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Revised

Design, synthesis, biological evaluation, and molecular docking study on triazine based derivatives as anti-inflammatory agents

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ABSTRACT

In an attempt to develop new anti-inflammatory agents, design, synthesis, pharmacological activities, and docking study of two groups of triazine-based derivatives were reported. Nine compounds (5a-5d and 10a-10e) consisting of triazine, vanillin, and phenylpyrazole were synthesized through the pharmacophore hybridization method. After confirmation of the structure of the synthesized compounds using spectroscopic methods (FT-IR, and NMR spectral data), their anti-inflammatory activity was evaluated using carrageenan-induced paw edema model in male Wistar rats (200–220 g) administered intraperitoneally at doses of 100 and 200 mg/kg. A group of rats received indomethacin (10 mg/kg) as the standard drug. Among compounds 5a to 5d, only compounds 5c and 5d showed a significant anti-inflammatory effect ($p < 0.01$). Also compound 10a at a dose of (200 mg/kg) and compounds 10b, 10c, 10d and 10e at both doses showed significant anti-inflammatory activity and this effect for 10a (200 mg/kg) and both doses of 10b and 10e was comparable with indomethacin. While indomethacin reduced paw edema by 90%, 10b as the most potent tested compound reduced edema by 93%. The synthesized compounds were docked into the binding sites of both cyclooxygenase-1- and 2- isoenzymes (COX-1 and COX-2) to explore their binding mode and possible interactions of these ligands.

1. Introduction

Non-steroidal anti-inflammatory drugs (NSAIDs) are the most widely used medicines for the treatment of different types of inflammations which exert their action by inhibition of cyclooxygenase enzymes [1,2]. Traditional NSAIDs, inhibit the COX-1 isoenzyme which is mainly responsible for the gastrointestinal side effects and/or inhibits both COX-1 and COX-2 isoenzymes [3,4]. Inhibition of the COX-2 isoenzyme leads to a reduction of prostaglandins and thromboxanes which are responsible for inflammatory effects [5,6]. Therefore, to have an anti-inflammatory drug without any gastrointestinal side effects, there is a continual need for a selective COX-2 inhibitor. Drugs such as celecoxib (Scheme 1), valdecoxib, and rofecoxib are the most important marketed selective COX-2 inhibitor drugs [7,8]. But their administration is associated with some myocardial thrombotic side effects. So it is an important goal in medicinal chemistry to find new anti-inflammatory agents with a potential for clinical use, associating with minimal or even without any adverse effects [9-12].

Merging two or more pharmacophores into a single molecule is an approach, frequently used in drug design and discovery that has recently been widely used to introduce new anti-inflammatory agents with potent activity and diminished side effects [13-15]. Triazines, as analog to the benzene ring, are heterocyclic scaffolds used in diverse compounds with a variety of biological activities such as analgesic, anti-tuberculosis, anti-fungal, anti-cancer, anti-protozoal, antimalarial, anti-viral, anti-microbial, and anti-inflammatory activity [16,17]. Due to their versatile structure (three isoforms depending on the positions of the nitrogen atoms) and also the different derivatives that can be synthesized from them, this heterocyclic ring has been increasingly studied in recent years. According to a review article published in 2019, about 35.17% of evaluated triazines were revealed promising anti-inflammatory properties [18]. So in designing new anti-inflammatory agents, the triazine ring could be a proper candidate. Additionally, vanillin and phenylpyrazole derivatives also represent important classes of compounds due to their highly pronounced pharmacological and biological activities such as anti-inflammatory effects [19-21].

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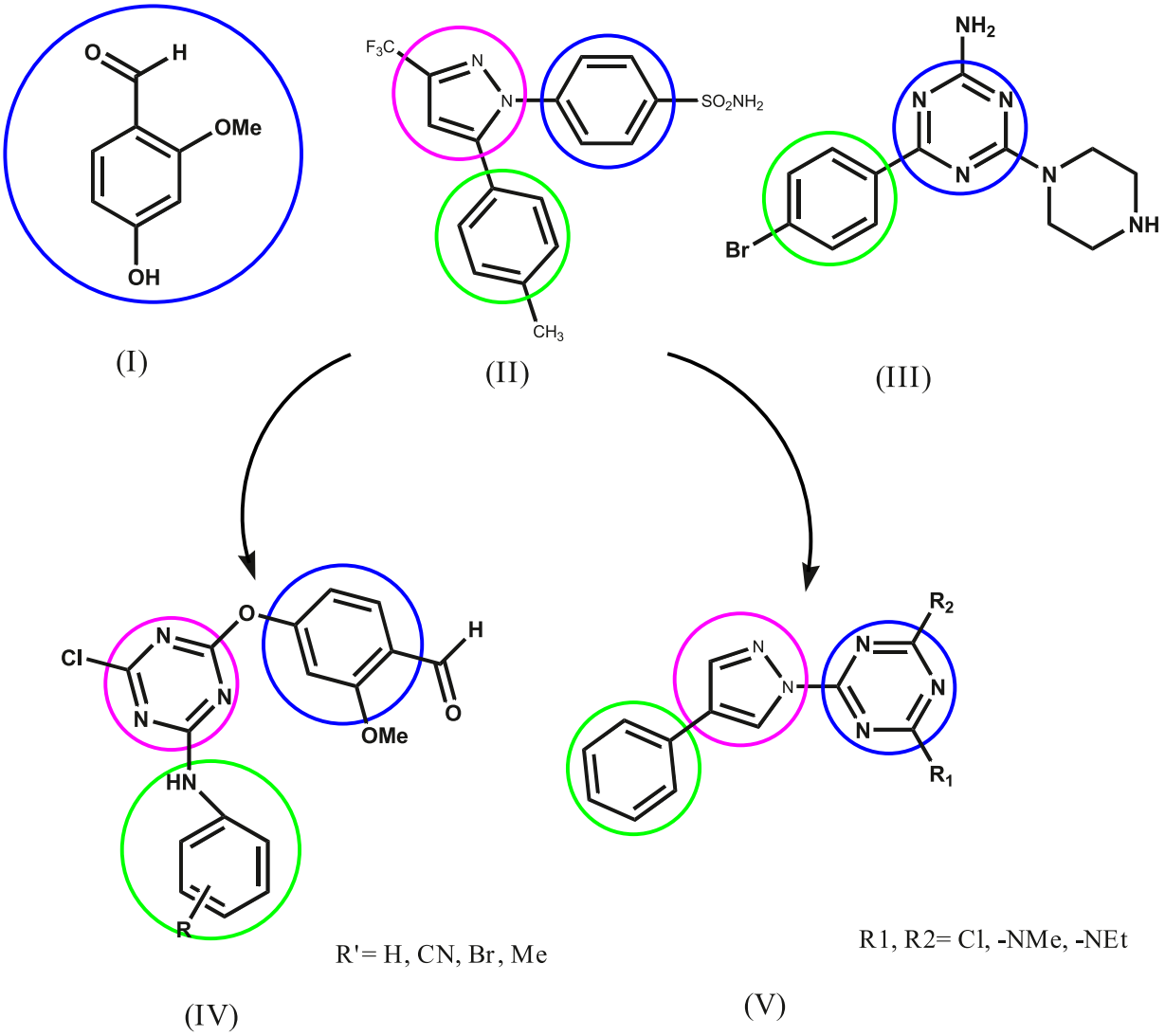


