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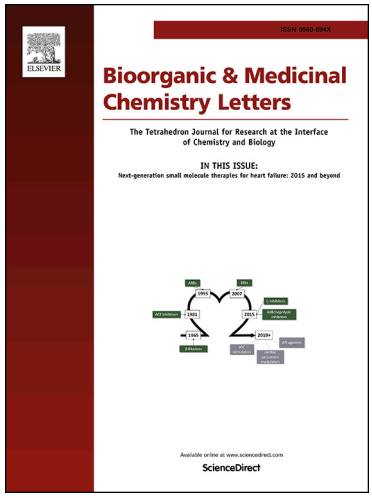

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Design, synthesis, evaluation, and molecular docking of ursolic acid derivatives containing a nitrogen heterocycle as anti-inflammatory agents

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Abstract

Ursolic acid derivatives containing oxadiazole, triazolone, and piperazine moieties were synthesized in an attempt to develop potent anti-inflammatory agents. Structures of the synthesized compounds were elucidated by ^{1}H NMR, ^{13}C NMR, and HRMS. Most of the synthesized compounds showed pronounced anti-inflammatory effects at 100 mg/kg. In particular, compound **11b**, which displayed the most potent anti-inflammatory activity of all of the compounds prepared, with 69.76% inhibition after intraperitoneal administration, was more potent than the reference drugs indomethacin and ibuprofen. The cytotoxicity of the compounds was also assessed by the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-*H*-tetrazolium bromide (MTT) assay, and no compounds showed any appreciable cytotoxic activity (IC_{50} > 100 μmol/L). Furthermore, molecular docking studies of the synthesized compounds were performed to rationalize the obtained biological results. Overall, the results indicate that compound **11b** could be a therapeutic candidate for the treatment of inflammation.

Keywords: Ursolic acid derivatives; Nitrogen heterocyclic; Anti-inflammatory activity; Cytotoxicity; Molecular docking

Inflammation is a complex biological process for modulation of the immune response against a diverse range of triggering factors, including infectious agents, allergens, free radicals, highly refined foods, and a sedentary lifestyle.[1] Exaggerated and prolonged inflammation may cause various diseases, which can seriously threaten human health, such as arthritis, sepsis, and even cancer.[2,3] At present, non-steroidal anti-inflammatory drugs (NSAIDs) are the most important class of widely used therapeutics for the treatment of inflammation and account for 35% of the global market for prescription pain medication.[4-6] Common NSAIDs, such as aspirin and indomethacin, can inhibit both cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2), causing significant toxicity to the gastrointestinal tract and kidney.[7-9] The gastrointestinal irritation, bleeding, and ulceration side effects of classical NSAIDs are caused by high COX-1 versus COX-2 selectivity, and may also be related to the acidity of classical NSAIDs.[10] The simple chemical derivatization (esterification or amidation) of the carboxylic function of representative NSAIDs has been shown to result, not only in reduction of the ulcerogenic effect, but also in increased anti-inflammatory activity.[11-17] Thus, there is a need to develop new anti-inflammatory drugs with decreased acidity and increased specificity for targeting COX-2.

Pentacyclic triterpenes are a very powerful class of natural products because of their wide range of biological activities and diversity of structures.[18,19] Ursolic acid (UA) is a well-known pentacyclic triterpene that is one of the major active components of many traditional Chinese medicines.[20] UA and its derivatives have been reported to have antihepatodamage,[21] anti-HIV,[22] antimalarial,[23] anti-inflammatory,[24] antidiabetic,[25] antimicrobial,[26] and antitumor[27] activity. However, the low bioavailability of UA *in vivo* restricts its clinical application. In recent years, chemical modification of UA has been widely investigated as a method of improving its biological activity and bioavailability. Research has shown that keeping a polar substituent at the C-3 position is essential for the pharmacological activity of UA.[28] The introduction of a nitrogen-containing heterocycle has been shown to be a useful tactic in the structural modification of natural products because the nitrogen atom can carry a positive charge and act as a hydrogen-bond acceptor or donor that can strongly

