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Full Length Research Paper

Pharmacological evaluation, molecular docking and dynamics simulation studies of salicyl alcohol nitrogen containing derivatives

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The current study was conducted to evaluate *in vivo* the anti-inflammatory, antinociceptive and antipyretic activities of salicyl alcohol nitrogen containing derivatives that are [4-(2-hydroxybenzyl) morpholin-4-iumchloride (I)], [1,4-bis (2-hydroxybenzyl) piperazine-1,4-diium chloride (II)]. The synthetic compound I, II and standard (aspirin) were evaluated in the laboratory animal model at three different dose levels for each activity. These compounds were examined for, anti-inflammatory activity in carrageenan induced paw edema model [50, 100 and 150 mg/kg intraperitoneally (i.p)], antinociceptive properties in acetic acid induced writhing model (15, 30 and 45 mg/kg i.p), hot plate test model (30 and 45 mg/kg i.p) and antipyretic activity in Brewer's yeast induced pyrexia model (50,100 and 150 mg/kg i.p), using Swiss albino mice. Result of this study indicated that these compounds; possess dose dependent statistically significant anti-inflammatory, antinociceptive and antipyretic properties, comparable to standard aspirin. Nonetheless, these compounds did not show antinociceptive properties in hot plate test when compared with centrally acting standard analgesic (morphine), thus signifying peripheral mechanism of action in the mediation of antinociception. In order to investigate receptor-compounds interactions in terms of the binding affinity, the molecules were subjected to molecular docking simulation analysis using FRED 2.1 software that showed better binding energy of the compounds with the Cyclooxygenase X (COX)- 2 enzyme, predicting these compounds as potential COX-2 inhibitors. As in actual cellular system there was a solvent which makes the enzyme to have a dynamic movement so, molecular dynamic (MD) simulation was carried out during 200 pico seconds (ps) to better understand the binding modes of these compounds with the receptor.

Key words: [4-(2-Hydroxybenzyl) morpholin-4-ium chloride], [1,4-bis (2-hydroxybenzyl) piperazine-1,4-diium chloride], anti-inflammatory, antinociceptive, antipyretic agents, molecular docking, MD simulation.

INTRODUCTION

Biochemical activities of drugs are clinically dependent upon their interaction with living systems. Most drugs exert their action through a complex mechanistic pathway but primarily it is the chemical skeleton which determines the drug response. Salicin (Figure 1), utilized in the treatment of acute rheumatism, was first reported by Machagan (Machagan, 1876). It is a glycoside of salicyl alcohol obtained from willow bark (*Salix alba*), chemically related to acetyl salicylic acid (aspirin) and exhibits almost similar activities in the body. Hydrolysis of salicin results

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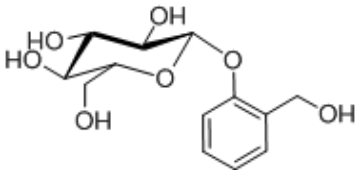

Figure 1. Salicin.

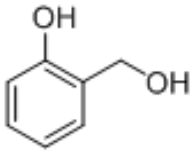

Figure 2. Salicyl alcohol.

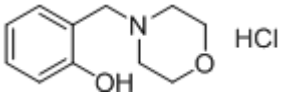

Figure 3. Compound I [4-(2-hydroxybenzyl) morpholin-4-ium chloride].

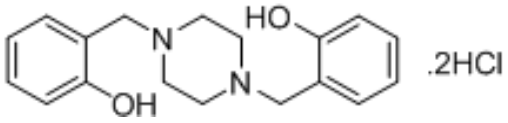

Figure 4. Compound II [1,4-bis (2-hydroxybenzyl) piperazine-1,4-diium chloride].

in liberation of salicyl alcohol (saligenin; 2-hydroxy methyl phenol) (Figure 2). Salicin has been reported to be useful as antipyretic pro-drug with no propensity for gastric damage (Akao et al., 2002). Saligenin which is a part of salicin, has been used for long time as a local anesthetic and has also been shown to have a local anesthetic property (Harischfelder et al., 1920; Harischfelder and Wynne, 1920). Furthermore, evidence of anesthetic and antipyretic activities of salicyl alcohol has been mentioned (Charles and Tony, 1956).

It has been reported that compounds containing piperazine moiety inhibit eicosanoid pathways (Coonrod et al., 2001) and possess different pharmacological properties (Berardi et al., 2008) including anti-inflammatory and antinociceptive activities (Jakubkien et al., 2003). In this connection, piperazine moiety is considered a critical core for novel drug design (Bali et al., 2010). Furthermore, anti-inflammatory and antinociceptive properties of heterocycles conjugated to morpholine have been also reported (Panneerselvam et al., 2009). Additionally, it has been argued that piperazine, piperidine and morpholine ring containing compounds having nitro, oxo or chloro substitution exhibit different biological activities (Folkes et al., 2007; Wermuth and Fontaine, 2003).

To manage inflammation, pain and pyrexia, nowadays Non-Steroidal Anti-Inflammatory Drugs (NSAIDs) are commonly prescribed worldwide whereas opioids are used for severe painful conditions like cancer pain that cannot be managed by typical NSAIDs. However, it is a well recognized fact that persistent use of NSAIDs exhibit several side effects notably; ulceration, perforation, hemorrhage of gastrointestinal tract, cardiovascular disorders and renal damage (Jones et al., 2008) while opioids cause drug tolerance and physical dependence (Mayer et al., 1995). Hence, there is an explicit need for alternative drugs to opioids and classical NSAIDs that possess effective anti-inflammatory, antinociceptive and antipyretic activities still devoid of such adverse effects. In this study, we have carried out selected pharmacological evaluation and molecular docking and dynamic simulation of our already synthesized derivatives carrying salicyl alcohol attached to morpholine and piperazine nuclei are; compound I [4-(2-hydroxybenzyl) morpholin-4-ium chloride] (Figure 3) and compound II [1,4-bis(2-hydroxybenzyl) piperazine-1,4-diium chloride] (Figure 4).

Keeping in view, the great importance of salicyl alcohol as a drug or a prodrug and the potential pharmacological activities of compounds obtained with morpholine and pipreazine substitutions and also their desirable solubility profile, we undertook targeted pharmacological evaluation of salicyl alcohol derivatives. In addition, to rationalize the pharmacological activities of these synthetic compounds at the molecular level, their interactions with the binding sites of COX's, were studied with molecular docking simulation approach using FRED 2.1 software.