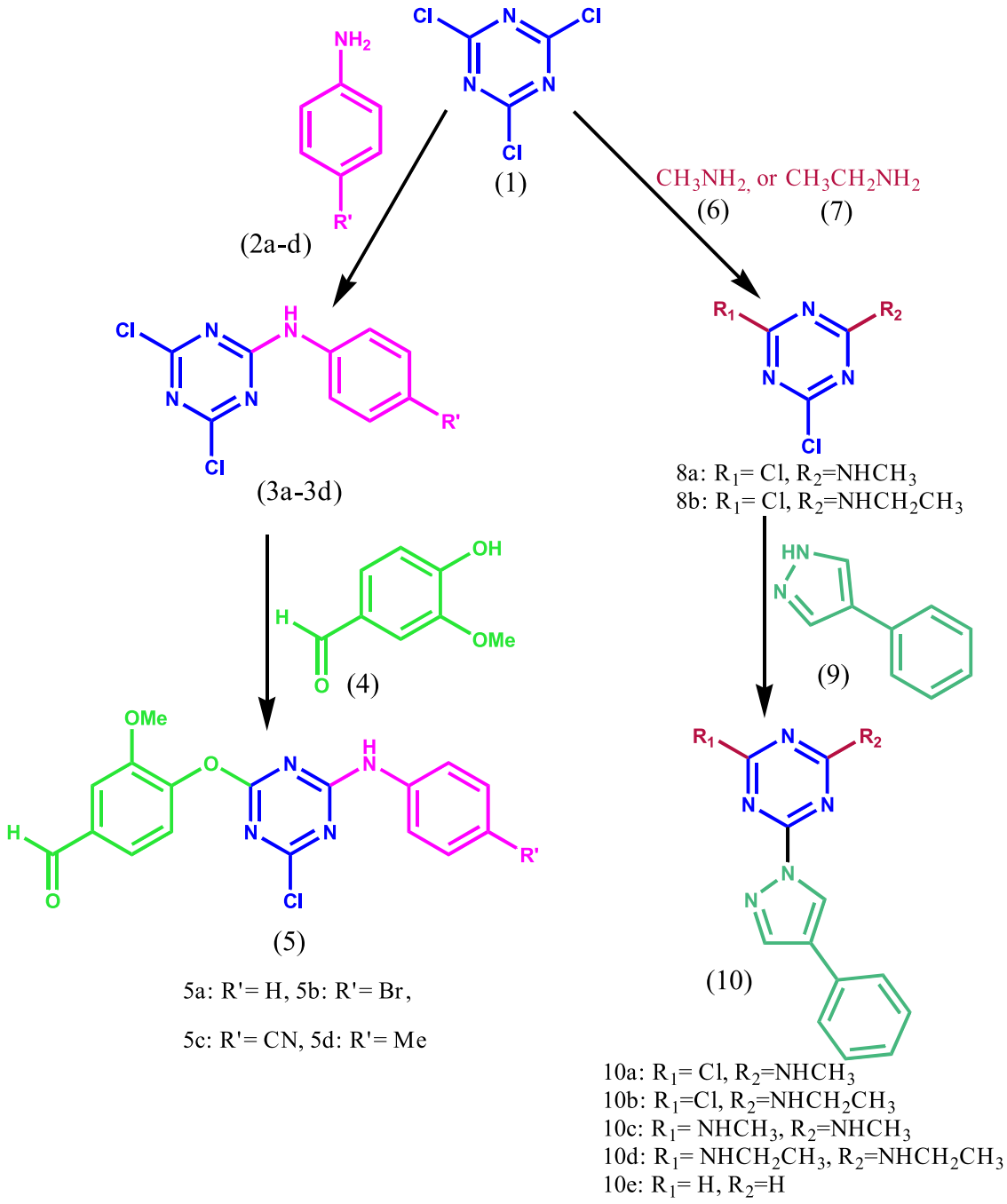
Scheme 1. Structures of vanillin (I), celecoxib (II), the anti-inflammatory derivative of triazine (III), and designed vanillin-triazine derivatives (IV) and phenylpyrazole-triazine derivatives (V).

The present work is a part of our ongoing research program aiming at developing a variety of heterocyclic systems for biological and pharmacological evaluation. Considering celecoxib as a selective COX-II inhibitor (Scheme 1) which is consisted of two aryl moieties attached to a central heteroaryl (pyrazole) ring, two groups of compounds were designed and synthesized. To design the target compounds, vanillin and triazine moieties were selected since the anti-inflammatory properties of vanillin as a natural substance have already been proven, and also this fact that triazine derivatives are key structures for the development of the new chemical class of anti-inflammatory agents [22,23]. In series 1, the central pyrazole ring of the celecoxib was replaced by the triazine ring, and one of the aryl rings was exchanged with vanillin (Scheme 1). In the other series, the triazine ring was placed instead of one of the two aryl moieties and binds to phenylpyrazole, which is present in the structure of celecoxib (Scheme 1). The structures of all synthesized compounds were characterized by their melting points, FT-IR, and NMR spectral data. The anti-inflammatory activity of the newly synthesized compounds was evaluated using the rat carrageenan-induced foot paw edema model [24], with the use of indomethacin as comparable reference drugs or positive control. Finally, synthesized compounds were docked into the binding sites of both COX-1 and COX-2 to explore their complementarity with the specified binding pocket.

2. Experimental

2.1. Material and method

Melting points were determined on an electrothermal 9200 melting point instrument (UK) and are uncorrected. Spectroscopic methods were applied for the characterization of the synthesized compounds. Infrared (IR) spectra were recorded as KBr plates using a Jasco-680 FT-IR spectrometer (Japan). ^{1}H NMR spectra were measured on an NMR Spectrophotometer (Bruker 500 MHz, Germany) with TMS as the internal standard, where J (coupling constant) values are estimated in Hertz (Hz). Thin-layer chromatography techniques with Merck silica gel 60 F254 plates (Germany) were used for evaluation of reaction progression and checking the purity of synthesized compounds.



Scheme 2. Synthesis of vanillin-triazine and phenylpyrazole-triazine derivatives.

2.2. Synthesis of vanillin-triazine and phenylpyrazole-triazine derivatives

The designed target compounds were synthesized according to scheme 2.

2.2.1. General procedure for the synthesis of 4,6-dichloro-1,3,5-triazine-2-ylphenylamine derivatives (3a-3d)

4,6-Dichloro-1,3,5-triazin-2-ylphenylamine derivatives were prepared following the earlier reported procedures [25]. A solution of cyanuric chloride (9.22 g, 0.05 mol) in 25 mL of acetone was placed in an ice-water bath to keep the temperature between 0 and 5 °C. Then 0.05 mol of different amines were added slowly and after 30 min 7.2 g (0.05 mol) of $NaHCO_3$ was added to the mixture at 0–5 °C. The resulting mixture was then stirred for a further 1 hour at the same temperature (0–5 °C) and finally, the solid product was filtered, washed with water, and dried under vacuum to obtain 4,6-dichloro-1,3,5-triazine-2-ylphenylamine derivatives as white solids.