influence the interaction between the molecule and its target.[29] Recent studies have used 1,3,4-oxadiazole as a pharmacophore because it is a good bioisostere of amides and esters, which can influence the pharmacokinetic properties of drugs by increasing the lipophilicity and thus the ability of drugs to reach their targets by transmembrane diffusion.[30] In addition, several 1,2,4-triazoles have been reported to possess potent antimicrobial[31] and anti-inflammatory[32] activity. Piperazine-based drug discovery has also attracted considerable attention in recent years. Studies have shown that the incorporation of a piperazine moiety can occasionally provide unexpected improvement in the bioactivity of compounds.[33-35] Furthermore, it has been reported that several ursolic acid, oxadiazole, triazole, and piperazine derivatives are COX-2 inhibitors and anti-inflammatory agents (Figure 1).[36-39] These findings suggest that introducing oxadiazole, triazole, and piperazine heterocycles into the C-28 position, and keeping an hydroxy substituent at the C-3 position of the ursolic acid scaffold may produce novel UA derivatives targeting COX-2 with improved anti-inflammatory properties.

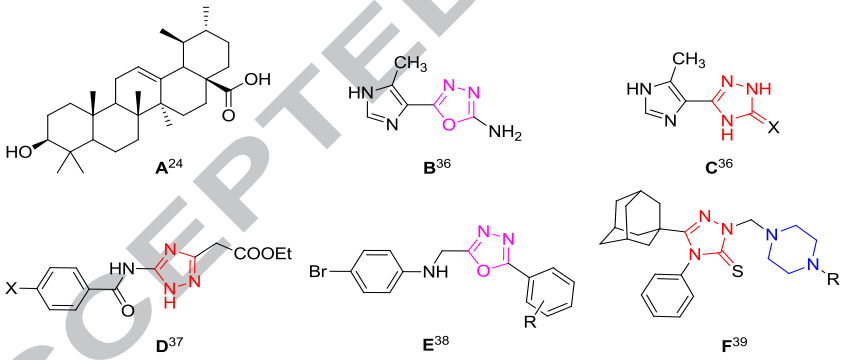
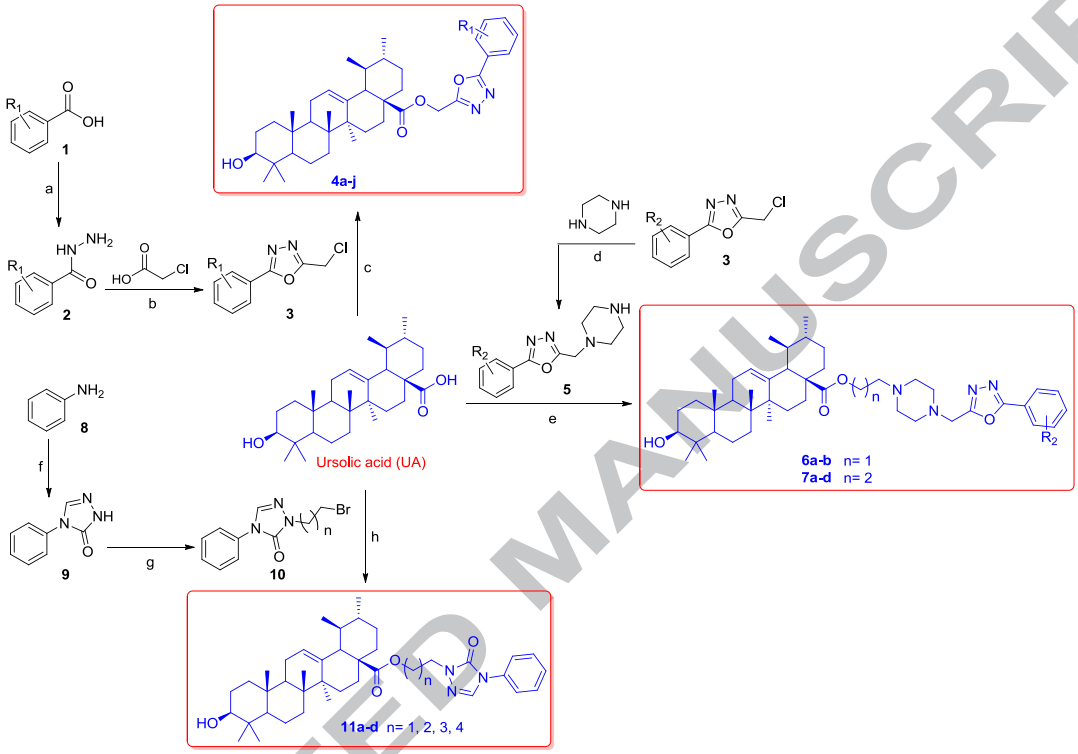


