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MICROWAVE ASSISTED SYNTHESIS, BIOLOGICAL EVALUATION AND DOCKING STUDIES OF NOVEL PYRAZOLINE DERIVATIVES AS POTENT ANTIINFLAMMATORY AND ANTIBACTERIAL AGENTS

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ABSTRACT

A novel series of 1-(4-chlorophenyl)-3-(4-substituted phenyl)-5-(5-(4-nitrophenyl) furan-2-yl)-4,5-dihydro-1H-pyrazole derivatives **(3a-e)** and (3-(4-substituted phenyl)-5-(5-(4-nitrophenyl) furan-2-yl)-4, 5-dihydropyrazol-1-yl) (pyridin-4-yl) methanone derivatives **(4a-e)** have been synthesized by conventional and microwave irradiation methods. The microwave irradiation method was found to be remarkably successful higher yields, environmental friendly and less reaction times when compared to conventional heating method. The synthesized compounds were characterized by FTIR, ^{1}H NMR and mass spectral data and screened for their *in vivo* antiinflammatory and *in vitro* antibacterial activity. Among the tested compounds, compounds **4b** and **4e** exhibited highest *in vivo* antiinflammatory activity. Some of compounds showed potent antibacterial activity. *In silico* prediction of toxicities and drug score profiles of synthesized compounds are found to be promising. Molecular docking results along with the biological data suggested that the compounds **(4a-e)** may be beneficial as molecular templates for antiinflammatory activity.

Key words: Pyrazoline, Antiinflammatory activity, Molecular docking, Microwave.

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INTRODUCTION

Inflammation is biological response of the tissue to an infection, irritation or foreign substances. It is body defense mechanism in order to remove or limit the spread of injurious agent as well to eliminate the necrosed cells[1]. Non steroidal antiinflammatory drugs (NSAIDs) have been recognized as important class of therapeutic agents for the treatment of inflammation and pain. The pharmacological effects of NSAIDs are due to inhibition of a membrane enzyme called cyclooxygenase (COX-1 and COX-2), which is involved in prostaglandin biosynthesis. COX-1 is predominantly expressed in most tissues, is responsible for the physiological production of prostaglandins, and COX-2, which is induced by mitogens, cytokines and endotoxins in inflammatory cells, is responsible for the elevated production of prostaglandins during inflammation process. For this reason, COX became an attractive target for the development of antiinflammatory drugs. Long term use of NSAIDs has been accompanied with several side effects such as gastrointestinal mucosal damage, intolerance, bleeding, renal toxicity and hepatotoxicity. Thus, development of novel anti-inflammatory agents with a fewer side effects is a major challenge to medicinal chemists.

Pyrazoline derivatives have been found to possess wide range of biological activities such as antiinflammatory[2], antitumuor[3], MAO-B inhibition[4], agonist of cannabinoid receptors[5], antimicrobial[6], antioxidant[7], antifungal[8], antitubercular[9], anticonvulsant[10], antidepressant[11], antiamoebic[12] and aniviral[13]. Further, pyrazoline is the core moiety in pharmaceutical compounds such as Celecoxib, SC-558, Antipyrine and Ramifenazone are shown in Fig. 1.

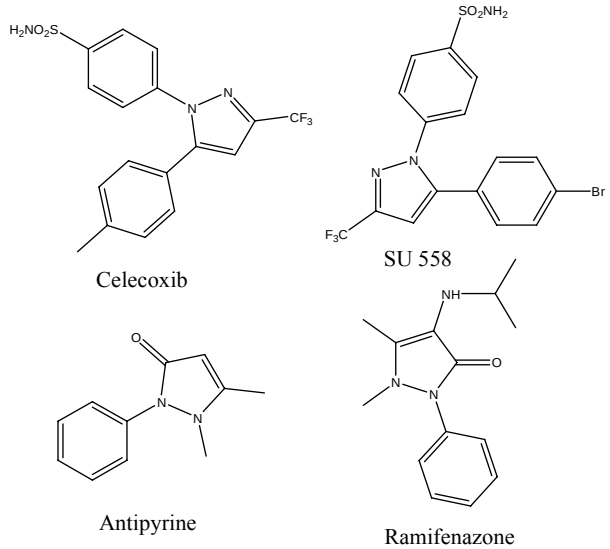


Fig. 1: Structures of the reported antiinflammatory agents

In recent times, microwave assisted organic reactions have received great interest and become very popular due to their enhanced reaction rate, ecofriendly nature, safety, and higher yields. Thus, keeping in view the advantages of this technique, and immense biological importance of pyrazolines, in the present study, we made an attempt to synthesize the title compounds by both the microwave and conventional methods and characterized by FTIR, ^{1}H NMR and mass spectral data. Further, the synthesized compounds were screened for their *in vivo* anti-inflammatory and *in vitro* antibacterial activity.

