Pharmacological Evaluation of Marginally Designed New Profen Analogues

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ABSTRACT

Objective: Pharmacological evaluate of hydrazide, marginal analogues of naproxen, flurbiprofen and ibuprofen, well established COX inhibitors, with the goal to identifying a Cyclooxygenase (COX) inhibitor(s) with optimum potency and efficacy. The synthesized and characterized analogues were tested for their acute toxicity, anti-inflammatory, analgesic and antipyretic activities as per reported bioassay models. Molecular docking analysis was also performed to predict ligand: protein interactions.

Method: Acute toxicity, anti-inflammatory, analgesic and antipyretic activities were performed in accordance with well-established reported bioassay models for the purposes.

Results: No acute toxicity was observed at intraperitoneal doses of 50, 100 and 150mg/Kg body weight. Biological assays revealed that test compounds -I to -IV have highly significant anti-inflammatory and peripheral analgesic activities. Antipyretic test revealed that test compounds I, II, III have highly significant antipyretic activity while IV in the doses of 20mg and 30 mg has significant and in the dose of 50 mg/Kb body weight has highly significant antipyretic activity. The molecular modelling revealed strong inhibitory interactions of the four compounds. The most suitable poses have higher docking score (energies) compared to flurbiprofen (reference ligand), that confirms highly significant anti-inflammatory and analgesic activities of the dimers.

Conclusion: The synthesized hydrazide analogues have highly significant pharmacological activities and have the potential to be further explored as new drug molecules.

Keywords: Analgesic, anti-inflammatory, antipyretic, molecular docking, COX

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INTRODUCTION

One of the inexhaustible areas for research is the modification of existing drug molecules, which may further be amplified, improved in potency, efficacy, or safety. Development and successful predictions of molecular computation of binding potential between the receptors (proteins) and ligands (drugs) further aided in the efforts of researchers even to design the most appropriate drug structures for both known and unknown proteins, drug discovery (1, 2).

Non-steroidal anti-inflammatory drugs (NSAIDs) is a class of drugs, which are the most widely prescribed and used medicines for their anti-inflammatory, analgesic and antipyretics activities. The selection of NSAIDs depends upon the clinical condition of patient, its Cyclooxygenase-1/2 (COX1/COX2) selectivity, duration of use, gastric ulcerative and cardiovascular risks etc., and (3-5). Till today, a number of NSAIDs are being introduced for the treatment of acute and chronic pain, inflammation and pyrexia of different origins, belonging to different chemical classes e.g. salicylates, fenamates, propionates, acetates, oxicams etc. (6) but inflammation, despite of this tremendous advancement in the field of pharmaceutical chemistry still presents a major challenge to the researchers.

Three members of profens, arylpropionic acid class of NSAIDs, i.e. *2(6-methoxynaphthalen-2-2yl) propanoic acid* (*Naproxen*) (7), *2-(2-flurobiphenyl-4-yl) propanoic acid* (*Flurbiprofen*) (8); *2-(4(2-methylproply) phenyl) propanoic acid* (*Ibuprofen*) (9) were selected for synthesis of their marginal (hydrazide) analogues to explore their pharmacological activities along with molecular docking analysis to predict its interaction potential, being one of the acceptable tools in drug discoveries (10). *In-vivo* pharmacological evaluation of the synthesised analogues with molecular docking analysis could provide a useful data for further augmentation of the existing drug molecules for optimal efficacy and safety because there is always a great need

for better and safer drugs; more active, selective and thus effective drugs with lesser side or toxic effects, for cure, symptomatic treatment or prophylaxis of inflammation and its associated disorders.

MATERIAL AND METHODS

General

All chemicals, reagents, and solvents were analytical grade, purchased from commercial suppliers, and used without purification unless otherwise specified. Ibuprofen, flurbiprofen and naproxen were provided by Polyfine Chemipharma (Pvt.) Ltd, Hayatabad, Peshawar, Pakistan. Ethanol and concentrated sulfuric acid were purchased from Sigma-Aldrich, USA), Lambda Carrageenan (Sigma, USA), glacial acetic acid (Panreac, Spain), Brewer yeast (Merck, Germany) and sterile normal saline I/V (Otsuka Japan), used as received.