MATERIALS AND METHODS

Equipments and chemicals

Anti-inflammatory activity was tested using digital plethysmometer (Model LE 7500 Plan lab *S.L.*), antinociceptive activity was tested using hot plate analgesiometer (Harvard apparatus, USA) and antipyretic with digital thermometer (Model CA92121, ACON Laboratories, USA). Lambda Carrageenan (Sigma, USA), acetyl-salicylic acid (Sigma, USA), glacial acetic acid (Panreac, Spain), Brewer yeast (Merck, Germany), Morphine sulphate obtained through proper channel from Punjab Drug House, Lahore Pakistan.

Animals

Mice (*Balb-c*), bred in the department of Pharmacy, University of Peshawar, animal house and bioassay laboratories, were used throughout experimental studies. Animals were housed in standard cages with free access to standard laboratory food and water available *ad libitum* except where experimental protocol restricted

to do so. All experimental procedures were carried out between 8.00 am-4.00 pm. The 12 h light and dark cycle was provided with temperature maintained at 22±2°C through a reversible air condition (AC). The animal studies were approved by Departmental ethical committee in its 3rd meeting held on June 15, 2011 and approval certificate obtained bearing number 15/EC/pharm. All the procedures were carried out in accordance with Animals Scientific Procedure Act (1986) UK.

Evaluation of anti-inflammatory activity (Carrageenan-induced paw edema model in mice)

Carrageenan-induced paw edema model was used for the determination of anti-inflammatory activity in mice as described previously (Winter et al., 1962). The animals of either sex (25-30 g) were fasted overnight with free access to water. For the experiments, the animals were randomly divided into four groups (I, II, III and IV), each group consisting of eight animals (n=8).

Group I: Control (untreated) group, received saline (intraperitoneal, i.p).
Group II: Standard treatment group (aspirin 50, 100 and 150 mg/kg kg i.p)
Group III: Test treatment group (Compound I 50, 100 and 150 mg/kg kg i.p)
Group IV: Test treatment group (Compound II 50, 100 and 150 mg/kg kg i.p)

Test samples, standard and vehicle control in their respective doses were administered intraperitoneally (i.p), 30 min prior to the injection of carrageenan. An equal volume of saline was received by saline control group. Then, each mouse was administered 0.05 ml of 1% carrageenan by subplanter injection in hind paw. A digital plethysmometer, which is a valuable instrument to quantify small volumes changes, was used to determine the anti-inflammatory activity by measuring edema (ml) before and after carrageenan injection at intervals of 1, 2, 3, 4 and 5 h. Inflammation was quantified according to the method described by Planichamy (1990).

% Inhibition = A-B/A × 100

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

Evaluation of antinociceptive activity

a. Acetic acid induced writhing test in mice

Albino mice (*Balb-C*), weighing 18-22 g of either sex were used in this study. Food was withdrawn from animals 2 h before the start of experiments. Writhing behavior was induced by intraperitoneal administration of 1% acetic acid (10 ml/kg).The number of writhes (abdominal muscles contraction, accompanied by an elongation of the body and hind limbs extension) occurring over a period of 20 min were counted after 5 min of administration of 1% acetic acid (Abbas et al., 2011). For the experiments, the animals were randomly divided into four groups (I, II, III and IV), each group consisting of eight animals (n=8).

Group I: Control (untreated) group, received saline (i.p).
Group II: Standard treatment group (aspirin 15, 30 and 45 mg/kg i.p)
Group III: Test treatment group (Compound I 15, 30, and 45 mg/kg i.p)
Group IV: Test treatment group (Compound II 15, 30, and 45 mg/kg i.p)

The test samples (compound I and compound II), standard (aspirin) and the saline control were administered i.p, 30 min prior to 1% acetic acid administration. Saline control group received an equal volume of saline. Percent protection against nociception was calculated with the help of the following formula.

% Protection = (1 - Mean number of writhes of treated drug/ Mean number of writhes of control) × 100

b. Hot plate test (thermal) in mice

Albino mice (*Balb-C*), weighing 18-22 g of either sex were used in this study. Prior to the start of experimental procedure, mice were habituated to laboratory environment at least for two hours. A transparent glass cylinder was used to restrict the animal to the surface of hot plate of analgesiometer and the temperature maintained at 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 (Abbas et al., 2011). A cut-off time of 30 s was fixed so that if an animal did not respond within the prescribed time, then they could be immediately removed from the hot plate surface to avoid tissue damage. For the experiments, the animals were randomly divided into four groups (I, II, III and IV), each group consisting of eight animals (n=8).

Group I: Control (untreated) group, received saline (i.p).
Group II: Standard treatment group (Morphine 5 mg/kg i.p)
Group IV: Test treatment group (Compound I 30 and 45 mg/kg i.p)
Group V: Test treatment group (Compound II 30 and 45 mg/kg i.p)

Thirty minutes after the pretest, control, standard and test samples were administered to their respective groups intraperitonelly (i.p). An equal volume of saline was received by saline control group. The animals were tested again after thirty minutes and response was recorded at 30 and 60 min on the hot plate of analgesiometer. Antinociception was calculated using the following formula.

% Antinociception = (Test latency – control latency)/ (Cut-off time – control latency) × 100

Evaluation of antipyretic study *[Brewer's yeast induced pyrexia test in mice]*

In this study, we used albino mice (*Balb-C*), weighing 25-30 g of either sex. For the experiments, the animals were fasted overnight with free access to water. The animals were randomly divided into four groups (I, II, III and IV), each group consisting of eight animals (n=8).

Group I: Control (untreated) group, received, water for injection (i.p).
Group II: Standard treatment group (aspirin 50, 100 and 150 mg/kg i.p)
Group III: Test treatment group (Compound I 50, 100 and 150 mg/kg i.p)
Group IV: Test treatment group (Compound II 50, 100 and 150 mg/kg i.p)

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 (Al-Ghamdi, 2001). An equal volume of saline was received by saline control group. Changes in rectal temperature were noted after 24 h of Brewer's yeast injection at 0.5, 1.0, and 1.5 h (Barkatullah et al., 2011). For insertion, rectal 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

Molecular docking study was conducted using FRED 2.1 (Khan et al., 2011). Molecular structure of the test compounds were generated by multi-conformer library. In order to get accurate results, compounds were exhaustively docked/scored to assess all possible positions for each ligand in the binding site of COX-2 (PDB ID: 3PGH). The exhaustive and extensive search of best binding mode is based on rotations of rigid and translations of all conformers inside the binding pocket defined by a default box implement in FRED. The filtered poses combine as an ensemble by omitting the poses ones, which clash with target protein. The final poses are then ranked or re-scored employing scoring functions available in FRED. In this study, Shapegauss was selected to determine the matching the shape of each ligand with the binding pocket. Default FRED protocol was employed except for the size of the searching box defining the boundaries of binding sites. In order to optimize the docking and scoring performance, we performed exhaustive docking with shapegauss using the "Optimization" mode of FRED. The "Optimization" mode includes a systematic solid body optimization of the top ranked poses from the exhaustive docking. Three different boxes were explored for enzyme. Three different simulations were carried out with an added value of 8 Å around the reference ligand.

