Anti-inflammatory Activity of Indole and Amide Derivatives of Ursolic Acid: Design, Synthesis, and Docking Studies

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Abstract

Ursolic acid (3-hydroxy-urs-12-ene-28-oic acid, UA) is one of the most notable pentacyclic triterpene which we have isolated from the biomass marc of the lavender plant *Lavandula angustifolia*. Then, UA was structurally modified by an introduction of indole ring at the C-3 position and amide group at the C-17 position with the aim to enhance its pharmacological potential. Firstly, we investigated the cytotoxic potential of all the synthesized derivatives in RAW 264.7 cells. The results of the study suggested that UA and its derivatives were non-cytotoxic to macrophages up to concentrations of 10.0 μM. In the present study we used Lipopolysaccharide (LPS), a potent activator of immune cells including macrophages consequently activating the inflammatory cascade. The nitric oxide (NO) inhibition was assessed for potential of these derivatives in LPS induced RAW 264.7 macrophages wherein the results demonstrated that among all, indole derivatives significantly reduced the levels of inorganic nitric oxide in cell culture supernatant in comparison to the parent molecule (UA) and as well as amide derivatives. Furthermore, compound 3 and 6 were evaluated for its cytokine inhibitory potential wherein, the results demonstrated that compound 3 and 6 significantly reduced the LPS-induced upregulation of pro-inflammatory cytokines (TNF-α, IL-6, IL-1β and PGE 2) and further upregulated the levels of anti-inflammatory cytokine (IL-10) in comparison to the UA in RAW 264.7 cells. The effect of these derivatives was also access on principal inflammatory mediators including iNOS, COX-2 and ROS. The results showed a reduction in the protein expression of iNOS and COX-2 indicating their potential role in dampening inflammation. Furthermore, ROS analysis demonstrated that both compound **3** and **6** significantly inhibited the LPS-induced reactive oxygen species. Additionally, molecular docking studies were performed to study the interaction of these

hits with NF-κB protein. Since, it is a critical transcription factor that plays a central role in the regulation of the immune and inflammatory responses. The results of molecular docking demonstrated that these potent compounds might have worked through NF-κB signalling pathway, which might be the mechanism of action.

Introduction

Inflammation is a complex and dynamic biological response that plays a fundamental role in the body's defence against injury, infection and various forms of stress. Being a double-edged sword inflammation is a finely regulated and coordinated process that involves a series of complex interactions between cells and molecules of the immune system. The primary purpose of inflammation is to eliminate the initiating cause of cell injury, clear out damaged cells and tissues and initiate the repair process[1]. The immune system's effectiveness in orchestrating and regulating inflammation is underpinned by its diverse array of specialized cells. Innate immune cells, including neutrophils and macrophages, serve as the first line of defence by swiftly recognizing and responding to pathogens and tissue damage. Macrophages being the pivotal players in the orchestration of inflammation. As key components of the innate immune system these versatile cells serve as dual role as both sentinels and effectors in the inflammatory response. On the other hand, adaptive immune cells, such as T and B lymphocytes, offer long-term immunity and precise regulation. T cells govern immune responses while B cells produce antibodies[2] [3]. Furthermore, in the orchestration of inflammation, immune cells release a myriad of signalling molecules, including cytokines and chemokines, which mediate immune cells and signalling molecules, ensures an effective response to pathogens and injury and ultimately, restoration of tissue homeostasis[4]. Subsequently, severe and persistent inflammation may cause several chronic inflammatory diseases including rheumatoid arthritis, cancer, diabetes and inflammatory bowel disease. Rheumatoid arthritis stands as a paradigmatic example of the intricate interplay between inflammation and chronic autoimmune disorders. This debilitating condition is characterized by persistent synovitis leading to progressive joint destruction and systemic manifestations[5]. The treatment of inflammation is a multifaceted field encompassing various strategies aimed at mitigating the detrimental effects of excessive or chronic inflammation while preserving its protective role in the body. Non-steroidal anti-inflammatory drugs (NSAIDs), such as aspirin, ibuprofen and indomethacin, are commonly used to alleviate pain and reduce acute inflammation by inhibiting the production of pro-inflammatory prostaglandins [6]. Glucocorticoids, like

prednisone, exert broad immunosuppressive effects and are used to manage severe inflammation in autoimmune diseases. Additionally, biologic therapies, including TNF-α inhibitors and interleukin blockers, target specific inflammatory mediators[7]. However, long-term use of these drugs is frequently accompanied by severe adverse effects such as gastrointestinal harm, cardiovascular, and substantial renal toxicity [8, 9]. Thus, we need to focus on advancing the development of innovative anti-inflammatory medications with minimal adverse effects and good efficacies. The inflammatory reaction is distinguished by the coordination of cytokines, chemokines and other inflammatory mediators that serve as the molecular architects of the intricate symphony that is inflammation. These signalling molecules play pivotal roles in orchestrating the immune response, regulating cell communication and maintaining tissue homeostasis. Cytokines, a broad category of small proteins, include both pro-inflammatory (TNF-α, IL-6 and IL-1β) and anti-inflammatory (IL-10) cytokines, which balance and modulate immune reactions [10]. TNF-α is a potent pro-inflammatory substance that controls several aspects of macrophage activity and it is immediately released after injury, illness, or contact with pathogens. TNF-α thus acts as a "master regulator" for synthesis of pro-inflammatory cytokines [11, 12]. Additionally, chemokines are specialized cytokines guiding the directional movement of immune cells to the site of inflammation, ensuring an organized and efficient immune response. Furthermore, nitric oxide (NO) and lipid mediators such as prostaglandins and leukotrienes play roles in vasodilation, increased vascular permeability and the amplification of inflammatory signals [13, 14]. The complex network of these mediators initiates and perpetuates the immune response, ensuring that immune cells are recruited, activated and coordinated to clear pathogens and restore tissue integrity. A characteristic pro-inflammatory signalling pathway, nuclear factor kappa B (NF-κB), has long been known and is a critical transcription factor with a central role in the regulation of inflammation. It is a key player in the cellular response to diverse stimuli, including infection, stress and inflammatory signals. In an inactive state, NF-κB is bound to inhibitory proteins (IκB) in the cytoplasm, preventing its translocation to the nucleus. Upon activation by pro-inflammatory cytokines, pathogens or other stressors, IκB is phosphorylated and degraded, allowing NF-κB to enter the nucleus and initiate the transcription of various gene involved in inflammation[15, 16]. Naturally occurring compounds are significant for the development of novel anti-inflammatory drugs because of their easy availability, safety and wide use in traditional medicine. Pentacyclic triterpenes are a large group of naturally occurring compounds that are extensively dispersed in

