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Molecular Docking and *In Vivo* Screening of Some Bioactive Phenoxyacetanilide Derivatives as Potent Non-Steroidal Anti-Inflammatory Drugs

Rajib Kumar Singh[1*], Arun Kumar Mishra[1], Pranesh Kumar[2], Debarshi Kar Mahapatra[3]

[1]Drug Design Laboratory, Faculty of Pharmacy, IFTM University, Moradabad 244001, Uttar Pradesh, India; [2]Department of Pharmacology, Aryakul College of Pharmacy & Research, Lucknow 226002, Uttar Pradesh, India; [3]Department of Pharmaceutical Chemistry, Dadasaheb Balpande College of Pharmacy, Nagpur 440037, Maharashtra, India.

ABSTRACT

Introduction: Cyclooxygenases (COXs) are the enzymes that specifically influence the production (minute quantities) of prostaglandins and their derivatives, which are responsible for discomfort, inflammation, and other symptoms. The study aimed to develop certain new phenoxyacetanilide derivatives in multi-step synthesis and to screen their anti-inflammatory and analgesic perspectives along with docking studies against main inflammatory target COX-2 enzyme as well as enzyme-linked immunosorbent assay (ELISA) analysis against two prominent inflammatory mediators; Interleukin-6 (IL-6) and COX-2 enzyme.

Methods: The newly synthesized substances were developed through multi-step schemes and were analyzed thoroughly employing advanced analytical techniques such as Fourier-Transformed Infrared Spectroscopy (FT-IR), Proton-Nuclear Magnetic Resonance Spectroscopy (^{1}H-NMR), Mass spectroscopy, and Elemental Analysis. The possibility of a binding site and binding strength of new acetanilide derivatives were discovered using a molecular docking study against COX-2 enzyme using AutoDock Vina software. All of the synthesized derivatives were tested for analgesic activity (using Eddy's hot plate method) and anti-inflammatory effects (employing the carrageenan-induced paw edema method).

Results: As compared to the standard drug diclofenac sodium, (2-[2-methoxy-4-(prop-2-en-1-yl)phenoxy]-*N*-(2-methylphenyl) acetamide) (RKS-1) expressed both strong analgesic as well as anti-inflammatory activity along with demonstrating the lowest docking score (-8.9 Kcal/mol) against COX-2 enzyme.

Conclusion: RKS-1 was found to be the most potent lead. The *in vivo* results were found to be successfully correlated with the obtained *in silico* data. The current study will draw the attention of global chemists towards the rational development of newer synthetic phenoxyacetanilide derivatives with pronounced non-steroidal anti-inflammatory drugs (NSAID) activity.

Key Words: Phenoxyacetanilide Derivatives, Anti-inflammatory, Analgesic, NSAID, Molecular Docking, Inhibitors

INTRODUCTION

Cyclooxygenase-1 and Cyclooxygenase-2, commonly recognized as COX-1 and COX-2 enzymes, have a strong effect on the production of prostaglandins. Prostaglandins and their derivatives are formed in minute quantities and are primarily responsible for discomfort, inflammation, and other symptoms. Prostaglandins do not circulate in significant concentrations in the bloodstream. Analgesic reaction, inflammation, and other physiological actions are all regulated by COX enzymes.[1]

Non-steroidal anti-inflammatory drugs (NSAIDs) are one of the most widely prescribed medications in the world, and they are primarily used to treat and prevent pain and inflammation caused by a variety of diseases. Diclofenac, naproxen, tolfenamic acid, and other NSAIDs are examples. By inhibiting the activity of the COX enzyme, NSAIDs serve as a blocking agent in the biosynthesis of prostaglandins. COXs, also known as prostaglandin H_2 synthases, are membrane-bound bifunctional enzymes that interact with and catalyze a particular stage in prostanoid biosynthesis.[2]

Docking is the most recent method used in modern times to determine the mechanism and fit of bioactive substances with receptor enzymes. Molecular docking is an enticing thrust field of study that seeks to recognize drug

Corresponding Author:
Rajib Kumar Singh, Research Scholar, Drug Design Laboratory, Faculty of Pharmacy, IFTM University, Moradabad 244001, Uttar Pradesh, India; E-mail: tukupritiraj@gmail.com