2.2.2. General procedure for the synthesis of vanillin-triazine compounds 5a-5f

In this series of compounds, the second chlorine group of the cyanuric chloride was replaced by vanillin. To a solution of synthesized compounds **(3a-3d)** (25.5 mmol) in 15 mL of DMF, 3.8 g (25.5 mmol) of vanillin, and 3.5 g (25.5 mmol) potassium carbonate were added. The reaction mixture was then stirred at 60 °C for 12 h. The progress of the reaction was monitored by TLC. Upon

completion, the mixture was decanted in crushed ice; the separated solid was collected and recrystallized from ethanol.

2.2.2.1. 4-(4–chloro-6-(phenylamino)−1,3,5-triazin-2-yloxy)−2-methoxybenzaldehyde (5a). $C_{17}H_{13}ClN_4O_3$; Yield: 83%, White Solid, m.p. 145–146 °C, IR (KBr, Cm^{-1}), 3362 (N–H, st), 3062, (C–H, aromatic, st), 2932 (C–H, aliphatic, st), 2742 (C–H, aldehyde, st), 1714 (*C = O*, aldehyde, st), 1557 (*C = N*), 1415–1612 (*C = C* aromatic), 768 (C–Cl).^{1}H NMR: (400 MHz; DMSO: d_6): 11.2 (1H, s, CHO), 8.0 (1H, d, ArH, *j* = 8), 7.7 (1H, S, ArH),*δ* 7.4 (2H, d, ArH, *j* = 6.8), 7.2 (4H, m, ArH), 6.5 (1H, NH, br), 3.72 (3H, s, OCH3).

2.2.2.2. 4-(4-(4-bromophenylamino)−6–chloro-1,3,5-triazin-2-yloxy)−2-methoxybenzaldehyde (5b). $C_{17}H_{12}BrClN_4O_3$; Yield: 86%, Yellow Solid, m.p. 140–142 °C, IR (KBr, Cm^{-1}), 3350 (N–H, st), 3132, (C–H, aromatic, st), 2824 (C–H, aldehyde, st), 1711 (*C = O*, aldehyde, st), 1651 (*C = N*), 1391–1680 (*C = C* aromatic), 1259 (C–O, st), 749 (C–Cl, st). ^{1}H NMR: (400 MHz; DMSO: d_6): 11.12 (1H, s, CHO), 8.1 (1H, d, ArH, *j* = 8), 7.7(3H, m, ArH), 7.42 (2H, d, ArH, *j* = 7), 7.2 (1H, d, ArH, *j* = 8), 6.7 (1H, NH, br), 3.69 (3H, s, OCH_3).

2.2.2.3. 4-(4-(4-formyl-3-methoxyphenoxy)−6–chloro-1,3,5-triazin-2-ylamino)benzonitrile (5c). $C_{18}H_{12}ClN_5O_3$; Yield: 83%, White Solid, m.p. 150–152 °C, IR (KBr, Cm^{-1}), 3270 (N–H, st), 3087, (C–H, aromatic, st), 2925 (C–H, aliphatic, st) 2784 (C–H, aldehyde, st), 2259 (C≡N, st), 1717 (*C = O*, aldehyde, st), 1571 (*C = N*), 1400–1600 (*C = C* aromatic), 1310 (C–O, st), 750 (C–Cl, st). ^{1}H NMR: (400 MHz; DMSO: d_6): 11.18 (1H, s, CHO), 8.1 (1H, d, ArH, *j* = 8), 7.7 (3H, m, ArH), *δ* 7.36 (2H, d, ArH, *j* = 6.8), 7.2 (1H, d, ArH, *j* = 8), 6.4 (1H, NH, br), 3.70 (3H, s, OCH_3).

2.2.2.4. 4-(4-(p-tolylamino)−6–chloro-1,3,5-triazin-2-yloxy)−2-methoxybenzaldehyde (5d). $C_{18}H_{15}ClN_4O_3$; Yield: 87%, White Solid, m.p. 140–143 °C, IR (KBr, Cm^{-1}), 3363 (N–H, st), 3067, (C–H, aromatic, st), 2936 (C–H, aliphatic, st) 2816 (C–H, aldehyde, st), 1717 (*C = O*, aldehyde, st), 1579 (*C = N*), 1371–1600 (*C = C* aromatic), 1252 (C–O, st), 770 (C–Cl, st). ^{1}H NMR: (400 MHz; DMSO: d_6): 11.12 (1H, s, CHO), 7.89(1H, d, ArH, *j* = 8), 7.73 (1H, s, ArH), *δ* 7.10–7.30 (5H, m, ArH), 5.7 (1H, NH, br), 3.70 (3H, s, OCH3), 1.25 (3H, s, CH_3).

2.2.3. General procedure for the synthesis of mono or diamino substituted triazine compounds 8a-8d

The mono amino (ethyl or methyl) substituted derivatives (**8a** and **8b**) were synthesized using the protocol reported for compound **3a**-**3d** [26]. In diamino substituted triazine compounds, two chlorine groups of the cyanuric chloride were replaced by amino (ethyl or methyl) groups. For this purpose, trichloro-1,3,5-triazine (5.0 g, 27 mmol) was dissolved in acetone (35 mL) and its temperature keeps at 0–5 °C by putting it in an ice-water bath. Then a solution of methylamine or ethylamine (54 mmol) was added in a dropwise manner and the solution was stirred for 30 min at room temperature. To this mixture, $NaHCO_3$ (54 mL, 2 N 108 mmol) was added and stirred for 6 h at 40 °C. Finally, the product as a white powder was filtered, washed with water, and dried under vacuum [26].

2.2.4. Synthesis of 4-Phenyl-1H-pyrazole (9)

4-Phenyl-1H-pyrazole **(9)** was synthesized by the earlier reported procedure [27]. To a dry and cooled DMF (25 mL), $POCl_3$ (10 mL) was added dropwise. Then phenylacetic acid (5 g, 37 mmol) was added and the solution was stirred at 85 °C for 3 h. After cooling to room temperature, the mixture was poured onto 50 mL of ice and kept for 0.5 h. Then a saturated solution of $NaBF_4$ (15 g in 10–15 mL of distilled water) was added to furnish a yellow solid which was filtered off and washed with ice-cold water. The obtained solid dissolved in a warm solution of NaOH (6 g in 100 mL of H_2O) at 50 °C and then the pH of the solution was adjusted to pH = 5 by dropwise addition of 10% HCl. In the next step hydrazine hydrate (4 mL, 0.13 mol) was added dropwise and the solution was stirred overnight. Finally, the resulting 4-phenyl-1H-pyrazole was filtered off as a white crystal and dried.

2.2.5. General procedure for the synthesis of phenylpyrazole-triazine derivatives (10a-10e)

For the synthesis of phenylpyrazole-triazine derivatives **10a-10e**, compounds **8a**-**8d** (20 mmol) were dissolved in DMF (20 mL) and then phenyl pyrazole (2.88 g, 20 mmol) was added and the solution was stirred at 60 °C for 3 h in presence of K_2CO_3 (2.7 g, 20 mmol). After completion of the reaction by monitoring with TLC, the reaction mixture was poured in water and the resulting precipitate was filtered and dried. As another derivative of this class, phenylpyrazole (2.88 g, 20 mmol) was reacted with an equimolar ratio with cyanuric chloride (3.70 g, 20 mmol) in acetone (15 ml) at room temperature. The product, which was precipitated in the mixture, was used for further investigations. The obtained products were recrystallized in ethyl acetate and n-heptane.