nature and possess a variety of biological activities. Ursolic acid, is naturally occurring pentacyclic triterpenoid, has garnered significant attention due to its diverse pharmacological properties and therapeutic potential. It is widely distributed in various botanical sources, including apple (*Malus domestica*) fruit peel, marjoram (*Origanum majorana*) leaves, oregano (*Origanum vulgare*) leaves, rosemary (*Rosmarinus officinalis*) leaves, sage (*Salvia officinalis*) leaves, lavender (*Lavandula angustifolia*) leaves and flowers, among others. Furthermore, UA has garnered attention for its antioxidant, anti-inflammatory, anti-HIV, anti-malaria, anti-cancer, anti- microbial and anti-diabetic properties [17, 18]. Numerous pre-clinical studies have highlighted the potential of UA in ameliorating rheumatoid arthritis. For instance, in an experimental model of RA using collagen-induced arthritis, UA treatment significantly reduced the production of key cytokines such as TNF-α, IL-6 and IL-1β as well as the expression of iNOS in the arthritic joints [19]. Despite the robust scientific support for UA's pharmacological properties *in vitro* and *in vivo*, its clinical application is hindered, primarily stemming from its restricted solubility in water and its limited ability to be effectively absorbed by biological system. Consequently, our objective is to synthesize derivatives of UA through chemical modifications to enhance its biological and pharmacological potential. Over the past few years, UA with novel structural frameworks has been developed and has shown greater pharmacological activity than the parent UA [20, 21]. It is fascinating that the majority of structural changes are mentioned on three active sites of UA, namely the hydroxy group at C-3, double bond at ring-C, and carboxylic acid at the C-17 position. According to several studies, maintaining a polar substituent at the C-3 position is necessary to retain the pharmacological activity of UA [22, 23]. Because nitrogen atoms can carry a positive charge and function as hydrogen bond acceptors or donors, they are an effective tool for changing the structural makeup of natural products. This can have a significant impact on how a molecule interacts with its target [24]. The introduction of an indole ring at the C-3 position and amide derivatives with piperazine at the C-17 position of UA has been reported to have antimicrobial and anticancer activity [23]. Jin et al. reported oleanolic acid indole derivatives as potential candidates for anti-inflammatory drug development [25]. Indoles are widely used in a variety of pathophysiological disorders, including cancer, bacterial and viral infections, inflammation, depression, migraines, emesis, and hypertension. Indomethacin is FDA approved anti-inflammatory drug that incorporate indole ring, a fused bicyclic ring with a wide range of functions, and plays a significant role in anti-inflammatory therapy[26]. In the present study, we have synthesized different indole-fused

derivatives as well as piperazine and morpholine amide derivatives of UA for the assessment of anti- inflammatory activity, which has not been reported in the literature.

In the present work, our data demonstrates the comparative evaluation of the anti-inflammatory potential of UA, the parent compound with its derivatives. The cytotoxicity of UA derivatives was determined using an MTT assay, and the nitric oxide (NO) inhibition potential was investigated in LPS-stimulated RAW 264.7 cells. The results of our study demonstrated that the indole derivatives of UA (3 and 6) showed significant NO inhibition and significant inhibition of pro-inflammatory cytokines (TNF-α and IL-6) at concentrations of 5 μM and 10 μM, respectively, compared to the parent compound (UA).

Experimental Section

The lavender marc was obtained (after hydro-distillation) and collected from the CSIR-IIIM botanical garden and authenticated from the CSIR-IIIM herbarium depository. All the chemicals and solvents used in the experiments were acquired from Sigma Aldrich®. All reaction processes were observed on 0.25 mm silica gel precoated Merck® TLC plates under ultraviolet fluorescence (UV 254 nm) using solvent ethyl acetate/n-hexane charring with the anisaldehyde-sulphuric acid reagent in which spots are converted into pick color spot or reddish colors after heating TLC. NMR (^{1}H and ^{13}C) spectra were captured using Bruker® Avance-400 spectrometer, deuterated chloroform ($CDCl_3$; reference solvent), and tetramethylsilane (TMS; internal standard). Molecular mass was performed on a Shimadzu® LCMS-2020. HRMS were calculated with Waters® Xevo-G2-XS QTOF mass spectrometer instrument. Silica gel (60–120 or 100–200) was used to purify the compounds through column chromatography. Characterization of all compounds has been provided in the supporting information.

General procedure for extraction and isolation of Ursolic Acid from Lavender Marc

De-oiled Marc (marc left after hydro distillation) lavender marc was extracted by DCM solvent to extract out the secondary metabolites by following the general procedure. The weight of 500 gm of marc was taken in a conical flask and solvent DCM (10 times the material; w/v) was added to it for 24 hrs. The solvent was decanted after 24 hrs. This process was repeated three times. The decanted solvent was concentrated by using rota-vapor with reduced pressure at 45°C. The total weight of the extract was obtained at 26.7 gm. Total extract was used to prepare the slurry in 60-

120 mesh and the same silica gel was used in column chromatography to the isolation of UA. Column chromatography was run in 20% EtOAc:Hexane to isolate the Ursolic acid. TLC was run in 30% EtOAc:Hexane which gives the Rf value 0.5. Ursolic acid is a UV inactive compound but it shows a pink color spot on TLC after charring with *p*-anisaldehyde sulfuric acid reagent.

Synthesis of Indole derivative of Ursolic acid

Take 1 gm of ursolic acid (**1**) into a round bottom flask and dissolved in acetone. Then, oxidize ursolic acid to obtain 3-oxo-ursolic acid by using Jone's reagent. Furthermore, Indole derivatives of UA were synthesized using substituted phenylhydrazine hydrochlorides via the Fisher indole synthesis reported procedure [25, 27].

Synthesis of piperazine, and morpholine amide derivatives of ursolic acid

The Hydroxy group of Ursolic acid at position C-3 was protected by acetylation and benzylation[28] to get compounds (8 and 9) as shown in scheme 2. 3-O-acetylursolic acid and 3-O-benzylursolic acid were added into DCM (dry) at 0ºC and oxalyl chloride was added dropwise and this mixture was stirred for 6h. Using a vacuum rotatory evaporator, the solvent was evaporated and concentrated till dry. After that obtained dry compound was soluble in DCM (HPLC grade) then, add triethylamine into it followed by piperazine or morpholine, and stirred at room temperature for 3h[29][30]. After completion of the reaction, acidified with 2N HCl, then filtered which was extracted with DCM and dried under vacuum pressure. This crude product was purified with column chromatography to get compounds (**10**, **11**, and, **12**).

