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Microwave synthesis of new naproxen analogues for chronic inflammation and their gastro-protective effect in post-operative model

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In the present study, novel naproxen analogues (NS9-NS12) were synthesized under microwave irradiation and characterized by ^{1}H NMR, ^{13}C NMR, and HR-ESI–MS advanced spectroscopic techniques. Their analgesic, anti-inflammatory and gastro-protective activities were evaluated in postoperative and chronic inflammatory pain models. Analgesic activity was determined by Eddy Hot plate and Acetic-induced Writhing tests using dose of 30 mg/kg (b.wt), while anti-inflammatory activity was determined by Egg induced method. Additionally, dose 1, 3, 10 and 30 mg/kg (b.wt) were used for post-operative pain and ulcerogenicity was assessed at 100 and 150 mg/kg(b.wt) after finding that all naproxen analogues were safe. Among all analogues, NS12 showed high significant ($p < 0.001$ increase in paw latency, inhibition in of writhing and significant ($p < 0.001$) edema reduction in subplantar area after 4 h of egg-induced edema. NS12 also significantly ($p < 0.001$) reduced postoperative pain and inflammation in both acute and repeated testing studies when compared to other analogues and untreated group. Histological and biochemical characteristics confirmed that naproxen derivatives exhibited minimal ulcerogenicity at 100 and 150 mg/kg (b.wt) in comparison to aspirin and naproxen. Based on these findings, synthesized naproxen analogues, particularly NS12 demonstrated potent analgesic and anti-inflammatory properties with enhanced gastric safety, indicating their potential as safer therapeutic alternatives to traditional naproxen for the treatment of pathological disorders associated with inflammation and pain.

Keywords Synthesis, Naproxen analogues, Microwave-Irradiation, Post-operative pain, Inflammatory pain, Ulcerogenic study

Non-steroidal anti-inflammatory drugs (NSAIDs) are extensively used globally to alleviate fever, pain and inflammation[1]. However, NSAIDs do not effectively alleviate pain in visceral organs as they may stimulate the release of cytokines that that trigger low-level inflammation[2]. The effects of NSAIDs arise from the inhibition of different types of prostaglandins that play a role in pain perception, inflammation, and the regulation of body

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temperature[3]. Among various NSAIDs, naproxen is widely used for pain relief by blocking the cyclooxygenase enzyme. This action leads to a reduction in prostaglandin levels across different tissues and fluids[4]. However, recent research has shown that prolong use or high doses of naproxen contribute to the formation of ulcers and bleeding, which may result in gastro-duodenal injury due to the presence of a free carboxylic group in the parent compound[4]. In order mitigate gastro-duodenal damage, research is concentrating on substituting the carboxylic free group with alternative groups that are less likely to cause gastric erosion. The replacement of the carboxyl group with amide methyl esters has shown reliable potential in combating inflammation[5,6]. The replacement of the carboxyl group in naproxen with glycolamide nitrate leads to reduced gastric damage while demonstrating while exhibiting significant anti-inflammatory properties[7]. Furthermore, a prodrug amide derived from naproxen has demonstrated a notable impact on inflammation when compared to naproxen[8]. Additionally, propane-amide derivatives of naproxen demonstrated a favorable effect against microbial infections, comparable to the medications used for treating these infections[9]. Other derivatives of naproxen have been reported to exhibit potential inhibitory effects on the colon cancer cell line.[9]. To the best of our understanding, there have been only a few studies that have investigated the substitution of the carboxyl group in naproxen with methyl ester, hydrazine, or imine groups. As a result, the development of new derivatives needs to be safe, effective and provide optimal analgesia for both postoperative and chronic inflammatory pain.

Derivatives of ibuprofen and flurbiprofen have been synthesized through a three-step reaction process. The first step involved esterification of carboxylic groups to form methyl esters. Second step involved transformation into hydrozoids using hydrazine monohydrate and subsequent condensation with benzaldehyde and salicylaldehyde, resulting two hydrazone derivatives derived from each original compound. These compounds play a significant role in alleviating pain relief, inflammation reduction, and providing gastro-protective effects, particularly in post-operative care[10,11] (Fig. 1).

Naproxen analogues (NS9-NS12) were synthesized under a new method specifically microwave irradiation and evaluated *in-vivo* in animal models of chronic inflammatory pain models and gastro-protective potential in post-operative model.