2.2.5.1. 4–chloro-N-methyl-6-(4-phenyl-1H-pyrazol-1-yl)−1,3,5-triazin-2-amine (10a). $C_{13}H_{11}ClN_6$; Yield: 61%, White Solid, m.p. 110–111 °C, IR (KBr, Cm^{-1}), 3221 (N–H, st), 3091 (C–H, aromatic, st), 2921 (C–H, aliphatic, st), 1552 (*C = N*), 1400–1600 (*C = C* aromatic), 770 (C–Cl, st). ^{1}H NMR: (400 MHz; DMSO: d_6): 8.1(1H, s, ArH, br), 7.9 (1H, s, ArH, br), 7.5 (2H, d, ArH, *j* = 7), 7.30 (2H, t, ArH, *j* = 7), 7.1 (1H, t, ArH, *j* = 7), 5.67 (1H, s, NH, br), 2.08 (3H, s, CH_3).

2.2.5.2. 4–chloro-N-ethyl-6-(4-phenyl-1H-pyrazol-1-yl)−1,3,5-triazin-2-amine(10b). $C_{14}H_{13}ClN_6$; Yield: 60%, White Solid, m.p. 125–126 °C, IR (KBr, Cm^{-1}), 3278 (N–H, st), 3103 (C–H, aromatic, st), 2948 (C–H, aliphatic, st), 1650 (*C = N*), 1400–1600 (*C = C* aromatic), 730 (C–Cl, st). ^{1}H NMR: (400 MHz; DMSO: d_6): 8.3 (1H, s, ArH, br), 7.9 (1H, s, ArH, br), 7.8 (2H, d, ArH, *j* = 6.9), 7.4 (2H, t, ArH, *j* = 6.9), 7.2 (1H, t, ArH, *j* = 6.9), 4.83 (1H, s, NH, br), 2.90 (2H, q, CH2, *j* = 3), 1.20 (3H, t, CH_3, *j* = 3).

2.2.5.3. N_2,N_4-dimethyl-6-(4-phenyl-1H-pyrazol-1-yl)−1,3,5-triazine-2,4-diamine (10c). $C_{14}H_{15}N_7$; Yield: 60%, White Solid, m.p. 115–116 °C, IR (KBr, Cm^{-1}), 3278 (N–H, st), 3086 (C–H, aromatic, st), 2948 (C–H, aliphatic, st), 1650 (*C = N*), 1400–1600 (*C = C* aromatic). ^{1}H NMR: (400 MHz; DMSO: d_6): 8.4 (1H, s, ArH, br), 8.1 (1H, s, ArH, br), 7.54 (2H, d, ArH, *j* = 8), 7.22 (2H, t, ArH, *j* = 8), 7.15 (1H, t, ArH, *j* = 8), 5.20 (2H, s, 2NH, br), 2.50 (6H, s, $2CH_3$).

2.2.5.4. N_2,N_4–diethyl-6-(4-phenyl-1H-pyrazol-1-yl)−1,3,5-triazine-2,4-diamine (10d). $C_{16}H_{19}N_7$; Yield: 56%, White Solid, m.p. 131–133 °C, IR (KBr, Cm^{-1}), 3266 (N–H, st), 3096 (C–H, aromatic, st), 2983 (C–H, aliphatic, st), 1618 (*C = N*), 1399–1550 (*C = C* aromatic). ^{1}H NMR: (400 MHz; DMSO: d_6): 8.1 (1H, s, ArH, br), 7.9(1H, s, ArH, br), 7.68(2H, d, ArH, *j* = 8), 7.4 (2H, t, ArH, *j* = 8), 7.2 (1H, t, ArH, *j* = 8), 4.53 (2H, s, 2NH, br), 3.00 (4H, q, $2CH_2$, *j* = 3), 1.10 (6H, t, $2CH_3$, *j* = 3).

2.2.5.5. 2,4-dichloro-6-(4-phenyl-1H-pyrazol-1-yl)−1,3,5-triazine (10e). $C_{12}H_7Cl_2N_5$; Yield: 60%, White Solid, m.p. 110–112 °C, IR (KBr, Cm^{-1}), 3093 (C–H, aromatic, st), 1560 (*C = N*), 1400–1600 (*C = C* aromatic), 742 (C–Cl). ^{1}H NMR: (400 MHz; DMSO: d_6): 8.4(1H, s, ArH), 8.1 (1H, s, ArH), 7.70 (2H, d, ArH, *j* = 7.9), 7.4 (2H, t, ArH, *j* = 7.9), 7.3 (1H, t, ArH, *j* = 7.9).

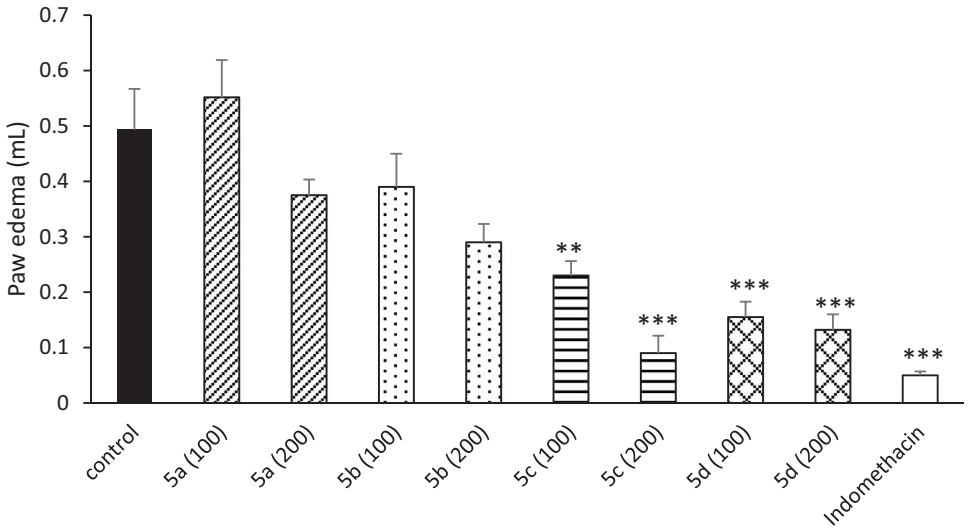


Fig. 1. The anti-inflammatory activity of vanillin-triazine derivatives in the carrageenan-induced paw edema test in the rat ($n = 6$). Vehicle (0.5% Tween 80 in saline) and two doses of each compound (100 and 200 mg/kg as shown in parenthesis) were administered 30 min before to sub plantar injection of carrageenan, and the volume of the paw (mL) was measured immediately before carrageenan injection and 4 h after injection. Indomethacin (10 mg/kg, i.p.) was used as the reference drug. Data are mean ± SEM of six animals in each group. $^{*}P$ <0.05 and $^{***}P$ < 0.001 and show significant difference from the control group (ANOVA with Scheffe post hoc test).