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biomolecular interactions in order to rationally implement the fundamentals of drug design in drug discovery. By inserting an atom (ligand) into the limiting location of the desired exact region of the protein, molecular docking elucidates the drug action process (receptor). The findings of the docking analysis can be used to infer that the binding energy, free energy, and stabilization of the drug-receptor complex are all positive. Molecular docking is currently being used to quantify the preliminary binding parameters of the ligand-receptor complex.[3]

The molecular docking mechanism necessitates a data bank to classify the target in PDB format and a system to schedule the ligand in PDB format. A broad range of applications (Discovery workshop, for example) is accessible in PDB format. These methods give you an understanding of ligands and their potential to attach to particular target receptors. In order to set the optimized conformation of the complex, molecular docking requires a pre-defined sampling of possible ligand conformations. The scoring feature of the software is commonly used to do this. The method of molecular docking can also be used to quantify ligand binding approaches that are dissimilar. This can be used to make medication applicants more successful, selective, and resourceful.[4]

Compounds of an acetamide nucleus have been shown to have a broad variety of medicinal effects in previous research over the last decade. Analgesic action, anti-inflammatory activity, anti-pyretic activity, anti-hyperglycemic activity, antioxidant activity, anti-cancer activity, anti-convulsant activity, and anti-microbial activity are also examples of this.[5,6]

The newly synthesized substances were developed through multi-step schemes and were analyzed thoroughly employing advanced analytical techniques such as Fourier-Transformed Infrared Spectroscopy (FT-IR), Proton-Nuclear Magnetic Resonance Spectroscopy (^{1}H-NMR), Mass spectroscopy, and Elemental Analysis. The possibility of a binding site and binding strength of new acetanilide derivatives were discovered using a molecular docking study against COX-2 enzyme using AutoDock Vina software. All of the synthesized derivatives were tested for analgesic activity (using Eddy's hot plate method) and anti-inflammatory effects (employing the carrageenan-induced paw edema method).

MATERIALS AND METHODS

Materials

Aniline, phenol, chloroacetyl chloride, potassium carbonate, acetone, ethyl methyl ketone, and other analytical quality chemicals and solvents were obtained from CDH Pvt. Ltd., New Delhi, and S. D. Fine Chemicals Ltd., Mumbai, India. In this analysis, double distilled water (Borosil®) was used.

Instrumentation

Recrystallization with a sufficient concentration of ethanol was used to purify the synthesized compounds. Merck® precoated Thin Layer Chromatography was used to test the integrity of freshly synthesized compounds (TLC). The traditional procedure (open capillary tubes method) was used to calculate the melting temperature of intermediates and compounds, and the results were reported as uncorrected. On an FT-IR spectrophotometer (Shimadzu® 8400SS), the FT-IR spectrum was recorded using the potassium bromide pellet process. The ^{1}H-NMR spectra were reported in $CDCl_3$ using trimethyl silane (TMS) as the internal norm (Bruker® Jeol at 300 MHz). Downfield from TMS, the ^{1}H chemical shifts were calculated in parts per million (ppm). A Shimadzu® GCMS-QP-2000 spectrometer was used to record the mass spectra. On an elemental analyzer (Carlo® Erba-1108), the elemental analysis was used to measure the % sum of carbon, oxygen, hydrogen, and nitrogen.

Animals

The animal trials were carried out under the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) guidelines, which were authorized by the Department Ethical Clearance (2017/837ac/Ph.D./02). Swiss albino rats, aged 5 to 6 weeks and weighing 160-250 g, were used in the anti-inflammatory and analgesic trials. In the animal house, the animals were housed in a regulated atmosphere (25–26°C temperature, 50–65% humidity, and 12 hours of light and 12 hours of darkness). The rodents were housed in groups of two, fed regular pellets, and allowed free access to water.

Synthesis method

Four phenoxyacetanilide derivatives were synthesized through a two-step reaction (**Table 1**).

Table 1: List of phenoxyacetanilide derivatives.