Results and discussion

Scheme 1 illustrates the synthesis of new naproxen analogues (NS9-NS12). The different naproxen analogues (NS9-NS12) were synthesized in three-step protocol, which began with naproxen (NS8) and included in three-step protocol, (i) esterification, (ii) hydrazide production (iii) via Schiff base reaction. In a first step, a good yield of naproxen esterified compound (NS9) in excellent yield (90%), obtained by a reaction of the free acid group of naproxen (NS8) with methanol in the presence of concentrated sulfuric acid (Con. H_2SO_4). In the second step, hydrazide production, naproxen esterified compound (NS9) reacted with hydrazine monohydrate in ethanol as a solvent for 2–3 h, yielding a high yield of 94% of naproxen hydrazide compound (NS10).

Finally, in the step 3, the naproxen hydrazide compound (NS10), which was produced by schiff's base reaction, was reacted with benzaldehyde and salicylaldehyde for 5-6 h to produce the desired naproxen analogues such as naproxen imine with benzaldehyde (NS11) and naproxen imine with salicylaldehyde (NS12) (94% & 96%), respectively. This reaction was carried out using ethanol as a solvent. High yields of new naproxen analogues (NS9-NS12) were obtained using three step protocol mentioned. The benefit of this novel methodology is that it ensures a high yield of products, new naproxen analogues (NS9-NS12) within a fast reaction time, as all reactions are carried out using microwave-irradiation.

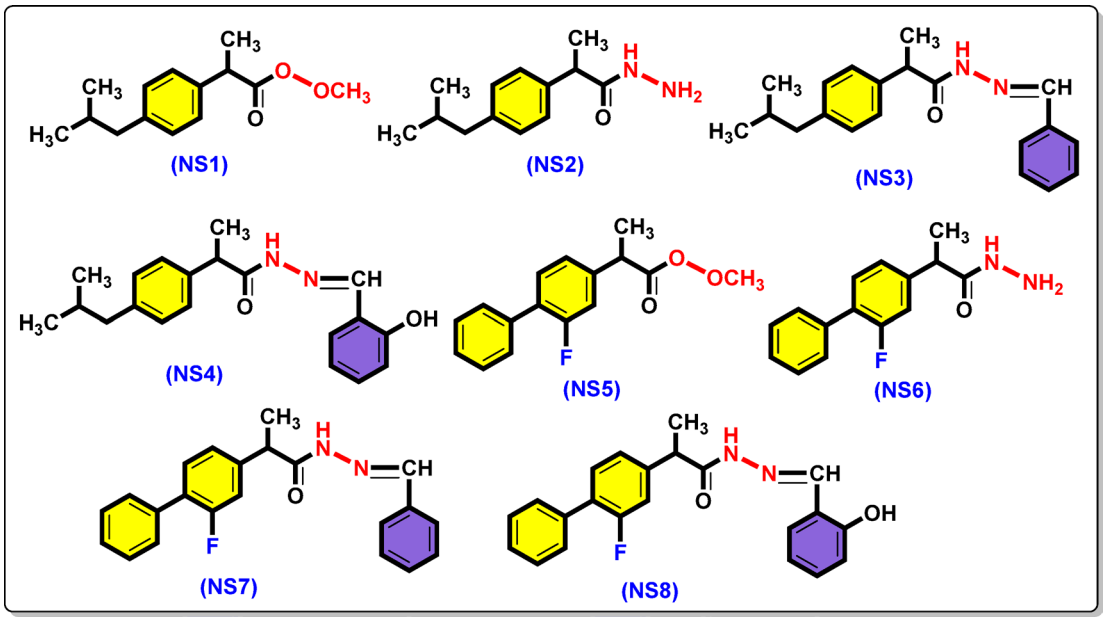
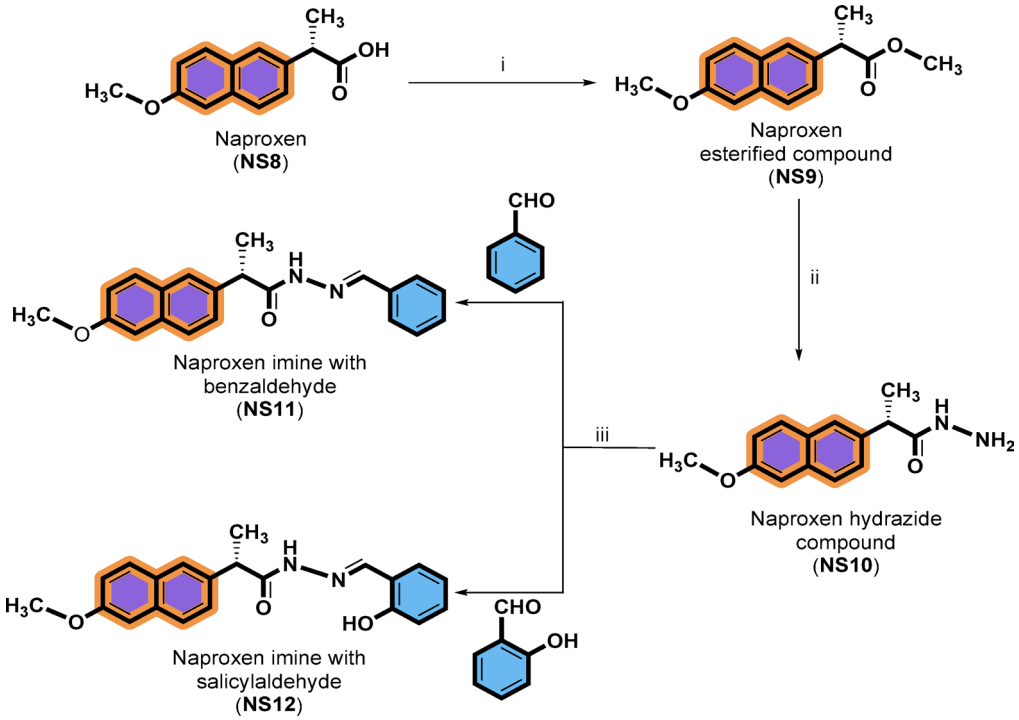


