

## SYNTHESIS, ANTI-INFLAMMATORY, ANALGESIC, MOLECULAR MODELING AND ADMET STUDIES OF NOVEL DICLOFENAC DERIVATIVES CONTAINING LEUCINYL MOIETY

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### ABSTRACT

The present work aims to synthesize novel diclofenac derivatives containing L-leucine moiety. The synthesized compounds docked into the active site to discover validated inhibitors of cyclooxygenases (COX-1 and COX-2). The calculations in-silico were predicted that, the compound with lowest energy of docked poses was interacted with residues of active site, perhaps could be making them possible selective inhibitors against (COX-2) and physiologically active. The binding score of compound compared with reference drug, and show extensive interactions with the targets, which may consider it a suitable selective inhibitor against (COX-2).

**Keywords:** Leucine, Diclofenac, Cox, Docking, Admet.

### 1. INTRODUCTION

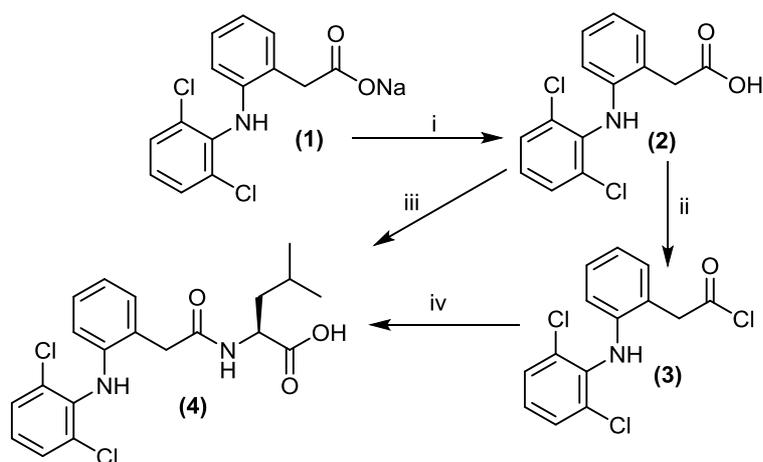
Non-steroidal anti-inflammatory drugs (NSAIDs) are widely employed in musculoskeletal disease, as well as their anti-inflammatory properties [1]. After widely evaluation, NSAIDs is efficacy in different clinical setting, and act as COX inhibitor (COX-1 and COX-2) through inhibiting the production of prostaglandins (PGs) [2, 3]. Diclofenac is a one from famous available members of these drug's class under current clinical usage [4], and suffer from a common toxicity of gastrointestinal drawback, due to inhibition non-selectively of cyclooxygenase enzyme [5, 6], also, its display anti-microbial [7, 8], ulcerogenic, analgesic, anti-inflammatory, lipid peroxidation [9, 10], antitumor [11] and inhibitor formation of transthyretin amyloid fibril properties [12]. Also, the amino acid derivatives especially containing amide and thioamide moieties possess diverse biological activities, such as anti-inflammatory, anti-tumor and antimicrobial activity [13, 14]. Furthermore, substituted 4-quinazolinone have antimicrobial [15], antifolates, antitumor [16], antimalarial [17], CNS depressant, anticonvulsants [18], anti-inflammatory [19] and analgesic activities [20]. Hence,

the present study aims to synthesis new series of leucinyldiclofenac derivatives acting as new potent anti-inflammatory agents without ulcerogenic effects, followed by molecular modeling to identify the structural features of these new series. The molecular docking was preformed, to predict the correct binding geometry for each ligand at the active site, which may be support that postulation, its active compounds may be act as a new NASIDs.

## 2. RESULTS AND DISCUSSION

### 2.1. Chemistry

The starting compound of 2-(2-((2,6-dichlorophenyl)amino)phenyl)acetyl chloride was carried out according steps depicted in (Scheme 1).



Scheme 1: reagent and conditions; i-  $\text{H}_2\text{O}/\text{dil-HCl}$ ., ii-  $\text{SOCl}_2$ , iii- L-Leu/fusion, iv- L-Leu/THF/TEA.

