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GRAPHICAL ABSTRACT

Indolyl-Isoxazolidines attenuates LPS-stimulated pro-inflammatory cytokines and increases survival in a mouse model of sepsis: Identification of potent lead

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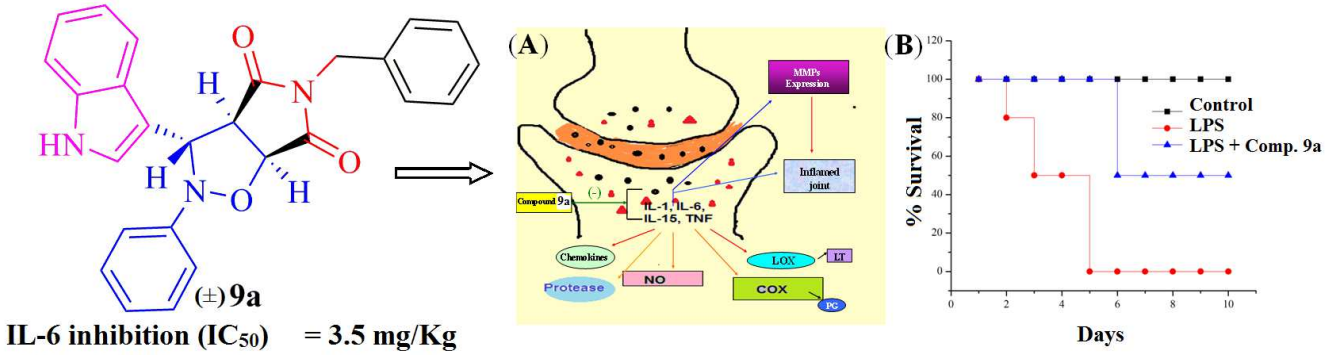

IL-6 inhibition (IC_{50}) = 3.5 mg/Kg

TNF-α inhibition (IC_{50}) = 9.4 mg/Kg

Indolyl-Isoxazolidines attenuates LPS-stimulated pro-inflammatory cytokines and increases survival in a mouse model of sepsis: Identification of potent lead

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Abstract: A library of indolyl-isoxazolidines (**6-9**) has been synthesized by regio- and stereoselective microwave irradiated 1,3-dipolar cycloadditions of *C*-(3-indolyl)-*N*-phenylnitrone (**2′**) with variedly substituted dipolarophiles (**3′-5′**) and screened for their anti-inflammatory activities through inhibition of pro-inflammatory cytokines such as TNF-*α* and IL-6. Amongst the evaluated compounds (**6-9**), bicyclic isoxazolidine (**9a**) was found to exhibit significant inhibitory potential against LPS induced human IL-6 and TNF-*α* in THP-1 cells. Compound **9a** was further assessed for *in vivo* analgesic and anti-inflammatory activities *via* acetic acid induced writhing and carrageenan induced paw edema models in mice, respectively. The results showed that compound possesses potent anti-inflammatory-analgesic activity comparable to indomethacin and did not show toxicity up to a 2000 mg Kg^{-1} dose as evidenced by histopathological studies. Consequently, the most active compound **9a** was also evaluated against LPS-induced septic death and exhibited a significant protection in *in vivo* mouse model. Taken all together, the results suggest that the compound **9a** is able to attenuate pro-inflammatory cytokines such as IL-6 and TNF-*α*; accelerate resolution of inflammation, and also increased survival rate of septic mice. Therefore, these "lead" isoxazolidines can be used as promising candidate for further analgesic/anti-inflammatory drug design and development.

Keywords: 1,3-dipolar cycloaddition; indolyl-isoxazolidines; pro-inflammatory cytokines; IL-6; TNF- *α*; anti-inflammatory-analgesic activity; sepsis.

Inflammation is an important mechanism contributing to the host's defense against pathogenic challenge and the restoration of normal tissue structure. Activated macrophages, the primary pro-inflammatory cells, mediate most cellular and molecular inflammation networks by producing NO, prostaglandin E2 (PGE2), and cytokines such as tumor necrosis factor-a (TNF-*α*) and interleukins (ILs) [1]. The dysregulated and elevated expression of cell adhesion molecules, such as ICAM-1, VCAM-1 and E-selectin, in response to pro-inflammatory cytokines such as TNF-*α*, IL-6, IL-1β and a bacterial polysaccharide, LPS on the luminal surface of vascular endothelial cells, are critical early event in inflammatory processes [2]. Therefore, reducing the expression

levels of LPS-inducible inflammatory mediators is considered as an effective way to attenuate a variety of disorders derived from the inflammation triggered by activated macrophages. It has also been demonstrated that IL-6 plays a vital role in synoviocytes proliferation and osteoclast differentiation through NF-kB expression [3]. The selective targeting of IL-6 over TNF-α has been considered beneficial, as TNF-*α* inhibitors (monoclonal antibodies) have a history of adverse side effects [4]. Therefore, inhibiting IL-6 by small molecules presents new opportunities to treat rheumatoid arthritis.

Consequently, enormous efforts are directed towards the structural design leading to the discovery of novel anti-inflammatory agents. Therapeutic importance of 3-heterosubstituted indoles has also been well established (**Figure-1**) [5]. The involvement of C3 functionalized indole nucleus has been used extensively to obtain biologically active compounds [6]. Even though it has varied actions, the role of indole in anti-inflammatory therapy is of prime interest which is prominently highlighted in the drug molecule indomethacin.

