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LIGAND-BASED VIRTUAL SCREENING AND ADME-TOX GUIDED APPROACH TO IDENTIFY NATURAL COMPOUNDS FROM *CONYZA SUMATRENSIS* (*RETZ.*) *E.H.WALKER* AS COX-2 INHIBITORS

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ABSTRACT

The present study was intended to evaluate the anti-inflammatory activity of fractions and compounds from *Conyza sumatrensis* (Retz) E.K. Walker (Cs). Extracts and three compounds **1**, **2** and **3** from Cs were tested for their anti-inflammatory activity through membrane red blood cell stabilisation test. *In silico* studies of compounds **1**, **2** and **3** towards Cyclooxygenase-2 (COX-2) enzyme was performed through Autodock Vina. Cs exhibited significant anti-inflammatory activity. Among the extracts tested, ethylacetate extract was the most potent while compound **1** exhibited the highest activity among the compounds assessed. *In silico* studies showed that compound **3** displayed the highest binding affinity (-7.7 kcal/mol) with the target COX-2, followed by compounds **1** (-6.5 kcal/mol) and **2** (-6.1 kcal/mol). Compounds **1**, **2** and **3** were also predicted free of toxicity. These results suggested that Cs might yield valuable adjunctive therapy for the treatment of inflammatory disorders.

Key words: *Conyza sumatrensis*; Inflammation; *In-silico*; Cycloxygenase enzyme; Toxicity.

INTRODUCTION

Non steroidal anti-inflammatory drugs (NSAIDs) belong to one of the most common therapeutic agents used worldwide for the treatment of pain, fever and inflammation. NSAIDs exert their therapeutic effect by inhibiting the activity of the enzyme cycloxygenase (COX) resulting in prevention of prostaglandin synthesis. There are three forms of COX enxymes including COX-1, COX-2 and COX-3. COX-1 is constitutive and provides cytoprotection in the gastrointestinal tract. COX-2 is inducible and mediates inflammation, whereas COX-3 is a variant of COX-1 and non functional in humans[1,2)]. However, the long-term administration of NSAIDs induces gastro-intestinal ulcers, bleeding, and renal disorders due to their non-selective inhibition of both constitutive (COX-1) and inducible (COX-2) isoforms of the cyclooxygenases enzymes[3)]. On the other hand, fully selective COX-2 inhibitors with reduced gastro-intestinal toxicity have been associated with adverse cardiovascular effects[4)]. Due to the deleterious side effects attributed to the prolonged use of NSAIDs and their ineffectiveness

in some cases, the control of inflammatory pain is still a major challenge.

Conyza sumatrensis (Retz.) E.H.Walker (*Cs*) also referred to *Erigeron floribundus* is a plant of Asteraceae family, which is used for the traditional treatment of inflammatory disorders including headache, dental pain and rheumatoid arthritis[5,6]. De Las Heras et al.[7] reported the anti-inflammatory activity of the ethanol extract from Cs in mice. Furthermore, Asongalem et al.[8] reported the anti-inflammatory activity of the aqueous extract of this plant in mice and rat models.

However, the anti-inflammatory activity of fractions and compounds from Cs is still unexplored. Hence, the main objective of the present study was to evaluate the anti-inflammatory activity of fractions and compounds from Cs.

In detailed fashion, this study was an attempt to overcome the following specific objectives:

-Evaluation of the *in vitro* anti-inflammatory potential of fractions and compounds from Cs through membrane red blood cell stabilisation test.

-*In silico* prediction of the binding affinity and the toxicity profile of compounds **1**, **2** and **3** with respect to the target enzyme COX-2.

MATERIAL AND METHODS

Collection and authentication of *Conyza sumatrensis*: The fresh leaves of Cs were collected in the month of February 2011 from the adjoining area of Dschang, located at latitude 5.4500° N and longitude 10.0667° E in the West region of Cameroon. The specimen was identified by Dr. S.C. Singh, Taxonomist, Central Institute of Medicinal and Aromatic Plants, Lucknow, India where a herbarium specimen with voucher number 13780 has been deposited.

Extraction of *Conyza* sumatrensis: The shade dried leaves (250g) were coarsely powdered and extracted thrice with methanol (3x 1.5L). The combined MeOH extract was concentrated under vacuum at 40°C, which afforded 30g of MeOH extract.