Molecular dynamics simulation

The molecular dynamics (MD) simulation was performed using MOE-dynamic implemented in MOE-2010.11 (www.chemcomp.com). The utilized data were enzyme-ligand complexes from the docking results. Before doing MD simulation, enzyme-ligand complexes were optimized with partial charge menu and energy minimization was carried out until RMS gradient 0.05. System was subsequently solvated with TIP3P water using cubic octahedron box extending to 7Å from the system and molecular dynamics were performed after that at 300 K. MD simulation was done by choosing MMFF94x force field and NVT (N, total atom; V, volume; T, temperature) ensemble with 0.002 ps time step and sampling every 0.5 ps. The other parameter was set on default value, which was ensemble NVT and NPA algorithm for creating ensemble trajectory. The simulation observation was done by examining the enzyme-ligand complex interaction between ligand atoms and enzyme atoms with the end of simulation (200 ps).

Solubility

Our synthetic compounds were checked for solubility pattern in different solvents including water according to descriptive terminologies of solubility (Vallender, 2009).

Statistical analysis

Data were computed for statistical analysis by using Graph Pad Prism Software, version 5, for multiple comparisons by one-way analysis of variance (ANOVA) with Dunnett's 't'test. Results were considered statistically significant at $p < 0.05$.

RESULTS

Anti-inflammatory activity of aspirin (standard), compound I [4-(2-hydroxybenzyl) morpholin-4-ium chloride] and compound II [1,4-bis (2-hydroxybenzyl) piperazine-1,4-diium chloride] in Carrageenan induced Paw edema model in mice

As depicted in Figure 5a, aspirin used as a standard, (Figure 5b) compound I and (Figure 5c) compound II, showed significant reduction in paw edema induced by carrageenan injection. One way ANOVA followed by Dunnett's post-hoc analysis revealed dose dependent decrease in paw edema. The effect was significant in standard aspirin, compound I and compound II treated groups at doses of 50 mg/kg (*p< 0.05), 100 mg/kg (**P<0.01) and 150 mg/kg (***P<0.001).

Antinociceptive activity of aspirin (standard), compound I [4-(2-hydroxybenzyl) morpholin-4-ium chloride] and compound II [1,4-bis (2-hydroxybenzyl) piperazine-1,4- diium chloride] in acetic acid induced writhing test in mice

As shown in Figure 6a (aspirin), 6b (compound I) and 6c (compound II), all the treated groups tested, showed significant reduction in acetic acid induced writhes. ANOVA followed by Dunnett's post-hoc analysis of aspirin and compound II revealed significant decrease in writhes at doses 15, 30 and 45 mg/kg (***P<0.001) while compound I exhibited dose dependent decrease in writhes at doses 15, 30 mg/kg (**P<0.01) and 45 mg/kg body weight (***P<0.001).

Antinociceptive activity of morphine (standard), compound I [4-(2-hydroxybenzyl) morpholin-4-ium chloride] and compound II [1,4-bis (2-hydroxybenzyl) piperazine-1,4-diium chloride] in Hot plate test (Thermally induced) in mice

As depicted in Figure 7a and 7b , morphine used as a standard at a dose level of 5 mg/Kg, significantly increase the latency time on hot plate while compound I and II failed to increase the latency time significantly. ANOVA followed by Dunnett's post-hoc analysis of morphine revealed increase in latency time (**P<0.01, ***P<0.001) while compound I and II failed to increase the latency time in the hot plate test.

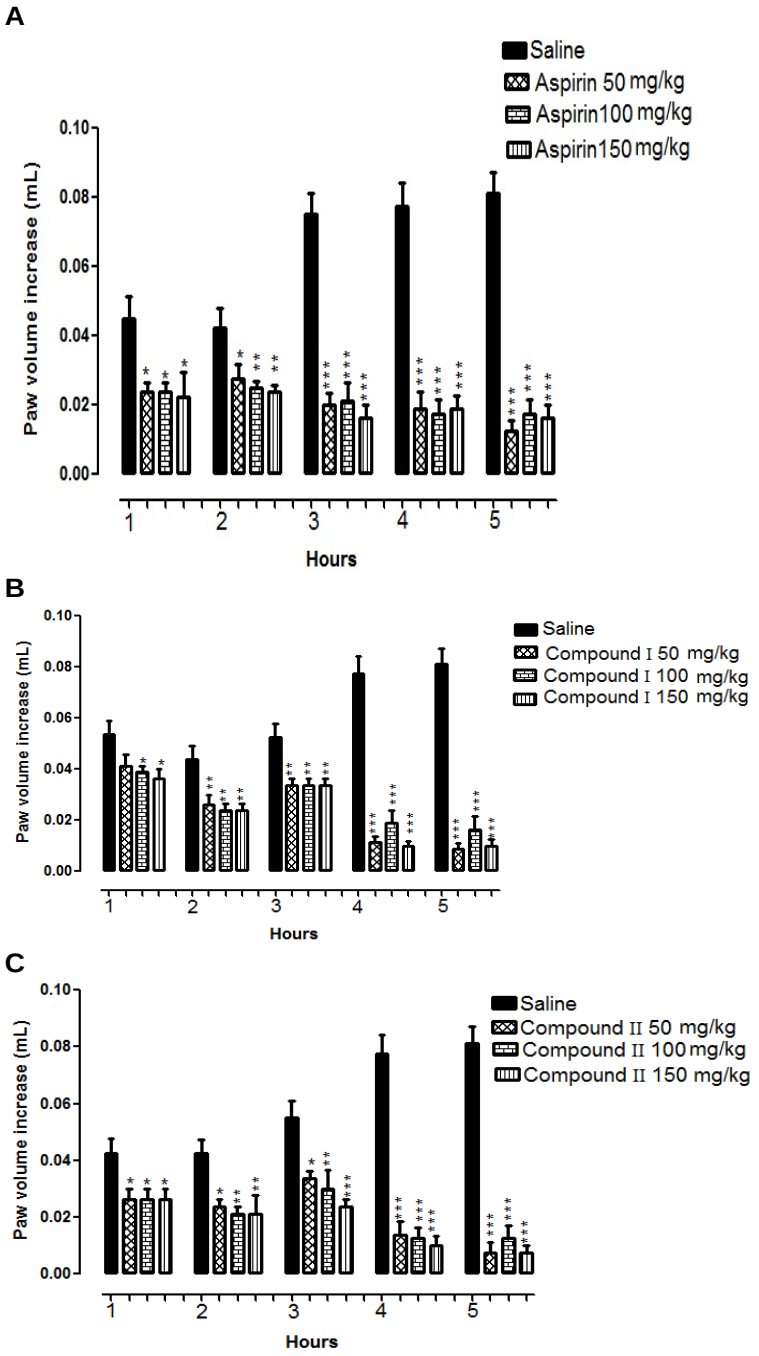

Figure 5. A, Anti-inflammatory activity of aspirin in Carrageenan induced paw edema test; **B,** Anti-inflammatory activity of compound I in Carrageenan induced paw edema test; C**,** Anti-inflammatory activity of compound II in Carrageenan induced paw edema test.