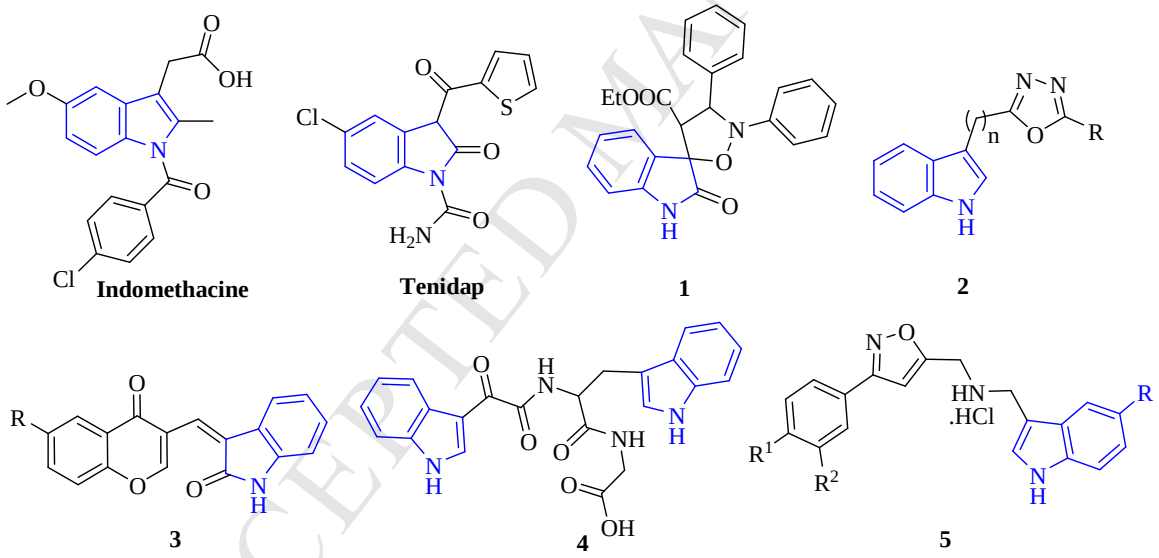


Figure 1: C-3 functionalized indoles as anti-inflammatory-analgesic agents

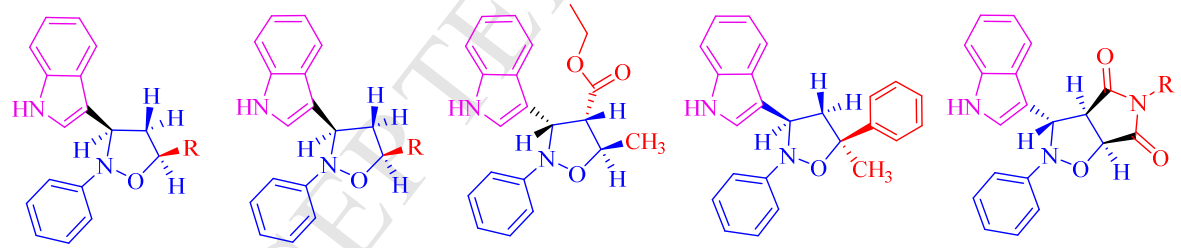
Isoxazolidines belong to an important class of heterocyclic compounds having a wide range of biological activities such as anti-inflammatory, anti-viral and anti-microbial [7]. We have recently reported pyridyl-isoxazolidines as nicotinic analogs for their nootropic activities [8] and chromanyl–isoxazolidines as apoptosis inducers through the mitochondrial-dependent pathway in HL-60 cells [9]. Therefore, taking cognizance of therapeutic potential of C3 functionalized indoles as anti-inflammatory agents and extensive pharmacological activities of isoxazolidines; it

was decided to evaluate previously synthesized indolyl-isoxazolidines [10] along with new derivatives, as inhibitors of pro-inflammatory cytokines through *in vitro* and *in vivo* expression of TNF-*α*, and IL-6, along with *in vivo* analgesic-anti-inflammatory activities.

Material and Methods:

Animals: Swiss albino mice (2-3 months old) of either sex, weighing 25-35 g (Animal House Facility, Guru Nanak Dev University, Amritsar, India) were habituated to the temperature, humidity and lighting (12 h light/dark cycle) controlled housing facility and group housed for at least 3 days before behavioral studies began. Animals had free access to food (standard laboratory rodent's chow) and water throughout the study. Before beginning the experiments, animals were acclimated to the laboratory environment for at least 30 min. All animal experimental protocols were approved by the Institutional Animal Ethical Committee (IAEC) of CPCSEA. All efforts were made to minimize animal suffering and to decrease the number of animals used.

Drugs and chemicals: All the chemicals and drugs used such as carboxymethylcellulose, lipopolysaccharide (LPS), MTT and indomethacin were purchased from Sigma Aldrich, USA; whereas, evaluated indolyl-isoxazolidines (**6-9**) were obtained by 1,3-dipolar cycloaddition reactions of *C*-(3-indolyl)-*N*-phenylnitrone (**2′**) with monosubstituted (**3a-f**), di-substituted (**4′a-b**) and cyclic dipolarophiles (**5′a-d)** as described earlier (Scheme-**1**) [10]; authenticated using NMR spectroscopy and mass spectrometry. A suspension of various doses of the compounds was prepared in 0.1% CMC solution. 0.9% saline was used as vehicle control. All the drugs were administered intraperitoneally (i.p.).



(±)**6 a-f**
R; **a** = -Ph,
b = -p-Me-Ph,
c = -Pyridyl
d = -CO_2Me
e = -CO_2Et
f = -CO_2Bu

(±)**6' d'-f'**
R; **d'** = -CO_2Me
e' = -CO_2Et
f' = -CO_2Bu

(±) **7**

(±) **8**

(±) **9 a-d**
R; **a** = -CH_2Ph
b = -Ph
c = -Et
d = -H

Figure-2: Variedly substituted indolyl-isoxazolidine analogs (**6-9**).

***In vitro* effect on cytokines level in THP-1 cells**: Human leukemia THP-1 cells were seeded in a 24-well plate at a density of 3.5 x 105 cells/ml of RPMI medium per well. THP-1 cells were then treated with PMA (20 ng/ml) for differentiation into macrophages for 18 h followed by rest period of 48 h. Cells were

further co-treated with 5 µM of each test compound and 1 µg/ml of lipopolysaccharide (LPS) in a serum-free medium. Culture supernatant was harvested after 24 h for analysis of different cytokines by using OptEIA ELISA Kits from BD Biosciences. Total protein content for all the samples was calculated by using Bradford reagent from Bio-Rad Laboratories. All the samples were normalized by dividing cytokine concentration with total quantity of protein.

Cell proliferation assay using MTT: THP-1 cells were plated in 96-well plate in 100 µL of medium and treated on the same day with various concentrations of test compounds **8**, **9a**, and **9b**, and incubated for 24−48 h. The MTT dye, at a concentration of 2.5 mg/mL, was added for 4 h at 37 °C. The supernatant was aspirated, and MTT−formazon crystals were dissolved in 150 µL of DMSO. The OD was measured at λ540 on an ELISA microplate reader. Cell growth was calculated by comparing the absorbance of treated versus untreated cells.

Effect of compounds on the production of LPS-induced pro-inflammatory cytokines TNF-α and IL-6 *in vivo* model: The effect of compounds **9a** and **9b** was investigated on the secretion of cytokines (TNF-α/ IL-6) in Swiss albino mice of either sex weighing 25-35g. Mice were then randomly divided into 8 groups (n = 4 per group). The treatment to each group was given in following format: group I (vehicle), group II (LPS, 1 mg/kg), groups III, IV and V (compound **9a** at doses 5, 10 and 20 mg/kg, respectively), groups VI, VII and VIII (compound **9b** at doses 5, 10 and 20 mg/kg, respectively). Blood sample was collected by cardiac puncture using heparin as anticoagulant and immediately centrifuged at 5000 rpm for 10 minutes. Blood plasma was collected and frozen at -20°C until activity. Cytokines (TNF-α/ IL-6) concentration was determined by using enzyme linked immunosorbent assay (ELISA), using commercial kits from Krishgen Biosystems, Mumbai-India, as per manufacturer's instructions. Blood plasma cytokine concentrations are expressed as picogram per milliliter (pg/mL).