Cell culture

For *in vitro* studies, murine macrophage RAW 264.7 cells were used and purchased from ATCC, Manassas, VA, USA. The cells were cultured in DMEM supplemented with 10% FBS and penicillin-streptomycin and were maintained at 37°C in a 5% CO2 humidified incubator.

Cell viability assay

The cytotoxic effect of the compounds was assessed in RAW 264.7 cells using an MTT reduction assay. Briefly, RAW 264.7 cells (104 cells/well) were treated with different concentrations (1 and 10μM) of the indole derivatives for 48h in flat-bottomed 96-well microtiter plates. MTT solution (2.5 mg/ml) prepared in PBS was added to each well 4 h before termination. DMSO was added to

each well to solubilize the formazan crystals and the absorbance was measured at 570 nm using a Synergy Mx plate reader.

NO Inhibition assays

Inhibition of nitric oxide (NO) in LPS-stimulated RAW 264.7 cells was used to screen the compounds for their anti-inflammatory potential. RAW 264.7 cells were induced with LPS (1µg/ml) to express high levels of nitric oxide followed by treatment with UA derivatives at a concentration of 5µM and 10µM for 24h. Griess reagent was used to evaluate the total amount of inorganic nitric oxide produced in the culture supernatant. The culture supernatant was incubated with an equal volume of Griess reagent at room temperature and the absorbance was measured at 540 nm using a Synergy Mx plate reader. L-NAME was used as a standard inhibitor of NO.

***In-vitro* cytokine estimation TNFα and IL-6 assay**

The endotoxin, LPS is a potent pro-inflammatory activator of macrophages and was used for the stimulation of RAW 264.7 cells for cytokine production. The effect of ursolic acid and active indole derivatives (**3**, **4** and **6**) was investigated on the inhibition of cytokine production. RAW 264.7 cells were treated with ursolic acid, compounds **3**, 4, and 6 at a concentration of 5 µM and 10µM for 1h followed by stimulation with 1µg/ml LPS for 24h. Supernatants were then harvested to quantify TNF-α and IL-6 concentration using ELISA Kits.

Molecular docking

Molecular docking was performed by using AutoDock Tool 1.5.7 software. The crystal structure of protein 2AZ5 (TNF-α) 1IKN (NF-κB) was obtained from Protein Data Bank (https://www.rcsb.org/). Ligand was prepared by using ChemBio3D software and converted into PDB format. Water molecules were removed and polar hydrogen was added to the protein. Both protein and ligand were converted into PDBQT format for docking. Docking structures were obtained by using Discovery Studio visualizer software.

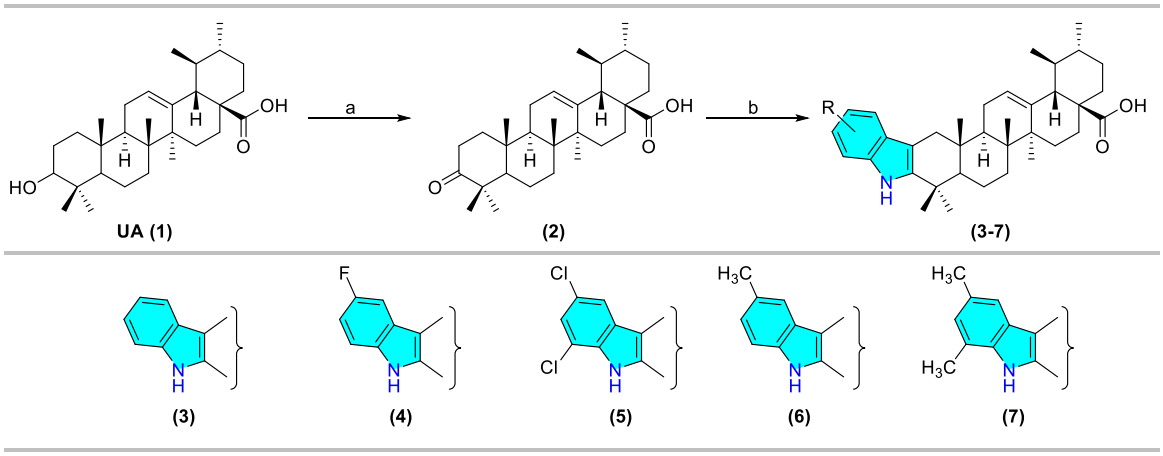
Statistical analysis

Data are expressed as mean ± standard deviation (SD) from three independent experiments. Results were considered significant at *$p < 0.05$, **$p < 0.01$. and ***$p<0.001$. Data were analyzed by using GraphPad Prism Software 2.