The purity of the products was checked on thin layer chromatographic plates of aluminum coated with silica gel (Kieselgel 60 F25, Merck, Germany), Melting points on a Bicot apparatus (Bibby Scientific Ltd UK), ^{1}H NMR on Bruker AV 300 and 400 MHz instruments using dimethyl sulfoxide (DMSO) as solvent, Electron impact mass spectra (EI MS) on a "JEOL MS Route" spectrometer (JMS 600-H), Infrared (IR) spectra on a "JASCO IR-A-302" spectrometer, UV spectra on Thermo Electron Vision Pro (V4.10), and C:H:N analyses were determined on a "Carlo ErbaStrumentazione" (Mod-1106) Italy.

Anti-inflammatory activity test was conducted on plethysmometer (Model LE 7500 Plan lab S.L.), anti-nociceptive activity on Hot Plate Analgesiometer (Harvard apparatus, USA) and antipyretic through digital thermometer (Model CA92121, ACON Laboratories, USA.

Statistical analysis

GraphPad Prism® (5.01) was used for statistical analysis, *in vivo* pharmacological activates data were analyzed by applying one-way ANOVA followed by Dunnett's post-hoc analysis.

General procedure for the synthesis of the compounds

Ibuprofen, naproxen and flurbiprofen were first converted to ethyl ester derivatives through reflux condensation with ethyl alcohol (11). The esters thus synthesized were treated with hydrazine hydrate through reflux condensation, for 24-48 hours, in ethyl alcohol as a solvent medium (12). The completion of reaction was monitored through TLC, using ethyl acetate and hexane as mobile phase. The reactions were completed in 12-18 hrs. The resultant solid products (Table 1) were filtered, washed with hexane (200-400 mL) and distilled water (400-500 mL) and dried.

Pharmacology evaluation

Mice (*BALB-c*), either sex, were used throughout the experimental studies. Animals were purchased from the National Institute of Health (NIH) Islamabad, Pakistan. The animals were kept under the standard laboratory conditions at $25 \pm 2\,°C$ and fed with standard food *ad libitum*, free access to drinking water with a 12 hr dark / 12 hr light cycle. Animals' studies were approved by the ethical committee of the Department of Pharmacy, University of Peshawar, Pakistan (No. 01/EC-15/Pharm dated 10-04-2015).

Acute toxicity

For the toxicity study, the test compounds at the dose of 50, 100 and 500 mg/Kg body weight were administered intraperitoneally (ip) through injection to four groups having six (n=6) mice in each group. While the control group was treated with normal saline. Animal were observed for 6 hours for any behavioural changes while the mortality rates were observed at 24 hours after the administration of test compounds (13).

Anti-inflammatory Activity Study *[Carrageenan-induced paw oedema test]*:

Mice were divided into five groups each containing five mice (n=6). Acute inflammation was induced by injecting 0.05 ml of (1%) carrageenan into plantar surface of mouse hind paw (14). Test groups I, II, and III were treated with 10, 20 and 30 mg/kg of the test compound, group-IV (control) was treated with normal saline (1 ml/kg) while the Group-V (reference standard) was with Ketoprofen (10 mg/kg), 30 min before carrageenan injection. The paw volume was measured at 0, 1, 2, 3, 4 and 5 hours using digital plethysmometer. The difference between the readings at time 0 h and different time interval was taken as the degree of oedema while was quantified as;

$$\% \text{ Inhibition } = \frac{\alpha - \beta}{\alpha} \times 100$$

Where, α and β indicate, increase in paw volume of control and drug-treated animals, respectively.

Evaluation of Anti-Nociceptive Activity

Acetic acid induced writhing test in mice

Mice were divided into five groups each containing six (n=6) mice. The test groups, I, II, and III were treated with 10, 20, 30 mg/kg body weight, i.p. of the test compound, group IV (control) received normal saline (10ml/kg, i.p.) while the Group-V (reference standard) was treated with diclofenac at the dose of 10mg/ kg, i.p. After 30 minutes of the administration of the aforesaid compounds, normal saline and drug, respectively, all the five groups were treated with 0.6% acetic acid at 10ml/kg, i.p. (15). Five minutes after acetic acid injection, mice were placed in individual cages and the numbers of writhes (abdominal contractions) were counted for each mouse for a period of 30 minutes after 5 minutes' latency period, and the percentage inhibition of writhing was calculated.

