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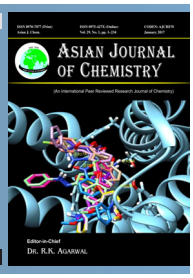

in silico Study of Furocoumarins from *Caryopteris odorata*: Moderate Inhibitors of Butyryl Cholinesterase and Lipoxygenase

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In the present investigation, five known furocoumarins (**1-5**), isolated from the chloroform soluble fraction of *Caryopteris odorata*, were evaluated for their inhibitory potential against two enzymes, namely, butyryl cholinesterase (BChE) and lipoxygenase (LOX). It was inferred from the results that all the five furocoumarins were moderate inhibitors of butyryl cholinesterase. Similarly, amongst the five, four furocoumarins also possessed moderate inhibitory potential against lipoxygenase. To investigate the binding modes of the isolated molecules, these were docked into the active sites of butyryl cholinesterase and lipoxygenase, whereby these molecules exhibited significant interactions and good correlations with the experimental data.

Keywords: *Caryopteris odorata*, Furocoumarins, Butyryl cholinesterase, Lipoxygenase, Molecular docking.

INTRODUCTION

Caryopteris odorata (Ham. Ex Roxb.) belongs to the family Verbenaceae. This family comprises about 100 genera and nearly 2600 species, which are mostly tropical and subtropical in distribution [1]. It is represented by almost 17 genera and about 35 species in Pakistan [2]. The powdered leaves and flowers of this plant are used in diabetic foot ulcer, tumors and wounds [3].

Furocoumarins are the natural constituents of some plants having utility as food or in phytomedicines, herbal teas and cosmetics. These molecules have gained considerable interest due to their phototoxic and photomutagenic properties [4]. The pure furocoumarins psoralen and 8-methoxy psoralen have been used as therapeutic drugs for the treatment of psoriasis in combination with UVA irradiation. This therapy is referred as psoralene UVA therapy and it is able to combat skin tumors under certain circumstances [5,6]. The furocoumarins have attained many new potential therapeutic applications as well. For example, some psoralen derivatives, such as 8-methoxy-psoralen, have exhibited anticonvulsant properties [7].

Butyrylcholinesterase (BChE, EC 3.1.1.8) enzyme belongs to a family of serine hydrolases. The active site of this enzyme contains different amino acid residues which promote the specifications against various inhibitors of this enzyme. The enzyme system is greatly involved in the termination of acetylcholine at cholinergic synapses [8]. The cholinesterase inhibitors promote acetylcholine for neuronal and neuromuscular transmission reversibly or irreversibly. It has been found that BChE (E.C 3.1.1.8) inhibition is an effective tool for the treatment of Alzheimer's disease and related dementias. The amount of BChE is significantly high in Alzheimer's plaques when compared in plaques present among normal age-related brains without dementia. BChE is produced in the liver and it enriches the blood circulation. It is also present in adipose tissue and can also be found in the intestine, smooth muscle cells, white matter of the brain and in many other tissues. So it is important to search new cholinesterase inhibitors for the treatment of Alzheimer's and related diseases [9].

Lipoxygenases (LOX, EC 1.13.11.12) constitute a family of non-haem iron containing dioxygenases that are widely distributed in animals and plants. In lipoxygenase, the iron present in the divalent state is oxidized to the catalytically active trivalent state by the reaction product, 15-hydroperoxy-eicosatetraenoic acid (15-HPETE) and leukotrienes from arachidonic acid as a substrate and 13-hydroperoxy-octadecadienoic acid (13-HPODE) from linoleic acid as a substrate. Leukotrienes are important biologically active intercessors in a variety of inflammatory events. It has been found that all these LOX products have an important role in many disorders such as bronchial asthma, inflammation *etc*. [10,11]. Therefore, lipoxygenases are credible target for the coherent drug design and discovery of

mechanism–based inhibitors for the treatment of bronchial asthma, inflammation, cancer and autoimmune diseases.