Figure 1. Chemical structures of some previous reported compounds as COX-2 inhibitors and anti-inflammatory agents

On the basis of the abovementioned reports, the present work aimed to synthesize novel derivatives of UA by introducing an 1,3,4-oxadiazole moiety, a piperazine ring, or a 4-phenyl-1*H*-1,2,4-triazol-5(4*H*)-one moiety to the UA nucleus with the objective of discovering potent anti-inflammatory agents that are devoid of gastrointestinal side effects. The substituents on the phenyl ring were simultaneously altered to investigate their contribution to the biological activity. Hence, four novel

series of ursolic acid derivatives were synthesized and evaluated for anti-inflammatory activity. Furthermore, the cytotoxicity of the ursolic acid derivatives was assessed by the MTT assay and the molecular docking with COX-2 was also studied.



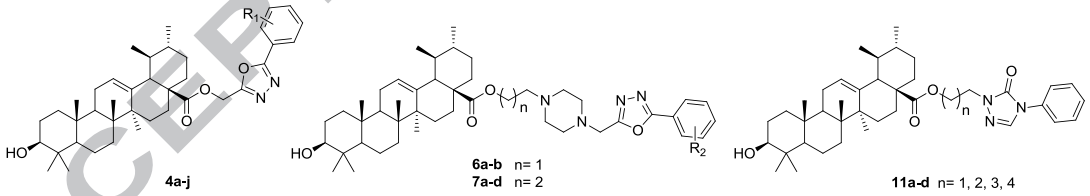
Scheme 1. Synthetic scheme for the synthesis of compounds **4a-j**, **6a-b**, **7a-d** and **11a-d**. Reagents and conditions: (a) MeOH, H_2SO_4; $NH_2NH_2.H_2O$; (b) $POCl_3$; (c) K_2CO_3, KI, acetone, reflux, 10 h; (d) K_2CO_3, KI; (e) $Br(CH_2)_nBr$; K_2CO_3, KI, acetone, reflux, 10 h; (f) $NH_2NHCOOCH_3$, $CH(OC_2H_5)_3$; EtOH, MeONa, reflux, 48 h; (g) $Br(CH_2)_nBr$; K_2CO_3, KI; (h) K_2CO_3, KI, acetone, reflux, 10 h

The synthesis of target compounds is outlined in Scheme 1. The appropriate benzohydrazide **2** was prepared according to the reported method.[40,41] An equimolar mixture of chloroacetic acid and benzohydrazide **2** was refluxed in phosphorus oxychloride (105–110 °C) for 5–6 h to afford compound **3**. The resulting mixture was cooled to room temperature and poured into ice water to give a solid, which was filtered and washed several times with cold water.[42] Then, reaction with anhydrous piperazine in refluxing acetone in the presence of KI/K_2CO_3 afforded the key intermediate **5**. Compound **10** was synthesized by reacting aniline with methyl

hydrazinecarboxylate in refluxing ethanol, in the presence of triethyl orthoformate and sodium methoxide, followed by reaction with a dibromoalkane in the presence of potassium carbonate and potassium iodide in refluxing acetone, as previously described.[43] The target compounds **4a–j**, **6a**–**b**, 7**a**–**d**, and **11a**–**d** were obtained in 60%–90% yields by reacting UA with the intermediates **3**, **5**, or **10** in refluxing acetone in the presence of KI/K_2CO_3.[35] The structures of the target compounds were characterized by ^1H NMR, ^{13}C NMR, and HRMS.