Acetic acid-induced abdominal writhing: The acetic acid-induced abdominal writhing test was used as described previously by Koster *et al*. Four experimental groups of mice were treated (I.P) with vehicle (0.1% carboxy methyl cellulose), compounds **9a** and **9b** (12.5 mg/kg), indomethacin (10 mg/kg) 30 min, before the administration of acetic acid solution (0.6 % v/v, 10 ml/kg, i.p.). The number of writhing was calculated for each mice, completed a period of 30 min after acetic acid injection, and the results were expressed as the means ± SEM of number of writhing.

Carrageenan-induced paw edema assay: For anti-inflammatory activity, carrageenan induced paw edema model was used as previously reported [5b]. Experimental groups of animals were treated with vehicle (0.1% carboxy methyl cellulose), indomethacin (10 mg/kg, p.o.), and compound **9a**, 30 minutes before the injection of 50 µL of carrageenan (1% w/v) in the right hind paw. Then the paw edema was measured by vernier caliper (Precise) at 5 min, 15 min, 30 min, 1 h, 2 h, 3 h, 4 h and 6 h after injection of carrageenan.

Involvement of cyclooxygenase or opioid receptor pathways: Six groups of mice with 5 animals in each group were taken for exploring the mechanism of action of compound **9a**, the animals were pretreated with misoprostol, for studying the involvement of the cyclooxygenase pathway and to check the involvement of the opioid receptors during the inhibition of algesia, the animals were pretreated with naloxon. Details of the protocols are shown in **Figure-3**.

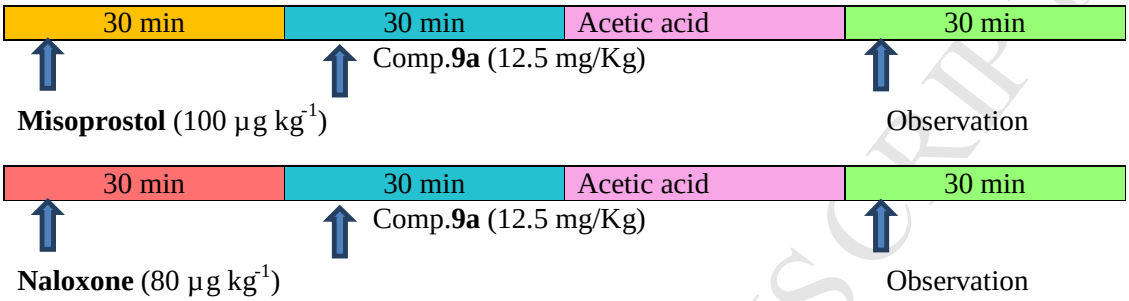


Figure-3: Schematic representation of experimental protocol for exploring the involvement of cyclooxygenase pathway or opioid receptor in the observed analgesic effect of compound **9a.**

Compound 9a attenuated LPS-induced inflammatory mortality in mouse model: Mice weighing 25-35 g were treated with compound **9a** (12.5 mg/kg) by *i.v.* injection either 15 min before or 15 min after the *i.v.* injection of LPS (20 mg/kg). Control animals received a similar volume of vehicle. Body weight change and mortality were recorded for 10 days.

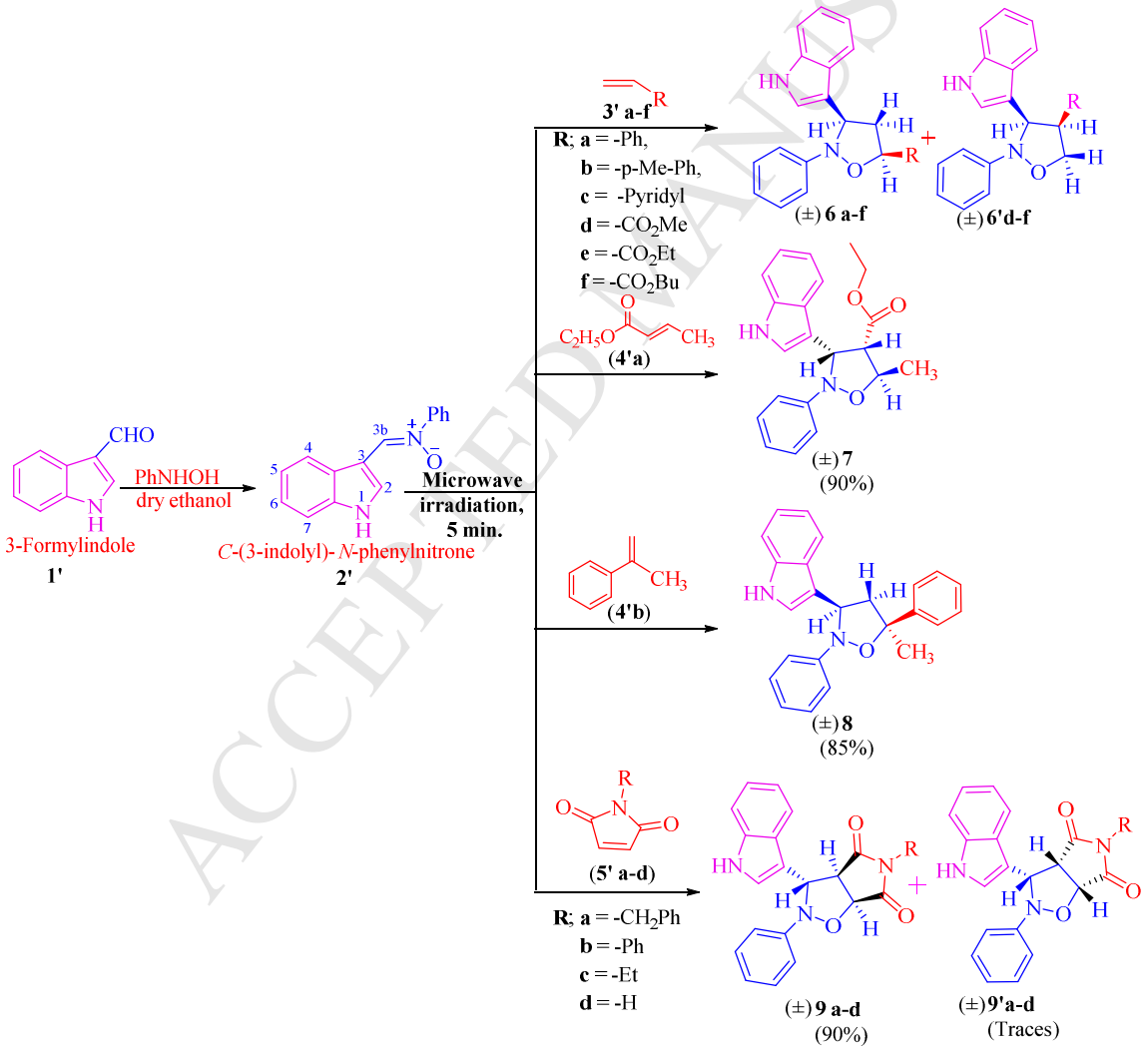
Statistical analysis: Statistical analysis was performed by one way analysis of variance (ANOVA) followed by Tukey's multiple comparison test as the post hoc test. All results are expressed as mean ± standard error of mean (SEM). The IC_{50} calculations (*in vivo*) were carried out using Design-Expert software, Stat-EasInc., Minneapolis, USA.