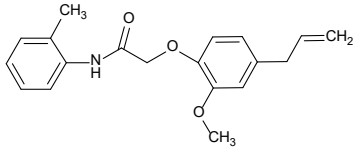
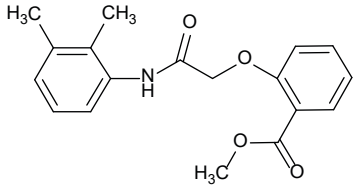
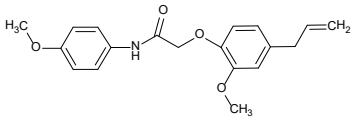
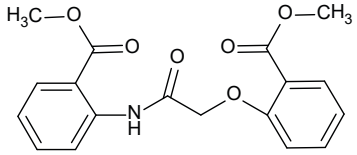
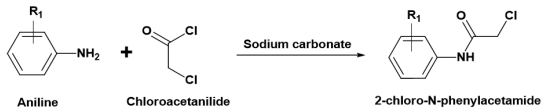
Code	Compounds Structure	Molecular Formula	Name of compounds
RKS1		$C_{19}H_{21}NO_3$	2-[2-methoxy-4-(prop-2-en-1-yl) phenoxy]-*N*-(2-methylphenyl) acetamide

Table 1: ***(Continued)***

Code	Compounds Structure	Molecular Formula	Name of compounds
RKS2		$C_{18}H_{19}NO_4$	Methyl 2-{2-[(2, 3-dimethylphenyl) amino]-2-oxoethoxy}benzoate
RKS3		$C_{19}H_{21}NO_4$	*N*-(4-methoxyphenyl)-2-[2-methoxy-4-(prop-2-en-1-yl)phenoxy]acetamide
RKS4		$C_{18}H_{17}NO_6$	Methyl 2-({[2-(methoxycarbonyl) phenoxy]acetyl}amino)benzoate

Ω-Chloroacetanilide (2-chloro-N-phenylacetamide)

The compound was produced using aniline and chloroacetyl chloride in the presence of ethyl methyl ketone and sodium carbonate, as defined in the literature (**Scheme 1**).

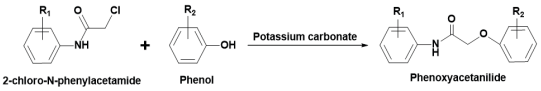


Scheme 1: Synthetic reaction scheme to synthesize Ω-Chloroacetanilide (2-chloro-*N*-phenylacetamide).

Distilled aniline or aniline derivatives were combined with methyl ethyl ketone in a 500 mL three-necked round-bottomed flask (RBF) with a mechanical stirrer and two 100 mL dropping funnels, which were kept in an ice bath with salt. Then, via the two dropping funnels RBF, solutions of chloroacetyl chloride in methyl ethyl ketone and sodium carbonate in distilled water were eventually applied. The sodium carbonate solution was first applied to the reaction flask, accompanied by the simultaneous dropwise addition of the chloroacetyl chloride (in methyl ethyl ketone) solution and the sodium carbonate solution from the falling funnels. The temperature of the reaction mixture was held between 7 and 10 degrees Celsius. The method of inserting the contents of the flask should be simple at the end (pH 7-8). About half an hour, the assembly was kept in its current state. The two falling funnels, as well as the ice water bath, were gone. The aqueous coating was separated after the contents of the flask were passed to a separating funnel. The organic coating was washed twice with water before being moved to a 250 mL conical flask and incubated overnight with sodium sulfate (Na_2SO_4). Under reduced pressure, the organic layer was decanted into a 250 mL round-bottomed flask and the solvent was extracted. The ethanol was used to recrystallize the strong.[7]

Phenoxyacetanilide derivatives

According to the recorded protocol, 2-chloro-N-phenylacetamide and substituted phenol were refluxed in dry acetone for 14-16 hrs with potassium iodide and anhydrous potassium carbonate to acquire phenoxyacetanilide (**Scheme 2**).



Scheme 2: Synthetic reaction scheme for phenoxyacetanilide derivatives.

A water bath was used to heat a reaction assembly consisting of a 250 mL three-necked round-bottomed flask with a mechanical stirrer and a condenser. The RBF was loaded with the chloro-compound (0.01 M) and dry acetone (40 mL). The RBF was then loaded with phenol or replacement phenol (0.01 M), potassium iodide (0.2 g), and anhydrous potassium carbonate. For 14-16 hours, the reaction mixture was stirred and refluxed. TLC was used to monitor the reaction's development. The reaction mixture was permitted to cool to room temperature before being purified at the end of the reaction cycle. Acetone was used to clean the residue. Under decreased heat, the solvent was eliminated. When the residue was permitted to settle, it solidified. To extract unreacted phenol, the solid was treated for 1 hour with a warm (45°C) 10% sodium carbonate solution when stirring continuously. The latest phenoxyacetanilide derivatives were

filtered, washed with acid, cleaned, and re-crystallized in an appropriate solvent.[8]

2-[2-methoxy-4-(prop-2-en-1-yl)phenoxy]-N-(2-methylphenyl)acetamide (RKS-1)

The following components were heated under reflux in dry acetone (30 mL) for 5 hrs: ω-chloro-(2-methyl)acetanilide 1.975 g (0.01 M); eugenol 1.64 g (0.01 M); potassium carbonate 3.5 g; and potassium iodide 0.2 g. After cooling and refining the reaction mixture, the filtrate was condensed under a vacuum system to form the crude solid product. Ethanol was used to recrystallize the crude solid.

