at HF/6-31G level. The structure of the active site was taken from Brookhaven Protein Data Bank (PDB entry 1XX2) for *E. cloacae* (E.C. 3.5.2.6) and designed according to hydrolytic enzyme type Serine [8, 9].

RESULTS AND DISCUSSION

The kinetic studies were realized with the chalcones (1)-(10) [10] as inhibitors, Amoxicilina® (Amoxycillin) and Clavulin® (Amoxycillin:Clavulanic acid) as reference antibiotics at 37 °C and all of them were monitored each 5 min during 10 h [11]. The kinetic parameters and the catalytic efficiency (K_{cat}/K_M) [12] are shown in the Table **2**.

The enzyme was inhibited by all the chalcones and clavulanic acid at different concentrations. These compounds were competitive inhibitors and the chalcones were better inhibitors than clavulanic acid because they showed apparent Michaelis-Menten constant, K_M', higher than K_M of clavulanic acid and the reaction is directed to formation of the enzyme-chalcone complex. The duration of the experiment indicated that the inhibition remained for 10 h and the enzyme-chalcone complexes did not break down during the

*Address correspondence to this author at the Laboratorio de Sustancias Bioactivas, Sede de Investigación Universitaria, Universidad de Antioquia. Carr. 53 #61-30. Medellín, Colombia. Departamento de Farmacología y Toxicología, Facultad de Medicina, Universidad de Antioquia. A.A. 1226, Spain; E-mail: consuelo.jaramillo@gmail.com

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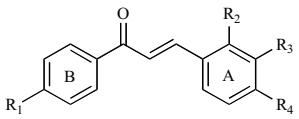
Table 1. Substituents of the Chalcones on the A and B Rings [10]

	R_1	R_2	R_3	R_4
(1)	CH_3	OCH_3	OCH_3	H
(2)	CH_3	OCH_3	H	OCH_3
(3)	Cl	OCH_3	H	OCH_3
(4)	CH_3	H	H	NO_2
(5)	Cl	H	H	H
(6)	Cl	H	H	Cl
(7)	Cl	OCH_3	OCH_3	H
(8)	CH_3	H	H	H
(9)	CH_3	H	H	OCH_3
(10)	Cl	H	H	NO_2

experiment which is in accord with observations made in the reference [11], that is to said, it might form a stable enzyme-chalcone complex that is showed in the low Ki values compared to Ki value of clavulanic acid. The low values of catalytic efficiency of the chalcones indicate that are potent inhibitors of the enzyme. The catalytic efficiency values of the chalcones are lower than the values reported for other inhibitors [6, 11, 13-15].

The chalcone (1) was the best inhibitor among all the chalcones, its K_{cat}/K_M is smaller than Clavulin®, that is to say, the chalcone (1) allowed enzymatic degradation of smaller quantity of amoxycillin than the other inhibitors.

Although both chalcones (1) and (2) are isomers, the chalcone (2) showed higher K_{cat}/K_M than the chalcone (1).

The K_{cat}/K_M of the chalcones changes in accordance with the substituents on the A and B rings. The chalcone (1) has a CH_3 group in *para* position of B ring and OCH_3 groups in *ortho* and *meta* positions of the A ring. These electron-donating groups contribute to their inductive effect on aro-

Table 2. Inhibition Parameters of Chalcones (1)-(10), Amoxicilina® and Clavulin® for Class C β-lactamase Enzyme of *Enterobacter cloacae* P99 [11, 12, 15]