The *in vivo* anti-inflammatory activities of the synthesized compounds were evaluated using the *in vivo* para-xylene-induced mice ear-swelling model. Dimethyl sulfoxide was used as the vehicle for the primary screening of the synthesized compounds and all the target compounds were administered at a dose of 100 mg/kg. Indomethacin (100 mg/kg) and ibuprofen (100 mg/kg) were used as reference drugs, and UA (100 mg/kg) was used as a positive control. The anti-inflammatory activity was assessed based on the ability of the tested compounds to prevent edema,[44] and the results are shown in Table 1.

Table 1. Anti-inflammatory activities of compounds **4a**–**j**, **6a**–**b**, **7a**–**d** and **11a**–**d** following i.p. administration.



	R	Dose (mg/kg)	Number of mice	Edema mean ± S.D. (mg)	Inhibition rate (%)	Cytotoxic activity IC_{50} (μM) [a]
DMSO	—	100	10	13.23 ± 0.81	—	NT [b]
Indometacin	—	100	10	9.68 ± 0.94***	26.83	NT
Ibuprofen	—	100	10	9.90 ± 0.61***	25.17	NT
UA	—	100	10	5.60 ± 1.27***	57.67	23.8
4a	H	100	10	4.98 ± 1.22***	62.31	>100
4b	2-Cl	100	10	8.10 ± 1.36***	38.77	>100
4c	3-Cl	100	10	4.61 ± 0.65***	65.44	>100
4d	4-Cl	100	10	13.30 ± 0.78**	—	>100
4e	2-Br	100	10	5.90 ± 0.84***	55.27	>100
4f	3-Br	100	10	6.12 ± 1.06***	53.74	>100
4g	4-Br	100	10	4.13 ± 1.40***	68.86	>100

4h	2-CH_3	100	10	4.56 ± 0.66***	57.16	>100
4i	3-CH_3	100	10	13.52 ± 0.69*	—	>100
4j	4-CH_3	100	10	5.53 ± 1.00***	58.23	>100
6a	H	100	10	5.41 ± 0.63	59.08	>100
6b	3-Br	100	10	5.16 ± 1.20***	60.99	>100
7a	H	100	10	6.90 ± 1.11	47.75	>100
7b	4-F	100	10	5.43 ± 1.50**	58.74	>100
7c	2-Cl	100	10	9.12 ± 1.50**	31.96	>100
7d	3-Br	100	10	4.40 ± 1.06***	66.49	>100
11a	—	100	10	4.73 ± 0.82***	64.22	>100
11b	—	100	10	4.00 ± 0.74***	69.76	>100
11c	—	100	10	4.48 ± 0.41***	66.09	>100
11d	—	100	10	5.73 ± 0.85***	56.66	>100

*: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$ compared with a vehicle group.

—: no anti-inflammatory activity

[a] The cytotoxic activity of compounds was evaluated by MTT assay after 24 h treatment of HCT116 cells. Values were shown as mean, n=3.

[b] Not tested.