Hot plate (thermal) test in mice

Mice, of either sex, weighing 18-22 g were used in this study. As per protocol, two hours habituated animals to the laboratory environment were randomly divided into five groups, six (n=6) animals in each group.The test groups I, II, and III were treated with 10, 20, 30 mg/kg/i.p. of the test compound, group-IV (control) was treated with normal saline (10ml/kg, i.p.) while the Group-V (reference standard) with tramadol at the dose of 5 mg/ kg, i.p.The animals were tested after 30 and 60 min on the hot plate of analgesiometer maintained at temperature of 54.0 ± 0.1°C. Hot plate reaction time (latency to response in seconds) was observed by noting licking, flicking of hind limb or jumping from cylinder (16). A cut-off time of 30 secs was fixed for animal's reaction to avoid tissue damage for longer exposure to hot plate. Anti-nociception was calculated using the following formula.

$$\% \text{ Analgesia} = \left(\frac{\text{Test latency} - \text{Control latency}}{\text{Cutoff time} - \text{Control latency}}\right) \times 100$$

Anti-pyretic Evaluation study *(Brewer's yeast induced pyrexia test in mice):*

Albino mice (*BALB-c*), weighing 25-30 g of either sex, were used in this study. As per protocol prior to experiments, the animals were fasted overnight with free access to water. The animals were randomly divided into five groups each group consisting of six animals (n=6). Test groups I, II, and III were treated with 10, 20 and 30 mg/kg of the test compound, group-IV (control) was treated with normal saline (10 mL/kg) while the Group-V (reference standard) was with Paracetamol (150mg/kg). Hyperpyrexia was induced by aqueous suspension of 20% Brewer's yeast (10 ml/kg body weight s.c) below the nape of neck in the back of mice. Changes in rectal temperature were noted after 24 hours of Brewer's yeast injection at 1, 2, 3, 4 and 5 hours (17). For insertion in animal's rectum, probe of digital thermometer was lubricated with olive oil and

maintained for thirty seconds for recording temperature. Those animals were selected for study that showed a minimum rise of at least 0.3-0.5°C rectal temperature.

Molecular docking simulation analysis

Molecular docking simulates the best-fit orientation of legend (drug) at the specific binding site of a receptor protein that signifies the pharmacological activity and drug designing rational with reference to that very receptor protein (18). COX-2, due to biosynthesis of prostaglandins (PGs), is one of the key enzymes involved in inflammation. Amino acid residues at the binding site (cavity), especially Arg_{120} and Tyr_{355} play a central part in designing or virtual screening for discovery of new COX inhibitors (19). Standard docking procedure for rigid protein and a flexible ligand was performed through AutoDock (4.0.1.) (20), along with AutoDock Tools using a Lamarckian generic logarithm. A grid (60x60x60 points) in three dimensions was built, centred on the centre of M loop of Cyclooxygenase-2 (COX2) (PDB ID: 3PGH). A grid spacing of 0.375 Å and a distance-dependent function of the dielectric constant were used for the calculation of the energy map. Default setting was used for other parameters. The most favorable free binding energy, after successful completion of docking, was selected as resultant ligand protein complex structure (19). To validate the accuracy of the binding mode and binding affinity flurbiprofen was used as a reference and co-crystallized ligand (19).

RESULTS

Acute toxicity

No acute toxicity was observed during 24 hours at doses of 50, 100, 150 mg/Kg body weight, of all the test compounds.

Anti-inflammatory activities

The anti-inflammatory activities data depicted (Figure 1) that TC-II in the dose of 20mg/Kg body weight have statistically significant (**p <0.005) while TC-III in all the three doses have highly significant anti-inflammatory effect (***p <0.0001) with reference to normal saline (10mL/Kg) as control and Ketoprofen 10mg/Kg, as reference standard. The doses of the three compounds were selected on the bases of average doses of the legend (drug) reported in literature.

Evaluation of anti-nociceptive activity

Hot plate (thermal) test in mice

The hot plate tests were found negative for the three test compounds the data is, therefore, not reported.