Molecular docking studies play an important role in the drug design as well as in mechanistic studies by placing a molecule into the binding site of the target macromolecule. We have docked the synthesized compounds into active site of the COX-2 enzyme (PDB ID: 3Q7D) by using MOE 2008.10 software and consequently to rationalize the obtained pharmacological data. Furthermore, *in silico* toxicity, C Log P, and drug score of synthesized compounds were determined by Osiris program (www.chemexper.com).

EXPERIMENTAL

Chemistry

All reagents were purchased from commercial sources. Melting points (m.p.) were uncorrected and determined in one end open capillary tubes using Analab melting point apparatus. The IR spectra were recorded on Shimadzu FTIR 8400 S spectrophotometer and expressed in wave numbers (cm^{-1}), using 1% potassium bromide disc. ^{1}H NMR spectra were recorded on Varian 400 MHz spectrometer using DMSO-d_6 as solvent and tetramethylsilane as an internal standard and mass spectra were recorded on Agilent 6430 triple quadruple LC-MS system. TLC was performed using E.Merck 0.25 mm silica gel plates and visualization of spots was accomplished with UV light.

Synthesis of 5-(4-nitrophenyl)-2-furfuraldehyde (1)

4-nitro aniline (0.13 mol) was dissolved in a mixture of water (25 mL) and concentrated HCl (33 mL). The solution was cooled to 0-5^{o}C and diazotized with sodium nitrite (0. 13 mol) in water (25 mL). The solution was stirred for 10 min and then CuCl (0. 04 mol) was added as catalyst. To this mixture, furfuraldehyde (0. 16 mol) was added and stirred for 4 hrs at the temperature of 40^{o}C. The progress of the reaction was monitored by TLC. The compound thus formed was filtered, washed with water, dried and recrystallized form methanol. The product obtained was confirmed by melting point (203-205^{o}C).

General procedure for the synthesis of chalcones (2a-e)

To a mixture of substituted acetophenone (0.01 mol) and 5-(4-nitro phenyl)-2-

furfuraldehyde (**1**) (0.01 mol) was dissolved in ethanol (30 mL), was added 10% NaOH solution (10 mL) and stirred for 8-10 hrs at room temperature. The progress of the reaction was monitored by TLC. The solid obtained was filtered, washed with ice cold water, dried and recrystallized from methanol. (2*E*)-3-[5-(4-nitrophenyl)-2-furyl]-1-phenylprop-2-en-1-one (**2a**): Yield 86%, m.p. 158-160ºC; TLC solvent system; toluene: ethyl acetate (7:3), R_f value: 0.7. IR (KBr, cm^{-1}): 3080 (aromatic C–H), 1650 (C=O), 1595 (C=C), 1512, 1324 (N-O), 1108 (C-O).

Conventional method

General procedure for the synthesis of 1-(4-chlorophenyl)-3-(4-substituted phenyl)-5-(5-(4-nitrophenyl) furan-2-yl)-4,5-dihydro-1H-pyrazole derivatives (3a-e)

A mixture of substituted chalcone (**2a-e**) (0.01 mol) and 4-chlorophenyl hydrazine (0.02 mol) was taken in 50 mL of ethanol and 3-4 pellets of KOH was added and refluxed for 12-15 hrs. The progress of the reaction was monitored by TLC. The reaction mixture was then cooled and kept overnight. The solid thus separated was filtered, washed with cold water, dried and recrystallized from ethanol.