Acute toxicity studies: Four groups of animals with three animals per group were taken. The first group was administered the vehicle and served as the control group; the second, third, and fourth groups were treated with compound **9a** at doses of 50, 300, and 2000 mg kg^{-1}, respectively. All of the treatments were administered after 4 h of fasting. Thereafter, the animals were observed continuously for the first 4 h and periodically thereafter for 24 h. After 14 days, one animal each in the control and highest dose (2000 mg kg^{-1}) groups was sacrificed, and histological studies were conducted using H and E staining.

Results and Discussion

Initially, the 1,3-dipolar cycloaddition reactions of *C*-(3-indolyl)-*N*-phenylnitrone (**2′**) with monosubstituted (**3′a-f**) and di-substituted (**4′a-b**) dipolarophiles were carried out by irradiating equimolar amount of the addends (neat) in a focused monomode microwave reactor, leading to the synthesis of variedly substituted isoxazolidines (**6-8**). A perusal of literature reveals that

bicyclic isoxazolidines are widely distributed in natural products and valuable pharmaceutical agents as well as synthetic intermediates. A number of desirable biologically properties [10d-f] have been reported for molecules containing this bicyclic scaffold. Therefore, keeping in view the high therapeutic importance of bicyclic isoxazolidines, the reaction of *C*-(3-indolyl)-*N*-phenylnitrone (**2′**) with various cyclic dipolarophiles (**5′a-d**) were carried out under similar set of conditions to obtain bicyclic isoxazolidines (**9a-d**). After completion of the reaction (TLC) the residues obtained were resolved by column chromatography over silica gel to obtain the cycloadducts (**6-9**, Scheme **1**) [10]. We had earlier reported characterization of the regio- and stereoisomeric indolyl-isoxazolidines on the basis of NMR and X-ray crystallographic studies [10a-c].



Scheme-1: Reaction of nitrone (**2′**) with variedly substituted dipolarophiles (**3-5**)

***In vitro* effect on cytokines level**: The release of pro-inflammatory cytokines is an important mechanism by which the immune cells regulate the inflammatory responses and contribute to various inflammatory and autoimmune disorders. All the synthesized compounds (**6-9**) were analyzed for their ability to decrease lipopolysaccharide (LPS) induced tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) production in THP-1 cells (**Table-1**).

Table 1: Inhibitory effect of compounds **6-9** on LPS induced pro-inflammatory cytokines IL-6 and TNF-α.

Compound No.	Concentration	IL-6 inhibition	TNF-α inhibition
UT Control		0.51 ± 0.43	1.40 ± 0.66
LPS	**1 µg/ml**	**6.06 ± 0.55**	**6.04 ± 0.54**
6a	5µM	5.40 ± 0.44	14.93 ± 1.70
6b	5µM	1.12 ± 0.62	4.58 ± 0.72
6c	5µM	2.77 ± 0.51	3.92 ± 1.11
6d	5µM	4.08 ± 0.44	7.27 ± 0.15
6e	5µM	3.20 ± 0.32	7.77 ± 0.43
6f	5µM	2.06 ± 0.46	3.98 ± 0.44
6′d	5µM	1.67 ± 0.57	3.00 ± 0.43
6′e	5µM	1.15 ± 0.51	4.37 ± 1.12
6′f	5µM	3.03 ± 0.42	2.59 ± 0.31
7	5µM	3.01 ± 1.18	3.98 ± 0.11
8	5µM	**1.13 ± 0.20**	**2.74 ± 1.71**
9a	5µM	**1.34 ± 0.91**	**2.37 ± 0.76**
9b	5µM	**1.43 ± 0.53**	**2.21 ± 0.40**
9c	5µM	3.09 ± 0.39	2.42 ± 0.29
9d	5µM	1.65 ± 0.32	4.28 ± 0.12

Notes: Human leukemia THP-1 cells after differentiation with PMA, were subjected to co-treatment with the test compounds (**6-9**; 5 µM) and LPS (1 µg/ml) for 24 h. Supernatant was used for the analysis of cytokines by OptEIA ELISA kits from BD Biosciences, according to the manufacturer's protocol. All the samples were normalized by dividing cytokine's concentration with total protein content. Data shown here are average of three similar experiments ± SD.

Several compounds from this series of molecules exhibited significant inhibitory activity against LPS induced human IL-6 and TNF-*α* in THP-1 cells. However, compounds **6b-c** bearing *p*-tolyl and pyridyl groups at C5- of isoxazolidine moiety, respectively, **6′d-6′e** (methyl- and ethyl-ester functionality at C4- position of isoxazolidine moiety), **8** (di-substituted isoxazolidine), **9a-b**, and **9d** (bicyclic isoxazolidines) at 5μM, showed particularly high activity against the induction of IL-6, where, the induction was reduced by 4-5 folds. Some of the compounds also reduced the induction of TNF-*α*; though the inhibitory effect was not as strong as against IL-6. Before the *in vivo* evaluation, the most potent compounds (**8**, **9a** and **9b**) were checked for their cytotoxic effect on THP-1 cells, to confirm that the suppressive effect of the these active compounds from *in vitro* assay is not because of cytotoxicity and the IC_{50} of these compounds **8**, **9a** and **9b** against THP-1 cell line was found to be >100 μM (24 h) and 74, 87, and 78 μM (48 h), respectively. This indicates that these compounds possess excellent therapeutic window for IL-6 and TNF-*α* inhibition with respect to THP-1 cell cytotoxicity. Therefore, the most active compounds were further chosen for *in vivo* evaluation, to determine whether, the lead compounds identified from *in vitro* studies were able to replicate the activity in animal models.

Effect of compounds on the production of LPS-induced pro-inflammatory cytokines TNF-*α* and IL-6 *in vivo* model: Based on the results of preliminary cytokine inhibition screening, two most active derivatives **9a** and **9b** were chosen for *in vivo* evaluation (dose-dependent inhibitory effects) against LPS-induced TNF-*α* and IL-6 release. The mice were fed with compounds at three doses (5, 10 and 20 mg/kg), followed by stimulation with LPS. Blood samples were collected and cytokine levels were measured in plasma (Figure **4** and **5**).