Acetic acid induced writhing test in mice

The data of anti-nociceptive activities, acetic acid induced writing test, of the test compounds (TC-I, -II & -III) with reference to normal saline 10mL/kg as control and Diclofenac 10mg/Kg as reference standard, depicted, statistically, that the results of the three test compounds were highly significant (***p<0.001) [Fig. 2]. The result also showed that there was a direct relation of percent pain inhibition with increase in dose of the test compounds.

Anti-pyretic evaluation study

The statistical analysis of the data of *Brewer's yeast induced pyrexia test* revealed that antipyretic activities of the test compounds were highly significant (*** p < 0.0001) in all the tested doses as compared to control (normal saline 10 mL/Kg) and Paracetamol reference standard (150mg/Kg body weight) [Fig. 3].

Molecular docking simulations

In order to get validated and accurate binding modes and binding affinities of the test compounds, flurbiprofen was used as a reference ligand to determine its RMSD value. RMSD value of reference compound was found to be 0.49, which is a satisfactory score for validation. The analysis of the docking data revealed that the best scoring conformations of the TC-I, -II and -III were found much closer to the best scoring confirmations of flurbiprofen (Figs. 4 to 7). All the test compounds showed considerable molecular interactions with main binding pocket of the enzyme. The binding energy of protein-ligand interactions are given in Table 2.

DISCUSSION

Molecular docking analysis focused on COX2 enzyme gave reasonable explanation of different anti-inflammatory activities of test compounds. The carbonyl oxygen and nitrogen of the hydrazide substitute of TC-I were involved in hydrogen binding with Tyr 355 with hydrogen bond energy -2.5 kcal/mol each, unlike flurbiprofen (standard used) although both the compounds anchored in the same pocket. The overall binding energy of TC-I was -96.862 kcal/mol. The inhibition was strong but not significantly increased. TC-II fit in the binding pocket anchoring on the Try355 and Arg120 residues having similar hydrogen bonding energy value of -2.5kcal/mol while the overall binding energy was -103.301 kcal/mol. TC-III also anchored via Try355 and Arg120 residues with Try355 hydrogen binding energy as -1.043 kcal/mol less than values of hydrogen bonds of the TC-I and TC-II.

The most favorable confirmations of the test compounds when combined in the binding site of COX-2 receptor, it revealed that all the test compounds packed in the same docking positons with almost similar behavior (21). The carbonyl moiety of TC-I and -III and the oxygen of ethoxymoiety of TC-II anchored on both Arg_{120} and Tyr_{355} (1st anchoring site), via hydrogen bonding interactions with in a narrow range of binding length difference (Table 2) and all were found considerable superimposable at anchoring site of the binding pocket (22) Figure 8.

The opposite side of the carbonyl group of the test compounds and flurbiprofen (i.e. the second phenyl ring of TC-I and TC-II and the isobutyl group of TC-III) were oriented in a hydrophobic cleft, 2nd anchoring site, lined with Leu352, Ser530, Val523, Try384, Phe381, Tyr385, Met522, Gly526 and Trp387, residues (22). The entire test compounds showed good binding energies.

CONCLUSION

As the flurbiprofen has more selectivity towards COX2, its hydrazide analogue (TC-III) has shown highly significant anti-inflammatory activity *in vivo* followed by high binding energy / score. While the ibuprofen and naproxen being equipotent for both COX1/COX2 and their analogues (TC-I and TC-II, respectively) have shown inhibition of both isomers *in vivo*. It was observed that by the addition of hydrazide group there is no change in COX2 selectivity of TC-I and –II, but their peripheral analgesic and antipyretic activities were statistically highly significant. TC-II have also shown statistically significant anti-inflammatory activity as well. The docking result revealed promising molecular interactions (docking score / energies) of the compounds in the binding pocket, especially with Arg-120 and Try-355 amino acids residue of COX2 protein, which is one

of the explanations for highly significant pharmacological activities, especially anti-inflammatory and analgesic activities of the TCs.

AUTHORS' NOTE

SMA Halimi, PhD Research Scholar, has designed and conducted the whole research and wrote the paper. Muhammad Saeed conceived paper, supervise the research work as PhD supervisor of the research scholar, critically revised manuscript and approved final version. K Muhammed Ahmed guided, facilitated the synthesis of analogues and interpretation of its characterization data. Safi Ullah provided assistance in synthesis, characterization and pharmacological evaluation. The authors declare that they have no conflicts of interest.