In continuation of our previous efforts to investigate the bioactive natural molecules from *Caryopteris odorata* (Ham. Ex Roxb.) [12-15], hereby, we report the butyryl cholinesterase and lipoxygenase inhibition study of the isolated fuorocoumarins, followed by their molecular docking (*in silico*) study.

EXPERIMENTAL

For column chromatography (CC), silica gel (70-230 mesh) and for flash chromatography (FC), silica gel (230-400 mesh) was used. TLC was performed on pre-coated silica gel G-25-UV_{254} plates. Detection was carried out at 254 nm and by ceric sulphate reagent. Purity was checked on TLC with different solvent systems using methanol, acetone and $CHCl_3$ giving single spot. The IR spectra were recorded Jasco-320-A spectrophotometer. ^1H NMR and ^{13}C NMR Spectra were run on Bruker spectrometers operating at 500 and 125 MHz, respectively. The chemical shifts are given in d in ppm and coupling constants in Hz. For further assistance, the literature survey has been referenced for the spectral data of each fuorocoumarin.

The plant *Caryopteris odorata* (Ham. ex Roxb.) was collected from hills of Pakistan in July 2012 and identified by Mr. Muhammad Ajaib (Taxonomist), Department of Botany, GC University, Lahore. A voucher specimen No. (G.C. Herb. Bot. 862) has been deposited in the herbarium of the same university.

Extraction, fractionation and isolation: The shade-dried ground whole plant (6.8 kg) was exhaustively extracted with methanol (12 L × 4) at room temperature. The extract was evaporated to yield the residue (633 g), which was dissolved in distilled water (1.5 L) and partitioned with *n*-hexane (1 L × 4), chloroform (1 L × 4), ethyl acetate (1 L × 4) and *n*-butanol (3 L × 4), respectively. These fractions were concentrated separately on rotary evaporator. The chloroform soluble fraction (274 g) was subjected to the CC over the silica gel using solvent system of *n*-hexane with the gradient of $CHCl_3$ (0 to 100 %) to obtain fifteen fractions (Fr. 1-15). Fraction 12 (1.3 g) was subjected to CC and eluted with *n*-hexane:chloroform (78:22) to isolate furocoumarin **1** (8.5 mg). The furocoumarin **2** (9.4 mg) was obtained from fraction 13 (6.8 mg) eluted with *n*-hexane: chloroform (74:26). Fraction 14 (1.1 g) was loaded on silica gel and eluted with *n*-hexane:chloroform (70:30) to purify compound **3** (2.9 mg). The fraction 15 (1.9 g) was subjected to flash chromatography slowly and continuously with *n*-hexane:chloroform (65:35) to afford molecule **4** (12 mg) from the head and furocoumarin **5** (8.3 mg) from the tail elution of the column.

Spectral characterization: The five isolated compounds were identified and characterized by NMR spectroscopy as psoralen [16] (**1**), methoxsalen [17] (**2**), oxypeucedanin [18] (**3**), isoimperatorin [19] (**4**) and bergamottin (5-geranyloxy psoralen) [20] (**5**). Their spectral data has been found in accordance with the reported one. These furocoumarin molecules were then utilized for the enzyme inhibition studies.

Butyrylcholinesterase assay: The BChE inhibition study was performed according to the established method [21]. The percent inhibition was calculated by the following equation:

$$\text{Inhibition (\%)} = \frac{\text{Control} - \text{Test}}{\text{Control}} \times 100$$

IC_{50} values (concentration at which there is 50 % in enzyme catalyzed reaction) compounds were calculated using EZ-Fit Enzyme Kinetics Software (Perrella Scientific Inc. Amherst, USA).