Fig. 1. Synthesis of Ibuprofen and flurbiprofen derivatives (NS1-NS8).



Scheme 1. Synthesis of new naproxen analogues (NS9-NS12).

Scheme 1 Reagents and conditions: (i) Con. H_2SO_4, MeOH, Microwave-Irradiation (MWI), at 85 ℃, 12 min, 90%; (ii) Hydrazine monohydrate, EtOH, Microwave-Irradiation (MWI), at 90 ℃, 25 min, 94%; (iii) Benzaldehyde or Salicylaldehyde, EtOH, Microwave-Irradiation (MWI), at 85 ℃, 20 min, 94% (NS11) & 96% (NS12).

All reactions were carried out in microwave-irradiations to get high-yields of naproxen analogues for the first time to minimize time for optimization of action. This is one of the new synthetic methodologies used to synthesize naproxen analogues. The physical data for naproxen analogues were summarized in Table 1.

Hydrolytic stabilities of naproxen derivatives

Hydrolytic stability is the major step in determining the new compounds as potential candidate to be used as drug molecule along with potency. Simulated fluids are employed at three pH levels to mimic gastric, plasma and intestinal environment effect on drugs stability. These systems were specifically designed to include the enzymes pepsin and trypsin. The compounds were studied at 1 mg/ml concentrations in triplicate and it was observed that all analogues presented considerable stabilities at all pH levels during the 24 h study time.

Acute toxicity studies of naproxen derivatives

Acute toxicological assessment of naproxen derivatives was conducted in accordance with OECD guideline 423, where a limit test dose of 2000 mg/kg (b.wt) was used. Following oral administration of naproxen derivatives, no treatment related toxic symptoms were recorded. All analogues exhibited no mortality and there were no observable changes in behavior, urinary function, or significant indicators related to the circulatory system, ocular health, or mucous membranes (nasal). Additionally, there were no changes in respiratory rate, skin and fur condition, or autonomic responses such as salivation, perspiration, piloerection, and central nervous system effects, including drowsiness, gait abnormalities, tremors, or convulsions. Furthermore, no adverse effects on breathing, skin condition, water intake, food consumption, or body temperature were noted. Consequently, these analogues appear to be safe at the dosage of 2000 mg/kg, with the LD_{50} estimated to be greater than 2000 mg/kg. It has been established that any pharmaceutical agent or compound with an oral LD_{50} exceeding 1000 mg/kg is generally regarded as safe and of low toxicity confirming, that naproxen derivatives are safe[26].