2.3. Biological activity

2.3.1. Anti-inflammatory activity

The anti-inflammatory activity of the newly synthesized compounds was evaluated using the carrageenan-induced paw edema model in rats [28]. Experiments were performed on male Wistar rats, weighing 200–220 g. All animals were maintained under standard laboratory conditions in the animal house of the School of Pharmacy, Isfahan University of Medical Sciences (Isfahan, Iran). These animals were euthanized immediately after each experiment. All experiments were carried out by local guidelines for the care of laboratory animals of Isfahan University of Medical Sciences (Isfahan, Iran). Animals are weighed, randomized into 6 groups, and kept for 1 week to acclimatize to the laboratory conditions and to minimize the stress level. The synthesized compounds were administered intraperitoneally (i.p.) at doses of 100 and 200 mg/kg to rats. The control group received only vehicles. The reference group or positive control received indomethacin (10 mg/kg). Thirty minutes after administration, the rats received a sub-plantar injection of 100 μL of a 1% (w/v) suspension of carrageenan lambda in the right hind paw [29]. The volume of the paw was measured by a mercury plethysmometer (Ugo Basil, Italy) immediately before and 4 h after carrageenan injection. The data were expressed as mean ± SEM of the volume difference (mL) of carrageenan-treated and control paw. The data obtained in the experimental groups were analyzed by one-way analysis of variance (ANOVA) followed by a Scheffe post hoc test. P <0.05was considered significant. The result of the anti-inflammatory activity of compounds is shown in Figs. 1 and 2.

2.4. Molecular docking

Molecular docking studies were performed with AutoDock 4.2. The methodology used for molecular docking protocol was similar to our previous reports [30]. The structures of COX-2 (PDB code: 3LN1, resolution: 2.4 Å) and COX-1 (PDB code: 3KK6, resolution: 2.75 Å) were obtained from the protein databank. Celecoxib as a co-crystallized inhibitor and water molecules were removed together with β-octylglucoside and *N*-acetyl-D-glucosamine in the structure; at the same time. The charge of the Fe atom was set to +2 manually. This structure was protonated using the AutoDock Tools program and was used for docking. The synthesized compounds **5a-5d** and **10a-10e** were drawn with the hyperchem software and optimized by using the semi-empirical PM3 method. Then the most stable conformation was utilized in docking calculation. The AutoDock Tools program was used to generate the docking input files. For docking of the synthesized compounds into the COX-1 and COX-2 structures, Auto Dock 1.5.6 software was used [31]. The size of the binding pocket was detected 50 × 50 × 50 points for both enzymes. According to the position of celecoxib in these structures, for the COX-2 enzyme, x (31.144), y (−22.960) and z (−16.605) dimensions were built, while x (−32.344), y (43.754), and z (−4.433) dimensions were used in COX-1 enzyme docking studies. The grid and docking parameter was set according to default parameters and finally, the obtained results files were analyzed using Accelrys Discovery Studio Visualizer 3.0 program and PyMOL Molecular Graphics System. By re-docking of celecoxib as a co-crystallized inhibitor back into respective enzymes COX-2 and COX-1 with root mean square deviation (RMSD) of below 1 Å values, the molecular docking protocol was validated. The free energies of binding (ΔG_b) and inhibition constants (K_i) and H-bond interaction obtained from the docking studies of the compounds as well as celecoxib as reference ligand with COX-1 and COX-2 by using Autodock4 is presented in Table 1.

3. Results and discussion

3.1. Preparation of novel vanillin-triazine and phenylpyrazole-triazine derivatives

Both vanillin-triazine and phenylpyrazole-triazine derivatives were prepared from the multistep condensation reaction of cyanuric chloride **(1)** and different nucleophiles as shown in **Scheme 2**. Because of the different reactivity of three chloro groups on triazines, the reactions were performed at different temperatures. At low temperature (below 5 °C) only the first chloro of cyanuric chloride can be substituted, while at room temperature two of them can participate in substitution reactions, and under high temperatures, all three chloro groups can be replaced with nucleophiles [32]

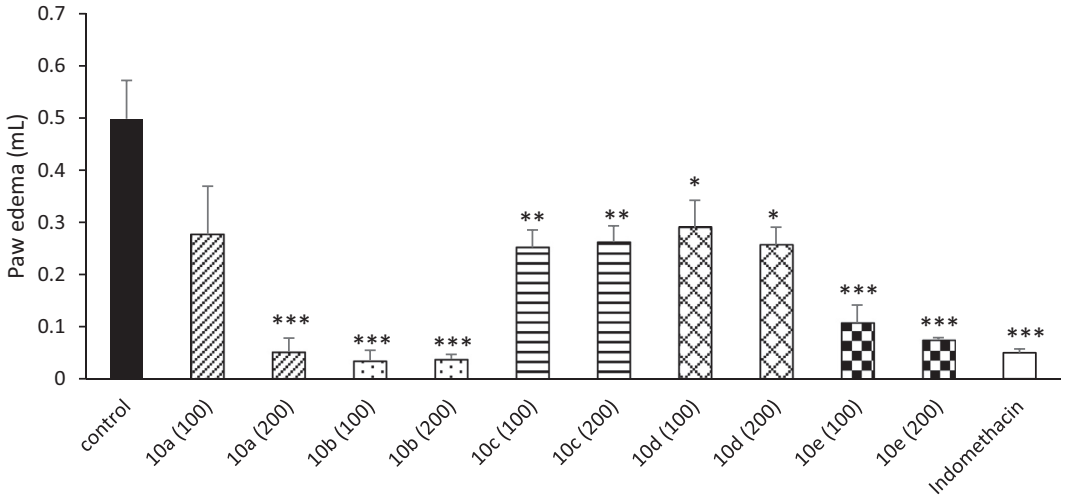


Fig. 2. The anti-inflammatory activity of phenylpyrazole-triazine derivatives in the carrageenan-induced paw edema test in the rat ($n = 6$). Vehicle (0.5% Tween 80 in saline) and two different doses of each compound (100 and 200 mg /kg as shown in parenthesis) were administered 30 min before to subplantar injection of carrageenan and the volume of the paw (mL) was measured immediately before carrageenan injection and 4 h afterward. Indomethacin (10 mg/kg, i.p.) was used as the reference drug. Data are mean ± SEM of six animals in each group. $^{*}P < 0.05$ and $^{***}P < 0.001$ show significant differences from the control group (ANOVA with the Scheffe post hoc test).

Table 1
Free binding energy (kcal/mol), interactions as well as inhibition constants (Ki) of synthesized compounds calculated by AutoDock.

compound	COX2 Binding energy[1]	Inhibition constants	H-bond	Hydrophobic interaction	COX1 Binding energy[1]	Inhibition constants	H-bond
5a	−8.69	423.9 [2]	Arg499	Tyr355, Ser530, Leu352	−8.34	768.30 [2]	–
5b	−8.78	367.85 [2]	Arg 499	Ser530, Tyr348, Val349	−7.27	4.73(μm)	–
5c	−9.23	172.25 [2]	PHe504	Leu352, Ser530, Val349	−8.38	423.08 [2]	–
5d	−9.12	205.36 [2]	Arg499	His90, Ser530, Leu352, tyr348	−7.74	2.11 [3]	–
10a	−7.14	5.87[3]		Ser530, Phe518, Leu531	−6.6	18.68[3]	–
10b	−7.4	3.77[3]		Leu352, Ile523, Tyr355, Trp387	−5.89	54.51[3]	Tyr371
10c	−7.27	4.66 [3]	Ser530	Leu352, Tyr355, Ile523, Trp387	−5.98	41.22[3]	Tyr371
10d	−7.41	3.69 [3]	Ser530	Phe518, Ile523, Tyr355, Ala527	−6.21	28.18[3]	–
10e	−8.41	668.67 [3]		Ser530, His90, Ser353, Tyr355,	−6.41	21.10[3]	–

[1] : (kcal/mol),.
[2] : nanomolar,.
[3] : micromolar.