Yield: 72.47%; m.p.: 83-85°C; R_f: 0.78; FTIR (KBr) υ (cm^{-1}): 3249 (-NH, stretching), 3061 (aromatic), 1712 (C=O), 1645 (-NH, bending), 1602 (C=C, aromatic), 1456 (-CH_3, bending), 1276 (C-O); ^{1}H-NMR (δ, ppm, $CDCl_3$): 2.20 (3H, s), 2.27 (2H, s), 3.33 (3H, s), 3.57(2H, s), 4.68(1H, d, J = 8.2Hz), 4.88(1H, d, J = 8.2Hz), 5.68(1H, q), 7.12 (1H, ddd, J = 8.3, 1.3, 0.4 Hz), 7.27 (1H, ddd, J = 8.1, 7.4, 1.3 Hz), 7.68 (1H, ddd, J = 8.3, 7.4, 1.4 Hz), 7.94 (1H, ddd, J = 8.1, 1.4, 0.4 Hz), 10.12(NH, s); MS: M^+ 311. Anal. Calcd. for $C_{19}H_{21}NO_3$: C, 73.31; H, 6.70; N, 4.5. Found: C, 73.29; H, 6.8; N, 4.5.

Methyl 2-{2-[(2, 3-dimethylphenyl)amino]-2-oxoethoxy}benzoate (RKS-2)

The following components were heated under reflux in dry acetone (30 mL) for 6 hrs: ω-chloro-(2,3-dimethyl)acetanilide 1.975 g (0.01 M); methyl salicylate 1.52 g (0.01 M); potassium carbonate 3.5 g; and potassium iodide 0.2 g. After cooling and refining the reaction mixture, the filtrate was condensed under a vacuum system to form the crude solid product. Ethanol was used to recrystallize the crude solid.

Yield: 76.67%; m.p.: 85-86°C; R_f: 0.79; FTIR (KBr) υ (cm^{-1}): 3251 (-NH, stretching), 3080 (aromatic), 1793 (C=O), 1692 (-NH, bending), 1384 (-CH_3, bending), 1277 (C-O); ^{1}H-NMR (δ, ppm, $CDCl_3$): 2.42 (3H, s), 2.73 (3H, s), 3.82 (3H, s), 4.72 (2H, s), 7.53 (1H, dd, J = 8.2, 2.3 Hz), 7.60 (1H, dd, J = 7.8, 2.3 Hz), 7.85 (1H, dd, J = 8.2, 7.8 Hz), 7.91 (1H, ddd, J = 8.3, 1.3, 0.4 Hz, 7.95 (1H, ddd, J = 8.1, 7.4, 1.3 Hz), 7.96 (1H, ddd, J = 8.3, 7.4, 1.4 Hz), 8.06 (1H, ddd, J = 8.1, 1.4, 0.4 Hz), 10.45 (NH, s); MS: M^+ 313. Anal. Calcd. for $C_{18}H_{19}NO_4$: C, 68.99; H, 6.11; N, 4.47. Found: C, 66.58; H, 5.85; N, 4.14.

N-(4-methoxyphenyl)-2-[2-methoxy-4-(prop-2-en-1-yl)phenoxy]acetamide (RKS-3)

The following components were heated under reflux in dry acetone (30 mL) for 5 hrs: ω-chloro-4-methoxyacetanilide 1.995 g (0.01 M); eugenol 1.64 g (0.01 M); potassium carbonate 3.5 g; and potassium iodide 0.2 g. After cooling and refining the reaction mixture, the filtrate was condensed under a vacuum system to form the crude solid product. Ethanol was used to recrystallize the crude solid.