Most of the synthesized compounds showed potent anti-inflammatory activities at 100 mg/kg with inhibition rates ranging from 31.96% to 69.76%. It is noteworthy that compound **11b** exhibited the most pronounced anti-inflammatory activity of all the compounds with an activity of 69.76%, which was higher than that of UA (57.67%), ibuprofen (26.83%), and indomethacin (25.17%). Disappointingly, compounds **4d** and **4i** did not exhibit any anti-inflammatory activity at the same dose, and compound **7c** only showed weak anti-inflammatory activity with an activity of 31.96%. Preliminary structure activity relationship (SAR) analysis can be drawn from the results for anti-inflammatory activity. The *in vivo* anti-inflammatory activity of compounds **4a**, **4c**, **4g** and **4j** was more potent than that of UA, which implies that introduction of an oxadiazole group can improve activity. The presence of different substituents on the phenyl ring of the 1,3,4-oxadiazole group was found to exert an appreciable influence on the observed inhibitory effect on anti-inflammatory activity in series **4**, but no clear pattern could be found for the SAR. The unsubstituted compound **4a** exhibited potent inhibitory activity with an activity of 62.31%. For the halogen substituted derivatives, the activity was in the order 3-Cl > 2-Cl > 4-Cl for the chlorinated compounds (**4c** > **4b** > **4d**) and 4-Br > 2-Br > 3-Br for the brominated

compounds (**4g** > **4e** > **4f**). The compounds (**4j**, **4h**, and **4i**) bearing an electron-donating group showed an activity order of 4-CH_3 > 2-CH_3 > 3-CH_3 (**4j** > **4h** > **4i**). For the **6** and **7** series, there was an obvious enhancement in activity when a piperazine ring was introduced. Compounds **6a** (59.08%), **6b** (60.99%), **7b** (58.74%) and **7d** (66.49%) exhibited more potent inhibitory activity than UA (57.67%), respectively. Comparing compounds **4f**, **6b** and **7d** with 3-Br substitutions, the *in vivo* anti-inflammatory activity was **7d** > **6b** > **4f**. These results imply that in some cases the incorporation of a piperazine moiety to ursolic acid could provide unexpected improvement in the bioactivity of UA derivatives. For the compounds in series **11**, compounds **11a**, **11b**, **11c**, and **11d** exhibited significant anti-inflammatory activity with inhibition rates ranging from 56.66% to 69.76%, 2-fold more potent than ibuprofen and indomethacin and more potent than that of UA. In addition, the order of activity for the different carbon chain lengths was C3 > C4 > C2 > C5 and the order of *in vivo* anti-inflammatory activity was **11b** > **11c** > **11a** > **11d**. These results indicate that incorporation of a triazolone moiety to ursolic acid can improve the anti-inflammatory activity. In general, the *in vivo* anti-inflammatory activity was in the order: series **11** > series **6** ~ series **7** > series **4**, indicating that the triazolone moiety was favorable for anti-inflammatory activity, and introducing a piperazine ring to the oxadiazole structure can also enhance the biological activity of the UA compounds.

Considering its promising anti-inflammatory activity, compound **11b** was chosen for further evaluation. A dose of 100 mg/kg was orally administered at different time intervals (1, 2, 3, 4, 5, and 24 h) after xylene application. As shown in Table 2, the activity profile of compound **11b** was consistent with that of indomethacin. Notably, the activity of compound **11b** (41.17%) reached its peak at 2 h and was found to be more potent than indomethacin at this time point (30.94%). The effect of the dosage on the activity of compound **11b** was also evaluated at concentrations of 25, 50, 100 and 125 mg/kg at 2 h after oral administration as shown in Table 3. The results showed a maximal effect, with an ear inflammation inhibition rate of 50.12%, at 125 mg/kg, which was more potent than indomethacin (38.74%). However, the inhibitory rate of

125 mg/kg doses was not be effectively measured due to the cytotoxicity of the compound which resulted in the death of the test mice. The inhibition rate of 125 mg/kg doses did not increase significantly compare with 100 mg/kg group, but the cytotoxicity was significantly increased. In a nutshell, these results indicated that the 100 mg/kg group of compound **11b** had the best inhibition (47.86%) under the safe dose.

Table 2. Anti-inflammatory activity of compound **11b** administered orally at different times after xylene application.