Fractionation of the methanol extract: Crude methanol extract from *Conyza sumatrensis* was suspended in water and then fractionated with organic solvents in order of increasing polarity to afford *n*-hexane, chloroform, ethylacetate, *n*-butanol and water soluble fractions[9].

Compounds considered for the study: The compounds **1**, **2** and **3** considered for the study were isolated from Cs in our earlier investigation[10].

***In vitro* anti-inflammatory assay:** Membrane stability of red blood cells incubated with different plant extracts, fractions at (500 μg/ml, 250 μg/ml and 125 μg/ml) and pure molecules at 50μg/ml doses was tested according to an *in vitro* model previously described by Trnavsky (1974) in Prasanna et al.[11] with minor modifications. Briefly, fresh blood (6 ml) was collected from median cubital vein of healthy volunteers in a tube containing 6 ml of sterile Alsevier solution (2% Dextrose, 0.8% Sodium Citrate, 0.05% Citric acid, and 0.42% Sodium chloride). Separation of red blood cells was performed by centrifuging the blood samples at 2000 rpm for 15 min with three washes with isosaline solution. Finally, a 10% v/v suspension of RBC working solution was prepared by adding 54 ml of isosaline solution to the pellet remaining in the tube. A reaction mixture (4.5 ml) consisting of 2 ml hypotonic saline (0.36% w: v NaCl), 1 ml 0.15 M phosphate buffer saline (pH 7.4), and varying concentration of the plant extracts/fractions (500, 250 and 125 μg/ml) and pure molecules thereof (50 μg/ml) in DMSO was added with 0.5 ml of 10% HRBC in isosaline. Two controls were performed: one with 1.0 ml of hyposaline instead of drug ($control_1$) and another with 1 ml of extracts/fractions/pure molecules solution without red blood cells ($control_2$). The mixture was incubated at 37°C for 30 min. The mixture was centrifuged, and the absorbance of the supernatants was read at 560 nm. Diclofenac sodium (Sigma Aldrich, USA) was used at 50μg/ml as the reference drug.

Percentage inhibition of the inflammation was expressed as:

100-[(Absorbance of test sample-$control_2$)/$control_1$] x 100

Molecular docking studies: Drawing and geometry cleaning of compounds **1**, **2** and **3** was performed through ChemBioDraw-Ultra-v12.0 (http://www.cam-bridgesoft.com/). The 2D structures were transformed into 3D structures by using converter module of ChemBioDraw. The 3D structures were subjected to energy minimization in two steps. In the first step, the energy was minimized by using molecular mechanics-2

(MM2) until the root mean square (RMS) gradient value became smaller than 0.100 kcal/mol.Å^2. In second step, minimized MM2 (dynamics) compounds were subjected to re-optimization through MOPAC (Molecular Orbital Package) method until the RMS gradient attained a value smaller than 0.0001 kcal/mol Å. The 3D crystallographic structures of the anti-inflammatory target COX-2 were retrieved through Brookhaven Protein Data Bank (http://www.pdb.org). The complex structure, PDB ID: (PDB ID: 1PXX) was taken for this study because of its binding affinity to diclofenac, the standard anti-inflammatory agent. The valence and hydrogen bonding of the ligands as well as target protein were subsequently satisfied. An extended PDB format, termed as PDBQT file was used for the coordinate files including the atomic partial charges. The software converted automatically the PDB file into PDBQT which was further used for the docking[12]. Polar hydrogen atoms were added to the protein target to achieve the correct ionisation and tautomeric states of amino acid residues such as His, Asp, Ser and Glu. The docking of ligands with receptor COX-2 was performed in triplicate and the average of consecutive similar results was taken as the final binding energy. The binding pose with the lowest docked energy was considered as the top-ranked cluster and was subsequently selected as the final model for post-docking analysis with PyMol Chimera softwares. Lipinski's 'Rule of Five' (Lipinski et al. [13]) was used to evaluate the oral bioavailability and drug likeness of the inhibitors. The prediction of the toxicity risk parameters such as mutagenicity, tumorogenicity, irritation and reproductive toxicity risk of compounds **1**, **2** and **3** was performed through Osiris software. Diclofenac was taken as positive control against the receptor COX-2.

Statistical analysis: The data, expressed as Mean±SE, were subjected to Kruskal-Wallis one way analysis of variance (ANOVA) through Graphpad PRISM Software. Inter group comparisons were made by Duns-test (non parametric) for only those responses which yielded significant treatment effects in the ANOVA test. $p< 0.05$ was considered statistically significant.