Yield: 67.27%; m.p.: 75-76°C; R_f: 0.67; FTIR (KBr) υ (cm^{-1}): 3263 (-NH, stretching), 3061 (aromatic), 1715 (C=O), 1637 (-NH, bending), 1603 (C=C, aromatic), 1492 (-CH_3, bending), 1262 (C-O); ^{1}H-NMR (δ, ppm, $CDCl_3$): 2.20 (3H, s), 3.30 (3H, s), 3.50 (2H, s), 3.73 (2H, s), 4.48 (1H, d), 4.68 (1H, d), 5.18 (1H, q), 7.28-7.46 (5H, 6.63 (dd, J = 8.5, 0.5 Hz), 6.64 (ddd, J = 8.8, 2.7, 0.5 Hz), 6.60 (dd, J = 2.8, 0.5 Hz), 6.55 (dd, J = 8.5, 0.5 Hz), 7.28 (2H, ddd, J = 8.8, 1.7, 0.5 Hz); MS: M^+ 327. Anal. Calcd. for $C_{19}H_{21}NO_4$: C, 69.71; H, 6.47; N, 4.28. Found: C, 67.62; H, 5.93; N, 3.88.

Methyl 2-({[2-(methoxycarbonyl)phenoxy]acetyl}amino)benzoate (RKS-4)

The following components were heated under reflux in dry acetone (30 mL) for 5 hrs: ω-chloro-2-carbomethoxyacetanilide 2.275 g (0.01 M); methyl salicylate 1.52 g (0.01 M); potassium carbonate 3.5 g; and potassium iodide 0.2 g. After cooling and refining the reaction mixture, the filtrate was condensed under a vacuum system to form the crude solid product. Ethanol was used to recrystallize the crude solid.

Yield: 78.71%; m.p.: 102-104°C; R_f: 0.63; FTIR (KBr) υ (cm^{-1}): 3216 (-NH, stretching), 3069 (aromatic), 1715 (C=O), 1670 (-NH, bending), 1605 (C=C, aromatic), 1535 (-CH_3, bending), 1278 (C-O); ^{1}H-NMR (δ, ppm, $CDCl_3$): 3.80 (6H, s), 4.92 (2H, s), 7.53-7.73 (3H, 7.62 (ddd, J = 8.1, 7.5, 1.5 Hz), 7.56 (ddd, J = 7.9, 1.5, 0.5 Hz), 7.68 (ddd, J = 8.3, 7.4, 1.4 Hz), 8.14 (1H, ddd, J = 8.1, 1.4, 0.4 Hz); MS: M^+ 343. Anal. Calcd. for $C_{18}H_{17}NO_6$: C, 62.97; H, 4.99; N, 4.08. Found: C, 59.63; H, 4.58; N, 3.73.

Molecular Modelling Studies

The intended enzyme (COX-2) PDB file (5KIR) was retrieved from the Protein Data Bank (PDB). Docking trials for phenoxyacetanilide derivatives with anti-inflammatory and analgesic effects have been conducted. By inhibiting the COX-2 enzyme, these compounds were tested for anti-inflammatory and analgesic efficacy. ChemDraw® editor was used to constructing two-dimensional (2D) configurations of newly synthesized molecules. The MOL file format was used to save these 2D constructs. The 2D structures were then transformed into three-dimensional (3D) structures, with the structural geometry tailored to the lowest energy state. Finally, the PDB format was used to save secure 3D constructs. It was subjected to docking analysis after preparing PDB files of ligand compounds and protein structures. The Lamarckian genetic algorithm was used to perform the docking analysis, which simulates molecular interaction studies of ligand and protein using AutoDock Vina software. AutoDock Vina outperforms standard AutoDock applications in terms of precision and numerical efficiency. Docking took into consideration the whole protein composition. The best-created pose was used for analysis in each docking sample. Each docking

experiment yielded negative binding affinity values in the kilocalorie per mol (Kcal/mol) unit.[9]

BIOLOGICAL EVALUATION

Preparation of Test Compounds

The research tests and comparison drugs were made in a double-distilled water solution. The first group (control) was given an intraperitoneal dosage of normal saline (0.9% concentration). The second group (standard) was given a 10 mg/kg b.w. dosage of diclofenac sodium solution. The dosage of final synthesized compounds was issued to the research groups at a dose of 20 mg/kg of b.w.