Time (h)	Dose (mg/kg)	Number of mice	Inhibition (%)	
			11b	Indometacin
1	100	10	22.56	11.96
2	100	10	41.17**	30.94**
3	100	10	29.73	18.65
4	100	10	20.25	15.63
5	100	10	16.37	10.28
24	100	10	—	—

**: $p < 0.01$ compared with a vehicle group.

—: no anti-inflammatory activity

Table 3. Anti-inflammatory activity of compound **11b** administered orally at different doses.

Time (h)	Dose (mg/kg)	Number of mice	Inhibition (%)	
			11b	Indometacin
2	125	10	50.12	38.74*
2	100	10	47.86**	33.32**
2	50	10	35.24	22.76
2	25	10	19.73*	4.85*

*: $0.01 < p < 0.05$ compared with a vehicle group. **: $p < 0.01$ compared with a vehicle group.

The cytotoxicity of these compounds was assessed in HCT116 cells by the MTT assay, and the results are shown in Table 1. None of the compounds showed any appreciable cytotoxic activity ($IC_{50} > 100$ μmol/L), except for UA ($IC_{50} = 23.8$ μmol/L). Furthermore, to determine whether the synthesized compounds were selectively toxic towards normal or cancer cells, we evaluated the cytotoxicity of the most potent compound **11b** using the MTT assay. As shown in Table 4, compound **11b** gave IC_{50} values of 100, 200, and 200 μM against HCT116, AGS, and L02 cells,

respectively, which indicated that compound **11b** showed no significant cytotoxicity towards normal and cancer cells at concentrations less than 100 μM and did not show much selectivity.

Table 4. Cytotoxic activity (IC_{50}[a], μg/mL) of compound **11b** against human cell lines.

Compound	Substituents	*In vitro* cytotoxicity IC_{50} [a] (μM)		
	R	HCT116 [b]	AGS [c]	L02 [d]
11b	—	> 100	> 200	> 200

[a] IC_{50} is the concentration required to inhibit the cell growth by 50%. Data represent the average of three independent experiments running in triplicate. Variation was generally between 5–10%.

[b] Human colon cancer cells.

[c] Human lung cancer cells.

[d] Human normal hepatic cells.

The superior anti-inflammatory activity and low cytotoxicity of the target compounds led us to study the molecular docking with the COX-2 enzyme to rationalize the observed anti-inflammatory activity and elucidate a possible mechanism of action of these compounds. All the calculations were performed using MOE 2008.10 software form Discovery studio 3.1 installed on 2.4G Core i5. The crystal structure of the COX-2 enzyme obtained from the protein data bank (PDB ID: 5FDQ)[45] was used for the molecular docking. Enzyme structures were checked for missing atoms, bonds, and contacts. Hydrogen atoms were added to the enzyme structure. Water molecules and bound ligands were manually deleted. The active site of the enzyme was defined to include residues within a 10.0 Å radius to any of the inhibitor atoms. The automated molecular docking program of MOE 2008.10 was used to dock compounds **4a**, **11b**, and UA on the active site of the COX-2 enzyme. The most stable molecular-docked model was selected according to the best scored conformation predicted by the MOE scoring function. The results indicated that the anti-inflammatory activities of the compounds might correlate with the results for docking with COX-2 (Table 5). Compounds **4a** and **11b** with better anti-inflammatory activities showed higher docking scores (150.19 and 151.72 kcal/mol) than UA (126.69 kcal/mol).

Table 5. The Libdock scores of docking (compounds **4a**, **11b** and **UA**) with COX-2 (PDB ID: 5FDQ).