Results and Discussion

Chemistry

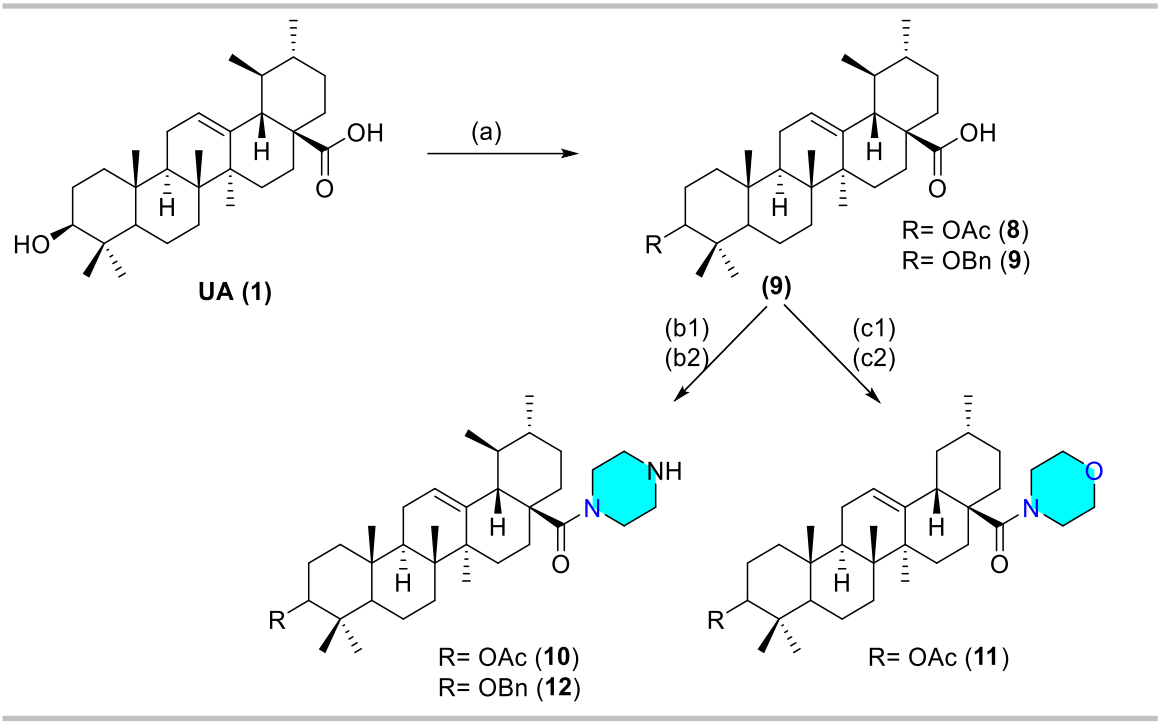
Ursolic acid (UA, 1) was isolated from a DCM extract of lavender marc and used as a starting material for the structural modification of indole and amide derivatives. Compounds **3**, **4**, **5**, **6**, and **7** were synthesized through structural modification at the C-2/C-3 position of UA using the Fischer indole synthesis reaction. The synthesis of indole derivatives was completed in two steps (Scheme 1). The first step involves the oxidation of the hydroxy group at the C-3 position of UA using the Jone's reagent to obtain 3-oxo-ursolic acid (**2**). In the second step, compound **2** was reacted with substituted phenylhydrazine hydrochloride to give desired compounds (**3-7**). All reactions were performed using ethanol: water (1:1) under reflux condition for 2-3 h to get–60-75 % yield[31] [25] [27]. All compounds were purified by column chromatography using silica gel 60-120 mesh and ethyl acetate hexane as a solvent. Compounds were characterized by spectroscopic analyses (^{1}H NMR, ^{13}C NMR, and HRMS).



Scheme 1. Synthesis and substrate scope for Indole derivatives of ursolic acid; Reagents and conditions: (a) Jone's reagent, acetone, 0°C, 2h, 98% (**2**); (b) Substituted-phenylhydrazine HCl (1.5 equiv.), EtOH: H_2O (1: 1), reflux, 2h, 60-75% (**3-7**).

In ^{1}H NMR, characteristic peaks of the indole-fused derivatives of UA were a singlet at δ 7.72-7.39 ppm, and in ^{13}C NMR signals in the range of 158.83 - 102.96 ppm showed in the aromatic region which confirms the indole ring formation. For the synthesis of the amide derivatives of UA, the hydroxy group of UA was protected using acetyl chloride to give compound 8, and benzyl chloride gave compound **9** (Scheme 2). The protected UA was subjected to a reaction with oxalyl chloride after the reaction with piperazine and morpholine to obtain compounds 10, 11, and 12.

Distinctive peaks of the amide derivatives in ^{1}H NMR showed a singlet at 3.58-3.48 ppm of 8 protons of piperazine and morpholine derivative which confirmed the product formation as shown in Scheme 2.



Scheme 2. Synthesis of piperazine, and morpholine amide derivatives of ursolic acid. Reagents and conditions : (a) $(CH_3CO)_2O$, Et_3N, DMAP (cat.), rt, 24h, 98% (**8**) and Benzyl chloride, NaH, DMF, rt 6h, 95% (**9**) ; (b1) Dry DCM, oxalyl chloride, Et3N, DMF(cat.), 5h; (b2) DCM, Et3N, DMAP(cat.), piperazine, 0^{o}C, 3h, 75% (**10**, **12**); (c1) Dry DCM, oxalyl chloride, Et3N, DMF(cat.), 5h; (c2) DCM, Et3N, DMAP(cat.), morpholine, 0^{o}C, 3h, 78% (**11**).

In vitro cytotoxicity by using MTT assay

In vitro, cytotoxicity of the synthesized derivatives of UA was determined by using an MTT assay in RAW 264.7 cells to work out the concentrations of the compounds to be used for the investigation of the anti-inflammatory potential without affecting cell survival [17].The results of the cell viability analysis demonstrated that indole and amide derivatives of UA along with ursolic acid were non-cytotoxic to macrophage concentrations of 10.0 µM wherein the cell viability was more than 90% (Table 1). The outcomes also imply that the anti-inflammatory potential of the derivatives must be investigated within the non-cytotoxic concentrations.

Table 1. *In vitro* cell viability of ursolic acid and its derivatives

Compound	% Cell Viability (conc. 1.0 µM)	% Cell Viability (conc. 10.0 µM)
Ursolic acid (**1**)	99.1 ± 0.30	95 ± 2.4
PK-UA-IN-1 (**3**)	95.8 ± 5.8	91.8 ± 5.4
PK-UA-IN-2 (**4**)	98.1 ± 2.4	94.6 ± 7.1
PK-UA-IN-3 (**5**)	95.5 ± 4.3	90.8 ± 7.9
PK-UA-IN-4 (**6**)	97.7 ± 0.6	93.4 ± 1.4
PK-UA-IN-8 (**7**)	97.8 ± 1.3	93.8 ± 2.1
PK-UA-Obn (**9**)	97.6 ± 2.3	93 ± 3.2
PK-UA-PIP (**10**)	96.3 ± 3.1	89.5 ± 6.4
PK-UA-MOR (**11**)	99 ± 1.1	95.1 ± 1.2
PK-UA-OBn-PIP (**12**)	98.2 ± 1.6	88.2 ± 4.1