Compound **4** was prepared with two strategy to afford (L)-2-(2-(2-(2,6-dichlorophenylamino)phenyl)acetamido)propanoic acid (**4**). The first strategy: by coupling of **3** with L-leucine in THF/TEA/ $\text{H}_2\text{O}$  media to give **4**, another strategy; through fused **2** with amino acid to afford the desired compound **4**. The IR spectrum of compound **4** indicated that presence of a OH and NH function as broad band ( $3343\text{cm}^{-1}$ ), and it's the  $^1\text{H}$ NMR spectrum showed a singlet at ( $\delta\text{H}$  11.99 ppm) due to OH protons of carboxylic. In order to establish enantiomeric purity of isolated compound, the specific rotation values were determined, which remained unchanged after repeated crystallization for several times. Also, enantiomeric excess (ee) and diastereoisomeric excess (de) values were determined, these values based on the stereo configuration of amino acid of amide part of products **4**, which obtained through nucleophilic addition of free amino acids on diclofenac. Also, from TLC analysis, the optical purity of the resulting compounds was greater than 97%. Thus, as expected, stereo chemical configuration at  $\alpha$ -carbon atom of the acid was practically unaffected and this synthetic transformation from chiral  $\alpha$ -amino acid could be applied to a wide range of compounds without undergoing any significant loss of optical activity.

## 2.2. Biological Activity

### 2.2.1. Anti-Inflammatory Activity

The screening of the anti-inflammatory activity in vivo for the synthesized compounds were preformed, using the functional model of Carrageenan-induced rat paw edema assay [21], this test is a nonspecific inflammation but is highly sensitive to NSAIDs. Indomethacin and Diclofenac is one of the most potent NSAIDs, and was used as a reference drug, the percentage reduction of tested compounds for edema in comparison with the control was calculated (Table 1). The percentage edema reduction for the tested compounds compared with the control and reference drugs groups, showed significant value ranged (58.17%) for compounds 4, (Table 1). The tested compound showed, nearly reduction percentage of oedema compared with reference drugs (Table 1).

**Table-1.** Anti-inflammatory effect of synthesized compounds on carrageenan- induced paw edema in rats after 1, 2,3 and 4h of test drug administration.

Compounds	Volume of paw edema (in ml)*				%Inhibition**
	1 h	2 h	3 h	4 h	
Control	71.69±9.941	105.2±10.98	119.3±12.04	125.1±11.12	—
Indomethacin	12.76±2.795a	16.73±2.886 a	28.28±3.789 a	40.67±4.801 a	67.49
Diclofenac	12.55±1.35 a	19.16±1.23 a	27.77±2.123 a	43.56±5.306 a	65.17
4	18.36±1.21	25.27±2.156 a	45.75±6.04 a	52.32±3.35 a	58.17

\* Values are expressed as mean ± S.E.M (n = 6) and analyzed by ANOVA. Dose= 20 mg/kg.

\*\* Percentage Inhibition of paw edema from control group

a) Statistically significant at the corresponding time (p < 0.05).

### 2.2.2. Analgesic Activity

The analgesic activity of tested compounds was evaluated by using tail-flick test in mice [22], with the same dose as used for anti-inflammatory activity, the percent protection of the drugs was calculated in Table 2. The compound under investigation, showed analgesic activity in 19.54%, and showed analgesic potency nearly the analgesic effect of diclofenacsodium (Table 2).

### 2.2.3. Acute Ulcerogenesis

The compounds were screened for gastric irritation activity. The ulcerogenic effect of Indomethacin and newly synthesized compounds were studied at 20 mg /kg in rats. The data in table 2, the tested compound exhibited significant reduction in ulcerogenic activity compared with the indomethacin (standard drug), which showed high severity index of  $4.50 \pm 0.316$ , this data confirmed that, these compounds showed negligible ulcerogenic effect, and may be considered as safer drugs for treating inflammation conditions.

**Table-2.** Analgesic activity using tail-flick test and Ulcergastric of rats for tested compounds.

Compounds	Analgesic activity*				%protection**	Ulcerogenic index	
	Base line	30 Min	60 Min	90 Min		Ulcer severity	Ulcer Number
Control	3.640±0.445	3.750±0.256	4.320±0.489	5.340±0.356	—	0.0	0.0
Indomethacin	4.700±0.452	7.960±0.823	10.28±0.873a	13.68±0.471a	60.96	22.4	11.8 ±
Diclofenac	5.000±0.512	6.240±0.623	7.580±0.725	10.26±1.121	47.95	±1.652	0.985
<b>4</b>	60.93±1.38@	85.94±8.324@	93.50±10.320@	100.65±11.32@	19.54	0.0	0.0

\* Values are expressed as mean ± S.E.M (n = 6) and analyzed by ANOVA. Dose= 20 mg/kg.