In vivo anti-inflammatory activity

The newly synthesized compounds were tested *in vivo* for anti-inflammatory activity using the standard carrageenan-induced paw edema method. To minimize edema heterogeneity, the rats have fasted overnight. Individual rats were given 5 mL of purified water orally prior to the start of the experiment. The compounds were provided orally to the research community (n = 6) at a dosage of 20 mg/kg b.w. by suspending them in a 5% acacia solution until the onset of inflammation. The inflammation was triggered by injecting 0.1 mL of 1% carrageenan solution into the sub plantar area of the rats' right hind paw through the subcutaneous path. A black mark was created on each rat's left hind paw just above the tibiotarsal junction of the leg with a marker to mark the amount at which the paw should be dipped in the column to ensure a steady paw volume every time. The mercury displacement approach was used to assess each rat's initial paw volume. A plethysmometer was used to calculate the volume of the paw at 0 hr, 1 hr, 2 hr, and 3 hr after the carrageenan suspension injection.[10] The discrepancy in paw width between injected and non-injected paws offers details about the compounds' capacity to minimize edema. Orally, the test group was given a 5% acacia solution. The positive control was diclofenac sodium (10 mg/kg b.w.). The data were expressed as mean ± standard error. The percent inhibition was calculated by the following formula:

$$\% \text{ inhibition (I)} = (1 - Vt / Vc) \times 100$$

Where, Vt and Vc indicate the mean change in paw volume of rats (treated and control) respectively. The findings of the carrageenan-induced rat paw edema method are presented.

Screening of Analgesic action

Eddy's hot plate system was used to test all of the synthesized compounds for analgesic action. The hot plate system was used to scan the laboratory animals for the sensitivity examination. An electrically heated panel (copper plate/glass surface) with a temperature of 55-56°C is used in the application. The rats in the analgesic sample were those that raised their tails on the heated plate in less than 5 seconds. The research group (n = 6) of rats were given the compounds orally at a dosage of 20 mg/kg b.w. For each animal, the lower 5 cm of the rat tail was marked with a black marker. The mice were screened by placing their distal tail (5 cm) on a hot plate that was kept at 55-56°C. The time it took for mice to retract their tail was assessed at 0 hr, 1 hr, 2 hr, and 3 hr. To prevent damage to the tail, the analysis was stopped 15 seconds after they were placed on the hot plate. Regular saline (3 mL/kg b.w.) was provided to the control group. As a supportive monitor, diclofenac sodium (10 mg/kg b.w.) was used.[11]

ELISA assay of COX-2 and IL-6

Altered levels of pro-inflammatory cytokines, including different interleukins IL-6, and COX-2 in liver tissue will be examined by enzyme-linked immunosorbent assay (ELISA) by using the standard protocol defined by the manufacturer.[12]

RESULTS AND DISCUSSION

Chemistry

A synthetic technique, as seen in Schemes 1 and 2, was used to effectively synthesize both of the new phenoxyacetanilide derivatives. The discrepancy in melting point and retention period between the starting material and the formed molecules was used to assess the formation of the novel phenoxyacetanilide compounds (**Table 2**).

Table 2: Physical properties of synthesized compounds.

Code	Molecular Formula	Melting Point (°C)	Molecular Weight	Rf Value	% Yield	Elemental Analysis Calculated			
						C	H	N	O
RKS-1	$C_{19}H_{21}NO_3$	83-85	311	0.78	72.47	73.31	6.7	4.5	15.43
RKS-2	$C_{18}H_{19}NO_4$	85-86	313	0.79	76.67	69.01	6.07	4.47	20.45
RKS-3	$C_{19}H_{21}NO_4$	75-76	327	0.66	67.27	69.72	6.4	4.28	19.57
RKS-4	$C_{18}H_{17}NO_6$	102-104	343	0.65	78.71	62.97	4.96	4.08	27.98

Spectral data

The spectra obtained for all the four compounds remained fairly overlapping and appeared analogous. The two large aromatic parts were identified through FT-IR spectra where the C=C and C-H components are predominantly seen at 3000-3100 cm^{-1} and 1600-1650 cm^{-1}, respectively. The proton-NMR additionally confirmed the aromatic parts by the appearance of the aromatic protons in the range of 6.9-8.2 ppm in the spectra. Amide, the most promising element of the scaffold lies astonishingly in the FT-IR range of 3200-3300 cm^{-1} (stretching) and 1600-1650 cm^{-1} (bending). Methyl group was perceived both in FT-IR (1425-1450 cm^{-1}) and ^{1}H-NMR (3-3.5 ppm). Moreover, the mass spectra of the compounds provided ample proof for the compound formation owing to the manifestation of the base peak which corresponds exactly with the molecular mass of the compound. The emergence of M+2 also confirmed the presence of isotope chlorine forms. Furthermore, the ratios of carbon, hydrogen, and nitrogen provided an absolute confirmation on the development of the compounds.