General procedure for the synthesis (3-(4-substituted phenyl)-5-(5-(4-nitrophenyl) furan-2-yl)-4, 5-dihydropyrazol-1-yl) (pyridin-4-yl) methanone derivatives (4a-e)

A mixture of substituted chalcone **(2a-e)** (0.01 mol) and isonicotinic acid hydrazide (0.01 mol) was taken in 50 mL of acetic acid and refluxed for 12-14 hrs. The progress of the reaction was monitored by TLC. The reaction mixture was then cooled and neutralized with dilute ammonia solution. The solid thus separated was filtered, washed with cold water, dried and recrystallized from ethanol.

Microwave assisted irradiation method

A mixture of chalcone **(2a-e)** (0.01 mol) and 4-chlorophenyl hydrazine/isonicotinic acid hydrazide (0.01 mol) was subjected to microwave irradiation for time period of 5 to 8 min at a power level of 600 W in the presence of piperidine (1-2 mL) catalyst. Progress of the reaction was monitored by TLC. After cooling, the solution was poured into crushed ice and the product thus obtained was filtered and recrystallized from ethanol.

1-(4-chlorophenyl)-5-(5-(4-nitrophenyl) furan-2-yl)-3-phenyl-4, 5-dihydro-1H-pyrazole (3a)

TLC solvent system; acetone: methanol (4:1), R_f value: 0.8. IR (KBr, cm^{-1}): 3103 (aromatic C–H), 1600 (C=N), 1510, 1324 (N-O), 1323 (C-N), 752 (C-Cl). ^{1}H NMR (DMSO-

d_6, 400 MHz, δ ppm): 6.91-7.90 (m, 15H, Ar-H), 5.62 (dd, 1H, CH of pyrazoline), 3.52 (dd, 1H, CH_2 of pyrazoline), 3.22 (dd, 1H, CH_2 of pyrazoline). MS (m/z): 444 (MH^+).

(3-(4-chlorophenyl)-5-(5-(4-nitrophenyl) furan-2-yl)-4, 5-dihydropyrazol-1-yl) (pyridin-4-yl) methanone (4b)

TLC solvent system; acetone: methanol (4:1), R_f value: 0.7. IR (KBr, cm^{-1}): 3076 (aromatic C–H), 1660 (C=O), 1599 (C=N), 1515, 1328 (N-O), 1324 (C-N), 752 (C-Cl). ^1H NMR (DMSO-d_6, 400 MHz, δ ppm): 6.92-7.80 (m, 14H, Ar-H), 5.54 (dd, 1H, CH of pyrazoline), 3.62 (dd, 1H, CH_2 of pyrazoline), 3.20 (dd, 1H, CH_2 of pyrazoline). MS (m/z): 473 (MH^+).

Pharmacological activity

Animals and instruments

Adult male albino Wister rats weighing 150-180 g were used for study. Rats were maintained under constant laboratory conditions and were allowed free access to water and food throughout the period of investigation. The experimental protocol for the pharmacological screening was done in accordance with the guidelines prescribed by an Institutional Animal Ethics Committee (Reg. No: 1374/ac/10/CPCSEA). Edema was produced by using 1% w/v of carrageenan, foot volumes were measured in a plethysmograph, which is based on the principle of mercury displacement.

In vivo antiinflammatory activity

The antiinflammatory activity of the synthesized compounds was assessed by *in vivo* using the carrageenan induced paw edema method[14]. The rats were divided into 12 groups of six animals each. The control group received 1 % aq.CMC. Ibuprofen sodium (100 mg/kg, standard) and test compounds (**3a-e** and **4a-e**, 100 mg/kg) in 1% carboxy methyl cellulose (CMC) were administered orally 1 hr before induction of inflammation. Inflammation was induced in to the sub-plantar region of the right hind paw by subcutaneous injection of 0.1 mL of freshly prepared 1% carrageenan in saline solution. The right paw thickness was measured using the plethysmometer at 0.5, 1, 2, 3 hrs after the carrageenan injection. The average value of edema was calculated by taking the average of six animals at different hours. Percentage inhibition of inflammation was calculated for each group with respect to the control group.