Lipoxygenase assay: Lipoxygenase activity was assayed according to the cited method [22] with minor modifications. 200 µL lipoxygenase assay mixture containing 150 µL sodium phosphate buffer (100 mM, pH 8.0), 10 µL test compound and 15 µL purified lipoxygenase enzyme was prepared. The contents were mixed, pre-read at 234 nm and pre-incubated for 10 min at 25 °C. The reaction was initiated by addition of 25 µL substrate solution. The change in absorbance noticed after 6 min at 234 nm was used as index for the inhibition. All reactions were performed in triplicates. The positive and negative controls were incorporated in the assay. Quercetin (0.5 mM $well^{-1}$) was used as a positive control. The percentage inhibition (%) was calculated by the same procedure as for butyrylcholinesterase assay.

Statistical analysis: All the measurements were done in triplicate and statistical analysis was performed by Microsoft Excel 2010. Results are presented as mean ± sem.

***in silico* Study (molecular docking):** To find out the bioactive conformations, the isolated furocoumarins were docked into the active pockets of butyrylcholinesterase and lipoxygenase separately by using default parameters of MOE-Dock program. Chem Draw ultra 12.0 software was used to draw the structures of these five compounds, saved in mol file format and brought to MOE 2009-10. Energy minimization was preceded up to 0.05 gradients by using MMFF94x force field through default parameter of MOE energy minimization algorithm. Database was created in which all the compounds were saved in their 3D structures in the mdb file format. Protein molecules of butyrylcholinesterase (PDB code: 1POP) and Lipoxygenase (PDB code: 1IK3) were retrieved from Protein Data Bank. All the water molecules were released from receptor protein and 3D protonation was carried out by using Protonate 3D Option. Energies of protein molecules were minimized by the default parameters of MOE 2009-10 energy minimization algorithm (gradient: 0.05, Force Field: MMFF94X). At last, all these compounds were docked into the binding pocket of enzyme. Re-docking procedure was also applied to confirm the validity of docking protocol [23]. After docking with 30 conformations of each compound, the best 2D images were selected for their specific types of interactions and drawn their 3D images along with their bond lengths.

RESULTS AND DISCUSSION

Five known furocoumarins, **1-5**, have been isolated from the chloroform soluble fraction of *Caryopteris odorata* (Ham. ex Roxb.) and their structures (Fig. 1) were identified on the basis of spectroscopic studies and by comparison with the published data of the closely resembling compounds. These furocoumarins were identified as psoralen [16] (**1**), methoxsalen [17] (**2**), oxypeucedanin [18] (**3**), isoimperatorin [19] (**4**) and bergamottin [20] (**5**).

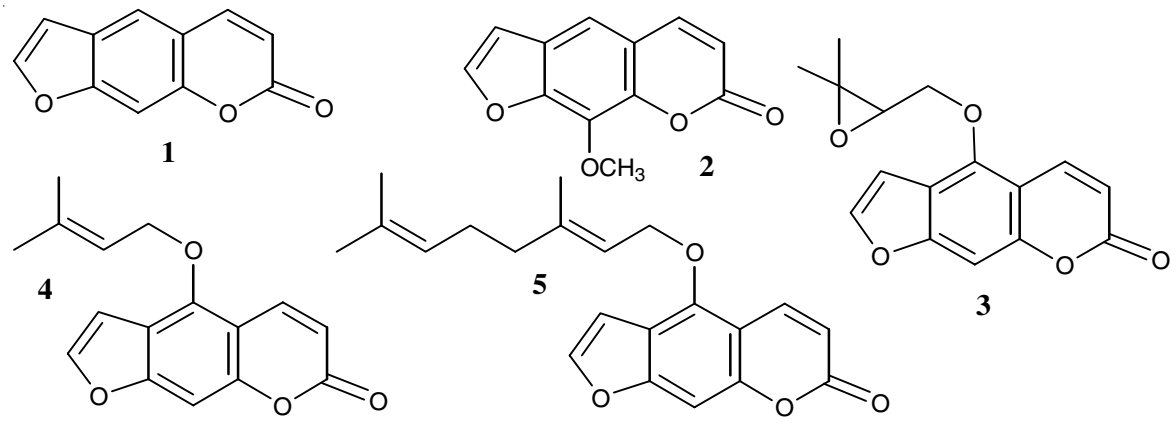

Fig. 1. Structures of fuorocoumarins (**1-5**)

Enzyme inhibition activity (*in vitro*): The isolated fuorocoumarins (**1-5**) were screened against butyryl cholinesterase (BChE) and lipoxygenase (LOX) enzymes to assess their enzyme inhibitory potential and the results are tabulated as percentage inhibition and IC_{50} values in Table-1. All the results were calculated as mean of three independent experiments and are presented as mean ± sem. It was revealed from results that these furocoumarins exhibited moderate inhibitory potential against both BChE and LOX enzymes.