Central and peripheral analgesic activities of naproxen derivatives

The findings presented in Table 2 indicate that treatment of animals with naproxen analogues, particularly NS11 and NS12, significantly extended the stay/latency time ($p < 0.001$) on hot plate when compared to the control group. Naproxen analogues e.g. NS 9 and NS10 showed a non-significant ($p > 0.05$) rise in latency time at 1 h, 2 h, and 3 h. However, NS 9 showed a slight significance ($p < 0.05$) increase in latency time at 3 h after which the latency began to decline. NS11 and NS12 showed highest increase in paw latency time, became significance ($p < 0.01$) at 1 h, 2 h and become more significant ($p < 0.001$) at 3 h of post-treatment in comparison to the untreated group before also starting to decline. Highest latency time for paracetamol ($p < 0.001$) was observed, started at 1 h of receiving respective drug followed by a decline. Additionally, peripheral analgesic activity induced by acetic acid

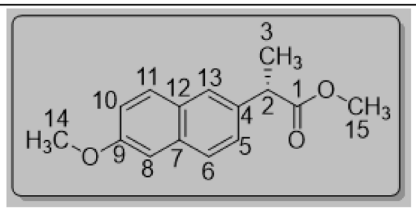
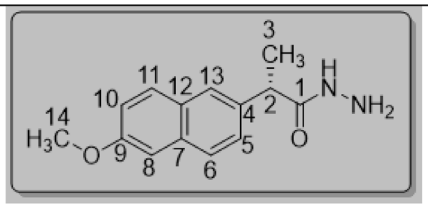
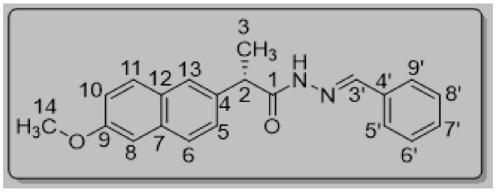
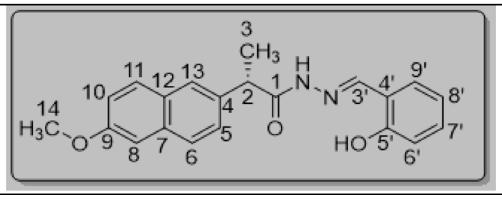
S. No.	Compound	Molecular Structure	M.P (°C)	Yield %	Colour
1.	NS-9		123-125	90	White solid
2.	NS-10		172-174	94	Light grey clay
3.	NS-11		177-179	94	Off white solid
4.	NS-12		181-183	96	Off white crystals

Table 1. Physical data of synthesized all new naproxen analogues

Latency time (sec)						
Treatment	**0 h**	**0.5 h**	**1 h**	**2 h**	**3 h**	**4 h**
Control	3.52 ± 0.1	5.5 ± 0.2	4.53 ± 0.7	4.58 ± 0.1	6.19 ± 0.1	5.83 ± 0.5
Paracetamol	4.12 ± 0.3	8.17 ± 0.5	12.9 ± 0.5**	20.8 ± 0.3***	22.5 ± 0.5***	18.5 ± 0.5
NS9	3.5 ± 0.1	4.52 ± 0.5	7.53 ± 0.2	8.59 ± 0.5	12.9 ± 0.5*	10.5 ± 0.5
NS10	3.9 ± 0.1	4.59 ± 0.5	8.51 ± 0.5	9.55 ± 0.2	11.5 ± 0.5	10.7 ± 0.5
NS11	3.57 ± 0.5	4.52 ± 0.5	11.3 ± 0.5**	12.8 ± 0.5**	16.5 ± 0.5***	15.5 ± 0.5
NS12	3.72 ± 0.5	4.5 ± 0.4	13.0 ± 0.4**	16.5 ± 0.2***	21.5 ± 0.5***	19.7 ± 0.5

Treatments	**No of writhing count/min**	**%Inhibition of writhing**
Control group	39.00 ± 2.00	0
Paracetamol	12.5 ± 1.5***	67.94%
NS9	26.00 ± 3.0*	33.33%
NS10	32.5 ± 2.5	16.66%
NS11	21.5 ± 1.5**	44.87%
NS12	16.5 ± 1.5***	57.69%

Table 2. Analgesic activity of Naproxen Analogue in Eddy Hot Plate and Acetic Acid-Induced Writhing method. Data are expressed as mean ± SEM, N = 5, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ significant compared with control. Two-way ANNOVA followed by Bonferroni tests for multiple comparison was used.