Compounds	Vmax (μM/min)	K_M, (μM)	K_i (μM)	R^2	K_{cat} (1/min)	K_{cat}/K_M (1/minM)
Amoxicilina®	$3.408x10^{-4}$	154.0	-------	0.993	5.680_x10^{-4}	3.688
Clavulin®	$3.400x10^{-4}$	211.9	14.363	0.982	$5.667x10^{-4}$	2.674
(1)	$3.411x10^{-4}$	406.7	3.291	0.865	$5.685x10^{-4}$	1.398
(2)	$3.412x10^{-4}$	389.6	3.530	0.910	$5.687x10^{-4}$	1.460
(3)	$3.415 x10^{-4}$	362.3	3.992	0.824	$5.692x10^{-4}$	1.571
(4)	$3.448 x10^{-4}$	360.2	4.033	0.876	$5.747 x10^{-4}$	1.595
(5)	$3.420x10^{-4}$	333.7	4.628	0.890	$5.700x10^{-4}$	1.708
(6)	$3.415 x10^{-4}$	298.4	5.759	0.967	$5.692x10^{-4}$	1.907
(7)	$3.408 x10^{-4}$	292.4	6.009	0.952	$5.680x10^{-4}$	1.942
(8)	$3.403x10^{-4}$	289.6	6.133	0.898	$5.672x10^{-4}$	1.958
(9)	$3.462 x10^{-4}$	258.0	7.996	0.917	$5.770x10^{-4}$	2.236
(10)	$3.404 x10^{-4}$	220.7	12.468	0.927	$5.673x10^{-4}$	2.571

matic system, and this inductive effect might be the predominant effect in the chalcones which would support the interaction with the enzymatic active site residues. Also, the inductive effect of the substituents increases the negative charge throughout the aromatic system of the chalconas [16].

The both substituents Cl on the B ring and the OCH_3 group in A ring in *para* position reduce the inhibitory activity of the chalconas because their resonance effect [16].

The molecular modelling method was applied in order to understand the interaction of the most active chalcones in the active site of β-Lactamase enzyme. The results supply convincing evidences of the importance of the hydrogen bond and the number of interactions of the chalcones in the active site of the enzyme [17-19].

The computational procedure was carried out using density funtional theory in order to determine the electronic properties of the chalcones (1)-(10) [20]. This perform helps us to interpret where and how the interaction between the chalcone and the active site could occur. The Fig. (**1**) shows the electrostatic potential map of the amoxycillin, clavulanic acid and chalcones. This map shows the regions of the molecules with higher electronic density, such regions are susceptible to stablish interaction with the residues of the active site in order to stabilize the enzyme-compound complex.

The range of total electronic density of the compounds is between -52.71 Kcal/mol and 52.71 Kcal/mol. The Fig. (**1**) shows the map of the compounds.

The total electronic density map of amoxycillin shows that the center of the most positive potential lies in the vecinity of the carbonyl carbon of the β-lactam ring what becomes this ring in electrophile susceptible to nucleophilic attack of a residue in the enzymatic active site. The aromatic ring shows a negative potential which improves the interaction with the active site of the enzyme because of electrostatic attraction between them, the electrostatic potential of amoxycillin is between -49.57 Kcal/mol and 49.57 Kcal/mol.

The most positive electrostatic potential of clavulanic acid lies on lactam ring, but it is less positive than amoxycillin. This property does that the clavulanic acid in the mixture Clavulin® competes for the active site of enzyme, the electrostatic potential of clavulanic acid is between -52.71 kcal/mol and 52.71 Kcal/mol.