Compound	Inhibition rate (%)	LibDock score (k.cal/mol)
4a	62.31	150.19
11b	69.76	151.72
UA	57.67	126.69

The preferred coordination modes of **4a**, **11b**, and UA with the COX-2 protein are presented in Figure 2. All the ligands showed a similar orientation in the COX-2 active site and the complex formed was stabilized by the formation of hydrogen bonds. It was observed that the oxadiazole fragment of compound **4a** fitted into the cavity formed by Thr212 and Met458 that is required for COX-2 fitting (Fig. 2A, 2B). H-bond interactions were formed between the nitrogen atom of the oxadiazole group and the amino acid Thr212. Compound **11b**, which had the highest docking score of 151.72 kcal/mol and the best anti-inflammatory activity (inhibition rate, 69.76%), showed two hydrogen bonds and two other chemical bonds with the amino acids Gln454, Val447, Ala446, and Ala450 of COX-2 (Fig. 2C, 2D). Only one hydrogen bond, with Ala99, was formed between UA and COX-2 (Fig. 2E, 2F). The preliminary docking results suggested that compounds **4a** and **11b** possibly cause their anti-inflammatory activity through the interaction with the COX-2 protein by targeting residues in the active cavities of COX-2. This may well explain why these compounds showed selective activity against the COX-2 enzyme. To validate the results of the molecular docking, further enzyme studies of these compounds with COX-2 is currently underway in our laboratory.