Inhibition of Nitric Oxide (NO) production

Numerous inflammatory diseases have nitric oxide (NO) release, which is produced by inducible nitric oxide synthase (iNOS), as an apathogenic factor [25]. NO is the key mediator of inflammation and is frequently used to assess the extent of the inflammatory response. NO has a major impact on inflammation through a process known as S-nitrosylation wherein, it attaches to cysteine thiols in different proteins. Therefore, increased nitrosylation during inflammatory reactions causes nitrosative stress, that ultimately promotes cell death [19] .We examined the NO inhibition properties of the UA derivatives against an inflammatory response generated by LPS-stimulated RAW 264.7 cells. Lipopolysaccharide (LPS) is a potent activator of immune cells including macrophages consequently activating the inflammatory cascade. NG-nitro-L-arginine methyl ester (L-NAME) has been used as a paradigmatic inhibitor of NO synthase as a positive inhibitor. The ursolic acid showed NO inhibition values of 31.2 ± 2.4% and 35.8 ± 3.2% at the concentration of 5.0 µM and 10.0 µM respectively, whereas indole derivatives of UA showed better NO inhibitory

activity than the parent compound (UA). From the results given in Table 2, It is evident that the highest NO inhibitory effects of compound **3**, **4**, and **6** was 45.5 ± 3.1%, 45.5 ± 2.6%, and 48.3 ± 3.1% at 5 µM and 54.8 ± 2.4%, 54.1 ± 4.2% and 54.3 ± 4.8% at 10µM concentration, respectively. Among all these compounds, amide derivatives of UA that contain morpholine (36.6 ± 7.2% and 42 ± 0.9%) and piperazine (32.5 ± 4.7% and 32.8 ± 3.9 %) moieties displayed NO inhibitory activity not much-improved activity than the parent compound (UA). The results of the study suggested that indole derivatives of UA are more active than the parent compound (UA) as well as amide derivatives of UA. This may be attributed to the inhibition of aberrant iNOS induction, an enzyme involved in the pathogenesis of numerous inflammatory diseases by causing nitrosative stress which consequently results in cellular injury.

Table 2. Study of the UA derivatives in the inhibition of NO production in LPS-stimulated RAW 264.7 cells

Compound	**% NO inhibition (conc.5.0µM)**	**% NO inhibition (conc.10.0µM)**
L-NAME	-	50.9 ± 2.3
Ursolic acid (**1**)	31.2 ± 2.4	35.8 ± 3.2
PK-UA-IN-1 (**3**)	45.5 ± 3.1	54.8 ± 2.4
PK-UA-IN-2 (**4**)	45.5 ± 2.6	54.1 ± 4.2
PK-UA-IN-3 (**5**)	36.8 ± 4.1	44.7 ± 1.5
PK-UA-IN-4 (**6**)	48.3 ± 3.1	54.3 ± 4.8
PK-UA-IN-8 (**7**)	33.8 ± 5.8	34.8 ± 3.2
PK-UA-Obn (**9**)	32.5 ± 6.0	34.5 ± 3.4
PK-UA-PIP (**10**)	32.5 ± 4.7	32.8 ± 3.9
PK-UA-MOR (**11**)	36.6 ± 7.2	42 ± 0.9
PK-UA-OBn-PIP (**12**)	35.7 ± 1.8	40 ± 0.6

Inhibition of pro-inflammatory cytokines (TNF-α and IL-6) in LPS-stimulated RAW 264.7 cells

Cytokines (IL-1, IL-6, and TNF-α, etc.) are the key mediators of inflammation and play essential roles in the development, maintenance, and resolution of inflammation that take the role in both acute and chronic inflammation through a complex and even conflicting network of interactions [32] [19]. TNF-α is a potent inflammatory mediator to the innate immune system's inflammatory response, inducing the generation of cytokines, activating or expressing adhesion molecules, and stimulating growth. The interleukin-6 (IL-6) family are pleiotropic cytokine that plays an essential role in host defense because it is a multifunction cytokine with a wide range of immunological and hematological functions as well as a robust potential to trigger the acute phase response [33]. So, we further investigated the compounds **3**, **4**, and, **6** that showed the best NO inhibition activity, so we selected these compounds along with parent (UA) for screening the expression of inflammatory cytokines in LPS-stimulated RAW 264.7 cells. The endotoxin, LPS is a potent pro-inflammatory activator of macrophages and was used for the stimulation of RAW 264.7 cells for cytokine production. The effect of ursolic acid and the selected indole derivatives was investigated on the inhibition of cytokine production. RAW 264.7 cells were treated with ursolic acid, **3**, **4**, and, **6** at a concentration of 5 μM and 10 μM for 1h followed by stimulation with 1μg/ml LPS for 24h. Supernatants were then harvested to quantify TNF-α and IL-6 concentration using ELISA Kits. TNF-α and IL-6 inhibition of compounds **3**, **4**, and, **6** are inevitably more potent than their parent substance (UA). A compound that shows more than 50% inhibition of TNF-α and IL-6 expressions is considered to be potent [32]. Compound **3** showed TNF-α inhibition 49.5 ± 0.04% and 70.1 ± 0.8% at 5.0 μM and 10 μM respectively and IL-6 inhibition 45.1 ± 0.09% and 73.3 ± 2.0% at the concentration of 5.0 μM and 10 μM, respectively, whereas compound **4** inhibit expression of TNF-α by 44.8 ± 1.8% at 5 μM and 66.9 ± 4.1% at 10 μM and along with the inhibition of IL-6 by 33.8 ± 3.6% and 66.2 ± 2.1% at the concentration of 5 μM and 10 μM respectively. The most active compound **6** displayed TNF-α inhibition by 49.2 ± 0.7% and 67.6 ± 4.9% at 5μM and 10 μM respectively and IL-6 inhibition by 46.7 ± 3.1 at 5μM and 72 ± 3.1 at 10μM that is almost two times more inhibition than the parent compound (UA) and also displayed 48.3 ± 0.1% and 54.3 ± 4.8% NO inhibition activity 5μM and 10 μM, respectively. The standard drug used for the treatment of inflammation, Dexamethasone showed inhibition of TNF-α (72 ± 4.2%) and inhibition of IL-6 (69.2 ± 5.1%) at 10 μM concentration whereas our synthesized compounds **3**

and **6** showed better activity than the standard compound by decreasing expression of IL-6 73.3 ± 2.0% and 72 ± 3.1% at 10µM concentration, respectively (Table 3). All of these findings suggested that compound **6** had better anti-inflammatory effects by decreasing the expression of pro-inflammatory cytokines (TNF-α, and IL-6).