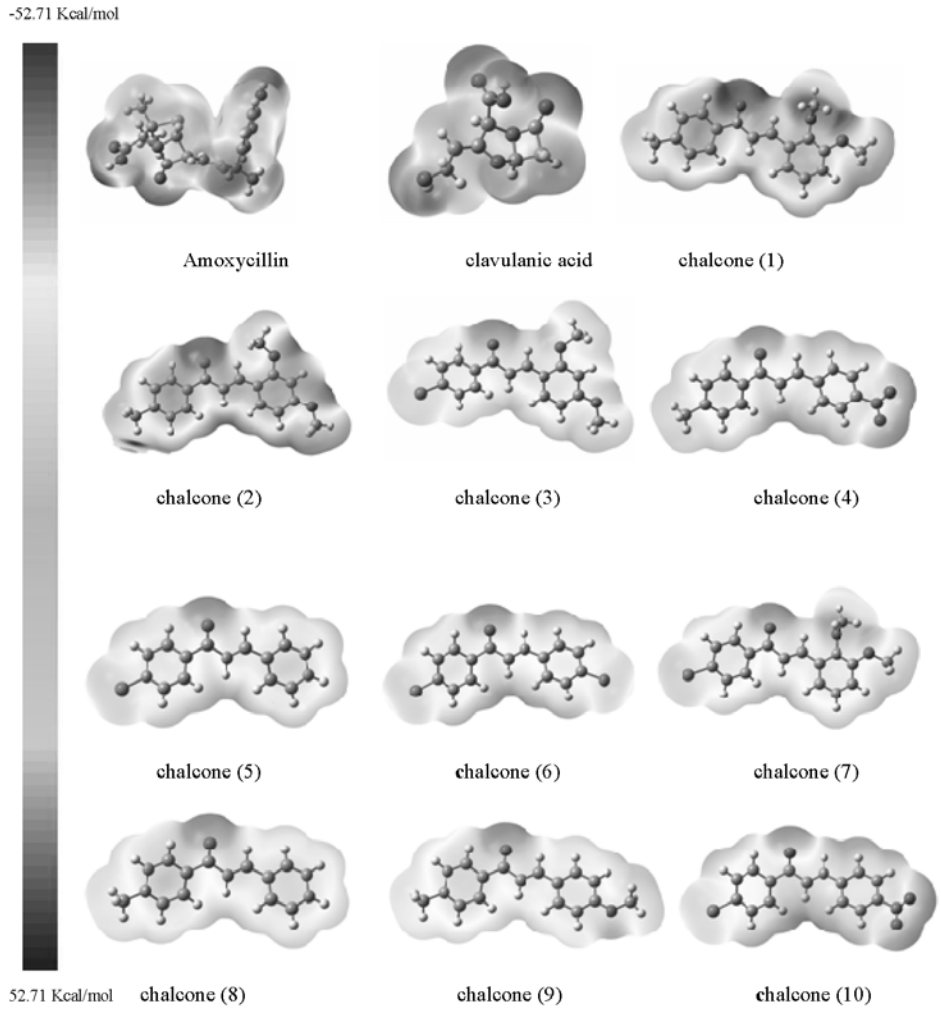


Fig. (1). Electronic density map of substrate and inhibitors calculated at HF/6-31G. Values of potential ranging vary from -52.71 Kcal/mol: nucleophiles to 52.71 Kcal/mol: electrophiles, intermediate values indicate regions between this extreme [20].