Antipyretic activity of aspirin (standard), compound I [4-(2-hydroxybenzyl) morpholin-4-ium chloride] and compound II [1,4-bis (2-hydroxybenzyl) piperazine-1,4-diium chloride] in Brewer's yeast induced pyrexia test in mice

As depicted in Figure 8a, aspirin used as a standard, Figure 8b) compound I and (Figure 8c) compound II, showed significant reduction in hyperpyrexia induced by Brewer's yeast. ANOVA followed by Dunnett's post-hoc analysis revealed dose dependent decrease in pyrexia. The effect was significant in standard aspirin, compound I and compound II treated groups at doses of 50 mg/kg (*p< 0.05), 100 mg/kg (**P<0.01) and 150 mg/kg (***P<0.001).

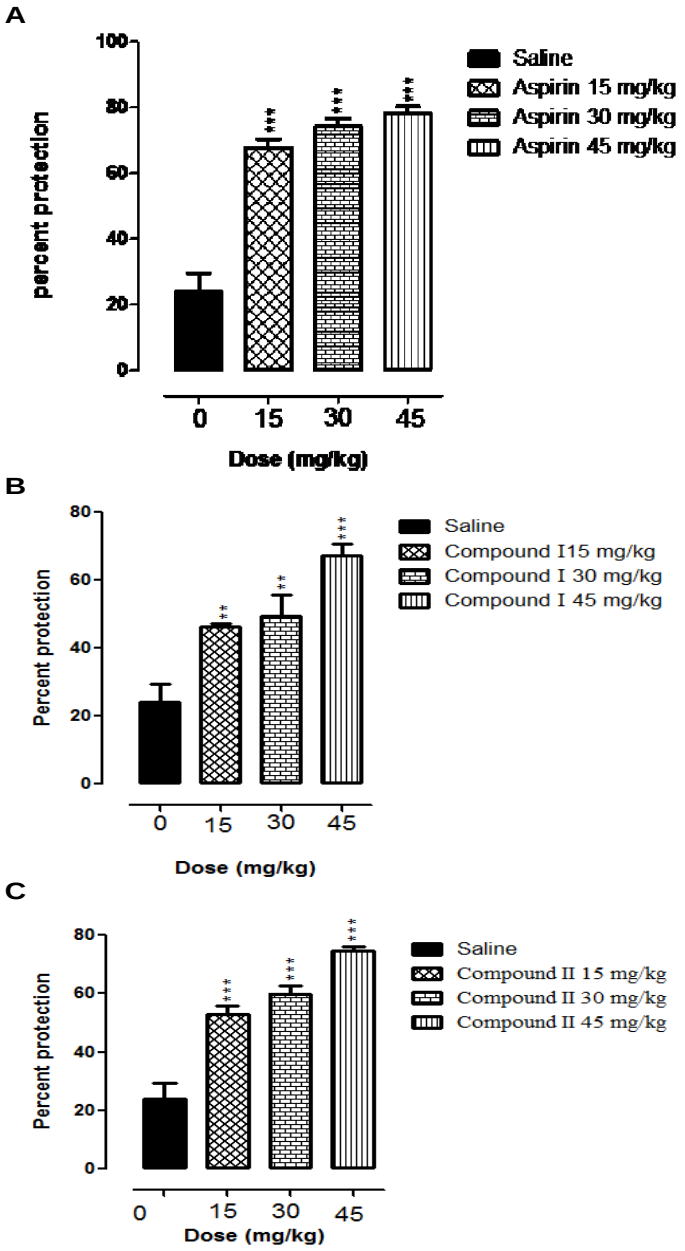

Figure 6. A, Antinociceptive activity of aspirin in acetic acid writhing test; B, Antinociceptive activity of compound I in acetic acid writhing test; C, Antinociceptive activity of compound II in acetic acid writhing test.

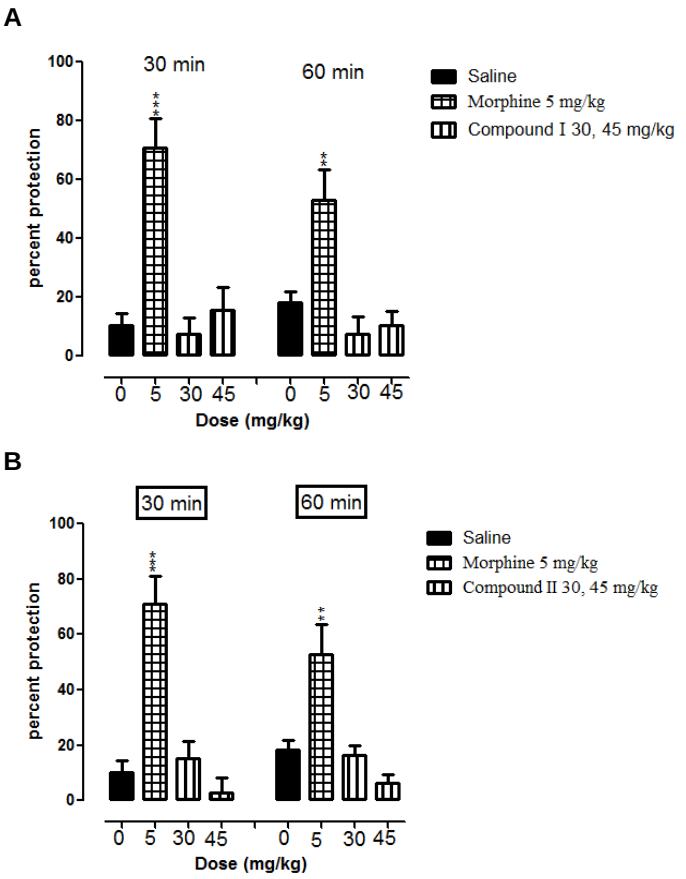

Figure 7. A, Antinociceptive activity of morphine and compound I in hot plate test; B, Antinociceptive activity of morphine and compound II in hot plate test.