Table 3. Effect of compounds **3**, **4**, and **6** on the expression of inflammatory cytokines (TNF-α and IL-6) in LPS-stimulated RAW 264.7 cells

Compound	**%TNF-α inhibition (conc.5.0µM)**	**%TNF-α inhibition (conc.10.0µM)**	**%IL-6 Inhibition (conc.5.0µM)**	**%IL-6 Inhibition (conc.10.0µM)**
Ursolic acid (1)	29.2 ± 1.6	46.3 ± 3.8	26.7 ± 1.2	49.9 ± 3.1
PK-UA-IN-1(3)	49.5 ± 1.9	70.1 ± 0.8	45.1 ± 2.4	73.3 ± 2.0
PK-UA-IN-2(4)	44.8 ± 1.8	66.9 ± 4.1	33.8 ± 3.6	66.2 ± 2.1
PK-UA-IN-4(6)	49.2 ± 0.7	67.6 ± 4.9	46.7 ± 3.1	72 ± 3.1
Dexamethasone	NP	72 ± 4.2	NP	69.2 ± 5.1

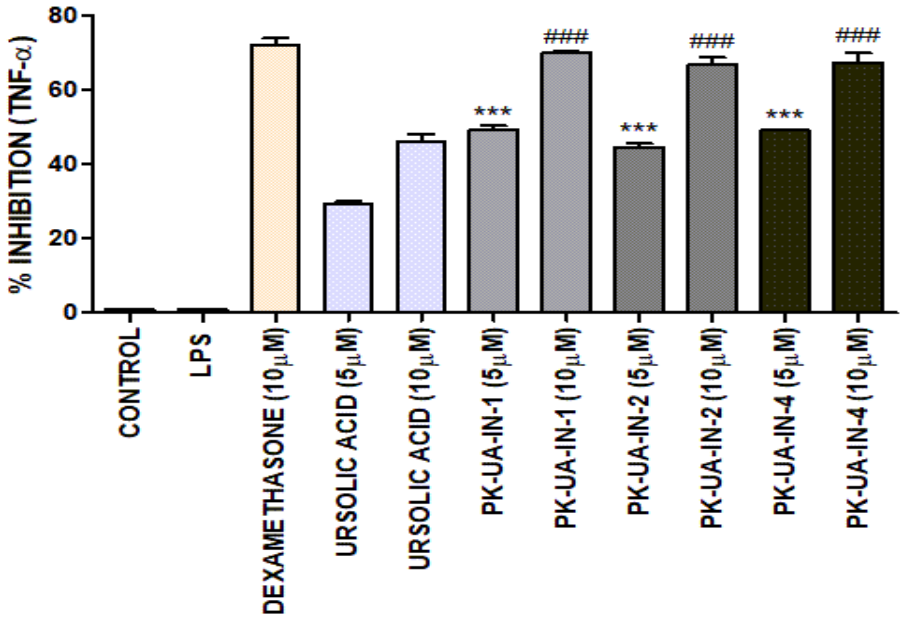


Figure 1. Graphical representation of compounds **3**, **4**, and **6** on the expression of inflammatory cytokines (TNF-α) in LPS-stimulated RAW 264.7 cells

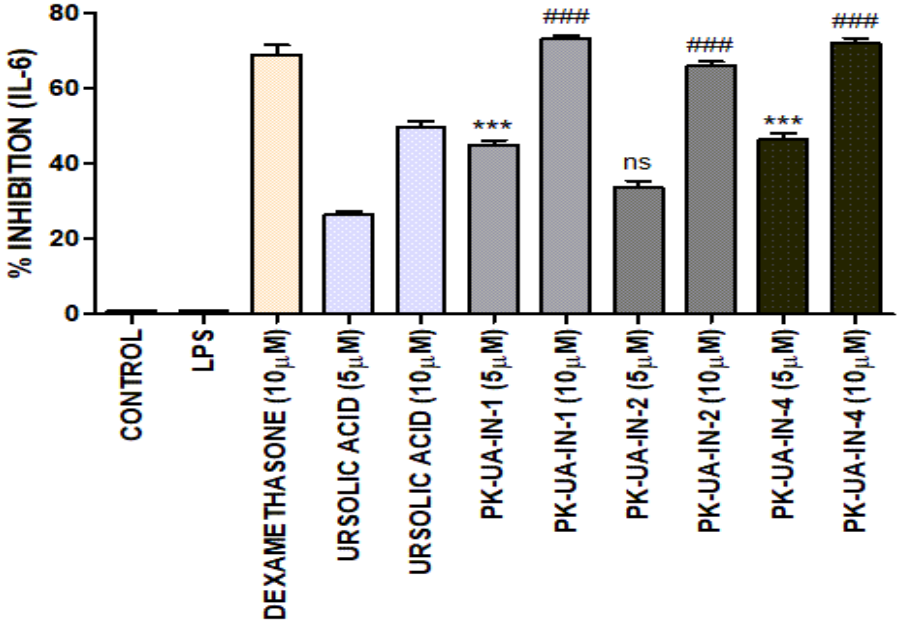


Figure 2. Graphical representation of compounds **3**, **4**, and **6** on the expression of inflammatory cytokines (IL-6) in LPS-stimulated RAW 264.7 cells

Structure-Activity Relationship

The chemistry of ursolic acid derivative design involves the strategic introduction of nitrogen-containing functional groups at specific positions, such as C-3, and carboxylic acid at the C-17 position on the ursolic acid scaffold to explore anti-inflammatory properties. For instance, indole derivatives were designed by converting the hydroxyl group at C-3 to a ketone functional group than, incorporate the indole moieties attached to ring A. For amide derivatives, we first allow the protection of the hydroxyl group at the C-3 position to avoid side products while converting it to an acyl or benzyl group and then to amide derivatives. This selection of appropriate nitrogen-containing moieties and their optimal placement within the molecular structure allowed us to obtain the desired products as designed. The choice of nitrogen-containing functional groups depends on their ability to interact with inflammatory targets or modulate the key pathways involved in inflammation. The position of the nitrogen-containing group within the ursolic acid molecule is critical for its activity and selectivity.

Based on the results of the NO inhibition assay, structure-activity relationship (SAR) analysis of ursolic acid derivatives **3–12** was performed. The formation of an indole ring (Compounds **3**, **4**, **5**, **6**, and **7**) at the C-3 position of ursolic acid significantly enhanced NO production inhibition, while amide bond formation at C-17 (-COOH group) was similar to that of the parent compound (UA) in LPS-stimulated RAW 264.7 cells. It has been observed that amide derivatives of UA containing benzyl group protection (**12**) at the C-3 position showed enhanced NO inhibition activity compared to the acyl-protected derivative (**10**). It was found that the insertion of functional groups such as alkyl and halogen groups in the indole ring improved the inhibitory effect when compared to UA, and derivatives **3**, **4**, and **6** of ursolic acid with indole, fluoro, and methyl groups, respectively, were the most active derivatives. Compound **6** contains a methyl group and was found the most potent compound with an NO inhibition of 48.3 ± 0.1% of NO inhibition in LPS-stimulated RAW 264.7 cells with low cytotoxicity compared with the parent compound (UA). Our SAR analysis showed that the overall increased anti-inflammatory activity might be attributed to the induction of an indole ring on the parent UA skeleton with an electron-donating group (methyl group) as shown below in figure 4.