Butyryl cholinesterase enzyme was inhibited by all the five furocoumarins taken into account and the most active one was methoxsalen (**2**) with IC_{50} value of 203.72 ± 0.27 μM relative to eserine, a reference standard, with IC_{50} value of 0.85 ± 0.0001 μM. The inhibitory potential of this molecule was probably because of three aromatic rings fused together having a methoxy group in middle ring and this may increase the π-π interactions of the molecule with the active site of the enzyme. The activity order of all the five fuorocoumarins against this enzyme was found to be as **2 > 5 > 3 > 4 > 1**.

TABLE-1
ENZYME INHIBITORY ACTIVITIES OF THE ISOLATED FUOROOUMARINS (**1-5**)

Compd.	Butyryl cholinesterase		Lipoxygenase	
	Inhibition (%)	IC_{50} (μM)	Inhibition (%)	IC_{50} (μM)
1	67.54±0.87	312.53±0.28	92.78±1.26	189.67±1.14
2	73.11±0.63	203.72±0.27	87.28±1.14	197.45±1.22
3	75.93±0.87	250.11±0,36	63.72±1.31	289.78±1.02
4	85.25±0.67	263.52±0.12	56.44±1.15	334.56±1.31
5	81.44±1.11	234.42±0.58	43.50±1.18	–
Control	82.82±1.09[a]	0.85±0.0001[a]	93.79±1.27[b]	22.4±1.3[b]

NOTE: All compounds were dissolved in methanol and experiments were performed in triplicate (mean ± sem, n = 3). a = Eserine, b = Baicalein.

Against lipoxygenase (LOX) enzyme, four fuorocoumarins, exhibited moderate inhibitory potential, while the most active one was found to be psoralen (**1**) having IC_{50} value of 189.67 ± 1.14 μM, relative to baicalein, a reference standard having an IC_{50} value of 22.4 ±1.3 μM. The inhibitory potential of this molecule might probably due to the three aromatic rings fused together and this might increase the π-π interactions of the molecule with the active site of the enzyme. The activity order of the four fuorocoumarins against this enzyme was experiential as **1 > 2 > 3 > 4**.

***in silico* study:** The molecular docking of these five ligands, **1-5**, was firstly performed into the active pocket of butyrylcholinesterase enzyme. It was inferred from the results that psoralen (**1**) was the molecule which has given strong hydrogen bond donor interaction with Val288 showing a bond distance of 1.95 Å Fig. 2 (2D & 3D). From the Fig. 3 (2D & 3D), it is clear that methoxsalen (**2**) has made three interactions.

