Ibuprofen is a commercially available and widely used non-steroidal anti-inflammatory drug being used in managing inflammatory disorders worldwide. This acid molecule has been subjected to esterification by coupling with geraniol as well as to amide formation by coupling with furfurylamine and isoamylamine for observing the changes in anti-inflammatory potential due to the addition of relatively long aliphatic chains. During these reactions, the reaction yields were within 88-94%. In this in vitro evaluation, the prevention of egg albumin denaturation and human red blood cell (HRBC) membrane stabilization were observed. For observing potential for preventing egg albumin denaturation, the synthesized compounds were applied as doses of 10 μg/ml, 20 μg/ml, 30 μg/ml, and 40 μg/ml. For observing the better response from ester derivative, 50 μg/ml, 100 μg/ml and 200 μg/ml doses were also tried. For observing the Human RBC membrane stabilization with amides, 50 μg/ml and 100 μg/ml doses were tried. Ibuprofen was taken as the reference compound and was applied in same doses in all of the experiments. The anti-inflammatory potential of the synthesized geranyl ester was remarkable in comparatively higher doses (16%, 45%, and 54% reduction in protein denaturation from 50 μg/ml, 100 μg/ml, and 200 μg/ml doses respectively). Isoamylamide was relatively more potent than geranyl ester in preventing egg albumin denaturation (reductions were 9%, 13%, 17%, and 31% from 10, 20, 30, and 40 μg/ml doses respectively). Furfurylamide was more potent than isoamylamide by showing 9%, 17%, 31%, and 46% inhibitions respectively. There were dose-dependent anti-inflammatory actions observed from both the geranyl ester and amides. The synthesized compounds were also subjected to the in silico study for observing the ligand-receptor interactions in the binding site of the cyclooxygenase enzyme.

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and various events, such as, increased protein denaturation, increased vascular permeability and membrane alteration \(^2,^3\).

Ibuprofen (1A) (Figure 1), a propionic acid type non-steroidal antiinflammatory drug (NSAID), is used broadly in treating various inflammatory disorders, like, arthritis, febrile illnesses and injuries. This is also a drug of choice in postoperative pains \(^4,^5\). This compound is a carboxylic acid itself and thereby has the capacity to cause hyperacidity in the gastrointestinal tract. Being a non-selective cyclooxygenase inhibitor, it may cause increased acid secretion and gastrointestinal bleeding thereby limiting the prolonged uses in managing chronic inflammatory disorders. It undergoes rapid biotransformation resulting in a serum half-life of only 1.8 to 2 hours. Thus this drug is completely eliminated within 24 hours and demands multiple daily doses \(^6\).

![Figure 1. Molecule structure of Ibuprofen](image)

Lipophilicity is a critical factor in drug discovery and development since it has vital role in determining pharmacokinetic properties of drug candidates. There are evidences suggesting necessity of controlling lipophilicity within a defined optimal range to improve chances of therapeutic success \(^7\). In case of ibuprofen, the short duration of action might be correlated with its low lipophilicity resulting from small logP value (calculated 3.72 by using ChemSketch v. 14.50). Thus esterification, especially by coupling with alcohols having non-polar chains, might increase the logP value resulting in increased duration of action. Similar changes can be expected by converting the free acid to the corresponding amide by using amines bearing relatively longer non-polar chains.

Geraniol (B), an alcohol having longer aliphatic chain, has been reported to possess diverse biological activities \(^8,^14\). Geraniol esters also have been reported to have pharmacological activities \(^9,^15\). Based on this idea, esterification with geraniol (1B) (Figure 2) have been tried in this study to get the new ester (01) of ibuprofen. With similar concepts, the furfurylamine (1C) and isoamylamine (1D) (Figure 2) were chosen for further derivatization.

![Figure 2. Molecule structure of Geraniol (1B), furfurylamine (1C) and isoamylamine (1D)](image)

Additionally, the desired ester (01) (Figure 3) has been calculated to have the logP value of 8.13 as predicted by ChemSketch, whereas, the amides (02 and 03) (Figure 3) were found to have the logP values of 3.74 and 4.17 respectively. The possible variations in the pharmacokinetic profile due to this varied logP values also gave us interests for observing the antiinflammatory potential and subsequent in vitro analysis for the interactions in binding site of cyclooxygenase enzyme.
2. Materials and Methods

2.1. Materials:

The necessary chemicals, reagents and catalysts were mainly collected from TCI and Sigma-Aldrich. Commercially available ibuprofen was collected from ACI Pharmaceuticals, Bangladesh, for this research project. The solvents used were from Duksan Pure Chemicals Co. Ltd and Daejung Chemicals, South Korea. Nuclear magnetic resonance (NMR) spectra were recorded by using Bruker 400 MHz NMR spectroscopy available in Bangladesh Council of Scientific and Industrial Research (BCSIR), Dhaka, Bangladesh. HRMS data were collected from Japan by using ion mode FAB+ and acetone as solvent.