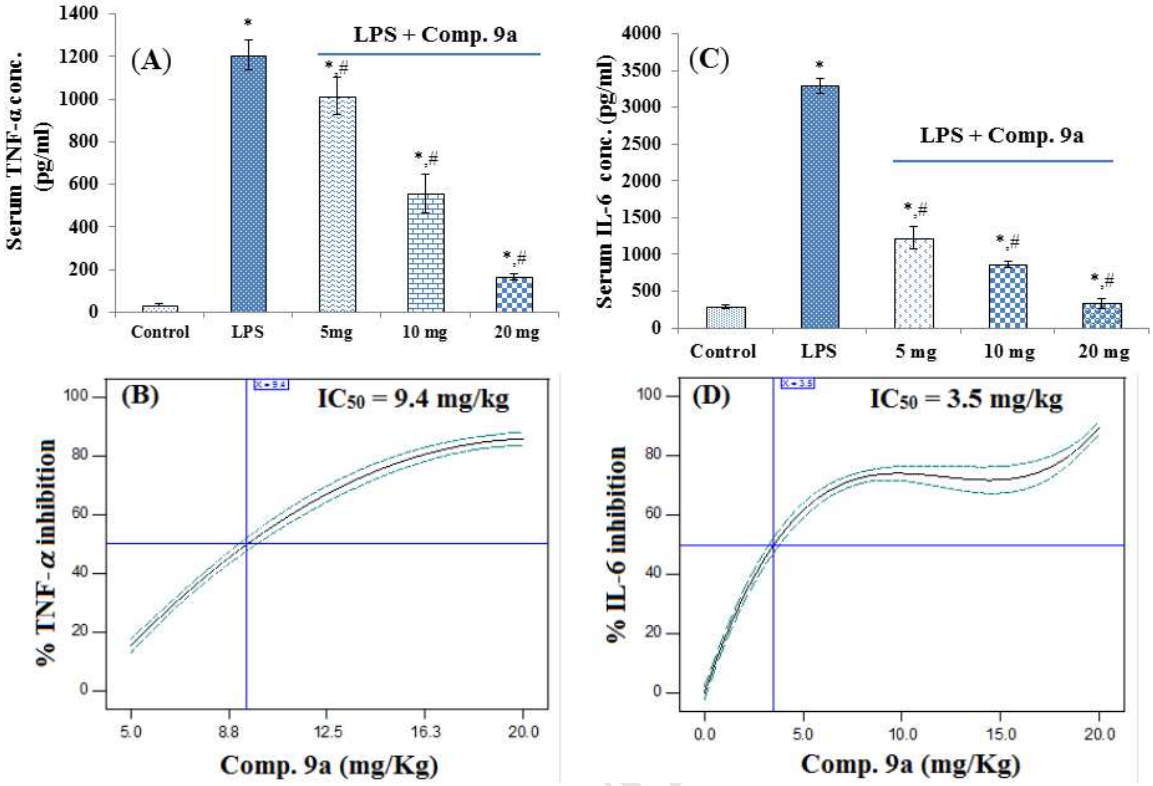


Figure-4: Effect of compound **9a** on production of TNF-α (**A**) and IL-6 (**C**) in Swiss albino mice; All values are expressed as mean ± S.E.M. Statistical differences were determined by one way analysis of variance (ANOVA) followed by Tukey's test *p<.005, *versus* control group and #p<0.01*versus* LPS treated group. (B) Second order fitted regression equation for IC_{50} (TNF-α) by varying the dose of comp. **9a**; (D) Second order fitted regression equation for IC_{50} (IL-6) by varying the dose of comp. **9a**

It was observed that the treatment with compound **9a** at the dose of 10 and 20 mg/kg, significantly reduced the plasma level of TNF-α upto 53.8 % and 86.06 % respectively, (**Figure-4A**) and IL-6 upto 73.71 % and 89.92 % respectively (**Figure-4C**). In order to determine the IC_{50} values for compound **9a** against TNF-α and IL-6, respectively, the experiment were run by varying the dose of investigational compound **9a** from 5 mg kg^{-1} to 20 mg kg^{-1} in triplicates. The dose response data of compound **9a** showing % inhibition of TNF-α, which was modeled using analysis of variance (ANOVA) with best fitted quadratic model. The model was highly significant at 99.99% (p-value <0.001) with non significant lack of fit and the IC_{50} value for compound **9a** against TNF-α inhibition is achieved at 9.4 mg/kg (**Figure 4B**). The fitted regression equation to find percentage inhibition against TNF-α (X_1) is given in equation 1.

$$\% \text{ inhibition } (X_1) = -37.640 + 12.064 \times A - 0.294\, A^2 \quad (1)$$

Where, A = Dose (compound **9a**) is in mg/kg of body weight.

Whereas, the dose response data of compound **9a** showing percent inhibition for IL-6 was best fitted using cubic model; the IC_{50} value against IL-6 for compound **9a** is achieved at 3.5 mg/kg (**Figure 4D**). Regression equation for percent inhibition of IL-6 (X_2) is given as equation **2**.

$$X_2 \text{ (\% inhibition of IL-6)} = -4.145 + 19.546 \times A - 1.673\ A^2 + 0.046 \times A^3 \quad (\mathbf{2})$$

where, A= Dose of compound **9a** (mg/kg of body weight).