Inflammation was expressed as the change in paw volume.

$$\text{Edema} = T_t - T_0$$

Where T_0 = Volume at '0' hrs

T_t = Volume at't' hrs

Percentage of edema inhibition = $(V_0 - V_t)/V_0 \times 100$

Where, V_0 = Volume of the paw of control at time't'

V_t = Volume of the paw of drug treated at time't'

Antibacterial activity

Antibacterial activity of all the test compounds was determined by Agar well diffusion method[15] against *Staphylococcus aureus*, *Bacillus subtilis*, *Escherichia coli* and *Pseudomonas aeruginosa* at 100 µg/mL concentration in dimethyl sulfoxide (DMSO) solvent. The fresh culture of bacteria are obtained by inoculating bacteria into nutrient broth media and incubated at 37°C for 24 hrs. This culture mixed with nutrient agar media was poured into petridishes under aseptic conditions. After solidification of media, bores were made by using sterile cork borer (8mm diameter). Into these cups standard drug (Ampicillin) and synthesized drugs are introduced and incubated for 24 hrs at 37°C. The zone of inhibition was measured in mm.

Molecular docking

Docking was performed on Windows 2002 using MOE 2008.10 version. COX-2 was retrieved from the protein data bank (PDB code: 3Q7D) and the receptor was visualized using sequence option and further co-factors were deleted. The partial charge of protein was adjusted with the help of force field method AMBER 99. Later, the protein was subjected to 3D protonation at cut off 12.0, and further hydrogen was added according to standard geometry and the receptor was energy minimized using force field MMFF94x at 0.01 KJ mole gradients. The ligand structures were written by using a builder module, and adjusting the partial charges using Hamilton MMFF94 force field method and subsequently 3D protonation and hydrogen addition was performed according to standard geometry. Ligands were energy minimized at cut off 12 using force field MMFF94x at 0.01 KJ mole gradient. Docking was performed using the option simulation followed by dock on selected active site amino acids using sequence option, and eventually docked using setting options such as receptor and solvent, alpha triangle, selected residues, affinity dG, force field refinement and best 30 pose. After obtaining docking results, out of the 30 best posed results for each chemical structure, best pose was retained. The resultant best pose score values in the series were used for analysis of docking and interaction[16].

Assessment of toxicity, lipophilicity and drug score profiles

Shredding of each molecule at every rotatable bond led to a set of core fragments. These in turn were used to reconstruct all possible larger fragments which could be the substructure of the original molecule. Afterwards, a search process of substructure determined the occurrence frequency of every one of the fragment (constructed and core fragments) within all traded dugs of 3300 as well as 15,000 commercially available chemicals (Fluka) to predict toxicity, C Log P, and drug score[17,18].