RESULTS

***In vitro* anti-inflammatory activity of extract, fractions and pure compounds:** The percentage protection of the human red blood cell of methanol extract, fractions and compounds isolated from Cs are summarized in Table 1. Methanol extract was found significantly potent with 61.78% of anti-inflammatory potential at 250 µg/ml. Chloroform, ethylacetate, *n*-butanol and water fractions at all the doses considered were more active than the reference drug, diclofenac sodium. Briefly, at 250 µg/ml, *n*-hexane, chloroform, ethyl acetate, *n*-butanol and water fractions exhibited 70.86%, 78.44%, 74.15%, 82.52% and 77.64% of anti-inflammatory potential respectively. However, such a difference in the activity may be due to the presence of mixture of compounds in the extracts and fractions when compared with a single compound in diclofenac sodium. In addition, compound **1** and **2** (Figure 1) were the most potent with 80% anti-inflammatory activity whereas compound **3** displayed 66.7% of anti-inflammatory activity (Table 1).

***In silico* studies**: Herein, we explored the orientation and binding affinity (in terms of total docking energy in kcal/mol) of compounds **1**, **2** and **3**. Before performing the docking, the standardisation of the software was done through blind docking of control diclofenac with its receptor COX-2. The redocking study showed that diclofenac bound to the same active site of the protein as described in the PDB database. The main interacting residues that facilitated the binding were TYR-385 and SER-530. The docking study of compounds **1**, **2** and **3** with the target revealed that all the ligands shared the same binding site. The fourteen residues located within the range of 4Å of diclofenac binding site were TYR-348, VAL-349, LEU-352, SER-353, TYR-355, LEU-384, TYR-385, TRP-387, PHE-518, MET-522, VAL-523, GLY-526, ALA-527, SER-530. These fourteen residues were found common in other interactions. Compound **3** shared all binding pocket residues with the target as noticed with diclofenac. The nature of the conserved pocket residues were aliphatic (VAL-349, LEU-352, LEU-384, VAL-523, ALA-527, GLY-526), aromatic (TYR-348, TYR-355, TYR-385, TRP-387, PHE-518), hydroxyl or sulphur-containing (SER-553, MET-522, SER-530) respectively. Compound **3** exhibited the highest binding affinity (-7.7kcal/mol), making 5 H-bonds followed by compounds **1** (-6.5 kcal/mol) and **2** (-6.1 kcal/mol) and making 3 and 2 H-bonds respectively with the receptor (Table 2, Figure 2).

Screening of active anti-inflammatory leads namely compounds 1, 2 and 3 for pharmacokinetic properties: Pharmacokinetic studies of the anti-inflammatory leads showed that compound **1** was violating 4 of the Lipinski's rules whilst compound **2** was violating 5. However, compound **3** was exempted of all the rules. Passively absorbed molecules with polar surface area (PSA)>140 $Å^2$ are considered to be less absorbed (bioavailability) when taken orally. Compounds **1** and **2** exhibited high total polar surface area (TPSA) (186.13 $Å^2$ and 168.93 $Å^2$ respectively) suggesting that they are poor in oral bioavailability while compound **3** (147.67 $Å^2$) in the normal TPSA range would be the most bioavailable compound via oral route (Table 3). Compounds **1** and **2** displayed high molecular weights, signifying that these compounds would be less excreted. Since the permeability of drugs drop at both low and high LogP (partition-coefficient), compound **3** would have less permeability across the cell membrane because of their low LogP value (Table 3). Meanwhile compounds **1** and **2** were exempted of this property. Higher lipophilicity of compounds lead to increased metabolism and poor absorption, along with an increased probability of binding to undesired hydrophobic macromolecules, thereby increasing the potential for toxicity.

Assessment of the toxicity parameters of compounds 1, 2 and 3: Compounds **1**, **2** and **3** displayed no risk for mutagenicity, tumorogenicity, irritation and reproductive and/or developmental toxicity due to high doses or long term use. However, diclofenac showed high risk for irritation (Table 4). These results suggested that compounds **1**, **2** and **3** might yield safe agents for the treatment of inflammatory disorders.