\*\* Percentage protection *tail/flick* tail from control group

a) Statistically significant at the corresponding time (p < 0.05).

#### 2.2.4. Determination of Acute Toxicity (LD50)

The LD50 for diclofenac sodium and isolated new compound **4** was screened, all rats were alive during the 24 h of observation when treated with up to 500 mg/kg for different compounds, and did not show a visible signs of acute toxicity, furthermore, Therapeutic index was calculated: as following (LD50 \ ED50). The LD50 of isolated new compound **4** was 820 mg/kg with therapeutic index 41.5, LD50 compared with value 530 mg/kg for diclofenac sodium was (therapeutic index= 25). So, the new isolated compound **4** have higher therapeutic index compared with diclofenac sodium, and may be appear to be relatively less toxic as anti-COX agents.

### 2.3. Molecular Modeling Studies

#### 2.3.1. Conformational Analysis

It is obvious that, a possibility existence of the synthesized amino acid derivatives **4** in L and D stereoisomer forms. In trying to achieve better insight into the molecular structure of the most preferentially stereoisomer forms for its compounds, conformational analysis of the target compounds has been performed using the MMFF94 force-field [23] (calculations in vacuo, bond dipole option for electrostatics, PolakeRibiere algorithm, RMS gradient of 0.01 kcal/A mol) implemented in MOE [24]. The most stable conformer for **4** were fully geometrical optimized with molecular orbital function PM3 semi-empirical *Hamiltonian* molecular orbital calculation MOPAC 7 package [25]. The computed molecular parameters, total energy, electronic energy, heat of formation, the highest occupied molecular orbital (HOMO) energies, the lowest unoccupied molecular orbital (LUMO) energies and the dipole moment for studied compounds were calculated (Table 3).

The calculated molecular parameters have been used to investigate the most stable stereoisomer form (table 3), and showed the most stable stereoisomer is the (L) form, this may be explained by slightly reduces its calculated energy, and leads to predominance this structures (L) form over (D) forms. The lowest minimization energy for the for new isolated structures exhibited (Fig .1), The higher HOMO energy values show the molecule is a good electron donor, in other hand, the lower HOMO energy values indicate that, a weaker ability of the

molecules for donating electron. LUMO energy presents the ability of a molecule for receiving electron [26], (Table 3).

**Table-3.** The Optimized Calculations Energies at PM3 molecular orbital for 1-4.

Cpd	L						
	E	E <sub>ele</sub>	HF	IP	HOMO	LUMO	$\mu$
<b>1</b>	-67011.1	-415646	-70.83	6.79	-6.09	-0.09	7.96
<b>3</b>	-74214.3	-403091	-2.454	8.75	-8.15	-0.31	3.17
<b>4</b>	-115987.68	-534052	-70.0305	8.407	-8.407	-1.37	4.96
Cpd	D						
	E	E <sub>ele</sub>	HF	IP	HOMO	LUMO	$\mu$
<b>1</b>	-68011.1	-425646	-71.83	6.79	-6.79	-0.09	7.96
<b>3</b>	-76214.3	-473091	-2.454	8.75	-8.75	-0.41	3.17
<b>4</b>	-11387.68	-564052	-72.2053	8.47	-8.20	-0.37	4.99

E: Total energy (kcal/mol), E<sub>ele</sub>:Electronic energy (kcal/mol),HF: Heat of formation (kcal/mol),IP: Ionization potential energy(kcal/mol), HOMO: Highest Occupied Molecular Orbital(eV), LUMO: Lowest Occupied Molecular Orbital(eV),  $\mu$ : Dipole moment(Deby).