In vanillin-triazine series, substituted 4,6-dichloro-1,3,5-triazin-2-ylphenylamine derivatives (**5a-d**) were synthesized by nucleophilic attack of different aromatic amines(**2a-2d**) to cyanuric chloride **(1)** at 0–5 °C. In the next step disubstituted s-triazine compounds (**5a-5d**) were obtained by the reaction of vanillin **(4)** with **3a-3d** derivatives. In phenylpyrazole-triazine compounds, one and/or two chlorine of cyanuric chloride **(1)** was replaced by methylamine **(6)** and/ or ethylamine **(7)** to produce **(8a-8f)** and the next chloro was replaced with 4-Phenyl-1H-pyrazole **(9)** which was prepared through Vilsmeier reaction, using a protocol reported in earlier work to obtain final products **(10a-10e).** For the preparation of tri-substituted triazines, the reactions were performed at 60 °C.

3.2. Characterizations of synthesized compounds

Novel vanillin-triazine and phenylpyrazole-triazine derivatives were characterized by spectral techniques such as ^{1}H NMR and FT-IR techniques (the results are presented in the experimental part).

In the vanillin-triazine series, the FT-IR spectrum of compound **5a-5d** showed absorptions at 3362–3270 and 1671–1557 cm^{-1} which indicate the presence of NH and $C = N$ groups, respectively. The vibrations in the range of 1717–1711cm^{-1} and also 2824–2742 cm^{-1}, showed stretch vibration of the aldehyde group of vanillin. The bands at 3132–3062 and 2936–2925 cm^{-1} also confirm stretch vibration of aromatic and aliphatic C–H, respectively. The absorptions at 2259 and 770–749cm^{-1} are related to C≡N and C–Cl groups, respectively. FT-IR spectrum of compound **5c** is shown as an example in Fig. 3. The ^{1}H NMR spectral data of the final vanillin-triazine products recorded in (D_6) DMSO along with its possible assignments was reported in the experimental part. All the aromatic and aliphatic H-atoms were found in their expected regions. The absorption of aromatic H-atoms of vanillin and phenyl rings appeared in the range of 8.1–7.2 ppm. The NH of the phenylamine ring exhibited a broad singlet at 6.7–5.7 ppm. The H-atoms of aldehyde and methoxy groups on the vanillin ring, present in derivatives, were found in the region at 11.20–11.12 and 3.72–3.69 ppm, respectively. ^{1}HNMR spectrum of compound **5c** is shown as an example in Fig. 4. It should be mentioned that the H-atoms of methyl substitution on the phenyl rings in **5d**, was found in the region at 1.25 ppm.

FT-IR spectrum of phenylpyrazole-triazine compounds **10a-10e** also confirmed the structure of synthesized compounds. The main IR vibrational bands of the compounds appeared around 1650–1552 and 1600–1400 confirm the presence of $C = N$ and $C = C$ groups, respectively. In derivatives containing the methylamine or ethylamine group, absorptions at 2983–2921 and 3278–3221 cm^{-1} indicated the presence of the aliphatic C–H and NH in the structures. FT-IR spectrum of compound **10b** is shown as an example in Fig. 3. The chemical structure of the synthesized phenylpyrazole-triazine compounds was further confirmed by the NMR technique.

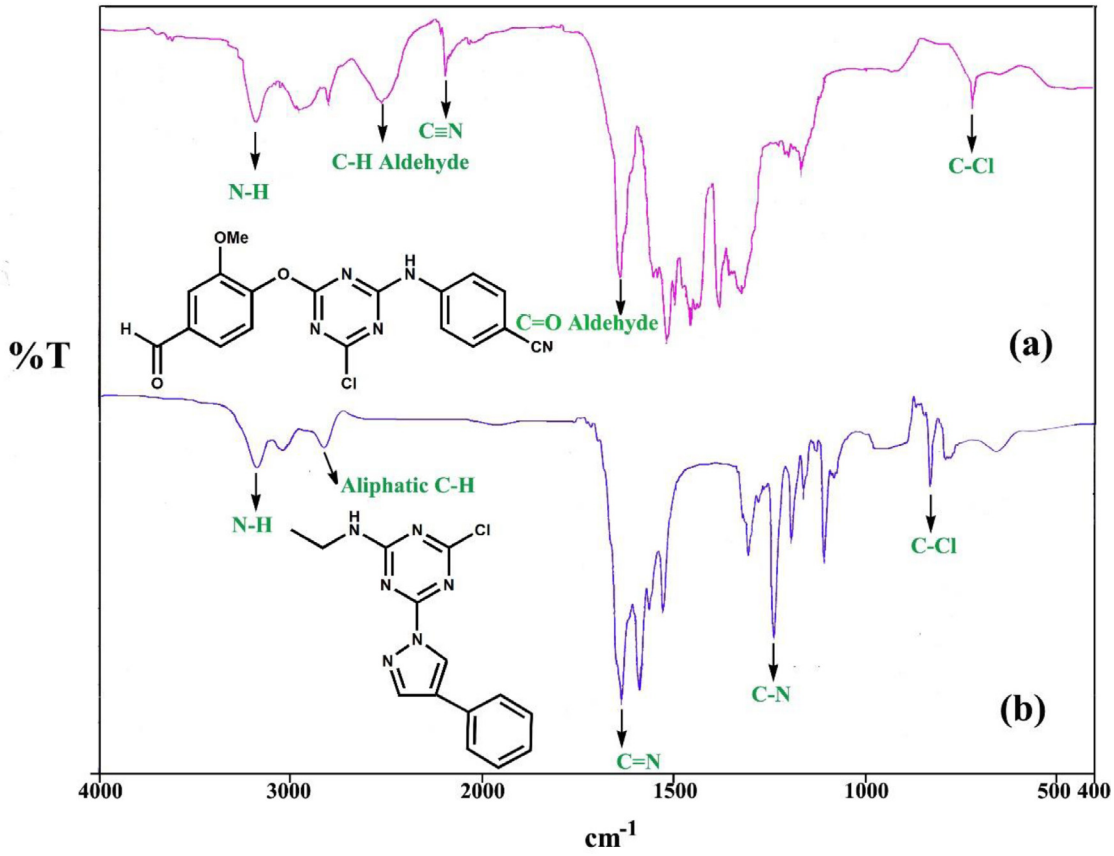


Fig. 3. FT-IR spectrum of compounds (a) **5c** and (b) **10b**.

In this series, the absorption of aromatic H-atoms of derivatives appeared in the range of 8.4–7.1 ppm. In the ^{1}H NMR spectrum of compounds **10a-10f**, the NH group of methyl and or ethylamine was presented at 5.67–4.53 ppm. Methyl and methylene groups related to methyl and ethylamine also were observed at 2.5–1.1 and 3.0–2.9 ppm, respectively. ^{1}HNMR spectrum of compound **10b** is shown as an example in Fig. 4. To summarize, the NMR spectra showed characteristic peaks for the synthesized hybrid compounds.