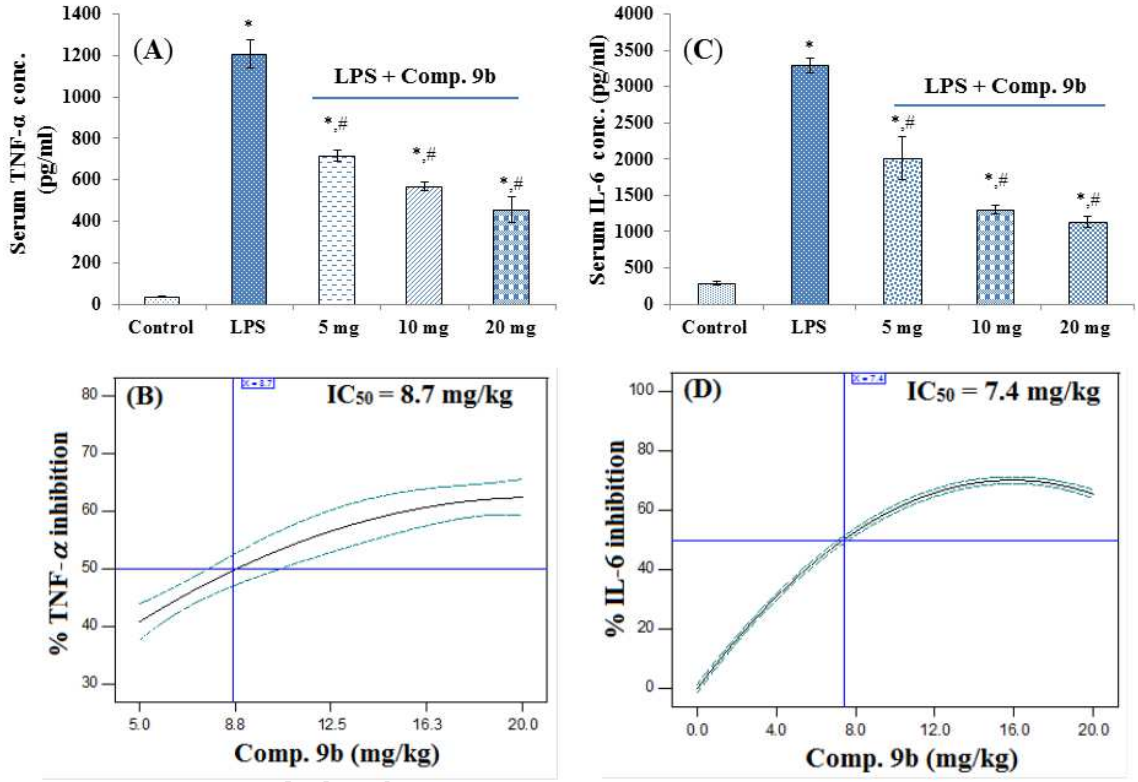


Figure-5: Effect of compound **9b** on production of TNF-α (**A**) and IL-6 (**C**) in serum *in vivo* LPS model; All values are expressed as mean ± S.E.M. Statistical differences were determined by one way analysis of variance (ANOVA) followed by Tukey's test *p<.005, *versus* control group and #p<0.01 *versus* LPS treated group; (B) Second order fitted regression equation for IC_{50} (TNF-α) by varying the dose of comp. **9b**; (D) Second order fitted regression equation for IC_{50} (IL-6) by varying the dose of comp. **9b**

However, at the same doses, compound **9b** inhibit the LPS induced plasma level of TNF-α (52.9 % and 62.1 %; **Figure-5A**) and IL-6 (60.46 % and 65.48 %; **Figure-5C**). Further, the IC_{50} values for compound **9b** (against TNF-α = 8.7 mg/kg and IL-6 = 7.4 mg/kg) were also determined in a similar set of experiment by varying the dose of investigational compound **9b** from 5 mg kg^{-1} to 20 mg kg^{-1} in triplicate and the dose response data of compound **9b** showing percent inhibition of

TNF-α (**Figure 5B**) and IL-6 (**Figure 5D**) was best fitted using quadratic model. The model was highly significant at 99.99% (p-value<0.001) with non significant lack of fit indicated model fitting. The fitted regression equations for percent inhibition of TNF-α (Y_1) and IL-6 (Y_2) are given in equation **3-4**, respectively.

Y_1 (% inhibition of TNF-α) = 24.781 + 3.645 × A – 0.088 A^2 (**3**)
where, A= Dose of compound **9b** (mg/kg) of body weight.

Y_2 (% inhibition of IL-6) = - 0.152 + 8.858 x A – 0.278 A^2 (**4**)
where, A= Dose of compound **9b** (mg/kg) of body weight.

On the basis of the promising efficacy of compound **9a** (IC_{50} value against TNF-α = 9.4 mg/kg and IL-6 = 3.5 mg/kg) and compound **9b** (IC_{50} value against TNF-α = 8.7 mg/kg and IL-6 = 7.4 mg/kg) in *in vivo* LPS models, revealed that both the compounds **9a** and **9b** showed prominent decrease in the levels of IL-6 in comparison to TNF-α; and further taken for studying *in vivo* analgesic/anti-inflammatory potential in animal models.

***In vivo* analgesic activity:** Acetic acid induced writhing test is used for detecting both central and peripheral analgesia. Intraperitoneal administration of acetic acid releases prostaglandins and sympathomimetic mediators such as PGE_2 and PGF2α and their levels are increased in the peritoneal fluid of the acetic acid administered mice [11]. The compounds such as **9a** and **9b** showing significant inhibition of LPS induced IL-6 and TNF-α were evaluated for their antinociceptive activity by the writhing test and results of writhing test (**Figure-6A**) indicate that compound **9a** exhibited most potent antinociceptive activity by reducing the number of abnormal muscle constrictions to 80.74% inhibition at 12.5 mg/kg dose, as compared to the standard drug indomethacin which cause 58.15% inhibition at 10 mg/kg.

***In vivo* anti-inflammatory activity**: Because, compound **9a** was found to inhibit the inductions of LPS induced IL-6 and TNF-α (both *in vitro* and *in vivo*) and possess significant analgesic activity as compared to indomethacin, it was decided to determine whether compound **9a** affects acute-phase inflammation in animal models. In this study, we used the carrageenan induced paw edema model as it is suitable experimental model for evaluating the anti-edematous and hyperalgesia effect and is believed to be biphasic; the first phase (1 h) involves the release of serotonin and histamines, and the second phase (>1 h) is mediated by prostaglandin, a cyclooxygenase product [12]. Carrageenan induces the release of TNF-α, which subsequently promotes IL-1β and IL-6 production in tissues, and *in vivo* anti-inflammatory results revealed

that compound **9a** (12.5 mg/kg) reduced edema by 36.92% at 6 hours, whereas, the positive control, indomethacin (10 mg/kg), reduced edema by 34.51% (**Figure-6B**). It was observed that the compound **9a** inhibited the carrageenan-induced paw oedema at all-time points (1–6 h from the carrageenan treatment, **Figure-6B**). These results suggest that the compound **9a** inhibited the biphasic inflammation induced by carrageenan.

Involvement of cyclooxygenase or opioid receptor pathways: To further understand the mechanism of action of compound **9a**, the animals were pretreated with misoprostol, for studying the involvement of the cyclooxygenase pathway and to check the involvement of the opioid receptors during the inhibition of algesia, the animals were pretreated with naloxone. The schematic representation of the experimental protocol is given in **Figure-3**.

The pretreatment of the animal with naloxone, an opioid receptor antagonist did not reverse the analgesic effect of compound **9a**, whereas, pretreatment with misoprostol, a prostaglandin analogue was found to decreased the analgesic effect of compound **9a**, although the difference was not statistically significant (**Figure-6C**).