Molecular docking simulation

After internal validation, molecular docking simulations of the test compounds were conducted (Khan et al., 2011). Both compound I [4-(2-hydroxybenzyl) morpholin-4-ium chloride] and compound II [1,4-bis (2-hydroxybenzyl) piperazine-1,4-diium chloride], showed considerable interactions (Figures 9 and 10) with the most important amino acid side chains (Arg120 and Tyr355), without any steric and electrostatic clash. Their comparable binding affinities with respect to NSAID (flurbiprofen) provided a strong clue for their significant anti-inflammatory and analgesic activity. The elongated, yet flexible skeleton of compound II showed considerable molecular interactions (Figure 10) with main binding pocket of the enzyme.

Molecular dynamics simulation

Molecular dynamics simulation was carried out using enzyme-ligand complexes which were produced from docking simulation. The utilized enzyme-ligand complexes were added with partial charges, optimized and energy minimized by using energy calculation (force field MMFF94x). The applied parameters were set to MOE default value, which were ensemble NVT, constant temperature 300K and 101kPa pressure. This parameter was useful as in real experiment; it is much easier to adjust temperature. The employed NPA Algorithm was the most accurate and sensitive algorithm and it could set up the ensemble correctly.

There are several ways to analyze MD simulation

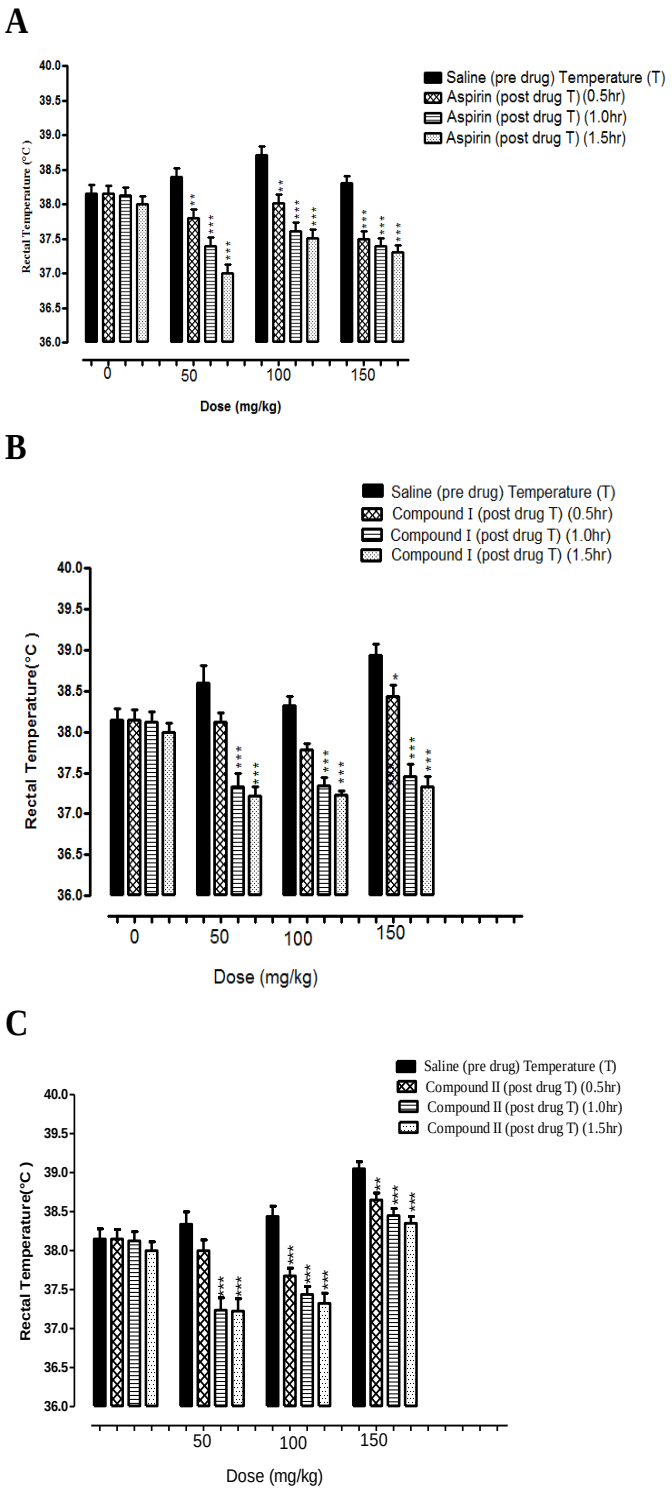

Figure 8. A, Antipyretic activity of aspirin in Brewer's yeast induced pyrexia test; B, Antipyretic activity of compound I in Brewer's yeast induced pyrexia test; C, Antipyretic activity of compound II in Brewer's yeast induced pyrexia test.

result. In this study we were concerned to review the total potential energy plot of complex conformation and ligand interaction to study the interaction between ligand and enzyme. Total potential energy plot could be used to

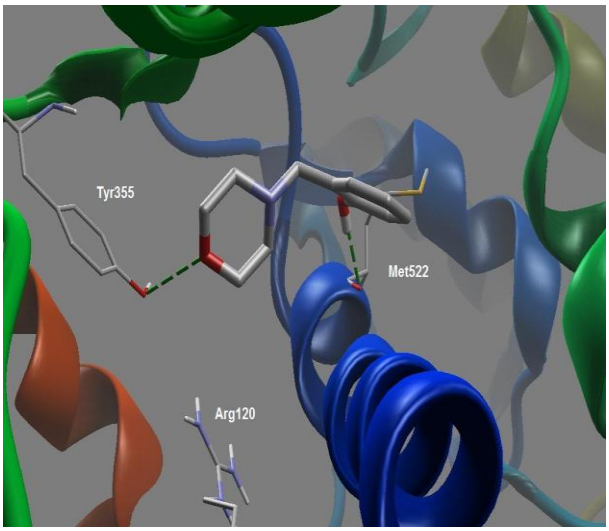

Figure 9. Molecular binding mode of Compound I [4-(2-hydroxybenzyl) morpholin-4-ium chloride] the inside main binding site of COX-2. Hydrogen bonding interactions are visible as green dotted lines. All hydrogen atoms except polar one are omitted for clarity.