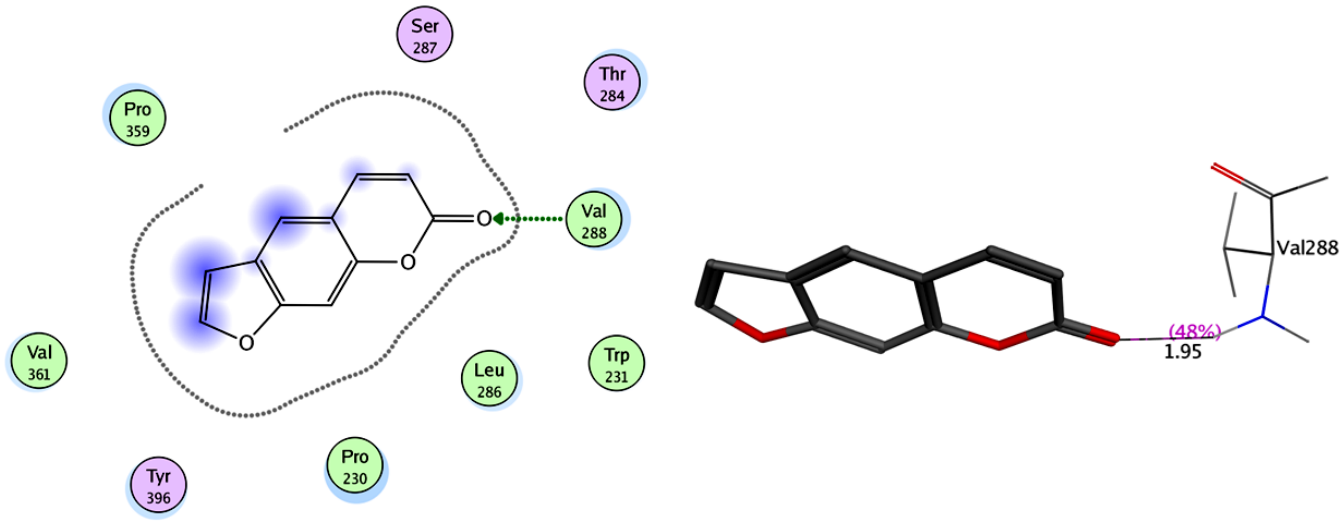

Fig. 2. 2D and 3D interaction analysis of psoralen (**1**) against BChE

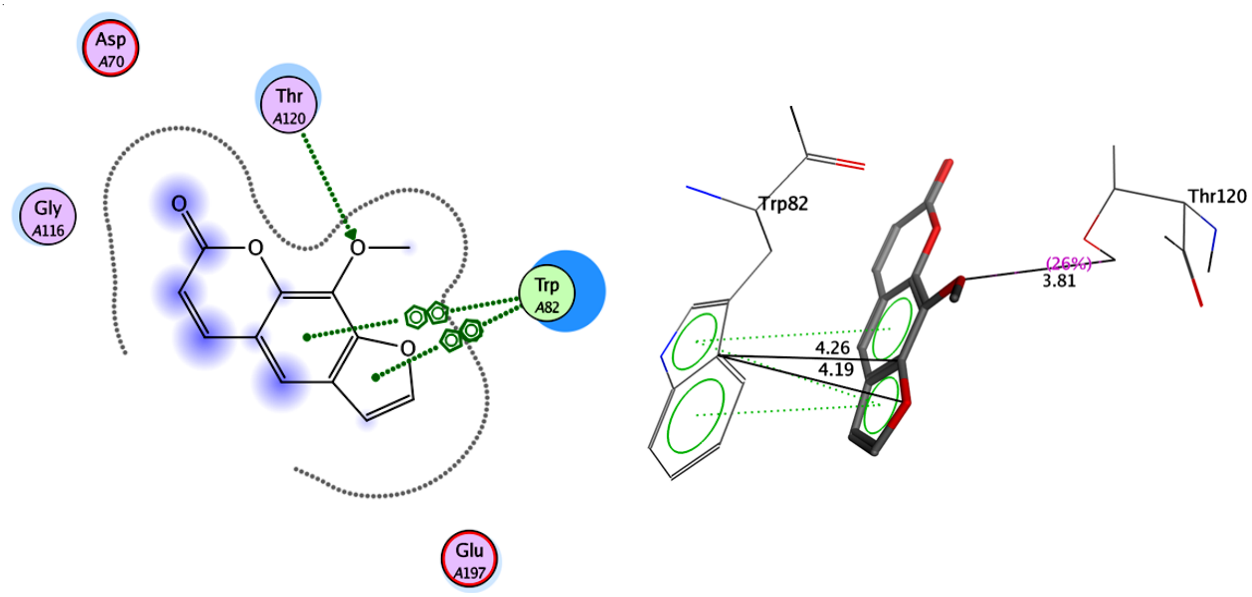

Fig. 3. 2D and 3D interaction analysis of methoxsalen (**2**) against BChE

First hydrogen bond donor interaction through its methoxy oxygen with Thr120 of the enzyme. The length of the distance identified was 3.81 Å. Likewise, Trp82 has given two π-π interactions with the rings of the ligand giving bond lengths of 4.19 Å and 4.26 Å. Similarly other compounds **3-5** were also docked and the results were correlated with the experimental data.