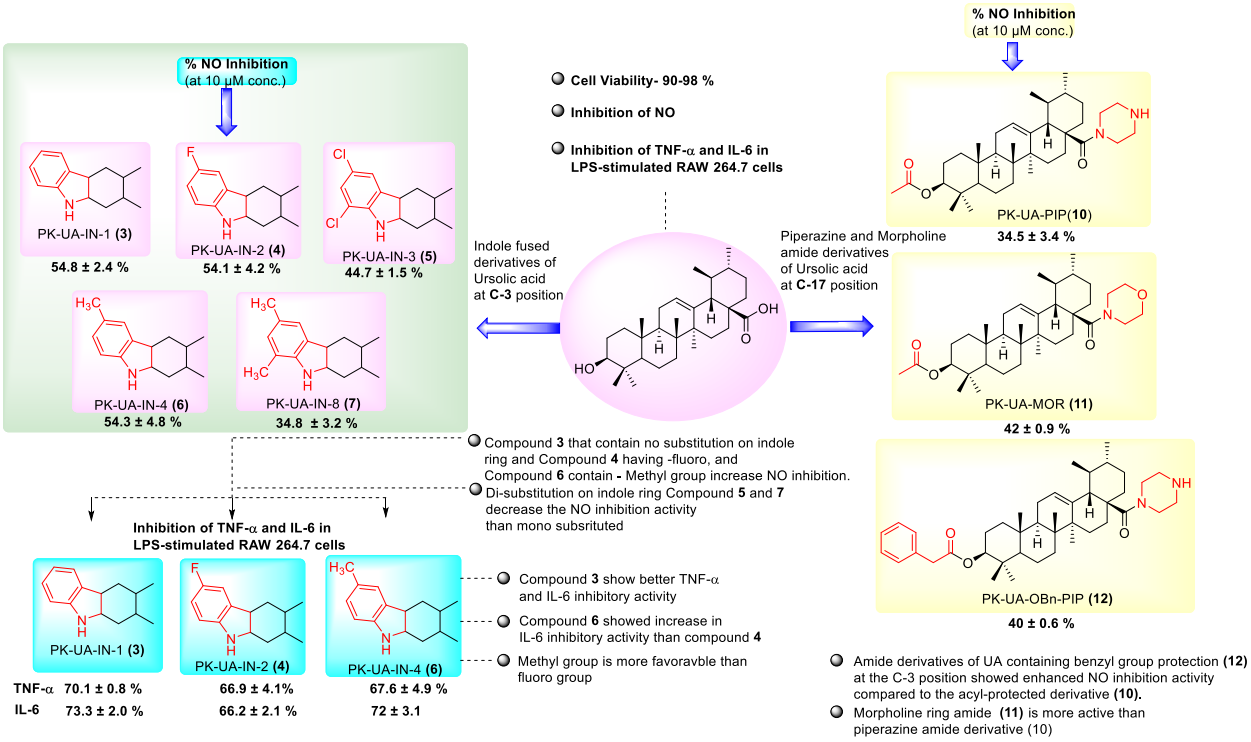


Figure 3. SAR study of indole and amide derivatives of Ursolic acid

Molecular Docking of compounds 3 and 6 with TNF-α and NF-κB proteins

To understand the better interaction between ligand and protein molecular docking analysis of compound **3** and **6** along with ursolic acid was performed with TNF-α pdb id: 2AZ5) and NF-κB (pdb id: 1IKN) protein [34]. While docking with TNF-α protein, compound **3** exhibited binding energy -10.7 kcal/mol that interacted with LEU157 with H-bonding and LYS11 with Pi-Alkyl bonding whereas docking of compound **3** with NF-κB protein displayed binding affinity -10.2 kcal/mol by interacting GLU225, THR52, PRO261 with conventional H-Bond, Pi-Alkyl bonding (**Fig. 5 A-D**). Additionally, with TNF-α protein, compound **6** showed binding affinity -10.5 kcal/mol through interaction with HIS15, TRY59, and LEU57 through Conventional H-Bond, Pi-Pi Stacked, and Pi-Alkyl, respectively while NF-κB protein, compound **6** displayed binding affinity -10.2 kcal/mol which interacts with GLN278, TRY254, and PHE141 by forming Conventional H-Bond, Unfavorable Aceptor-Aceptor, Pi-Pi T-Shaped, Pi-Alkyl bonding (Fig.6 F-H).