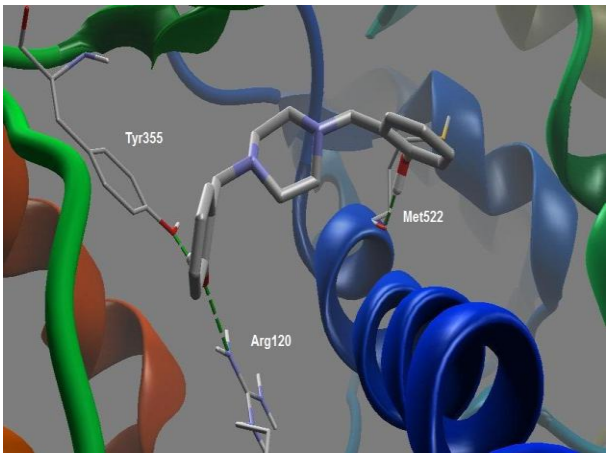

Figure 10. Molecular binding mode of compound II, [1,4-bis (2-hydroxybenzyl) piperazine-1,4-diium chloride], the inside main binding site of COX-2. Hydrogen bonding interactions are visible as green dotted lines. All hydrogen atoms except polar one are omitted for clarity.

overview system conformational changes during simulation. Any damage to enzyme structure such as denaturation will affect total potential energy plot. As shown in Figure 12, all system gave similar plot during simulation. It means that complexion with ligand did not damage enzyme's structure. Enzyme was stable with or without ligand complex on it.

Solubility

We have checked solubility of the synthesized compounds

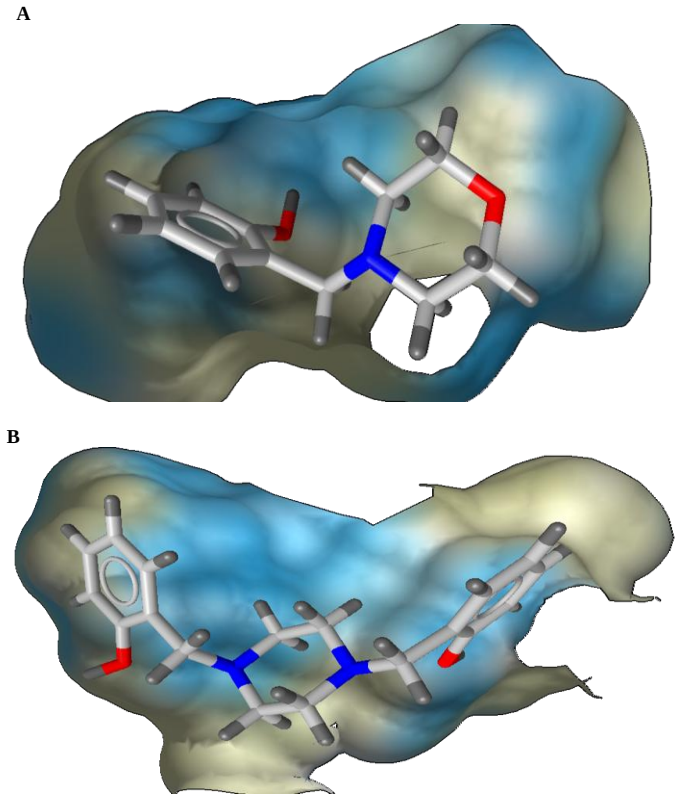

Figure 11. A and B, Hydrophobic (yellowish colored surface) and hydrophilic (blue colored surface) interactions between compound I [4-(2-hydroxybenzyl) morpholin-4-ium chloride], compound II [Compound II [1,4-bis (2-hydroxybenzyl) piperazine-1,4-diium chloride] and COX-2.

in different solvents according to the descriptive terminologies of solubility (Vallender, 2009). Due to the presence of ammonium moieties these compounds are freely soluble in water, methanol, Dimethyl formide (DMF) and Dimethyl sulfoxide (DMSO). The pH of aqueous solution of the compounds was determined to be approximately 7 (neutral).

DISCUSSION

Across the globe, medical management of pain and inflammation is still a major challenge for drug discovery researchers and clinicians with an economic impact of billion annually (Wilhelm et al., 2009). It has been reported that pain, inflammation and pyrexia share common pathways in their development and mediation and often occur simultaneously. The pain receptors are activated by mechanical, chemical, thermal or other stimuli. After injury, histamine, serotonin, bradykinin, prostaglandin and other mediators are released. Histamine takes part in early stage of inflammation. Serotonin and bradykinin activates pain receptors while prostaglandins produce fever and increase pain sensation (Davies et al., 1984). Therefore, drugs that inhibit these mediators act as analgesic, anti-inflammatory and antipyretic. The most widely accepted pathway involved in mediation of pain, inflammation and pyrexia is the cyclooxygenase pathway (Samad et al., 2002). Treatment strategies for these disorders involves inhibiting the enzymes; Cyclooxygenase-1 (COX-1), cyclooxygenase-11 (COX-11) and probably cyclooxygenase-111 (COX-111). In this respect, NSAIDs such as aspirin, flurbiprofen etc; the inhibitors of COX enzymes are in general practice for the clinical management of inflammation, pain and pyrexia (Steinmeyer, 2000). However, major serious limitation associated with chronic use of NSAIDs is the development of GIT disorders like ulceration, perforation and bleeding (Singh and Rosen Ramey, 1998), renal disorder (Bao et al., 2012), CVS disorders (David and Richard, 2005) and pulmonary distress (Camus, 1997), which arise due to their non selective inhibition of COX enzymes (Tapiero et al., 2002) and partly due to acidic nature of these drugs responsible for their local erosion properties. Among NSAIDs, aspirin is regarded a proto-typical drug and widely used despite of adverse effects.

This study aimed to evaluate *in vivo* and compare with aspirin, the derivatives bearing salicyl alcohol attached to morpholine and piprazine rings [4-(2-hydroxybenzyl) morpholin-4-ium chloride], [1,4-bis(2-hydroxybenzyl) piperazine-1,4-diium chloride] for selective pharmacological activities and molecular docking simulation studies.

Carrageenan-induced paw edema method (Winter et al., 1962) is widely used for testing anti-inflammatory activity of drugs. Inflammatory process proceed in two stages in rodents, the first stage, almost 1 h is associated with activity of biogenic amines, the second stage involves eicosanoide actions while the vasodilators peptides released in the process, maintain connection in the two stages (Vinegar et al., 1969). It has been demonstrated that the anti-edema effect of anti-inflammatory agents results due to inhibition of the cyclooxygenase (Cryer and Feldman, 1998) or lipooxygenase (De Simone et al., 2011) pathways, although such claim require further investigation. In this study, the anti-nflammatory activity of compound (I) and (II) and standard aspirin were tested in the carrageenan induced paw edema model. It was found that the compounds were significantly effective in reducing edema comparable to standard aspirin at doses 50, 100 and 150 mg/kg at 1, 2, 3, 4, and 5 h interval after carrageenan injection (Figure 5a, b, c). Acetic acid induced writhing and hot plate tests, were employed to study the anti-nociceptive activity of the compounds. Finding of this study indicated that both of the synthesized compounds (15, 30, 45 mg/kg) were effective in significantly ameliorating pain induced by acetic acid injected into the abdominal cavity but failed to show statistically significant effect in the thermally induced pain (Figures 6a, b, c and 7a, b). Basically, writhing test is used to evaluate both peripheral and central antinociceptive activity (Trongsakul et