DISCUSSION

The findings of the present study demonstrated that extracts and compounds from Cs are capable of stabilising red-blood cell membrane against hypotonic stress, indicating its ability to prevent rupture or haemolysis of RBCs. Since there is a close similarity of the red-blood cell membrane (RBC) to the lysosomal membrane, protection against hypotonicity or heat-induced lysis of RBC is often extrapolated to stabilisation of lysosomal membranes and used as a biochemical index of anti-inflammatory activity[14, 15]. Moreover, membrane stabilisation leads to the prevention of leakage of serum protein and fluids into the tissues during a period of increased permeability caused by inflammatory mediators[16]. Cs extracts and compounds perhaps stabilized the red blood cell membrane by preventing the release of lytic enzymes and active mediators of inflammation. Previous studies have shown that, flavonoids, phenolic compounds and most other secondary plant metabolites can exhibit anti-inflammatory effects as a result of their membrane stabilising actions[17,18]. Since the plants under study were found to contain phenolic compound such as compound **3**, it is not unreasonable to speculate that this may be one of possible chemical components responsible for the observed membrane stabilising action. *In silico* prediction of compounds **1** and **2** could be correlated with the observed *in vitro* anti-inflammatory potential while the prediction of compound **3** was inversely correlated with its *in vitro* activity. This observation might be due to the high lipophilicity and therefore the low availability and binding affinity of compounds **1** and **2** on the corresponding targets though they displayed the highest *in vitro* anti-inflammatory potential. None of the Lipinski rule was violated by compound **3,** suggesting that this compound might be the best with regards to its pharmacokinetic properties in the treatment of inflammatory conditions. All the screened compounds were found to possess no risk of toxicity.

CONCLUSION

In conclusion, Cs methanol extract, fractions and pure compounds exhibited significant anti-inflammatory activity. In addition, the molecular docking study predicted compounds **1**, **2** and **3** as potential ligands for the target enzyme cyclooxygenase (COX) which could be the mechanism by which the tested compounds might overcome the inflammation condition. Quantitative structure activity relationship in order to determine the chemical groups responsible for the activity of the compounds is envisaged.

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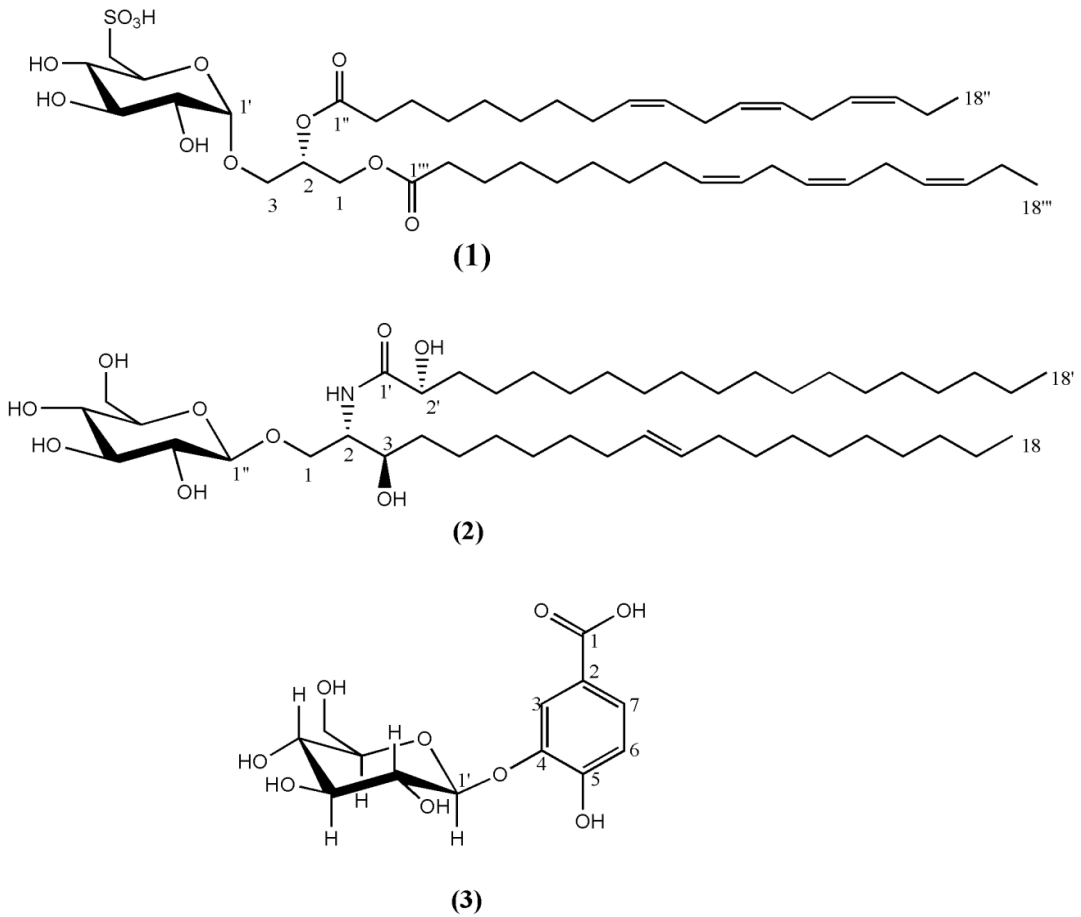