3.3. Anti-inflammatory activity

A well-known model of acute inflammation is Carrageenan-induced paw edema which consists of a biphasic inflammatory response produced by several inflammatory mediators [33]. After the injection of carrageenan into the rat paw, several mediators such as histamine, serotonin, and bradykinin are sequentially released in the initial phase (0–1 h), and then the production of prostaglandins (PGs) are increased through the activation of cyclooxygenase-2 (COX-2) and the release of nitric oxide (NO) in the second phase (1–6 h) which are considered as inflammatory factors and play important roles in the tissue damage by inflammation [33,34]. The earlier work showed that derivatives of s-triazine exerted anti-inflammatory effects. About 35.17% of evaluated triazines revealed promising which is related to the inhibition of the p38 MAPK pathway or in the production of several inflammatory mediators [18]

In this investigation Indomethacin as a standard anti-inflammatory drug inhibited carrageenan-induced paw edema by 90% [Figs. 1 and 2]. Compound **10e** as the best derivatives in the studied compounds showed significant anti-inflammatory activity (reduced carrageenan-induced paw inflammation by 93%) at doses of 100 and 200 mg/kg significantly ($P < 0.001$). In vanillin-triazine derivatives, compounds **5a** and **5b** didn't exert good anti-inflammatory properties in comparison to Indomethacin as illustrated in **Fig. 1**. In this series, compound **5c** at doses of 200 mg/kg significantly ($P < 0.001$) showed comparable anti-inflammatory effects to indomethacin by a significant reduction in paw size (81%) after carrageenan injection. This compound at a dose of 100 mg/kg inhibited carrageenan-induced paw edema by 54% (Fig. 1). In this series of compounds, **5d** doses of 100 and 200 mg/kg resulted in significantly ($P < 0.001$) inhibition of edema of 69% and 74% respectively. Phenylpyrazole-triazine derivatives (**10a-10e)** showed better anti-inflammatory effects than the vanillin-triazine derivatives **5a-5d** Compound **10a** at a dose of 200 mg/kg significantly ($P < 0.001$) showed comparable anti-inflammatory effects by a significant reduction in paw size (89.6%) after carrageenan injection. Compound **10b** at doses of 100 and 200 mg/kg significantly ($P < 0.001$) showed a greater inhibitory effect (93%) than indomethacin and it was the best compound in both series. Phenylpyrazole-triazine derivatives compound **10e** at doses of 200 mg/kg significantly ($P < 0.001$) showed comparable anti-inflammatory effects by a significant reduction in paw size (85%) after carrageenan injection.

Data from Carrageenan-induced paw edema models suggest that the designed triazine derivatives showed a very good anti-inflammatory effect compared to the similar previously studied compounds, reported in the Table 2. The anti-inflammatory effect of the compounds may be due to a decrease in the production of PGs, NO, bradykinin, or other inflammatory mediators. However, more investigations are needed to find out their exact mechanism of action.

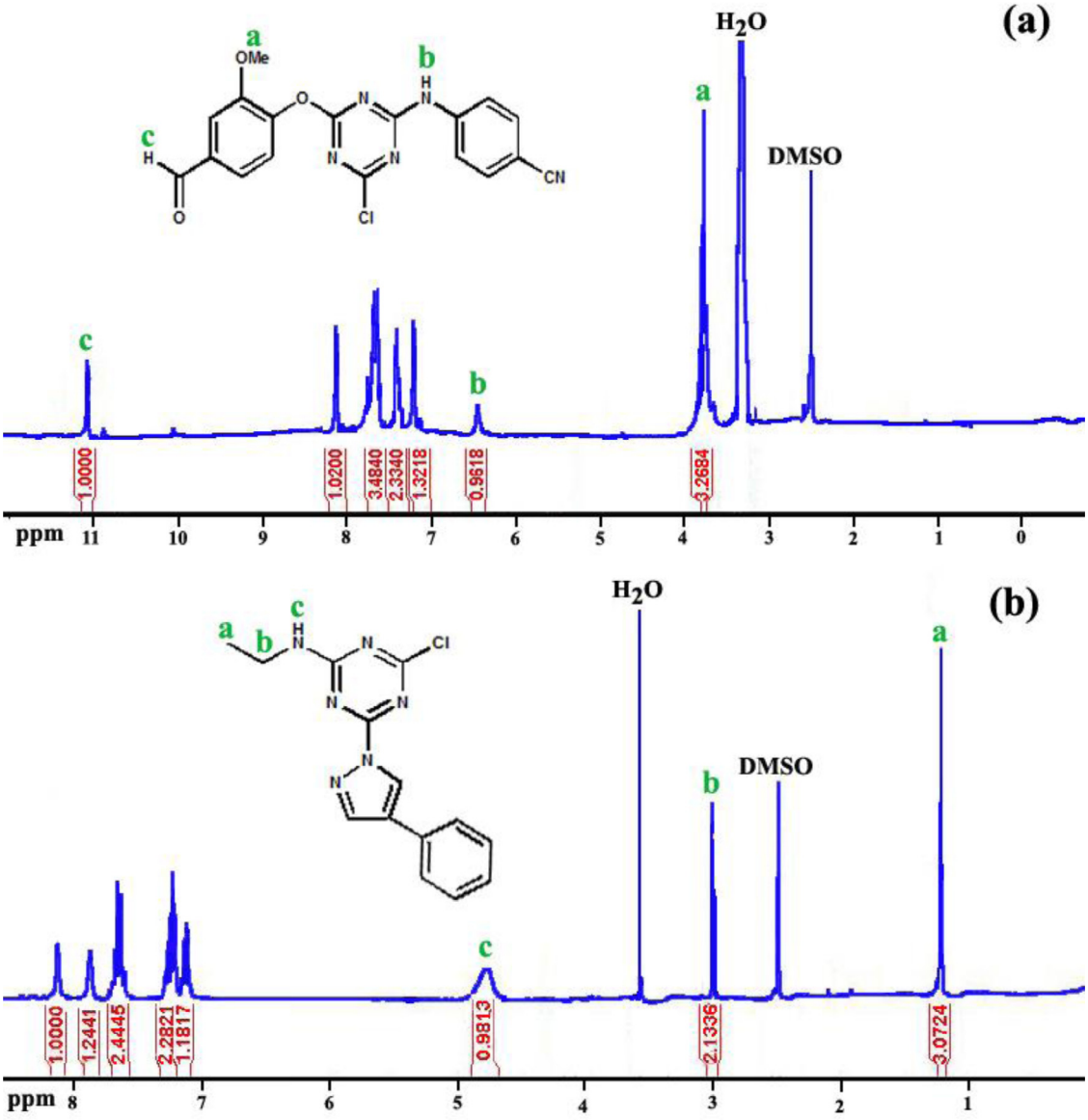


Fig. 4. [1]HNMR spectrum of compounds (a) **5c** and (b) **10b** in DMSO.