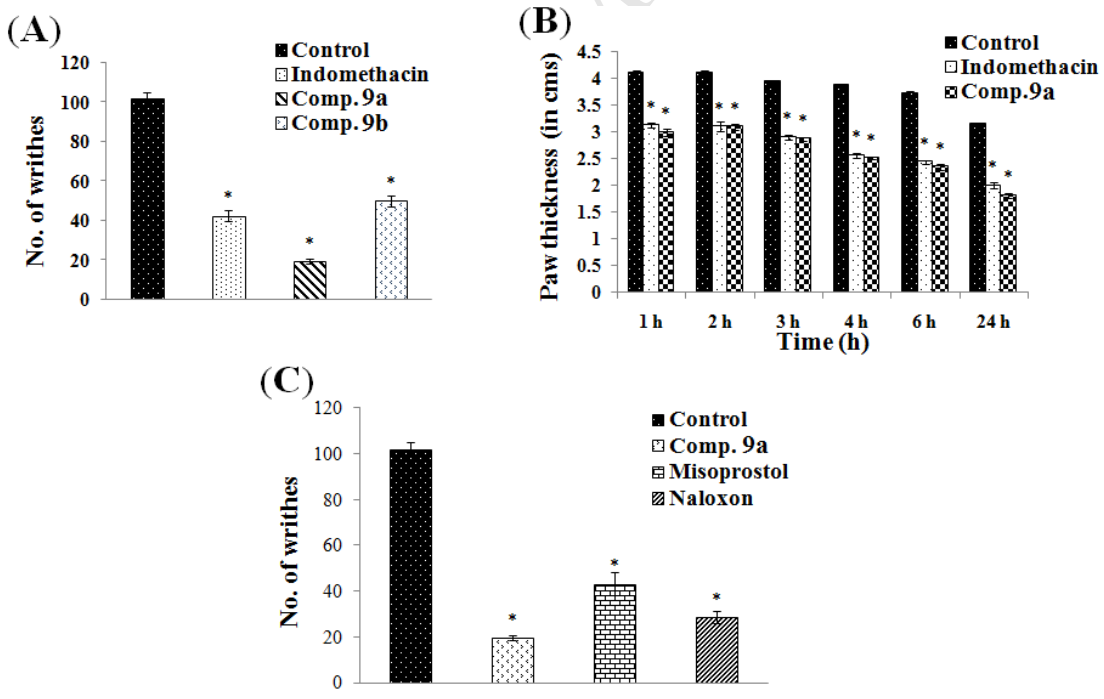


Figure-6: (**A**) Effect of compounds **9a** and **9b** on acetic acid induced writhing in mice; (**B**) Effect of compound **9a** on carrageenan induced rat paw inflammation in mice; (**C**) Effect of misoprostol and naloxone on analgesic effect of compound **9a** on acetic acid induced writhing in mice. All values are expressed as mean ±SEM. * $p<0.05$ Vs control.

It is well known that severe pain especially of neuropathic origin involves a complicated interplay of neuromodulators and cytokines. Although, the precise pathogenic pathway is not well understood, there is growing body of evidence that postulates the involvement of mediators such as eicosanoids, interleukins IL-6, bradykinin and TNF-α as the major culprits [13]. It has been postulated that in painful inflammatory conditions such as osteoarthritis, there is formation of pro-inflammatory cytokines including IL-1, IL-6, IL-15 and TNF-α which trigger a cascade of inflammatory reactions including activation of proteases, other chemokines as well as auto-stimulation, lipooxygenases, cyclooxygenases and metalloproteases (MMPs) etc. which lead to an exaggerated inflammatory reaction and pain [14]. The compound **9a** was found to decrease the lipopolysaccharide induced expression of IL-6 and TNF-α in human leukemia THP cell lines. Thus, it was essential also to check the effect of compound **9a** on the expression of COX-2 and iNOS (Figure 7). Results of western blotting experiment reveals that the expression of iNOS displayed a concentration dependent decline in the expression which confirms the negative regulatory effect of compound **9a** on NF-κB pathway, whereas, COX-2 expression was also reduced in a concentration dependent manner, indicating the broad anti-inflammatory effect of compound **9a**.

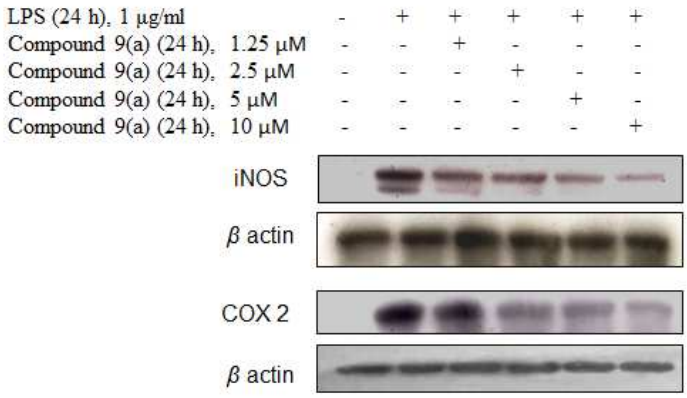


Figure 7: THP-1 cells were co-treated with LPS and different concentrations of compound **9a** for 24 h. Cells were lysed with RIPA and lysates were analyzed for the expression of COX-2 and iNOS by western blotting; beta actin was used as internal control.

In addition, the *in vivo* studies in mice revealed compound **9a** has a potent analgesic and anti-inflammatory activity, which was comparable to the standard drug indomethacin. Along with this, partial reversal of the observed analgesic response in acetic acid induced writhing was observed on pretreatment with misoprostol, a PGE_2 analogue. Therefore, it may tentatively be

suggested that compound **9a** is acting through interplay of pathways including inhibition of inflammatory cytokines as well as decreased formation of prostaglandins (**Figure-8A**).

Compound 9a enhances the survival rate in septic mouse model: As a major endotoxin, LPS from gram-negative bacteria has been implicated as a major cause of sepsis, thus, based on significant analgesic and anti-inflammatory effects of compound **9a** and for the potential clinical application, we have further determined whether compound **9a** attenuates endotoxin shock through inhibiting LPS-induced inflammatory response. Mice were injected with LPS at the dosage of 20 mg/kg intravenously in the presence or absence of compound **9a**, and their survival rates were monitored for 10 days, respectively, as shown in **Figure-8B**. All animals treated with LPS alone died as a result of the septic shock, whereas, animals treated with compound **9a** (12.5 mg/kg) 15 min after LPS injection (for therapeutic effect), the survival rates were significantly increased as compared to that of the positive control group (LPS treated, **Figure-8B**).

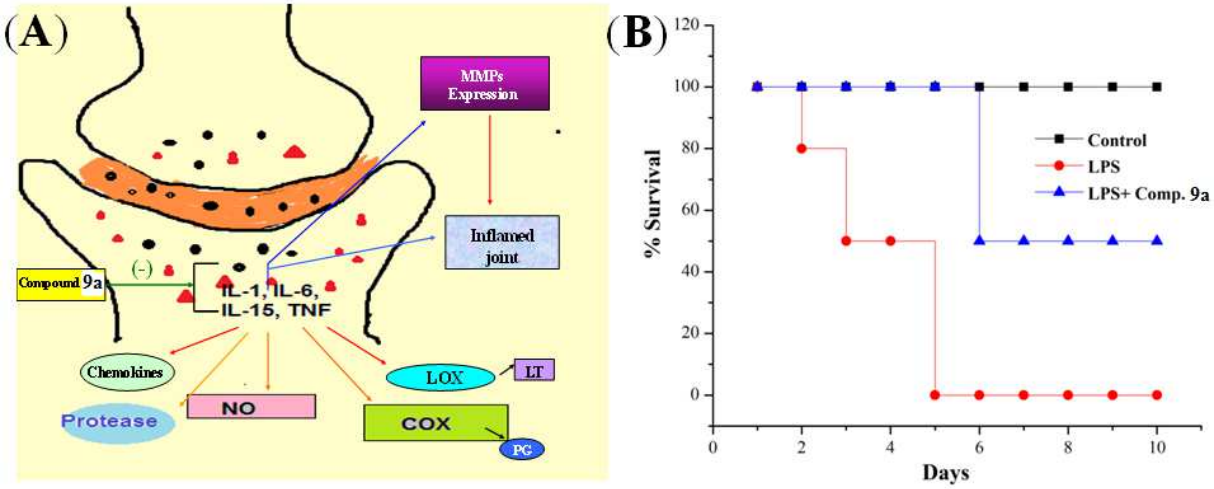


Figure-8: (**A**) Tentative mechanism of action of compound **9a**; (**B**) Compound **9a** improves survival of mice subjected to a lethal dose of LPS. Mice were treated with compound **9a** (12.5 mg/kg) 15 min after the injection of 20 mg/kg of LPS. Survival was recorded for 10 days after the LPS injection at the interval of 1 day.