Figure 1: Chemical structures of compounds (**1)** [(2S)-1,2-di-O-[(9Z,12Z,15Z)-octadeca-9,12,15-trienoyl]-3-O-(6-sulpho-alpha-D)-quinovopyranosyl glycerol]; **(2)** [1-O-beta-D-glucopyranosyl-(2S,3R,8E)-2-[(2'R)-2-hydroxy-palmitoylamino]-8-octadecene-1,3-diol] and (**3) [**3-O-beta-D-glucopyranosyl-3,4-dihydroxybenzoic acid] from *Conyza sumatrensis*.

Table 1: *In vitro* anti-inflammatory activity of *Conyza sumatrensis* through human red blood cell membrane stabilisation test.

Concentration (µg/ml)	**Activity (% protection)**									
	Methanol extract	Hexane fraction	Chloroform fraction	Ethyl acetate fraction	*n*-butanol fraction	Last water fraction	Com-pound **1**	Com-pound **2**	Com-pound **3**	Diclofenac
50	-	-	-	-	-	-	80.1± 0.004***	80.01± 0.0016***	66.71± 0.001**	70.06± 0.0001**
125	52.2± 0.02**	68.16± 0.008**	71.76± 0.004**	72.056± 0.012**	81.14± 0.0017***	76.55± 0.003***	-	-	-	-
250	61.78± 0.014**	70.86± 0.005**	78.44± 0.01***	74.15± 0.0026**	88.52± 0.0097***	77.64± 0.008***	-	-	-	-

*Note: Values are expressed as SEM, n= 3/concentration; NT, not tested due to meagre recovery. **p<0.01, ***p<0.001 control vs treatment.*

Table 2: Docking scores (kcal/mol) of positive control diclofenac and compounds from *Conyza sumatrensis* with respect to the anti-inflammatory target COX-2 (PDB 1PXX).

Compounds	Binding docking energy (kcal/mol)	Cyclooxygenase COX-2 (PDB: 1PXX) Binding pocket residues (4Å)	Number of H-bonds (Å)	Interacting residues
Diclofenac	-8.4	TYR-348, VAL-349, LEU-352, SER-353, TYR-355, LEU-384, TYR-385, TRP-387, PHE-518, MET-522, VAL-523, GLY-526, ALA-527, SER-530	2 (2.8 & 3.0)	TYR-385, SER-530
1	-6.5	VAL-89, LEU-93, ILE-112, VAL-116, ARG-120, GLN-192, PHE-205, PHE-209, PHE-210, HIS-226, GLY-227, VAL-228, VAL-344, TYR-348, VAL-349, LEU-352, SER-353, TYR-355, PHE-357, LEU-359, ASN-375, ARG-376, ILE-377, ALA-378, PHE-381, TYR-385, TRP-387, PHE-518, MET-522, VAL-523, GLY-526, ALA-527, PHE-529, SER-530, LEU-531, GLY-533, LEU-534	3 (3.0, 2.8, 2.6)	TYR-385 (3.0, 2.8) SER-530 (2.6)
2	-6.1	HIS-90, VAL-116, ARG-120, GLN-192, PHE-205, PHE-209, PHE-210, HIS-226, GLY-227, VAL-344, ILE-345, TYR-348, VAL-349, LEU-352, SER-353, TYR-355, LEU-359, ASN-375, ARG-376, ILE-377, ALA-378, PHE-381, LEU-384, TYR-385, TRP-387, ARG-513, ALA-516, ILE-517, PHE-518, MET-522, VAL-523, GLY-526, ALA-527, PHE-529, SER-530, LEU-531, LYS-532, GLY-533, LEU-534	2 (3.0, 3.1)	TYR-385, ALA-527
3	-7.7	VAL-116, ILE-345, VAL-349, LEU-352, SER-353, TYR-355, PHE-381, TYR-385, TRP-387, PHE-518, MET-522, VAL-523, GLY-526, ALA-527, PHE-529, SER-530, LEU-531, LEU-534	5 (3.2, 2.8, 3.0, 3.0, 3.0)	ARG-120 (3.2, 2.8), TYR-355 (3.0), TYR-385 (3.0), SER-530 (3.0)

PDB: Protein Data Bank.