RESULTS AND DISCUSSION

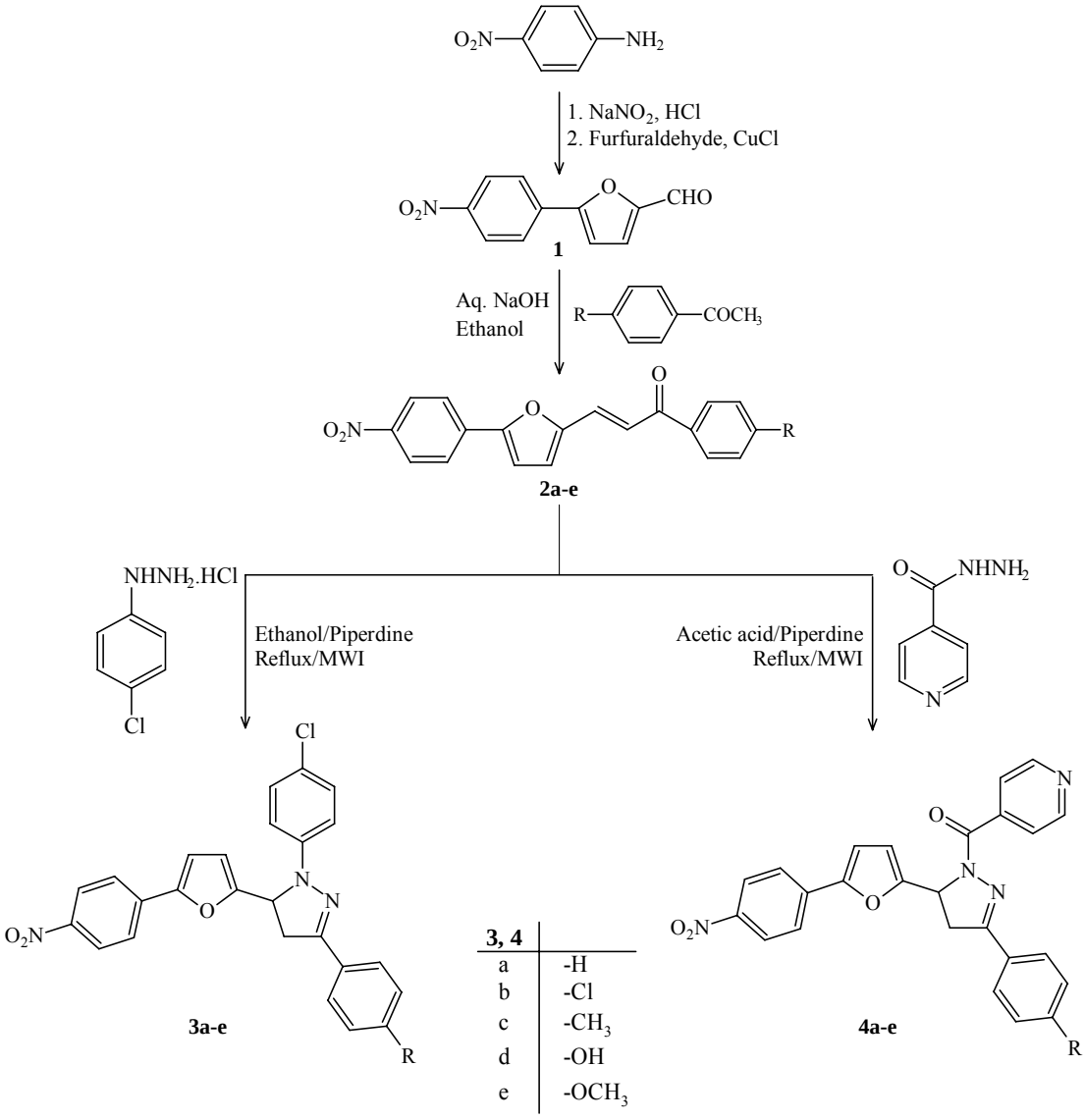
According to **Scheme 1**, 5-(4-nitrophenyl)-2-furfuraldehyde **(1)** was prepared by diazotization followed by sandmeyer reaction of para nitro aniline. Compound **(1)** was converted into chalcones **(2a-e)** by a base catalyzed Claisen–Schmidt condensation with different substituted acetophenones. The structures of chalcones **(2a-e)** were confirmed by the carbonyl peak around 1650 cm^{-1} and C=C stretching around 1600 cm^{-1} in IR spectra. Further, compounds **(2a-e)** were reacted with 4-chlorophenyl hydrazine to give first series of pyrazolines **(3a-e)** and other series of pyrazolines **(4a-g)** were obtained by reacting with isonicotinic acid hydrazide. The compounds were synthesized by both conventional and microwave irradiation methods. The physical data, reaction time, % yield in both the methods are compared and mentioned in the Table 1. The newly synthesized pyrazolines were characterized by FTIR, ^{1}H NMR and mass spectral data. In ^{1}H NMR, The $–CH_2CH$ of pyrazolines protons appeared as three double doublets around δ 3.2, δ 3.6 and δ 5.6, further the structures were confirmed by FTIR and mass spectral data.

Table 1: Physical characterization data of synthesized compounds

Compd.	M.P. (^{o}C)	Conventional time (hrs)	Yield (%)	Microwave time (min.)	Yield (%)
3a	143-145	12	70	6	79
3b	140-142	13	68	8	80
3c	141-143	11	72	6	82
3d	142-144	14	66	5	78
3e	138-140	15	69	8	82
4a	128-130	13	64	6	77
4b	131-133	14	69	7	83

Cont…

Compd.	M.P. (°C)	Conventional time (hrs)	Yield (%)	Microwave time (min.)	Yield (%)
4c	133-135	12	63	8	78
4d	135-137	13	68	6	84
4e	129-131	14	64	7	81



Scheme 1: Synthesis of titled compounds (3a-e) and (4a-e)

In vivo antiinflammatory activity

From the obtained results (Table 2), it was observed that compounds **4b** (p-chloro) and **4e** (p-methoxy) showed highest activity (80.19 % and 79.73% inhibition) after 3 hrs that may be attributed to the presence of pyridinoyl moiety on nitrogen of pyrazoline and the results are comparable to the standard drug, ibuprofen (88.26 % inhibition of edema).