Again, the same fuorocoumarins (**1-5**) were docked into the binding pocket of the LOX enzyme. In this case, psoralen (**1**) established one strongest metallic contact with the iron of enzyme, second side chain donor interaction with His518 and third arene-arene interaction through its furan ring with Trp519. The bond distances among these interactions were found as 2.46, 3.17 and 3.56 Å respectively. On the opposite side, iron is very closely attached with Ile857 and Asn713 at distance of 1.81 and 2.07 Å respectively as illustrated in Fig. 4 (2D & 3D). Methoxsalen (**2**) has made four contacts. First strongest metallic contact with the iron of enzyme through its methoxyl oxygen with a bond distance of 2.67 angstrom. Second side chain donor interaction with His518 giving a bond length of 3.18 angstrom. The remaining two π-π interactions were found between His523 and furan ring of the ligand giving bond lengths of 3.50 and 3.92 angstroms respectively, as shown in Fig. 5 (2D and 3D). In a similar way, the other furocoumarins (**3-5**) were also docked and the results were in agreement with the experimental enzyme inhibitory data.

Conclusion

It was concluded that all the isolated furocoumarins (**1-5**) have moderate inhibitory potential against BChE while four moleucles (**1-4**), also possess moderate potential against LOX enzymes and these results were fully supported by the molecular docking study. So, the studied molecules might be utilized as possible drug candidates for the treatment of those