### 2.3.2. Admet Factors Profiling

Oral bioavailability considered playing an important role for the development of bioactive molecules as therapeutic agents. Many potential therapeutic agents fail to reach the clinic, because of their ADMET (absorption, distribution, metabolism, elimination and toxic) Factors. Therefore, a computational study for prediction of ADMET properties of the molecules was performed for tested compounds **1-4**, by the determination of topological polar surface area (TPSA), a calculated percent absorption (%ABS) which was estimated by Zhao et al. equation [27], and “rule of five” formulated by Lipinski [28], which established that, chemical compound could be an orally active drug in humans, if no more than one violation of the following rule: i) ClogP (partition coefficient between water and octanol) < 5, ii) number of hydrogen bond donors sites  $\leq 5$ , iii) number of hydrogen bond acceptors sites  $\leq 10$ , iv), molecular weight <500 and molar refractivity should be between 40-130. In addition, the total polar surface area (TPSA) is another key property linked to drug bioavailability, the passively absorbed molecules with (TPSA>140) have low oral bioavailability [29]. All calculated descriptors were performed using MOE Package [24], and their results were disclosed in (Table 4). Our results revealed that, the CLogP (factor of the lipophilicity [30] was less than 5.0 except **3** and **20-23**, the molecular weight (MW< 500), hydrogen bond acceptors between (2-4), hydrogen bond donors between (1-4) and molar refractivity values ranged (~73-78),this data show these compounds fulfill Lipinski’s rule. Also, the percent in ranged between (~ 66-99%).

The HOMO and LUMO of a molecule play important roles in intermolecular interactions [31], through the interaction between the HOMO of the drug with the LUMO of the receptor and vice versa. The interactions stabilized inversely with energy gap between the interacting orbitals. Increasing HOMO energy and decreasing LUMO energy in the drug molecule lead to enhancement stabilizing interactions, and hence, binding with the receptor.

**Table-4.** Pharmacokinetic parameters important for good oral bioavailability of compounds 1-4:

CPD	HBD	HBA	LogP	V	Vol.	TPSA	%ABS	Log S	mr	$\Delta E$	$\eta$	S	$\chi$	$\sigma$	$\omega$
1	1	2	4.202	0	150	29.1	99.305	4.914	73.18	6.70	3.352	0.29	3.44	3.44	1.76
3	1	2	5.131	1	132.25	29.1	99.305	5.661	75.46	8.33	4.16	0.23	4.58	4.58	2.51
4	4	4	4.85	0	122.55	30.2	66.75	-7.70	78.60	7.02	3.51	0.28	4.89	4.89	3.40

TPSA: Polar surface area ( $\text{\AA}^2$ ), %ABS: Absorption percentage, Vol: Volume ( $\text{\AA}^3$ ), HBA: Number of hydrogen bond acceptor, HBD: Number of hydrogen bond donor, V: Number of violation from Lipinski's rule of five., Log P: Calculated lipophilicity., Log S: Solubility parameter, mr: Molar Refractivity,  $\Delta E$ : Energy Gaps(eV),  $\eta$ : Hardness(eV), S: Softness(eV),  $\chi$ : Electronegativity (eV),  $\sigma$ : chemical potential(eV),  $\omega$ : Electrophilicity (eV).

Furthermore, the global and local chemical reactivity descriptors for molecules have been defined (table 4), like softness (measures stability of molecules and chemical reactivity [32]), hardness (reciprocal of softness), chemical potential, electronegativity (strength atom for attracting electrons to itself), electrophilicity index (measuring lowering energy due to maximal flowing electron between donor and acceptor) [32, 33]. The results showed, the higher potency compound 4 have, higher electrophilicity, higher hardness, lower energy gap, lower softness, lower electronegativity and lower dipolemoment (Table 3), which may be explained the highest anti-inflammatory affinity of its compounds (Table 4).

### 2.3.3. Docking Studies

In brief, two isoforms of COX protein are known: COX-1, is responsible for the physiological production of prostaglandins, which is expressed in most tissues; and COX-2, is responsible for the increasing production of prostaglandins during process of inflammation, which is induced by endotoxins, cytokines and mitogens in inflammatory cells [34]. Recently, from analysis of X-ray cocrystal of arachidonic acid with COX-2 showed that, carboxylate coordinated with Tyr-385 and Ser-530 [35], as well as the action of NSAIDs, through interaction carboxylate group with Tyr-385 and Ser-530, which stabilize the negative charge of the tetrahedral intermediate [35], and demonstrated that, Tyr-385 and Ser-530 have a structural and functional evidence for the importance of them in the chelating of the ligands [35]. Molecular docking of the synthesized compounds into the active site of COX was performed, in order to understanding obtained biological data on a structural basis, through rationalized ligand-protein interaction behavior. All calculations for docking experiment preformed with MOE 2008.10 [24]. The tested compounds were evaluated in silico (docking), using X-ray crystal structures of COX-1 (ID: 3N8Y) [36] and COX-2 (ID: 1PXX) [35] complexes with Diclofenac as inhibitor. The tested compounds docked into active sites of both enzymes COX-1 and COX-2. The active site of the enzyme was defined to include residues within a 10.0  $\text{\AA}$  radius to any of the inhibitor atoms. MOE scoring function of the most stable docking model for tested compounds applied to evaluate the binding affinities between the inhibitors complexes with (COX) active site, Table (5). The complexes were energy-minimized with an MMFF94 force field [23] until the gradient