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Table 1: Hydrazide Analogues of Ibuprofen, Naproxen and Flurbiprofen
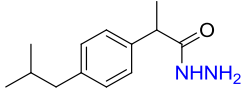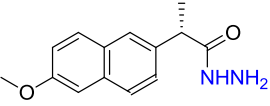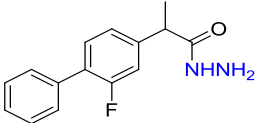
Test Compound	Structure	IUPAC name	M.W	Formula
TC-I		2-(4-isobutylphenyl) propanehydrazide	220.311	$C_{13}H_{20}N_2O$
TC-II		(S)-2-(6-methoxynaphthalen-2-yl) propanehydrazide	244.289	$C_{14}H_{16}N_2O_2$
TC-III		2-(2-fluorobiphenyl-4-yl) propanehydrazide	258.291	$C_{15}H_{15}FN_2O$

Table 2: Binding energies of highest scored confirmations of the test compounds and reference legend through docking analysis

Test Compounds	Binding Energy (kcal/mol)	Anchoring (Hydrogen Bonding) Length (Å)		Anchoring (Hydrogen Bonding) Energy (kcal/mol)	
		Arg_{120}	Tyr_{355}	Arg_{120}	Tyr_{355}
Flurbiprofen (Ref. Standard)	-119.141	3.064 2.963	3.090	-0.864 -1.482	-2.5
TC-I	-96.862	-	2.747 2.705	-	-2.5 -2.5
TC-II	-103.301	2.797	2.892	-2.5	-2.5
TC-III	-98.346	2.644	2.957	-2.5	-1.043
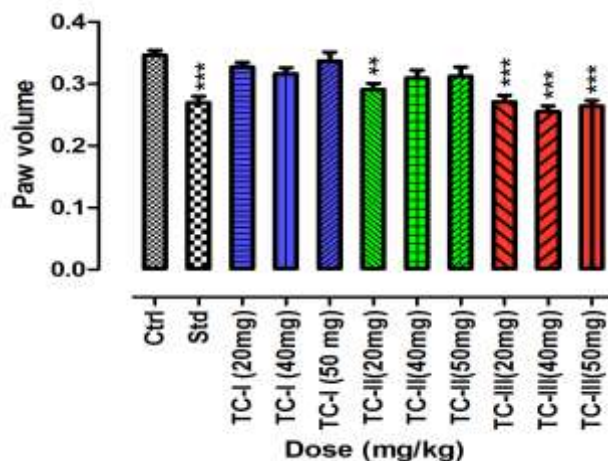
Fig. 1: Summarized comparison of anti-inflammatory activities of TC-I, TC-II and TC-III (20, 40, 50mg/kg) with control (normal saline 10 mL/kg) and reference standard (Diclofenac 10mg/Kg). Bars present as mean ± SEM of % inhibition in paw volume, for a group of five animals (n=6).
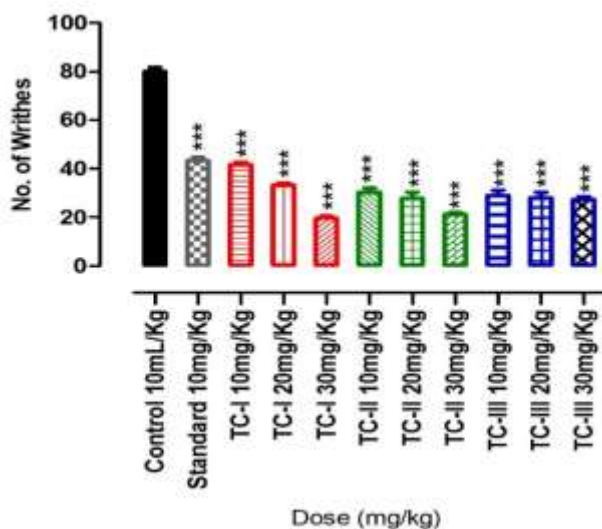