Acute Toxicity Studies: OECD guidelines (OECD, 2001) [15] were followed for acute toxicity studies of compound **9a**. Four groups of animals with three animals per group were taken, the first group was administered the vehicle and served as the control group; the second, third, and fourth groups were treated with compound **9a** at doses of 50, 300, and 2000 mg kg^{-1}, respectively. All of the treatments were administered after 4 h of fasting. Thereafter, the animals were observed continuously for the first 4 h and periodically for 24 h. After 14 days, one animal

each from the control and the highest dose (2000 mg kg^{-1}) group was sacrificed, and histological studies were conducted using H and E staining. Results revealed that gross behavioral abnormality was not observed in any of the four groups of animals and even no significant changes were evident in the myocardium, liver or kidney of the animals treated with the highest dose of compound **9a** (2000 mg kg^{-1}) as compared to those of the control group (**Figure-9**).

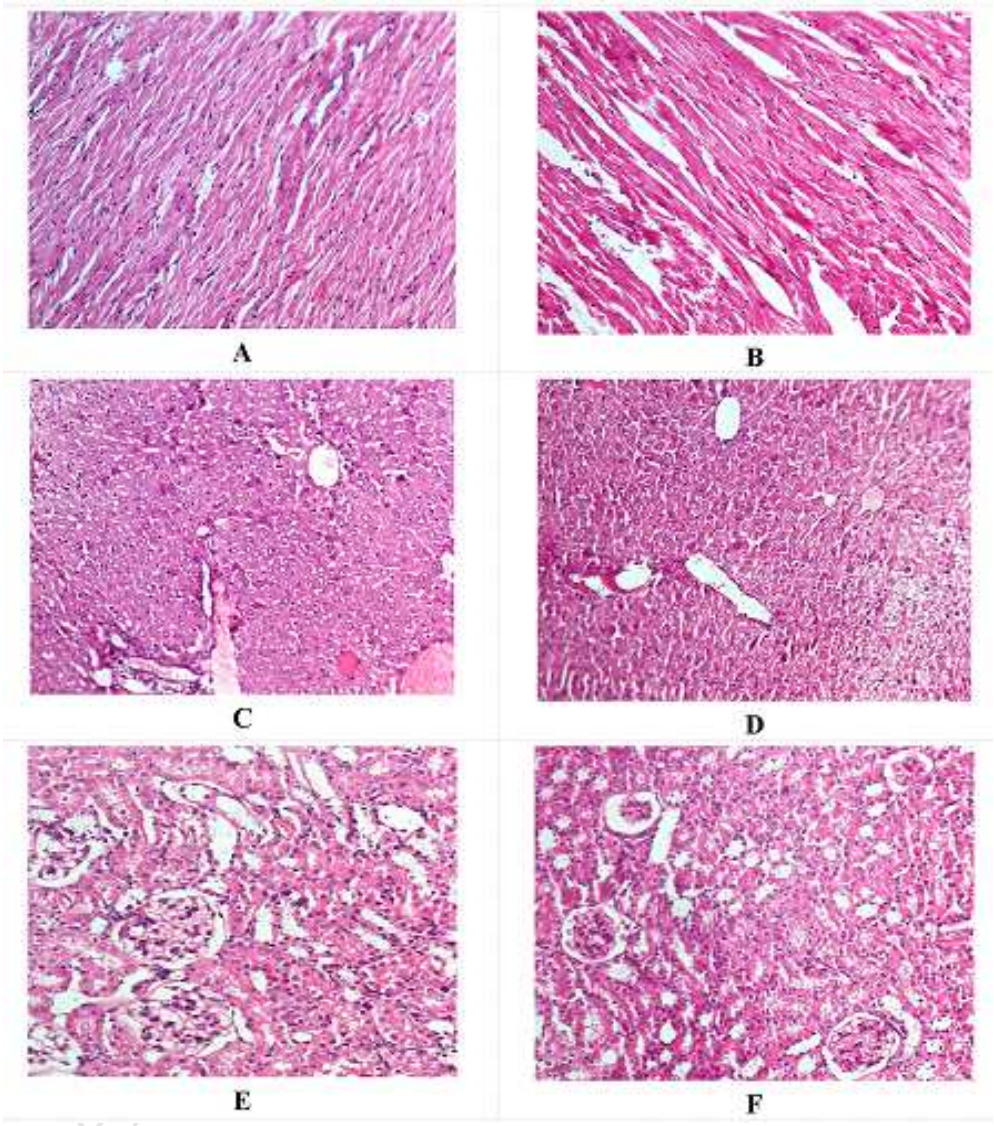


Figure-9: Histology of control (**A**) and compound **9a** treated heart (**B**), control (**C**) and compound **9a** treated liver (**D**), and control (**E**) and compound **9a** treated kidney (**F**).

Conclusions:

A focused library of substituted indolyl-isoxazolidines (**6**-**9**) has been synthesized by regio- and stereoselective 1,3-dipolar cycloaddition reactions and evaluated for their activity against pro-inflammatory cytokines IL-6 and TNF-α in LPS stimulated model. The *in vitro* studies showed that all the compounds possess significant IL-6 and TNF-α inhibition. In particular, compound **9a**, is most potent inhibitor of IL-6 and TNF-α. Testing of this compound on albino mice showed reversal of algesia and inflammation with much more efficacy as compared to the standard drug indomethacin. The mechanistic studies indicated that the anti-inflammatory and analgesic effects of compound **9a** share a common molecular pathway probably involving the inhibition of pro-inflammatory mediators. Thus, our findings clearly demonstrated that compound **9a** exhibited significant inhibitory effects on LPS-induced inflammation and had potential in the prevention and treatment for LPS-induced sepsis, and can be exploited as 'lead' compounds for the design of new analgesic/anti-inflammatory agents.

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Additional Information

Competing Interests: The authors declare no competing financial interests.

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Highlights of Manuscript

- Variedly substituted Indolyl-isoxazolidines (**6-9**) have been synthesized by regio- and stereoselective microwave irradiated 1,3-dipolar cycloaddition reactions.
- All the synthesized compounds were evaluated for inhibitory effect against LPS induced pro-inflammatory cytokines (TNF- α and IL-6) *in vitro* and *in vivo*.
- The most active compound **9a** (bicyclic isoxazolidine analog) possesses significant *in vivo* anti-inflammatory/analgesic activity comparable to indomethacin.
- Moreover, compound **9a**, also showed remarkable protective effect against septic shock in *in vivo* mouse model.