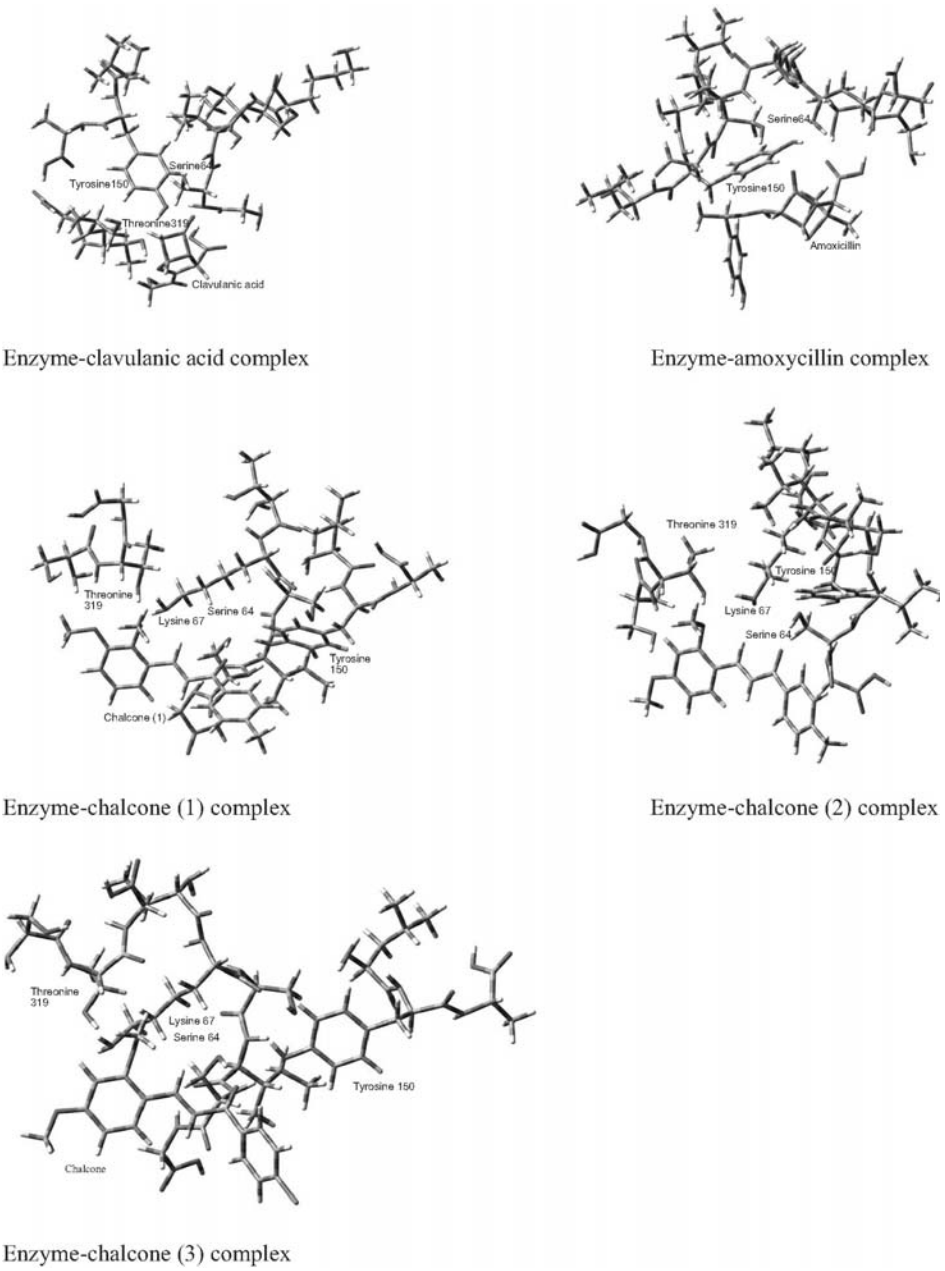


Fig. (2). Geometry optimization of enzyme-chalcone (1), enzyme-chalcone (2), enzyme-chalcone (3), enzymeamoxycillin and enzyme-clavulanic acid complexes at B3LYP/3-21G level [20].

In general, all inhibitors and substrates of β-lactamase enzyme are negatively charged in order to improve the electrostatic attraction to the active site which is positively charged [7, 8, 20].

The negative potential of the chalcones (1) and **(**2) lies on all aromatic system and if the rings have electron-donating groups, the negative potential throughout the molecule will be higher. Also, the closeness of the metoxy groups in the aromatic rings improves the inhibitory activity of the chalconas [8]. The chalcones show an electrostatic potential between -40.16 Kcal/mol and 40.16 Kcal/mol. The electronic potential of the chalcone (3) is lower than chalcone (2) because the chalcone (3) has an electron-withdrawing group in B ring, this group reduces the electronic density on the chalcone and this has its effect incresing the K_{cat}/K_M value, therefore, this chalcone would be less competitive for the active site of the enzyme.

In order to determine the interaction of the compound in the active site of the enzyme, it was carried out the molecular modelling of enzimatic complexes taking 4 residues of the active site: Ser64, Lys67, Tyr150 and Thr319, which would stablish interaction with the regions of higher electronic density of the compounds, the other residues used to modelling were a residue before and a residue after each residues that would stablish interaction. The peptides were: Gly63-Ser64-iLe65-Ser66-Lys67-Thr68, Leu149-Tyr150-Ala151, and

Ser318-Thr319-Gly320. The residues were chosen in according to the protein data bank, PDB (1XX2, P99 β-lactamase from *Enterobacter cloacae* (E.C. 3.5.2.6)) [3, 8, 10].