2.2. Synthesis of the geranyl ester of ibuprofen

The mixture of ibuprofen in thionyl chloride was refluxed for 1 hour (Figure 4). Excess thionyl was removed under rotary and the residue was suspended in dry dichloromethane. To the resultant suspension was added triethylamine (1.2 eq) under ice-bath system. Then, geraniol (1.1 eq.) was added slowly and the mixture was stirred at room temperature. At the end of the reaction, the solvent was removed by rotary and to the residue were added water and then ethyl acetate. The organic layer was collected, washed with brine and dried by using anhydrous sodium sulfate. Solvent was removed by rotary evaporator and the crude ester thus obtained was purified by flash column chromatography (94% yield).

2.3. Amide coupling of ibuprofen with furfurylamine and isoamylamine

As shown in Figure 5, thionyl chloride was added to ibuprofen and the mixture was refluxed for 1 hour. The excess thionyl was removed under rotary and the residue was suspended in dry dichloromethane. To the suspension was then added triethylamine (1.2 eq) under ice-bath system. Desired amine (1.1 eq.) was added slowly and the mixture was stirred at room temperature. At the end of the reaction, solvent was removed by rotary and to the residue were added water and ethyl acetate. The organic layer was collected, washed with 0.1 N HCl and brine and then was dried by anhydrous sodium sulfate. Solvent was removed under vacuum. The crude amide thus obtained were purified by flash column chromatography (88% yield).

Figure 3. Ester (01), Amides (02) and (03)

Figure 4. Synthesis scheme of geranyl ester (01) of ibuprofen

Figure 4. Synthesis scheme of geranyl ester (01) of ibuprofen
2.4. Observation of membrane stabilization effect on heat induced hemolysis

2.4.1. Preparation of Human Red Blood Cell (HRBC) suspension

Fresh whole human blood (10 ml) was mixed with heparin in centrifuge tube. The blood mix was then centrifuged at 3000 rpm for 10 min. Subsequently, the supernatant was discarded and the residue was washed with equal volume of normal saline. The washing was repeated for three times. The residual blood was collected and reconstituted as 10% v/v RBC suspension with normal saline for next applications.

2.4.2. Observation of heat induced hemolysis

For observing the membrane stabilization, the reported method \cite{16} method was applied with minor modifications. Isotonic buffer solution (5 mL each) was taken in centrifuge tubes containing 200 and 400 μg/mL of test drugs. The experiments were run by taking 6 sets for each concentration. Tubes containing 5 mL of the vehicle, but no test drug, were taken as negative control for this study. Ibuprofen was taken as reference standard and were taken as 2 sets containing 100 μg/mL of ibuprofen in 5 mL of solution. To each of the centrifuge tubes, were added 0.05 mL of RBC suspensions. AMer gentle mixing, one set of the various types of tubes were incubated at 54°C for 20 minutes and the remaining sets were maintained at 0–4°C for 20 minutes. At the end of heating, the centrifuge tubes were cooled by using water bath. The reaction mixtures were subsequently centrifuged at 3,000 rpm for 3 minutes and then the supernatants were collected for measuring absorbance (OD) at 540 nm using isotonic buffer solution as the blank. Analysis was done by the following two equations -

\[
\text{Percentage of hemolysis} = \frac{100 \times (\text{OD}_2-\text{OD}_1)}{(\text{OD}_3- \text{OD}_1)}
\]

Where, \(\text{OD}_1\) = absorbance of test sample unheated; \(\text{OD}_2\) = absorbance of test sample heated; \(\text{OD}_3\) = absorbance of control sample heated.

\[
\text{Percentage inhibition of hemolysis (\%)} = 100 - \text{hemolysis (\%)}
\]