Fig. 2: Summarized comparison showing anti-nociceptive activities of TC-I, -II, & -III (10, 20 and 30 mg/kg) *vs* normal saline (10mL/Kg) and Diclofenac 10 mg/kg as reference standard. Bars present as mean ± SEM of number of writhes, for a group of six animals. The effects are highly significant in all the three compounds (***$p < 0.0001$).
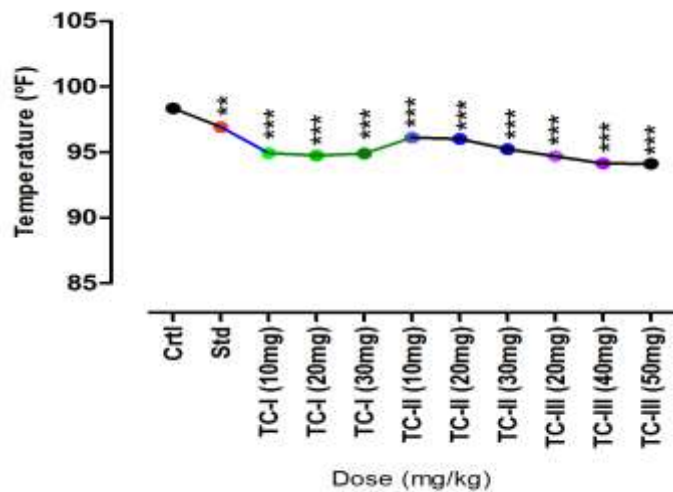
Fig.3: Summarized comparison showing antipyretic activity of different concentration of Test Compounds (TC-I, -II and –III) along with Control, normal saline (10mL/Kg) and reference standard (Paracetamol 150 mg/kg). The antipyretic activities of the test compound were found highly significant (***$p < 0.0001$).
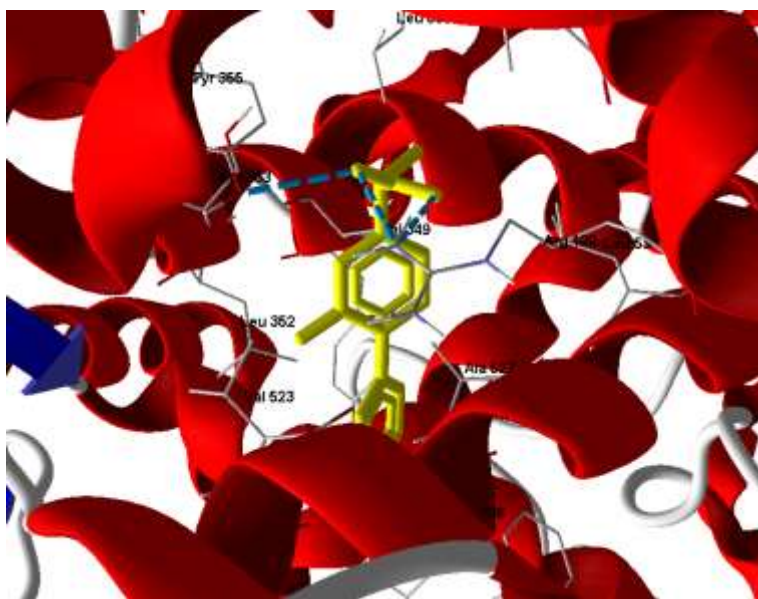

Fig. 4: Docking confirmations of Flurbiprofen (reference legend) in the binding pocket of COX2 enzyme protein.
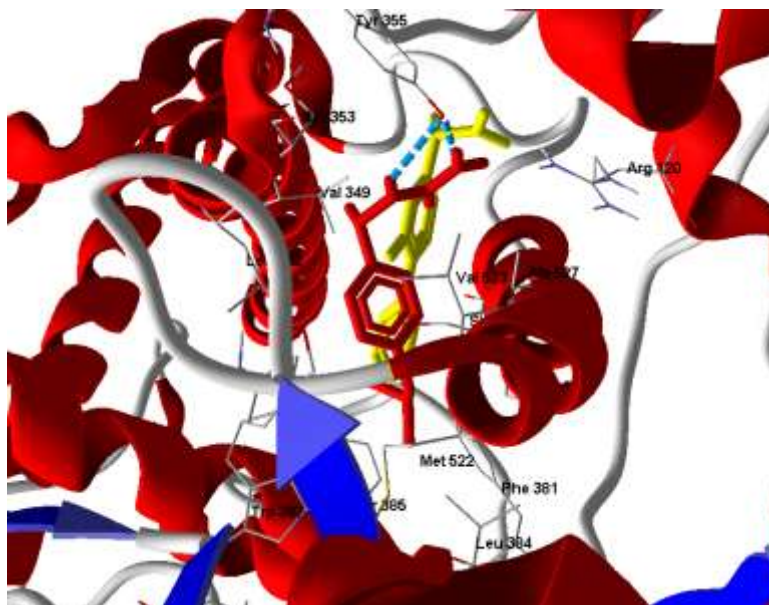
17