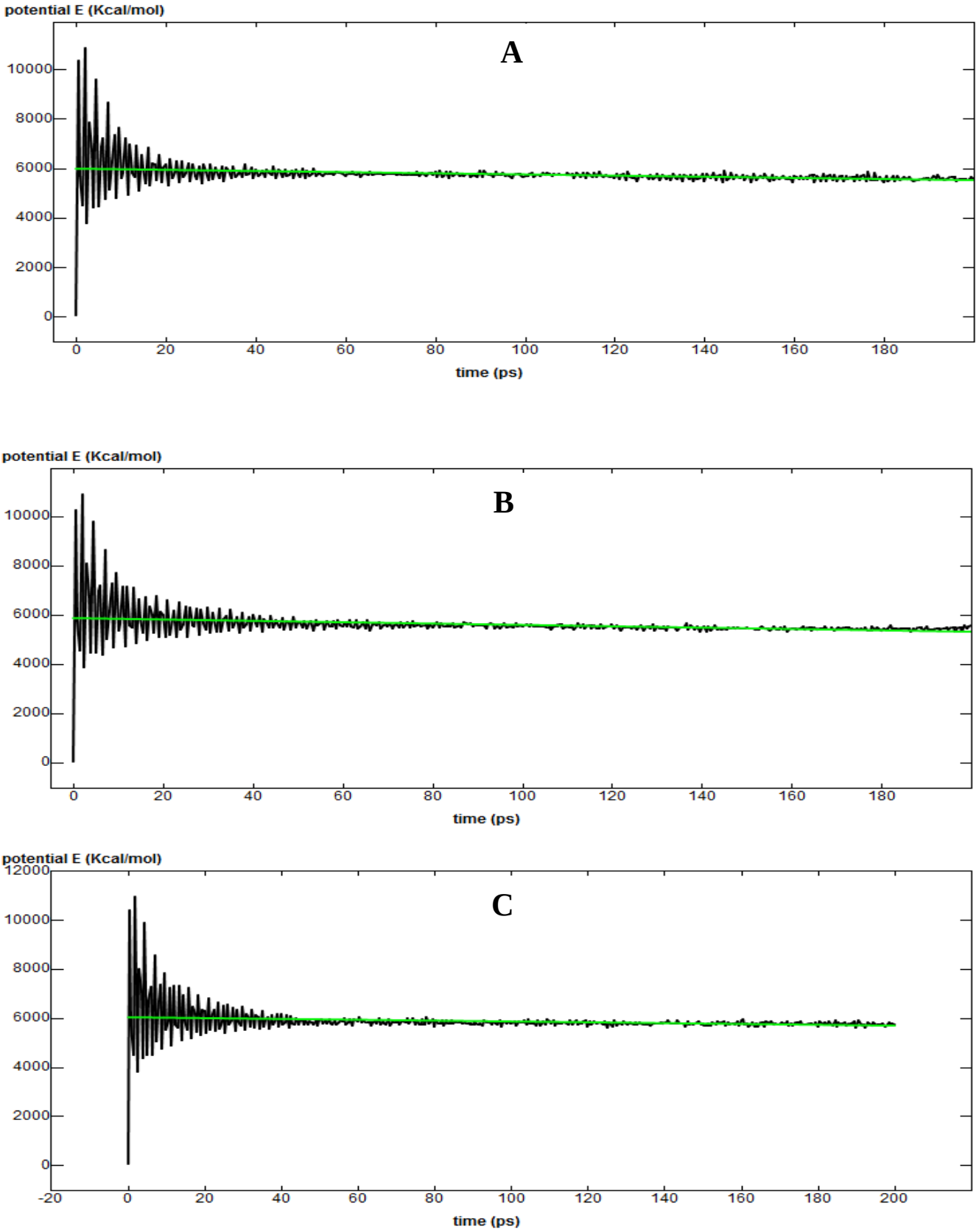

Figure 12. Total potential energy plot during MD simulation.
(A) Enzyme without ligand; (B) Enzyme complex with compound I; (C) Enzyme complex with compound II.

al., 2003).This test is very sen-sitive but it is not specific, so one cannot surely interpret that the activity is centrally or peripherally mediated (Chen et al., 1995). Accordingly, we tested our com-pounds (30, 45 mg/kg) using hot plate analgesia model that is suitable for centrally acting agents (Hosseinzadeh and Younesi, 2002). Our results clearly showed that the compounds were effective in acetic acid induced writhing test but ineffective in hot plate test thus implicating peripheral mechanisms in the antinociceptive effect like NSAIDs.

Brewer yeast induced pyrexia is considered as pathogenic and release prostaglandin to disturb the thermoregulatory center (Hullati and Sharada, 2007). In this study, we examined the antipyretic activity of these compounds using Brewer's yeast induced pyrexia model in mice. Our findings suggest significant antipyretic activity comparable to that of aspirin (Figure 8a, b, c). From our findings, it appears that these compounds may have inhibitory effect on the mediators of pyrexia in-cluding prostaglandin-biosynthesis, as prostaglandins are one of important regulators of pyrexia (Ranels and Griffin, 2003).

Accordingly, we carried out molecular modeling simulation study to see the binding mode of our synthetic compounds with cycloxygenase enzymes employing FRED 2.1 package (Khan et al., 2011). Molecular docking simulate and facilitate the study of binding of drug to a specific site of protein in the receptor of interest and thus signifying the best-fit orientation of ligand and rational drug design (Rowlinson et al., 2003). In-fact, COX-2 is one of the major enzymes involved in inflammation due to biosynthesis of prostaglandins (PGs) (Khan et al., 2011). Heme is involved in catalysis of PGs biosynthesis. Any substrate can only access the catalytic site (heme) via passing through a narrow pocket sur-rounded by the important amino acid residues especially Arg120 and Tyr355. These two amino acids play a central part in designing or virtual screening for discovery of new COX inhibitors. In this study, flurbiprofen was used as a reference and co-crystallized ligand, to validate the accuracy of binding mode and binding affinity (in terms of binding energy in Kcal/mol) of our compounds. Best scoring conformation of flurbiprofen was found very close to the experimental conformation (RMSD value: 0.453). In case of compound I [4-(2-hydroxybenzyl) morpholin-4-ium chloride], oxygen of morpholyl group (Figure 9) revealed hydrogen bonding with Tyr355 (at a distance of 3.55°A). However a weak dipole-dipole interaction was observed between the cyclic oxygen with Arg120 but no hydrogen was detected. Interestingly, the same compound I, was found in favorable contact with O (oxygen) of Met522 (2.94°A) via phenolic moiety. Surface area of the main binding pocket of COX-2 is generally polar in nature while hydrophobic regions (based on surrounding alkyl and aryl side chains) were also present. In both compounds, no unwanted hydrophobic-hydrophilic interactions were found (Figure 11; A and B), which supported our experimental activity.