2.5. Inhibition of protein denaturation:

For observing the inhibitory potential against the protein denaturation, the reported method \cite{17} has been applied with minor modifications. In this case, 0.2 ml of egg albumin was taken and 2.8 ml of phosphate buffer saline of pH 6.4 was added. Subsequently, 2 ml of varying concentrations of test compounds were added to these mixtures. Similar mixtures were made by replacing the test compound with ibuprofen to use as the reference positive controls. Similarly, phosphate buffer solution, without test compounds, was considered as the negative control. Denaturation value observed from these blank solutions were considered to represent 100% denaturation. The resultant
mixtures were incubated at 37ºC for 15 min and then was heated at 70ºC for additional 5 minutes. The test procedures were repeated for 5 times. After cooling the mixtures under tap water, absorbance values were recorded at 660 nm. The percentage inhibition of denaturation was represented by the absorbance values and were calculated by the following formula:

\[ \text{Percentage inhibition of denaturation} = 100 \times \left( \frac{OD1}{OD2} - 1 \right) \]

Where, \( OD1 \) = absorbance of the test sample, \( OD2 \) = absorbance of the control

2.5. In silico analysis

2.5.1. Protein preparation:

The published \(^{[18]}\) PDB entry 4PH9, having ibuprofen as the ligand, was chosen for this study. This ligand, as well as, other heteroatoms were first removed by using the Notepad editor to get the free protein molecule for docking activities. The protein PDB file was then converted to the corresponding PDBQT file format by using the Autodock Tools (version-1.5.6). Similarly, structures of the synthesized compounds drawn by ChemDraw (Version 12.0) were converted to the corresponding PDBQT files. Finally, these PDBQT files were taken for the docking \(^{[19]}\) by Autodock Vina (version-1.1.2).

2.5.2. Ligand preparation:

The structures of the desired molecules were first drawn in ChemDraw (Version-12.0). Subsequently, these structures were saved as PDB files. In the final stage, the PDB structures were saved as the PDBQT by applying the Autodock Tools (version 1.5.7). Subsequently the PDBQT files were taken for docking studies with the Autodock Vina.

2.5.3. Docking by using Autodock Vina:

Docking analysis was done by using Autodock Vina for predicting the protein-ligand interactions. For this purpose the Autodock Tools (Version-1.5.7) was applied. The pocket size \((X, Y, Z = 12 \, \text{Å}, 10 \, \text{Å} \text{and} \, 8 \, \text{Å} \text{respectively})\) and center \((x, y, z = 13.5, 23.0, 25 \text{respectively})\) were selected based on the space occupied by the reported ligand, ibuprofen, in the enzyme protein. The conformations having the lowest energy (highest affinities) were considered for analyzing the binding interactions.

3. Results and Discussion

3.1. Synthesis of desired compounds

\(^1\)H NMR spectra of (E)-3,7-dimethylocta-2,6-dien-1-yl 2-(4-isobutylphenyl) propanoate (01):

\[(400 \text{ MHz, CDCl}_3) \delta 0.88 (m, 9H), 1.35 (m, 14H), 1.70 (m, 2H), 4.25 (m, 5H), 7.55 (m, 2H), 7.73 (m, 2H).\]

\(^1\)H NMR spectra of N-(furan-2-ylmethyl)-2-(4-isobutylphenyl) propanamide (02):

\[(400 \text{ MHz, CDCl}_3) \delta 7.09-7.28 (m, 4H), 6.25 (s, 1H), 6.07 (s, 1H), 5.63 (br s, 1H), 4.30-4.43 (m, 2H), 3.55 (m, 1H), 2.43-2.45 (m, 3H), 1.80-1.87 (m, 1H), 0.82-0.89 (m, 6H). \text{HRMS observed m/z M}+1: 286.1802 \text{ (calculated actual mass, M: 285.1729). Elemental composition: M}+1: C_{18}H_{24}O_2N.\]

\(^1\)H NMR spectra of 2-(4-isobutylphenyl)-N-isopentylpropanamide (03):

\[(400 \text{ MHz, CDCl}_3) \delta 7.09-7.18 (m, 4H), 5.25 (br s, 1H), 3.47-3.65 (m, 1H), 3.15-3.20 (m, 2H), 2.43-2.45 (m, 2H), 1.84 (m, 1H), 1.40-1.51 (m, 3H), 1.23-1.29 (m, 2H), 0.81-0.89 (m, 12H). \text{HRMS observed m/z M}+1: 276.2331 \text{ (calculated actual mass, M: 275.2249). Elemental composition: M}+1: C_{18}H_{30}NO.\]