Fig. 5: Docking confirmations of TC-I in the binding pocket of COX2 enzyme protein.

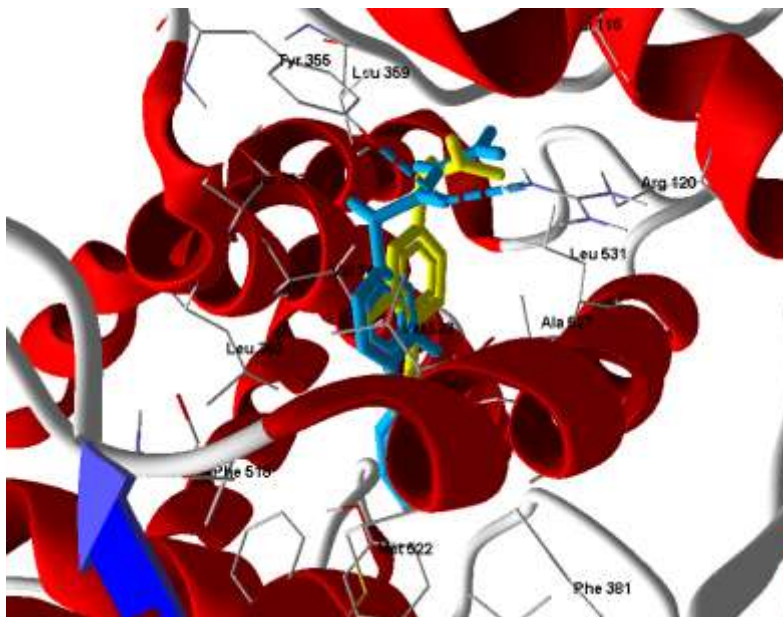Fig. 6: Docking confirmations of TC-II in the binding pocket of COX2 enzyme protein.

Fig. 7: Docking confirmations of TC-III in the binding pocket of COX2 enzyme protein.
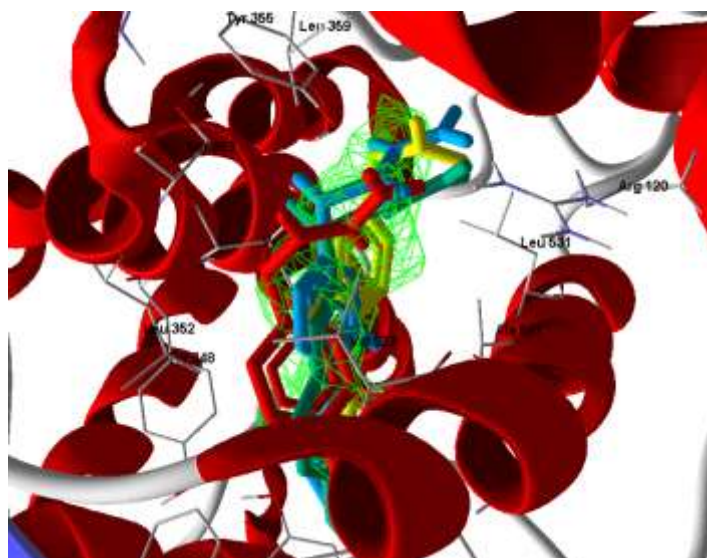

Fig. 8. Superimposition of test compounds TC-I (red), TC-II (green), TC-III (blue) and reference legend Flurbiprofen (yellow) in binding site. While the green mesh represents the binding cavity / pocket.