The combined favorable interactions based on dipole-dipole interactions and hydrogen bonding could be the major reason behind its experimental effect on inflame-mation and peripheral pain. The elongated yet flexible skeleton of compound II [1,4-bis (2-hydroxybenzyl) piperazine-1,4-diium chloride] showed considerable molecular interactions (Figure 10) with main binding pocket of the enzyme. Phenolic groups on both ends of the compound II revealed major role in the strong binding affinity for COX-2. On outer side of the binding pocket, phenolic group showed the same role as played by the carboxylic moiety of co-crystallized flurbiprofen. Phenolic group anchored both Arg120 and Tyr355 via hydrogen bonding interactions at 2.468°A and 2.75°A, respectively. On the other hand, the second phenolic group strongly held the O (oxygen) of Met522 using hydrogen bonding at a distance of 2.60°A. However, piperazine ring played a role of spacer group without showing any polar interactions. Molecular docking simulation studies have revealed special hydrogen bonding interactions with amino acid residue of COX-2, like Arg120 and Tyr355, thus implicating prostaglandin pathway with COX activity. Techniques which are frequently considered in "rational drug designing" include structure based designing, ligand based designing and De-Novo drug design (França et al., 2006). Structure based designing is the most consistent and prevailing practice for "lead compound" than others and involves explication of three dimensional (3D) structure of the target protein or macromolecular inside or outside the cell (receptors, enzymes, ion channels, DNA, RNA etc) (Tomlinson et al., 2009). The structure of target protein can be obtained by different methods. X-ray crystallography is the most accurate and unswerving source for the structure of target protein. However crystallization of isolated and purified target protein is a restraining factor. Comparative protein modeling study involves receptors or proteins of same family are modeled by the using their protein sequences and converting the primary protein sequence structures to the 3D secondary and tertiary structures following the pattern of related protein of known X-ray structure. Molecular docking and molecular dynamic simulations (MD-simulations) approach is used for the preliminary screening of molecules to be developed as potential drugs (Alonso et al., 2006).

Molecular dynamic simulations (MD-simulations) is a well-known approach to examine structural and dynamic features (molecular motion due to intra or inter molecular interactions and external stress) of proteins. Compound to be screened is docked in the active site for evaluation of its binding mode. Scoring of various binding modes is performed by various algorithms (methods) in terms of binding energy (that is Kj/mole). Molecules with lowest binding energy are selected while others are rejected. Libraries of compounds can be screened in a day using super computers. Selected molecules are subjected to pharmacological studies including in-*vivo* (Tambunan et al., 2011).

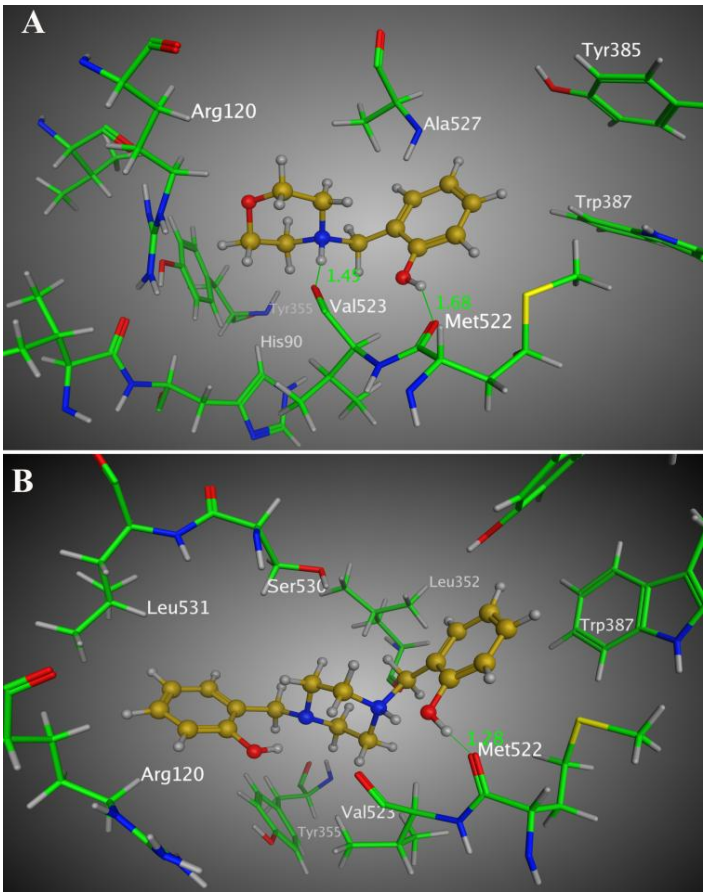

Figure 13. Ligand interaction after MD simulation COX-2 enzyme with (A) Compound I; (B) Compound II.

MD simulation, a number of possible complex conformations were being examined and we could assume that the last conformation was the best conformation. In Figure 13, the observation of MD simulation toward both ligands showed that both ligands had interaction with important active site residues of the COX-2 enzyme. We can see from Figure 13; A and B, that there were changes in ligand's orientation during MD simulation. These changes movement of enzyme.

After MD simulation phase, compound I showed two hydrogen bonds with active site residues of COX-2 enzyme (Figure 13A). Compound I bind with two important residues of COX-2 enzyme, which were Met 522 and Val 523. Compound II showed a little bit different result. Its hydrogen bond's to only one active site residue Met 522 (Figure 13B). Moreover, compounds, having ammonium moieties that are freely soluble in water and other solvents and having neutral characteristics, thus making them suitable for oral and parenteral formulations. In contrast, aspirin has low water solubility and highly acidic nature that impart it cellular erosive and ulcerogenic properties.

In summary, this study evaluates the anti-inflammatory, antinociceptive, antipyretic activities and molecular docking and dynamic studies of salicyl alcohol derivatives. Our findings indicate that these compounds possess significant antinociceptive, anti-inflammatory and antipyretics activities comparable to aspirin as supported by in-vivo animal and molecular docking and dynamic simulation studies. Moreover, compounds are freely water soluble with neutral properties thus making them suitable for oral liquid and parenteral formulations. However, further studies are warranted to find out any potential gastric ulcerogenic properties in comparison to aspirin to confirm their GIT tolerability.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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