3.2. Furfurylamide and isoamylamide of ibuprofen in preventing denaturation of egg albumin

For observing the \textit{in vitro} antiinflammatory activity, the prevention of denaturation of egg albumin was observed, since it has been reported to be correlated to the antiinflammatory activity. There were four different doses \((10, 20, 30, 40 \, \mu\text{g/mL})\) applied to observe the dosed relationship.
The isomylamide showed 9%, 13%, 17% and 31% prevention of the denaturation of the egg albumin respectively. But, the furfurylamide showed relatively higher rates of preventions (9%, 17%, 31% and 46% respectively). Interestingly, both the compounds offered dose-response relationships similar to the reference compound, ibuprofen. However, both of the amides showed reduced antiinflammatory activity as compared to the parent ibuprofen. The reductions were of comparatively low intensities when higher doses applied (Figure 6).

![Figure 6. Prevention Graph of egg albumin denaturation by isomylamide and furfurylamide of ibuprofen](image)

### 3.2. Geranyl ester in preventing denaturation of egg albumin

The observations from the *in vitro* antiinflammatory activity from the amides were further extended by trying with geranyl ester of ibuprofen. While applied 10, 20, 30, 40 μg/mL doses, as used for the amides in the earlier study, there was found no remarkable antiinflammatory activity. So larger doses were tried for these long chain ester derivative as shown in graph 2. In this case, the activity was found to be significantly reduced by the esterification when compared to parent ibuprofen. At the lowest dose applied, there was nearly 75% reduction of the antiinflammatory action because of this molecular modification. Interestingly, the reduction of the antiinflammatory activity was low in cases of higher doses applied (Figure 7).

![Figure 7. Prevention Graph of albumin denaturation by geranyl ester of ibuprofen](image)

### 3.4. Isoamylamide and furfurylamide in Human RBC membrane stabilization

The amides were taken for the observation of the *in vitro* antiinflammatory activity by using Human RBC membrane stabilization method for further confirmation. In this case, observation was made by using 50 μg/mL and 100 μg/mL doses. The isomylamide was found to be relatively more potent in both the applied doses as observed from the membrane stabilization of 55% and 68% from
50 μg/mL and 100 μg/mL doses respectively (Figure 8). Though there was significantly reduced antinflammatory potential from the amides as when compared to parent acid, there was dose dependent membrane stabilization in this study.

![Figure 8. Graph of Membrane stabilization by isoamylamide and furfurylamide of ibuprofen](image)

3.5. Molecular Docking Analysis

In the docking studies, the synthesized amides, 2-(4-isobutylphenyl)-N-isopentylpropanamide (02) and N-(furan-2-ylmethyl)-2-(4-isobutylphenyl)propanamide (03) were taking various orientations in the reported ibuprofen binding site of cyclooxygenase-2 enzyme. Among the observed possible orientations, the binding modes with the lowest energy were taken for making a comparative analysis. The N-isopentenyl group of compound 03 was projected to the site occupied by the isobutyl group of ibuprofen, whereas, the isobutyl group was projected to another non-polar gap surrounded by VAL117, LEU360 and LEU532 (Figure 1). It can be observed that this orientation was comparable to the reported orientation of ibuprofen (purple color) as given in Figure 9. On the other hand, the compound 02 was taking the orientations projecting its furanyl methyl group to the non-polar gap. Both the 02 and 03 were projecting the methyl group to the relatively non-polar position close to VAL524. While comparing with the orientation of ibuprofen, the polar acid function was projected to a site surrounded by polar residues, ARG121 and TYR365.
Both the amides 02 and 03, as shown in the figure 9, has taken orientations similar to the orientation of the reported ibuprofen in the binding site of cyclooxygenase-2 (COX-2) enzyme. However, because of slight changes in the orientations some interactions appeared to be different. While comparing with the reported molecule, the ARG120, TYR385 and SER530 have been reported to be involved in useful ligand-receptor, whereas the test compounds were closer to VAL117, LEU360 and LEU532, as shown in figure-1. These changes in interactions which might have contributed to the reduction in the antiinflammatory potentials of the synthesized compounds.

4. Conclusion

The furfurylamide and isoamylamide of ibuprofen were showing the dose dependent antiinflammatory activity in the in vitro study, though the potency was significantly reduced as compared to the parent acid. The reduction was found to be highest in case of geranyl ester and this might have been resulted from the introduction of relatively non-polar aliphatic chain of the geranyl alcohol. The amides were found to be relatively more potent as compared to the ester derivative.

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