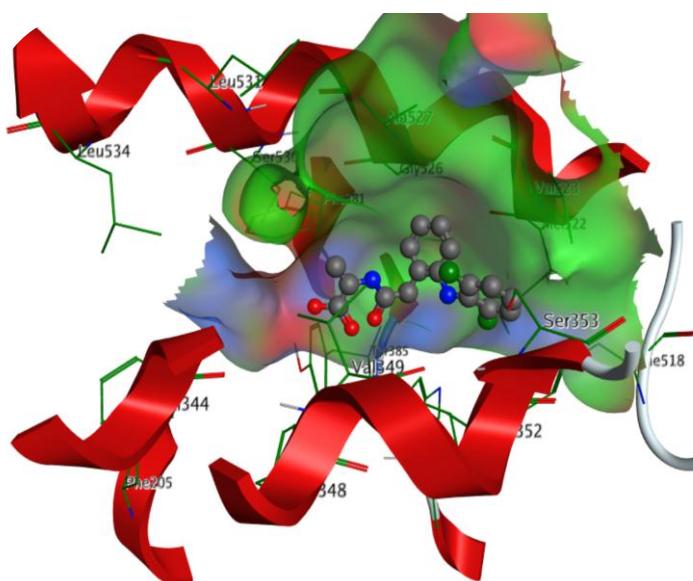
convergence 0.05 kcal/mol reached. The most active compounds docked successfully into the COX-1 active site.

**Table-5.** Docking energy scores (kcal/mol) derived from the MOE for new isolated ligands:

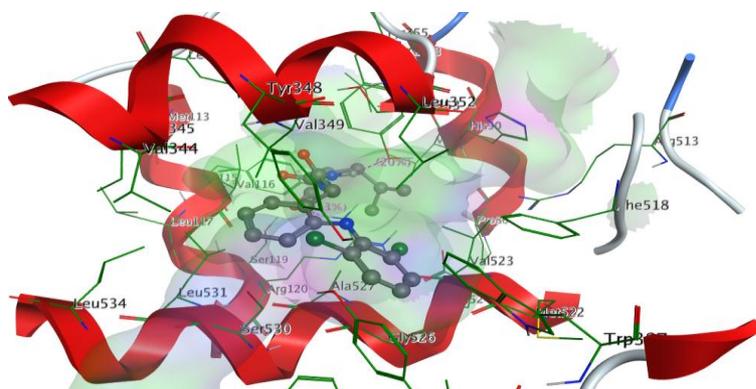
Cpd.	COX-1				
	dG	Int.	H.B.	Eele	Evdw
1	-9.37872	1.94646	-15.2523	-9.37872	-22.4701
4	-14.4626	-10.1012	-14.4626	-147.879	-14.4626
Cpd.	COX-2				
	dG	Int.	H.B.	Eele	Evdw
1	-98.8173	-22.018	-14.9099	-7.95803	-15.1421
4	-121.166	-23.6146	-25.3448	-15.14974	-21.5073

**d.G.:** free binding energy of the ligand from a given conformer, **Int.:** affinity binding energy of hydrogen bond interaction with receptor, **H.B.:** Hydrogen bonding energy between protein and ligand, **Eele:** the electrostatic interaction with the receptor, **Evdw:** van der Waals energies between the ligand and the receptor.