3.4. Discussion of docking

One of the computationally inexpensive methods for predicting if and how a ligand will bind to a protein binding pocket, followed by an estimation of how strong is the ligand-binding affinity, is molecular docking [40-42]. To explain the COX-1/COX-2 selectivity of newly synthesized compounds, a docking study was used to estimate the possible inhibitory activities and mechanism of binding to COX enzymes. In general, the binding energy of both synthetic compounds with cyclooxygenase II was higher than the cyclooxygenase I enzyme. The result of docking of vanillin-triazine derivatives showed a relatively better selectivity to COX-II active site which can be attributed to having more functional groups on this group of compounds. In vanillin-triazine derivatives by holding constant the aniline core, substitution of bromine, methyl, and nitrile groups on phenyl rings may increase binding energy through hydrophobic and H-bonding interactions. The aldehyde groups on the vanillin ring played a conserved role in stabilizing the docking of aniline, bromoaniline, and methylamine derivatives**,** and balanced its placing with the key amino acid, Arg499, by forming proper H-bonds. Meanwhile, some H-bonds are stabilized by the binding of the Phe504 amino acids with the carbonyl oxygen of the vanillin in aminobenzonitryl derivative. The phenyl ring of aniline derivatives and vanillin formed proper hydrophobic interactions with His90, Tyr355, Ser530, Tyr385, and Leu352.

Phenylpyrazole-triazine derivatives also showed better selectivity to COX-II active site. In this series, when two methylamine or two ethylamine groups are placed on a tri-azine ring, the compound can form a hydrogen bond with Ser530 amino acid. The triazine and phenylpyrazole ring in his group also formed proper hydrophobic interactions with His90, Tyr355, Typr387, and Leu352.

Molecular docking studies have shown that compounds 4-aminobenzonitryl derivative from vanillin-triazine series, **5c** ($\Delta G_{binding} = -9.23$) and aminoethyl triazine- phenylpyrazole, **10c** ($\Delta G_{binding} = -7.41$) are the best compounds in each category, respectively (Fig. 5).

Table 2
Anti-inflammatory effect of similar previously studied compounds.

Triazine based compound	Anti-inflammatory test	Route of administration	Std. drug	% Inhibition of Edema.	Degree of paw thickness	Ref.
7-methyl-4-phenylpyrazolo[1,5-α][1,3,5]triazine-2(8H)-thione	Carrageenan induced rat paw edema	oral	Phenylbutazone	Std.=60 Comp= 54.28	–	[35]
N-(2, 6-di (piperazin-1-yl) pyrimidin-4-yl)−1-(4, 8-dimethoxy-3-methylbenzo [1,2-b: 5,4-b'] difuran-2-yl)−1-(piperazin-1-yl) methanimine	Carrageenan induced rat paw edema	oral	Diclofenac	Std.=63.00 Comp=68.00	–	[36]
N-arylidene-N'- [5-(4-isobutylphenyl)-[1,2,4]-triazin-3-yl] hydrazines	Carrageenan induced rat paw edema	oral	Ibuprofen	Std.= 50.51 Comp=58.37	–	[37]
2- (5,6-diphenyl-3-thioxo-1,2,4-triazine-2-yl)−3,4,5-trimethoxy-phenilic acid	Carrageenan induced rat paw edema	oral	Indomethacin	Std.= 79.28 Comp=58.24	–	[38]
vanilline	Carrageenan induced rat paw edema	i.p.	diclofenac	–	Std.=0.4 Comp=0.4	[19]
(2,3-Dihydro-benzofuran-5-yl)-acetic acid [4-(4′-fluoro-biphenyl-4-ylmethoxy)−3-methoxybenzylidene]-hydrazide	Carrageenan induced rat paw edema	ip	Diclofenac sodium	–	Std.=1.16 Comp=1.00	[39]

ip: Intraperitoneal injection.
–: not reported.

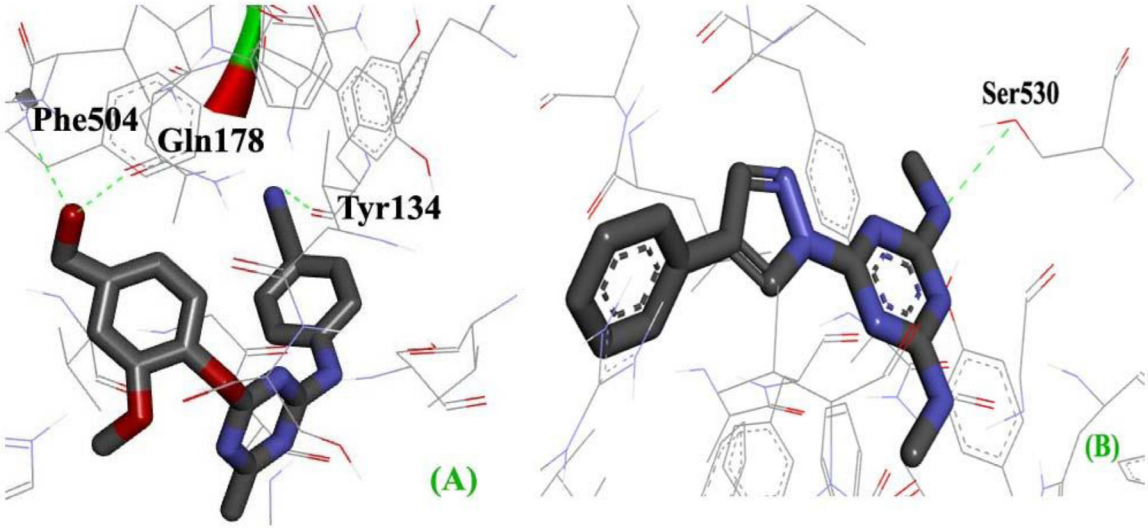


Fig. 5. The binding mode of compounds 5c (A) and 10c (B) in the active site of COX-II was obtained from autodock4.

4. Conclusion

Two series of triazine-based derivatives were synthesized and evaluated for *in vivo* anti-inflammatory activities. The structure of nine synthesized compounds (5a-5d and 10a-10e) consisting of triazine, vanillin, and phenylpyrazole was confirmed by spectroscopic methods (FT-IR, and NMR spectral data). The anti-inflammatory activity of these derivatives was evaluated using the carrageenan-induced paw edema model in male Wistar rats (200–220 g) administered intraperitoneally at doses of 100 and 200 mg/kg. Compounds **5c, 5d, 10a, 10e** at doses of 200 mg/kg significantly ($P < 0.001$) showed anti-inflammatory effects comparable to the

standard drug indomethacin (90%) by a significant reduction in paw size (74–89.6%) after carrageenan injection. Compound **10b** at doses of 100 and 200 mg/kg significantly ($P < 0.001$) showed a greater anti-inflammatory activity (reduced carrageenan-induced paw inflammation by 93%) than indomethacin and it was the best compound in both series. From the docking studies, compounds **5c** and **10c** are the most active analogs, with the lowest binding energies. These two compounds exhibited inconsequential recognition at the binding pocket of COX-II. Further studies are necessary to understand the underlying and implicated mechanisms of the observed pharmacologic effects.

Declaration of Competing Interest

None.

CRediT authorship contribution statement

Parvin Asadi: Software, Visualization, Writing – original draft, Conceptualization, Validation, Formal analysis, Resources, Writing – review & editing. **Mohsen Alvani:** Writing – original draft, Formal analysis, Investigation. **Valiollah Hajhashemi:** Conceptualization, Validation, Formal analysis, Writing – review & editing. **Mahboubeh Rostami:** Conceptualization, Validation, Formal analysis. **Ghadamali Khodarahmi:** Supervision, Project administration, Conceptualization, Validation, Resources, Writing – review & editing.

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