Table 2: Antiinflammatory activity of ibuprofen and synthesized compounds

Compd.	0.5 hr	1 hr	2 hrs	3 hrs	% Inhibition after 3 hrs
Control	2.62 ± 0.02	3.81 ± 0.05	4.24 ± 0.06	4.68 ± 0.03	
Ibuprofen	1.68 ± 0.03*	1.16 ± 0.02*	0.96 ± 0.02*	0.55 ± 0.04*	88.26
3a	1.95 ± 0.01*	2.15 ± 0.04*	1.97 ± 0.02*	1.68 ± 0.05*	64.05
3b	1.81 ± 0.03*	1.72 ± 0.06*	1.35 ± 0.03*	1.07 ± 0.03*	77.03
3c	1.84 ± 0.02*	1.84 ± 0.05*	1.76 ± 0.04*	1.52 ± 0.03*	67.47
3d	1.91 ± 0.06*	2.11 ± 0.04*	1.55 ± 0.03*	1.49 ± 0.04*	68.14
3e	1.80 ± 0.02*	1.69 ± 0.06*	1.48 ± 0.04*	1.19 ± 0.03*	74.58
4a	1.86 ± 0.02*	1.77 ± 0.07*	1.67 ± 0.03*	1.58 ± 0.05*	66.15
4b	1.80 ± 0.01*	1.72 ± 0.03*	1.28 ± 0.02*	0.92 ± 0.04*	80.19
4c	1.85 ± 0.01*	1.75 ± 0.05*	1.68 ± 0.04*	1.39 ± 0.03*	70.31
4d	1.76 ± 0.02*	1.68 ± 0.03*	1.48 ± 0.02*	1.22 ± 0.04*	73.83
4e	1.82 ± 0.01*	1.64 ± 0.02*	1.39 ± 0.02*	0.95 ± 0.02*	79.73

Each value represents mean ± SE of six animals; *$P < 0.05$ as compared to control; using one way ANOVA was done by Dunnett's t-test. Ibuprofen and test compounds were taken at a dose of 100 mg/Kg body weight.

Antibacterial activity

The synthesized compounds were evaluated for their antibacterial activity by agar well diffusion method. The results are summarized in Table 3. Compounds **4b** and **4e** exhibited potent antibacterial activity. Remaining compounds were found to possess slight to moderate antibacterial activity against all the four organisms when compared with standard drug, ampicillin.

Table 3: Antibacterial activity of ampicillin and synthesized compounds

Compound	Zone of inhibition (Diameter in mm)			
	S. aureus	***B. subtilis***	***E. coli***	***P. aeruginosa***
3a	15	16	14	13
3b	18	19	18	16
3c	17	18	13	15
3d	13	14	15	17
3e	15	17	16	15
4a	16	14	15	14
4b	22	23	25	23
4c	18	17	14	16
4d	15	16	14	15
4e	20	21	22	19
Ampicillin (50 μg/mL)	25	24	27	25

Test compounds were taken concentration of 100 μg/mL

Molecular docking

Molecular docking study was performed for further exploration of the mechanism of action of the titled compounds with COX-2 enzyme and to elucidate the observed biological results. Docking of compound **4b** showed one hydrogen bond interactions (oxygen of C=O and Tyr 355; d = 2.387 A^o and furan ring showed stacking interaction with Arg 120. Moreover, compound was surrounded by Val 523 and Ser 353, which are very similar to that of the interactions exhibited by the Known COX-2 inhibitor.

Similarly, compound **4e** showed one hydrogen bond interactions (oxygen of C=O and Tyr 355; d = 2.37 A^o and furan ring showed stacking interaction with Arg 120. Very similar sorts of docking interactions have been observed in remaining compounds. In summary, the incorporation of C=O group in the series **(4a-e)** provided additional hydrogen bond interactions with the active site amino acids of COX-2 and this might have contributed

for better activity of these series of compounds as compared to other series of compounds **(3a-e)**. Further, this is supported by results obtained in *in vivo* antiinflammatory activity. The two-dimensional and three-dimensional representations of compound **4b** were given in Fig. 2.