**Fig-2.** The compound (1) Docked into the active site of COX-2. using MOE tool.



the binding energies obtained from modeled of diclofenac **1** and **4** into active site of COX-2 are (~ 98.8 and -121.16), respectively, (Fig. 1 and 2, table 5), reference drug (**1**) and compound **4** stabilized in binding pocket by adjusting two phenyl ring in perpendicular with each other. In addition, the compounds (**1**) and (**4**) were arranged with binding pocket by perpendicular adjusting of chlorophenyl ring of ligands **1** and **4** with phenyl ring of Tyr-385.

**Fig-2.** The compound (4) Docked into the active site of COX-2. using MOE tool.

The compound 4 showed, strong hydrogen bond interaction of NH- Leu. with Ser-530, and carbonyl moiety of Leu with Tyr-385. This 4 stabilized in binding pocket by adjusting dichloro phenyl ring perpendicular with phenyl ring of Tyr-385.

The results obtained clearly reveals that, the amino acid residues close to the reference molecules diclofenac are mostly the same as those observed in the currently isolated synthesized compounds complexes with protein (Fig. 1 and 2). Compared with the original inhibitor 1, the higher binding energies and binding process interaction observed for 4in COX-2, and the lower binding energies of isolated compounds in COX-1, this results indicate that, this compound 4 act as selective inhibitor against COX-2 and considering most suitable more suitable COX-2 inhibitor than diclofenac, this could probably due to the presence hydrophobic amino acid residue in prepared ligand.

### 3. CONCLUSION

A series of Diclofenac derivatives containing L- leucine moiety (4) synthesized. The comparative docking experiment was carried out on isolated synthesized compound (4) compared with reference molecules 1, which indicate that, this compound was stabilized in the binding pocket of COX with a similar binding mode of diclofenac, and have higher binding score, and showed suitable selective inhibitors against COX-2. The compounds subjected to ADMET profile, which theoretically revealed that, this compounds should present good passive oral absorption. The ulcerogenic studies screened for isolated compound 4, and showed negligible ulcerogenic effect with higher safety and better therapeutic index than diclofenac, molecular docking studies supported with ulcerogenic effect, and understanding the various interactions for ligands and active sites of enzyme help to design novel potent selective COX-2 inhibitors.

## 4. EXPERIMENTAL

### 4.1. Instrumentation and materials

Melting points taken on a Griffin melting point apparatus and are uncorrected. Thin layer chromatography (Rf) for analytical purposes was carried out on silica gel and developed.

Benzidine, ninhydrin, and hydroxamate tests used for detection reactions. The IR spectra of the compounds recorded on a Perkin-Elmer spectrophotometer model 1430 as potassium bromide pellets and frequencies reported in  $\text{cm}^{-1}$ . The NMR spectra were observed on a Varian Genini-300 MHz spectrometer and chemical shifts ( $\delta$ ) are in ppm. The mass spectra recorded on a mass spectrometer HP model MS-QPL000EX (Shimadzu) at 70 eV. Elemental analyses (C, H, N) were carried out at the Microanalytical Centre of Cairo University, Giza, Egypt.

## 4.2. Synthesis

### 4.2.1. 2-[(2,6-Dichlorophenyl)amino]phenyl acetyl chloride (**3**).

Prepared by reported method and directly used in the next step (40, 41).

### 4.2.2. General procedures for synthesis L-aminoacid derivatives (**4**).

Path 1:

A mixture of (0.01 mol) L-leucine with (0.01 mol) of 2-(2-(2,6-dichlorophenyl-amino)phenyl)acetic acid **2**, was fused at  $280^\circ\text{C}$  in an oil bath for 15 min., the fused mass was dissolved in ethanol and poured onto cold water, the solid obtained was recrystallized from ethanol to give compound **4**

Path 2:

The mixture of (0.01 mol) L-leucine with 2-[(2,6-dichlorophenyl)amino]phenyl acetyl chloride (**3**) were dissolved in mixture of water (25 ml) and THF (15ml), triethylamine (2ml) was added, followed by portion-wise addition of acid chloride (**3**; 0.01 mmol) during 30 min, temperature of the reaction mixture was kept at  $10^\circ\text{C}$  during the addition. Stirring continued for 3 h at  $10^\circ\text{C}$ . THF was removed by concentration of the reaction mixture under reduced pressure; water (30 ml) was added and acidified with 1 N HCL to pH =5. The crude products filtered and re-crystallized from ethanol. The product **4** was chromatographically homogeneous by iodine and benzidine development. Brown crystal : yields=81%;Rf= 0.38 (CHCl<sub>3</sub>/EtOH=3/1); mp: 148-50 °C;  $[\alpha]_D^{25} = +49.1^\circ$  (EtOH); IR (KBr  $\text{cm}^{-1}$ ) v; 3343 broad band (OH,NH), 3044 (CH<sub>arom</sub>.), 2911 (CH<sub>ali</sub>.), 1692(CO), 1576(CONH)  $\text{cm}^{-1}$ ; <sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>)  $\delta$ = 11.99 (s, 1H-OH), 7.36 – 6.98 (m, 7H-Ar-H), 6.99. 01 (s, H-NH-Dic.), 6.59(s, H-NH-Ala), 3.86(s, 2H, CH<sub>2</sub>-Dic.), 1.13 (m, 1H, CHCH<sub>2</sub>-Leu), 0.92(t, 2H, CH<sub>2</sub>-Leu), 0.18 (m, CH(CH<sub>3</sub>)<sub>2</sub>-Leu), 0.014(s, 6H, (CH<sub>3</sub>)<sub>2</sub>-Leu) ; Anal./Calcd. for C<sub>20</sub>H<sub>22</sub>C<sub>12</sub>N<sub>2</sub>O<sub>5</sub> (408): C (58.82%), H (5.39%), N (6.86%). Found: C (58.69); H (5.42); N, (6.84).

## 4.3. Molecular Modeling Study

### 4.3.1. Generation of Ligand and Enzyme Structures

#### 4.3.1.1. Selection of COX structures

Docking study was carried out for the target compounds into (COX-1 (ID: 3N8Y) and COX-2 (ID: 1PXX) using MVD,4.0 and MOE,10. The crystal structure of the (COX) complexes with (1), which a selective inhibitor of COX-2 in co-crystallized form in the active site of the receptor. From X-ray crystal structure studies of the COX enzyme, the mouse enzyme expected to be very similar to the human [15], and used as model for human COX enzyme.

#### 4.3.2. Preparation of Small Molecule

Molecular modeling of the target compounds built using MOE, and minimized their energy with PM3 through MOPAC. Our compounds introduced into the (COX) binding site accordance the published crystal structures of (1) bound to kinase.

#### 4.3.3. Stepwise Docking Method

##### 4.3.3.1. MOE Stepwise

The crystal structure of the (COX) with a Diclofenac (1) as inhibitor molecule, was used for the receptor molecule, Water and inhibitor molecules were removed, and hydrogen atoms were added. The parameters and charges assigned with MMFF94x force field. After alpha-site spheres were generated using the SITE FINDER module of MOE. The optimized 3D structures of molecules were subjected to generate different poses of ligands using triangular matcher placement method, which generating poses by aligning ligand triplets of atoms on triplets of alpha spheres representing in the receptor site points, a random triplet of alpha sphere centers is used to determine the pose during each iteration. The pose generated rescored using London dG scoring function. The poses generated were refined with MMFF94x forcefield, also, the solvation effects were treated. The Born solvation model (GB/VI) used to calculate the final energy, and the finally assigned poses assigned a score based on the free energy in kJ/mol

#### 4.4. Pharmacology

##### 4.4.1. Determination of Acute Toxicity

The acute toxicity and lethality ( $LD_{50}$ ) for the new isolated compound estimated in albino mice (25–30 g). In a preliminary test, animals in groups of three, received one of 300, 500, 600 or 700 mg/kg for the tested compounds and diclofenac. Animals observed for 24 h for signs of toxicity and number of deaths. The  $LD_{50}$  calculated as the percentage mortality in each group was determined 24 h after administration [34].

##### 4.4.2. Acuteulcerogenesis

The studies carried out on healthy Albino rats at a dose 20 mg/kg. The animals were divided into different groups of six each, group I served as control and received vehicle only, groups II received pure indomethacin 20 mg/kg, the other groups were administered test compounds in dose molecularly equivalent to 20 mg/kg of indomethacin. Before 24 h administration of the tested compounds, Food not water removed; the rats fed normal diet for 17 h and then sacrificed after the drug treatment. The stomach removed and opened along the greater curvature, washed with distilled water and cleaned gently by dipping in saline. The mucosal damage examined by means of a magnifying glass. For each stomach, the mucosal damage was assessed according to the following scoring system [35].

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