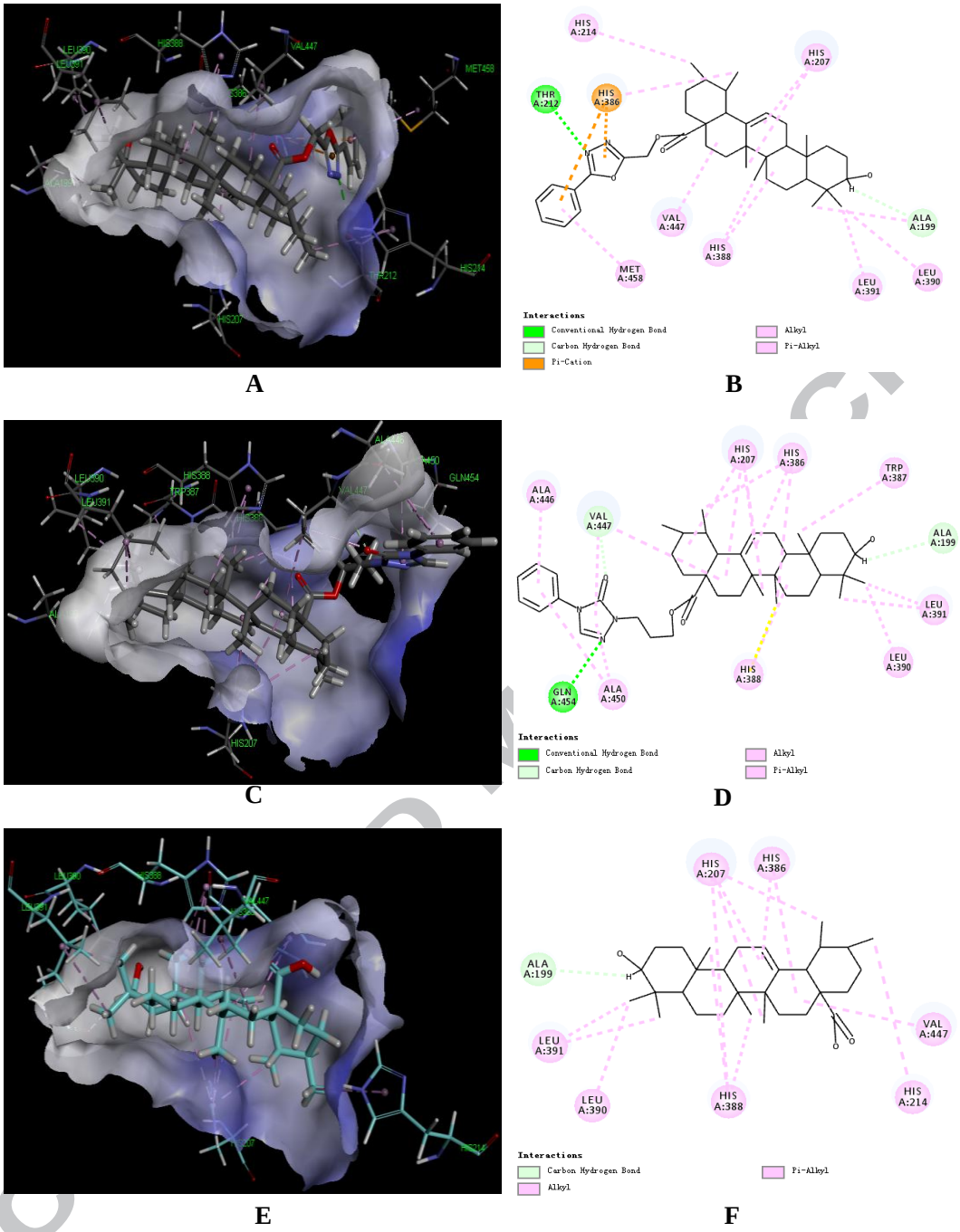


Figure 2. Docking result of compound **4a**, **11b** and **UA** with COX-2 (PDB ID: 5FDQ). (A) Key residues in binding site surrounding **4a**. (B) 2D molecular docking modeling of compound **4a** with 5FDQ. (C) Key residues in binding site surrounding **11b**. (D) 2D molecular docking modeling of compound **11b** with 5FDQ. (E) Key residues in binding site surrounding **UA**. (F) 2D molecular docking modeling of compound **UA** with 5FDQ.

In summary, 20 UA derivatives containing oxadiazole, triazolone, and piperazine moieties were synthesized and evaluated for anti-inflammatory activity. Compound **11b** showed the most potent inhibitory activity towards ear inflammation of all the

synthesized compounds (69.76%), which was higher than ibuprofen (25.17%) and indomethacin (26.83%) at 100 mg/kg (i.p.), and was 1- and 2-fold more potent than the standard drugs. The cytotoxicity of the compounds was assessed by the MTT assay, and no compounds showed any appreciable cytotoxic activity (IC_{50} > 100 μmol/L), in contrast to UA. Furthermore, the molecular docking results indicated that the UA derivatives exhibited high affinity for the COX-2 active site and possibly exhibit their anti-inflammatory potency via inhibiting COX-2. Our findings might provide information for the development of potentially new and safe COX-2 inhibitors as anti-inflammatory agents.

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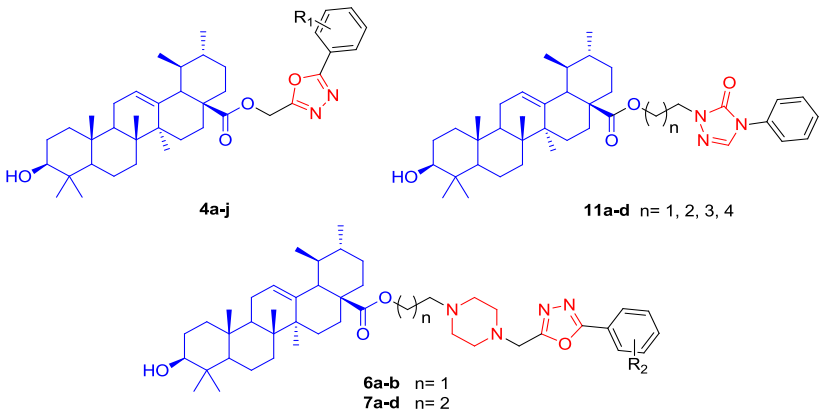

In an attempt to develop novel and potent anti-inflammatory agents, four novel series of ursolic acid derivatives containing oxadiazole, triazolone and piperazine moieties were designed, synthesized, and evaluated for anti-inflammatory activity. The cytotoxicity and molecular docking studies of these compounds was also assessed.

Highlights:

- Ursolic acid derivatives containing nitrogen heterocyclic were synthesized.
- The anti-inflammatory activity and cytotoxicity of compounds were evaluated.
- Compound **11b** is a promising anti-inflammatory agent**.**
- All compounds did not exhibit any remarkable cytotoxic activity.
- The molecular docking studies of **11b** was performed.