The molecular modelling of enzyme-chalcone (1), enzyme-chalcone (2), enzyme-chalcone (3), enzyme-amoxycillin and enzyme-clavulanic acid complexes showed that the chalcone (1) and (2) can stablish hydrogen bonds with Ser64, Lys67, Tyr150 and Thr319. The geometry optimization of enzyme-inhibitor and enzyme-substrate complexes is shown in (Fig. (**2**)).

The Ser64-OH residue forms hydrogen-bond with the carbonyl oxygen of substrate and inhibitors and all the compounds interact with Tyr150-OH residue.

The clavulanic acid forms 2 hydrogen-bonds with Ser64-OH, Tyr150-OH and Thr319-OH in the enzymatic complex. The residues of active site of the enzyme interact with the carbonyl oxygen of the lactam ring of amoxycillin. After these compounds are attracted to active site by electrostatic attraction, the residues Ser64 and Tyr150 are essential to stablish interaction and form the enzymatic complex, this result is according to reference [5, 7].

According to the geometric parameters of enzymatic complexes of the chalcones, the strongest hydrogen bond was established between the Ser64 residue and the chalcone (1). This chalcone forms 6 interactions with the carbonyl and *ortho*, *meta*-OCH_3 groups, but in the chalcones (2) and **(**3) were stablished 3 interactions. The number of the hydrogen bond could explain the high stability and irreversibility of the enzyme-chalcone (1) complex. The Lys67 residue establishes interaction with *o*-OCH_3 in A ring of chalcone (1), Thr319 establishes two hydrogen bonds with both OCH_3 groups in chalcone (1) and one hydrogen bond with *o*-OCH_3 of the chalcones (2) and (3). The number of interactions in the active site of the enzyme is increased by the electron-donating groups proximity, and a high number of interactions forms a stable enzymatic complex and a higher affinity of the chalcones to enzyme. The modelling showed interactions in which the proton forms two contacts with the acceptor oxygens, the interactions had been still reported [17, 18].

The Fig. (**3**) shows the scheme of the interactions between the residues of the active site and the chalcones (1), (2), (3), amoxycillin and clavulanic acid.

CONCLUSION

The modelling studies suggest that the hydrogen bonds and electrostatic attractions of the chalcones in the active site of β-Lactamase enzyme are favoured for proximity of electron-donating substituyents and the most negative electrostatic potential. The interactions that the residues stablish with the inhibitors are favoured for negative charge on the aromatic system of the chalcones. The carboxylic acid group increases the interactions in the active site of β-Lactamase enzyme in clavulanic acid and amoxycillin.