Molecular modeling

The target compounds were compared to the reference drugs diclofenac and indomethacin in a molecular docking analysis on the COX-2 enzyme. The four target compounds had lower docking scores on the COX-2 enzyme, indicating that they could have improved analgesic efficacy by interacting with amino acid residues; SER-530 and TRY-355 (**Figure 1**). RKS-1 was the most potent of the synthesized molecules, with the lowest docking score (-8.9 Kcal/mol) against the COX-2 enzyme. Of all the compounds synthesized, RKS-3 and RKS-2 were found to be one of the potent, with the lowest rank of docking scores (-8.7 Kcal/mol and -8.5 Kcal/mol) for the COX-2 enzyme (**Table 3**).

Table 3: Binding energy and Interaction Residue of Ligands with Cyclooxygenase-2.

Ligands	Binding energy (Kcal/mol)	Interaction residues
Indomethacin	-10.2	SER-530
Diclofenac Sodium	-8.5	SER-530
RKS-1	-8.9	TYR-355
RKS-2	-8.5	SER-530, TYR-355
RKS-3	-8.7	SER-530
RKS-4	-8.0	SER-530, TYR-355

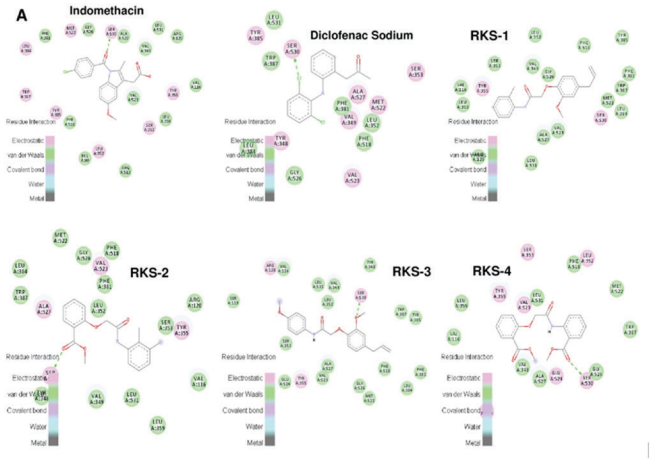


Figure 1: Molecular docking studies of Phenoxyacetanilide Derivatives as Cyclooxygenase-2 inhibitors.

Anti-inflammatory activity

Through the carrageenan-induced paw edema method, the fabricated compound was shown to have excellent *in vivo* anti-inflammatory efficacy as compared to the regular medication. Over the span of 3 hrs, the phenoxyacetanilide compounds displayed remarkable percent edema reduction (**Table 4**). The electron-donating substituents in the compound are assumed to suppress inflammatory mediators such as COX-1/2 and lipoxygenase (LOX).[13] However, it can be inferred from the analysis that the compound RKS-4 did not perform well in anti-inflammatory screening, which may be attributable to the molecule's extremely strong lipophilic characteristic. It's conceivable that the compound was spread across the body, with the active fraction hitting the inflamed region in small quantities, inhibiting the minimal amount of inflammatory mediators.[14] The collected *in silico* data was successfully compared with the *in vivo* findings.

Analgesic activity

After 3 hrs, the phenoxyacetanilide compounds demonstrated strong analgesic efficacy. In the Swiss albino rats, there was a substantial decrease in discomfort. Electron-donating substituents likely play a crucial role in mediating the analgesic effect. However, biological activity was observed to be smaller than that of diclofenac sodium, the normal medication (**Table 5**). The involvement of the substituents in the chalcone scaffold was assumed to minimize the expression of inflammatory mediators such as COX-1/2, LOX, 15-hydroxyprostaglandin dehydrogenase (15-PGDH), and other miscellaneous inflammatory mediators, all of which are essential in the mediation of algesia.[15]

Table 4: Anti-inflammatory effect of newly synthesized derivatives.