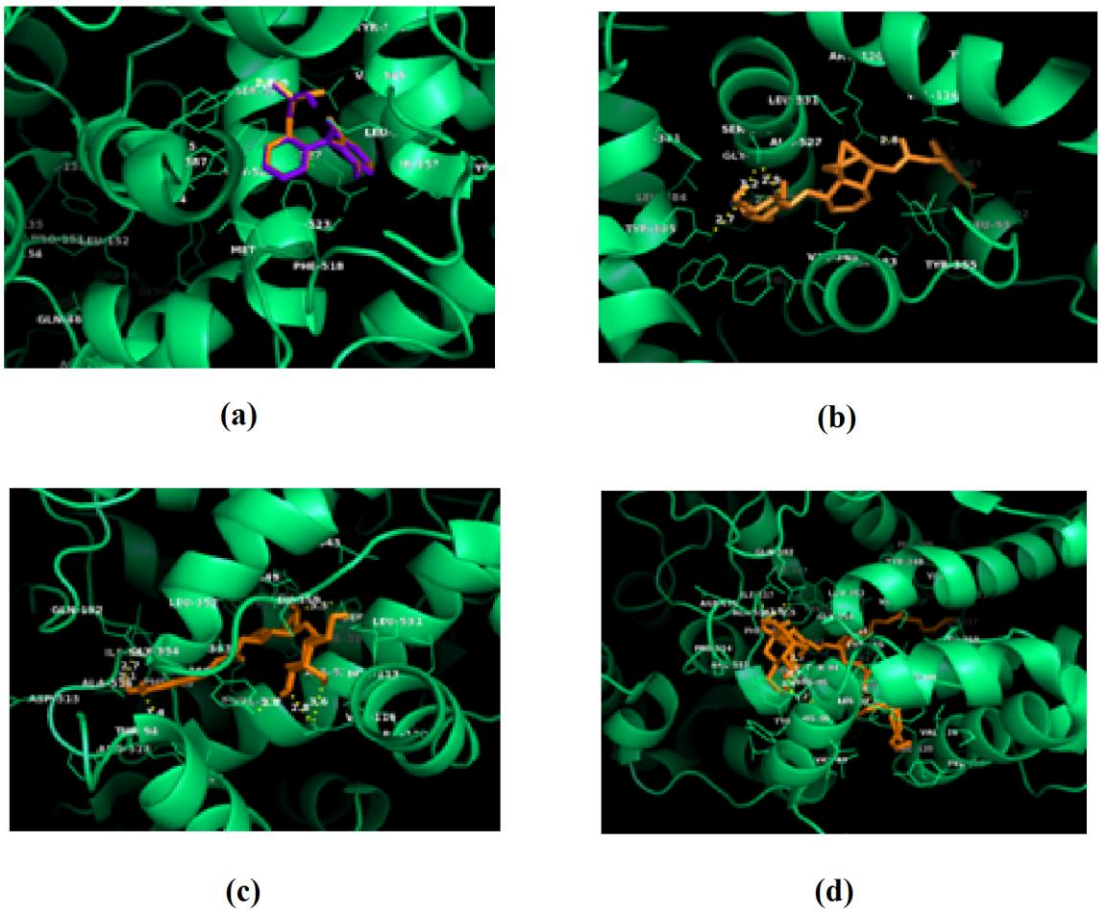

Figure 2: Diclofenac and compounds **1**, **2** & **3** docked into the receptor COX-2 (PDB: 1PXX).
(a) Diclofenac, **(b)** Compound **1**, **(c)** Compound **2** and **(d)** Compound **3** docked into the receptor COX-2 with binding energies of -8.4 kcal/mol, -6.9 kcal/mol, -7.7 kcal/mol and -6.5 kcal/mol respectively.

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