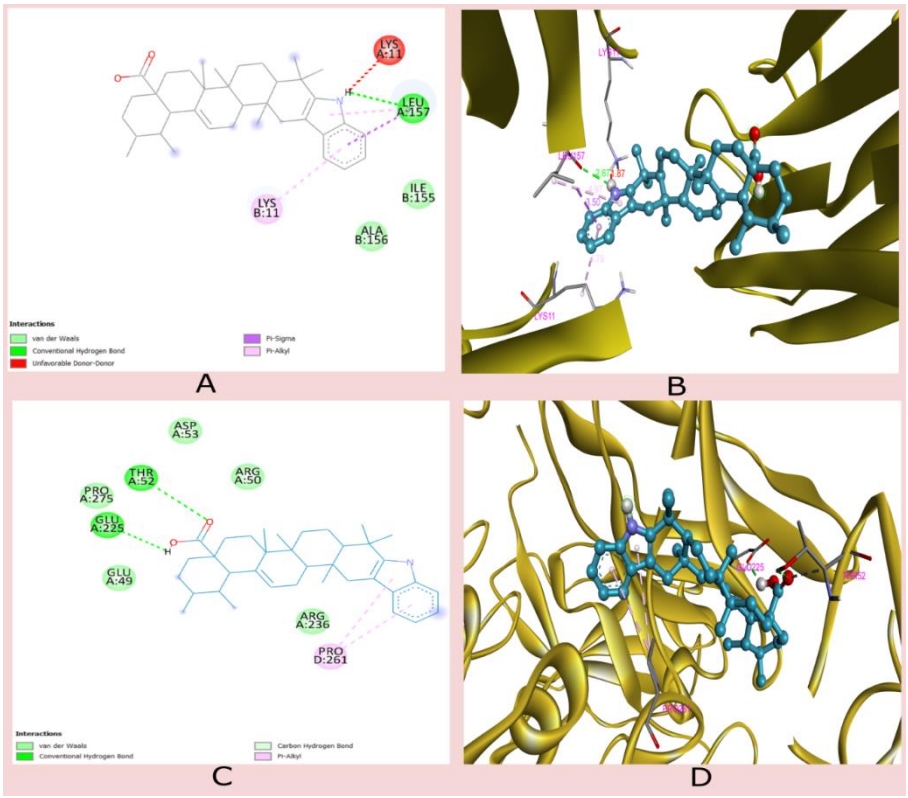


Figure 4. (A) 2D interaction of compound **3** with TNF- α (pdb id : 2AZ5) protein. (B) 3D interaction of compound **3** with TNF- α (pdb id : 2AZ5) protein. (C) 2D interaction of compound **3** with NF-κB (pdb id : 1IKN) protein. (D) 3D interaction of compound **3** with NF-κB (pdb id: 1IKN) protein.

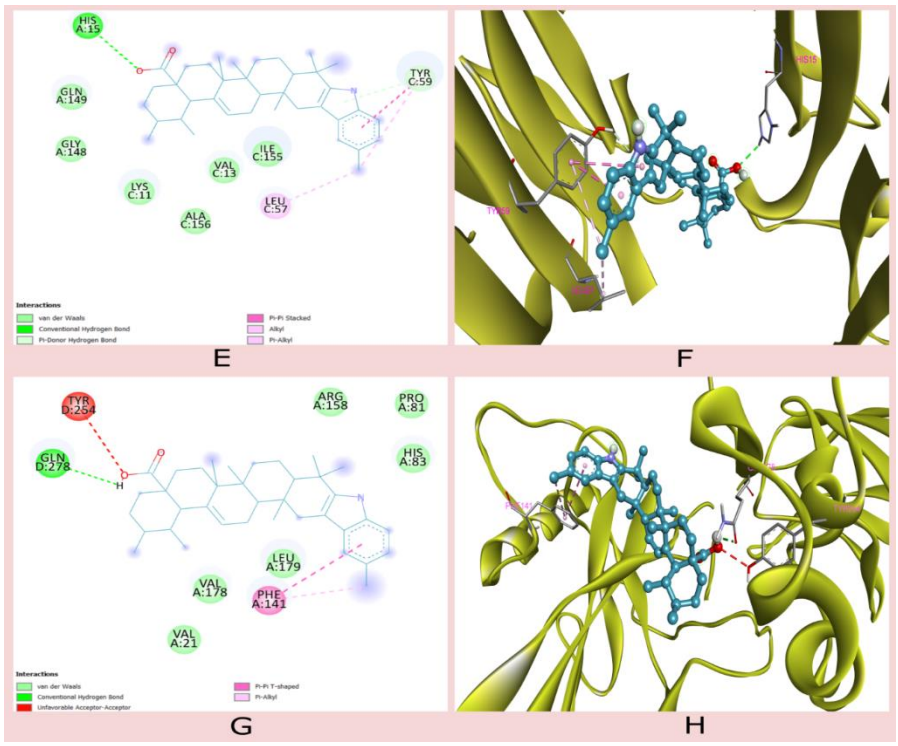


Figure 5. (E) 2D interaction of compound **6** with TNF-α protein (pdb id: 2AZ5). (F) 3D interaction of compound **6** with TNF-α protein (pdb id: 2AZ5).(G) 2D interaction of compound **6** with NF-κB (pdb id: 1IKN) protein. (H) 3D interaction of compound **6** with NF-κB (pdb id: 1IKN) protein

In silico studies of active compounds, **3** and **6** suggest the interaction of these compounds with the protein receptors. TNF-α is a pro-inflammatory cytokine that is responsible for the different aspects of inflammation and NF-κB is the signaling pathway, which is mainly dependent on the interleukin-1 (IL) and tumor necrosis factor-α (TNF-α) for the activation of signaling pathways. The crucial role of NF-κB as a central inflammatory mediator in the regulation of immune response and inflammatory disorders is widely accepted.

This transcription factor is involved in the production of a variety of inflammatory mediators including cytokines, and chemokines while also regulating the activation of the inflammasome. NF-κB is linked to a variety of inflammatory disorders both acute and chronic including rheumatoid arthritis, osteoporosis, and inflammatory bowel disease [35] [36].

Thus, meticulously designing derivatives of natural compounds targeting the NF-κB signaling is an attractive approach to developing novel anti-inflammatory agents. So, from the molecular docking study, it has been clear that the active compounds **3** and **6** are interacting with TNF-α protein and NF-κB protein which means compounds **3** and **6** are active molecules for anti-inflammatory activity. As evident from our docking studies, compound **6** which exhibits better inhibition activity than compound **3** confirms our experimental result would be due to additional interactions while docking the two most active compounds.

Conclusions

In conclusion, we have synthesized and screened indole and amide derivatives of ursolic acid (**1**) isolated from biomass waste marc of the lavender plant (*Lavandula angustifolia*). The investigation revealed that compounds **3** and **6** displayed lower cytotoxicity as well as NO inhibition activity. These compounds showed significant anti-inflammatory activities, effectively downregulating the expression of pro-inflammatory cytokines TNF-α and IL-6. Molecular docking analysis further supported the experimental results, highlighting the potential for optimizing protein binding sites with active molecules to TNF-α protein (2AZ5) and NF-κB protein (1IKN) targets. Of particular interest, compound **6** exhibited the most potent anti-inflammatory activity among all the synthesized compounds, making it a promising lead for the development of new

anti-inflammatory agents with low cytotoxicity. These findings will contribute to valuable insights into the design of novel therapeutic candidates targeting inflammation-related disorders. Overall, this research contributes to advancing the field of anti-inflammatory drug discovery and offers potential avenues for future investigations in this area.

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Author Contributions

Puneet Kumar performed the chemical synthesis and docking analysis. Rupali Choudhary performed the biological activities. Zabeer Ahmed, Gurleen Kour, and Jasha Momo H. Anal conceptualized the experiments, manuscript, and supervision. All authors read and approved the final manuscript.

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