Group	Adminis-tered dose (mg/kg)	Mean Paw Volume (mm., mean ± SEM)			
		0 min	60 min	120 min	180 min
Control	3 mL/kg	3.21±0.12	4.16±0.25	6.27±0.24	6.88±0.74
Diclofenac sodium	10	4.14±0.42	2.16±0.62	1.88±0.19	0.53±0.04
RKS-1	20	4.38±0.21	2.01±0.42	1.64±0.24	0.49±0.02
RKS-2	20	3.41±0.41	3.01±0.21	2.64±0.51	2.32±0.13
RKS-3	20	4.01±0.21	3.09±0.19	2.48±0.17	1.69±0.09
RKS-4	20	3.12±0.43	2.99±0.24	2.67±0.19	2.46±0.13

n = 6 for each group, one-way Analysis of Variance (ANOVA) test was used

Table 5: Analgesic effect of newly synthesized derivatives

Group	Administered dose (mg/kg)	Reaction time (min., mean ± SEM)			
		0 min	60 min	120 min	180 min
Control	3 mL/kg	1.26±0.02	1.03±0.01	1.43±0.04	1.29±0.09
Diclofenac sodium	10	1.62±0.12	2.96±0.32	3.34±0.12	3.59±0.14
RKS-1	20	1.53±0.24	3.19±0.14	3.85±0.17	4.39±0.32
RKS-2	20	1.44±0.19	2.98±0.54	3.11±0.41	3.25±0.21
RKS-3	20	1.51±0.14	3.01±0.14	3.67±0.19	3.99±0.14
RKS-4	20	1.54±0.03	1.94±.02	2.13±0.25	2.25±0.13

n = 6 for each group, one-way Analysis of Variance (ANOVA) test was used

ELISA Studies

ELISA estimated the concentration of pro-inflammatory cytokine (IL-6) as well as COX-2 in rat liver to study the impact of phenoxyacetanilide compounds on inflammatory mediators linked to carcinogenic involvement (**Figure 2**). Many of these inflammatory markers were shown to be stronger in the liver of the carcinogen control group. The compound RKS-1 was shown to have a greater healing potential than the standard drug diclofenac sodium.

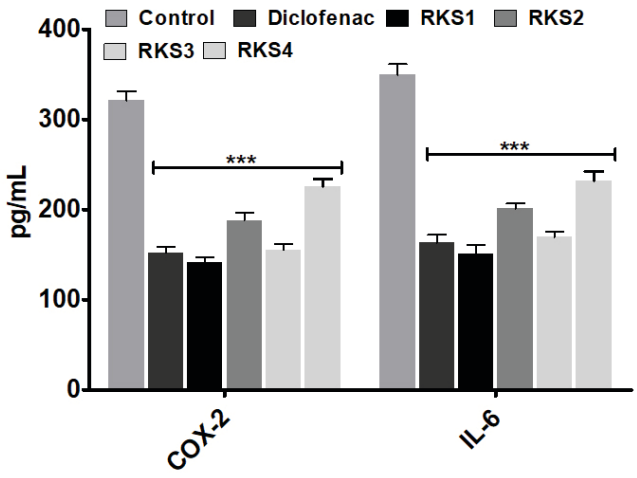


Figure 2: The inhibitory effects of phenoxyacetanilide compounds on inflammatory mediators.

CONCLUSION

The present study successfully expressed the hidden anti-inflammatory (explored through carrageenan-induced paw edema method) and analgesic (explored through Eddy's hot plate method) perspectives of the Phenoxyacetanilide derivatives (RKS-1 to RKS-4) which opens new avenues for applications as NSAIDs. The most active lead was discovered to be RKS-1 which was assumed to minimize the expression of inflammatory mediators such as COX-1/2, LOX, 15-PGDH, and other miscellaneous inflammatory mediators. The collected *in silico* data was successfully compared with the *in vivo* findings where both the studies concluded analogous findings. The RKS-4 did not do well in anti-inflammatory testing, which may be attributed to the molecule's strong lipophilic characteristics that might facilitate wide spreading across the body tissues. However, no structure-activity-relationships (SARs) can be expected from this analysis due to a limited number of molecules synthesized in the whole series, which necessitating further testing through the development of more such active inhibitors. The current research would focus global medicinal chemists' attention on the rational production of newer synthetic NSAID compounds of Phenoxyacetanilide series with significant anti-inflammatory response and edema-reducing efficacy.

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Conflict of Interest

The authors declare no conflict of interest.

Authors' Contribution

RKS: Performed the synthesis, characterization, and biological activities of compounds

AKM: Provided concept, guidance, and idea for this work

PK: Performed the molecular docking studies

DKM: Wrote the manuscript, removed plagiarism, and corrected grammar

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