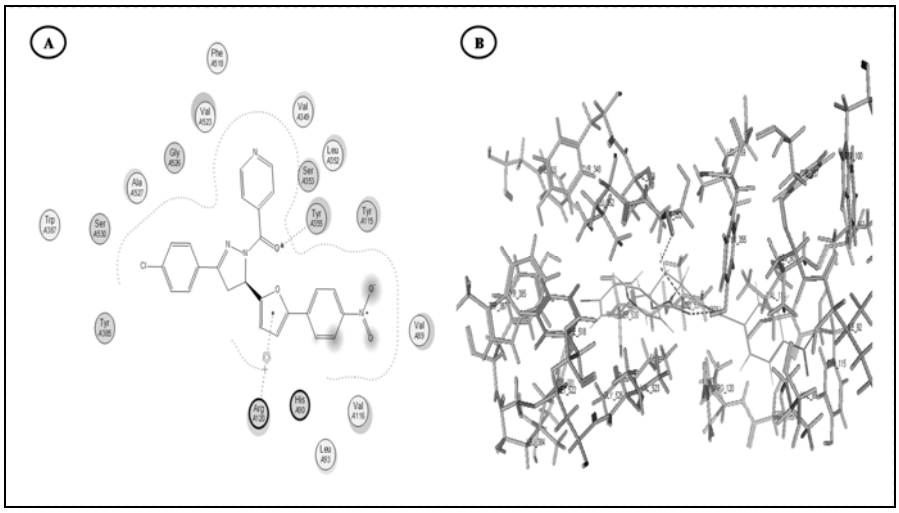


Fig. 2: (A) Two-dimensional representation of the interacting mode of 4b with COX-2 (B) Three-dimensional structural model of compound 4b (purple) into COX-2

Assessment of toxicity, lipophilicity and drug score profiles

Osiris program was used for prediction of the toxicity of the synthesized compounds. The prediction relies on a substructure search process determining the occurrence frequency of any fragment (constructed and core fragments) within any of toxicity classes. All synthesized compounds showed low *in silico* possible toxicity risks.

Osiris program was also used for prediction of the C log P of the synthesized compounds. C Log P is a well established parameter to measure the compound hydrophilicity. Compounds show reasonable probability of being well absorbed, when they have C log P value around 5.0. From the table, it was observed that all compounds have shown C log P around 5.0 indicating that the synthesized compounds could be possible drug candidates. The drug score combines C Log P, molecular weight and toxicity risks in one handy value that may be used to judge the compounds overall potential to qualify for a drug. Predictions of C log P, drug score and toxicity for the title compounds are given in Table 4 and almost all the compounds possess good values of drug score, C log P, and low probable toxicity risks as revealed by computational *in silico* studies.

Table 4: Computationally predicted toxicity risks, lipophilicity and drug scores of the title compounds

Compound	C log P[a)]	Drug score	Toxicity risks[b)]
3a	6.50	0.14	Negative
3b	7.12	0.12	Negative
3c	6.82	0.12	Negative
3d	6.20	0.14	Negative
3e	6.40	0.13	Negative
4a	5.48	0.17	Negative
4b	6.09	0.14	Negative
4c	5.80	0.18	Negative
4d	5.18	0.18	Negative
4e	5.38	0.16	Negative

[a]Calculated lipophilicity

[b]Mutagenicity, tumorigenicity, irritancy, and reproductive effects

CONCLUSION

A novel series of pyrazolines derivatives were synthesized by using conventional and microwave irradiation methods and all the compounds were characterized by physical and spectral data. The synthesized compounds were evaluated for *in vivo* antiinflammatory and antibacterial activity. Among the tested compounds, compounds **4b** (p-chloro) and **4e** (p-methoxy) exhibited highest *in vivo* antiinflammatory activity. Further, the same compounds have showed significant antibacterial activity. Assessment of toxicities and drug score profiles of synthesized compounds are promising. Moreover, the synthesized compounds showed significant docking interactions with COX-2 active site. Molecular docking results along with the biological data suggested that the tested compounds have the potential as valuable lead for antiinflammatory activity. However, further optimization is warranted.

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