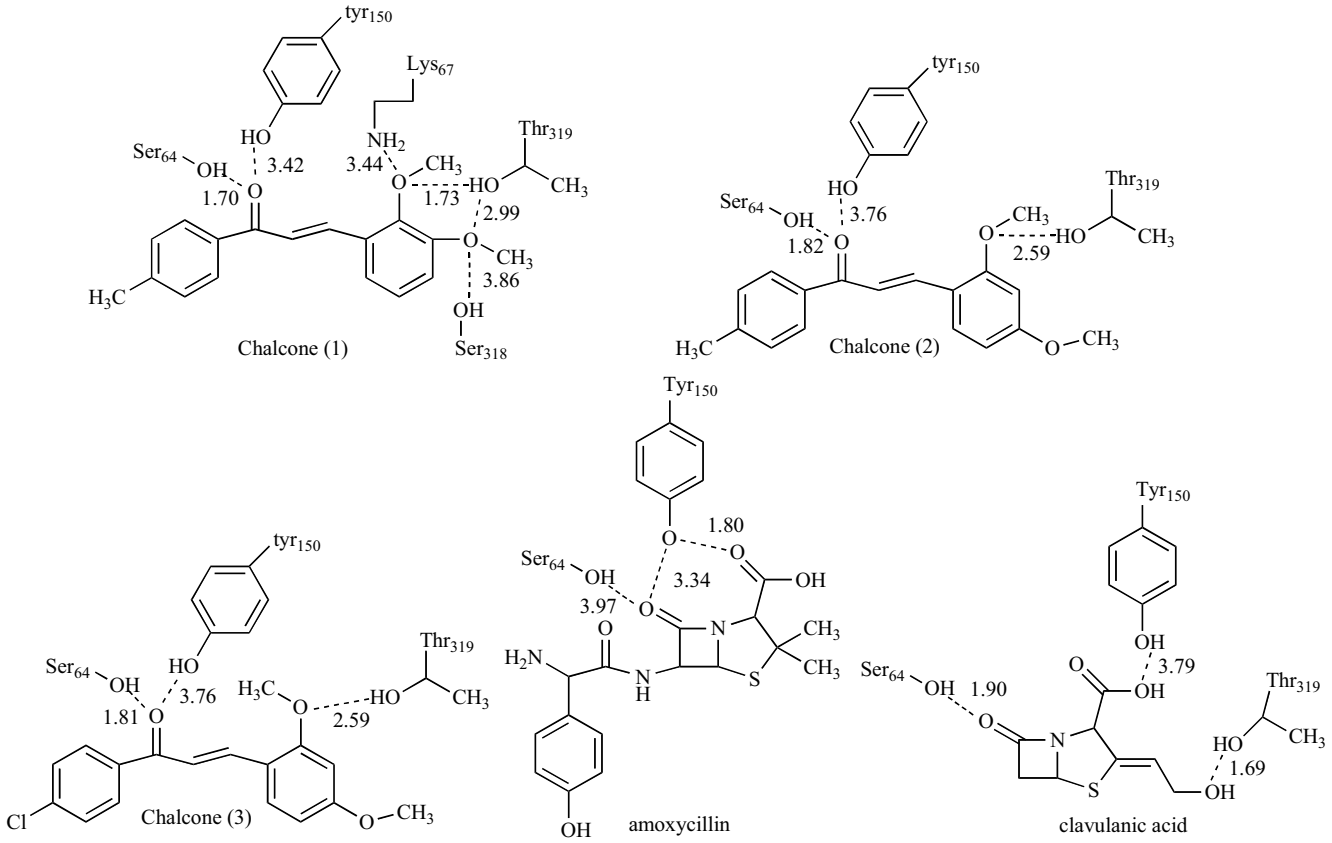


Fig. (3). Distances of the enzyme-chalcones (1)-(3), enzyme-amoxycillin and enzyme-clavulanic acid complexes in the active site of β-Lactamase enzyme. Distances in Å.

A good chalcone inhibitor of the β-lactamase enzyme has electron-donating groups with electron pairs which can establish hydrogen bonds with the residues in the active site, this electrons resonate on aromatic rings and increase the electronic density in order to favour the electrostatic attraction to active site and form the enzyme-chalcone complex.

EXPERIMENTAL

Enzymes and Substrates

Amoxicilina® and Clavulin® were obtained from commercial sources. The enzyme P99 β-lactamase (class C of *Enterobacter cloacae,* EC 3.5.2.6) was purchased from Sigma-Aldrich Co. The chalcones were synthesized according to reference [10]. Kinetic studies with the enzyme at 0.6 μM were performed in buffer phosphate (pH 7.3), and using Amoxycillin as substrate at 100 μM, and clavulanic acid and chalcones (1)-(10) as inhibitors at 5.4-108 μM. The hydrolysis of the substrate was monitored using spectrophotometer, UV-Varian 50-BIO, at 250 nm each 5 min for 10 h at 37 ºC [11]. The Vmax and K_M were determined using the software GraphPadPrism 5.00 for windows, San Diego, California, USA [10].

Molecular Modelling

The general procedure was carried out according to reference [8]. The residues of active site were taken of PDB (P99 β-lactamase of *Enterobacter cloacae,* entry 1XX2). The geometry optimization of the compounds and the enzymatic complexes were performed using density functional theory (DFT) with the HF and the Becke3-Lee-Yang-Parr (B3LYP) functional, and the 6-31G and the 2-31G basis set, respectively [8, 20].

ACKNOWLEDGEMENT

We thank Universidad de Antioquia for supporting this work.

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Received: 27 February, 2009 **Revised: 07 April, 2009** **Accepted: 10 April, 2009**

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