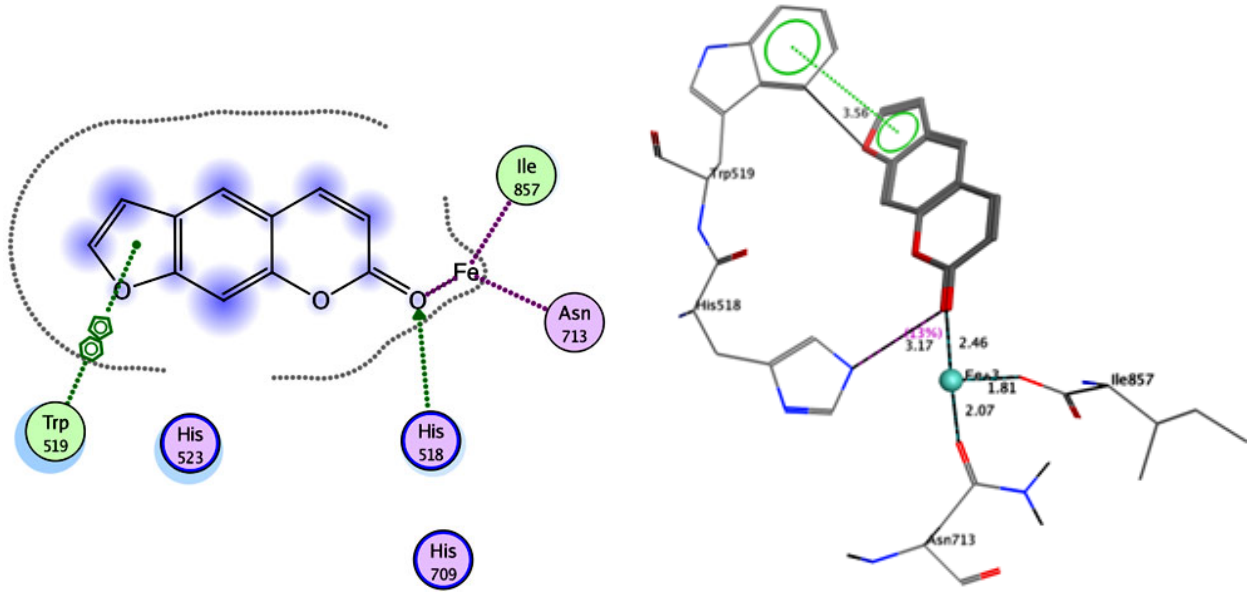

Fig. 4. 2D and 3D interaction analysis of psoralen (**1**) against LOX

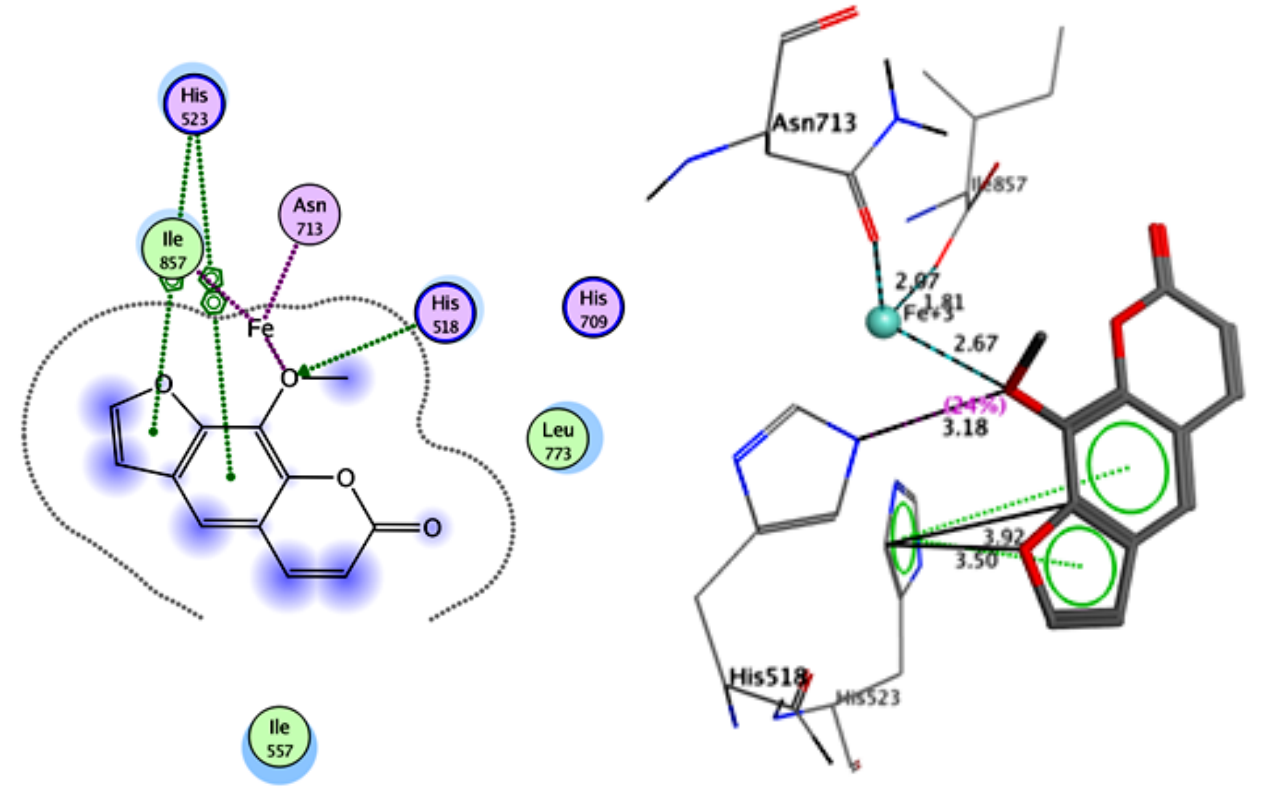

Fig. 5. 2D and 3D interaction analysis of methoxsalen (**2**) against LOX

diseases which are associated with the over expression of these enzymes.

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