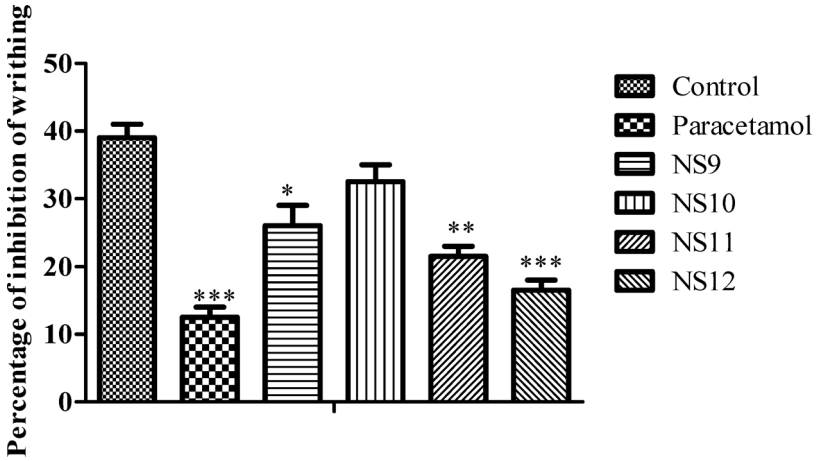


Fig. 2. Percentage of inhibition of Writhing of Naproxen (NS9-NS12).

Treatments	0 min	1 h	2 h	3 h	4 h
Egg Induced Paw Edema					
% Inhibition of Edema					
Un treated group	0	0	0	0	0
Diclofenac sodium	7.24%	16.36%	26.82%	35.23%	42.89%
NS9	7.24%	6.49%	15.85%	21.42%	18.07%
NS10	1.44%	2.33%	20.77%	26.19%	30.12%
NS11	5.21%	9.09%	17.03%	30.95%	33.73%
NS12	13.91%	9.09%	21.95%	33.33%	39.75%
Paw size of Egg Induced Paw Edema					
Paw size in cm					
Un treated group	3.45 ± 0.05	3.85 ± 0.05	4.10 ± 0.041	4.20 ± 0.02	4.15 ± 0.02
Diclofenac sodium	3.20 ± 0.02	3.22 ± 0.02	3.00 ± 0.025	2.72 ± 0.2	2.37 ± 0.02***
NS9	3.20 ± 0.02	3.60 ± 0.50	3.45 ± 0.029	3.30 ± 0.05	3.40 ± 0.04
NS10	3.40 ± 0.02	3.76 ± 0.50	3.25 ± 0.029	3.10 ± 0.05	2.90 ± 0.04*
NS11	3.27 ± 0.02	3.5 ± 0.04	3.4 ± 0.025	2.90 ± 0.02	2.75 ± 0.02**
NS12	2.97 ± 0.02	3.50 ± 0.50	3.20 ± 0.029*	2.80 ± 0.05**	2.50 ± 0.04***

Table 3. Effects of Naproxen Analogue against Egg-Induced Paw Edema. Data are expressed as mean ± SE of five for each group, N = 5, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ significant compared with control. Statistical analysis was performed by Two-way ANNOVA with Bonferroni post hoc tests for multiple comparison.

in Balb/c mice is summarized in Table 2 and Fig. 2. The results revealed that animals received NS12 at a dose of 30 mg/kg (b.wt) exhibited more significance ($p < 0.001$) reduction in writhing response compared to control group. NS11 also resulted in a significant reduction in writhing ($p < 0.01$) relative to the control group, while NS9 showed a slight reduction ($p < 0.05$) in writhing. The inhibition percentages were 57.69% for NS12, followed by 44.87% for NS11, 33.33% for NS9, and 16.66% for NS10. The positive control group receiving paracetamol demonstrated a more potent inhibition (67.94%, $p < 0.001$) in the writhing response compared to the group treated with normal saline.

Anti-inflammatory activity of naproxen derivatives

The findings presented in Table 3 indicate that animals receiving naproxen analogues exhibited a significant reduction in edema in the sub plantar region induced by egg. After 4 h of edema induction, NS12 significantly alleviated edema in the sub plantar area of the paw ($p < 0.001$), with a slight significance ($p < 0.05$) reduction at 2 h, becoming significance ($p < 0.01$) at 3 h. In contrast NS11, NS10 showed slight significance ($p < 0.05$) reduction in edema at 4 h compared to untreated group, while NS9 showed non-significant effect ($p > 0.05$) following 4 h of edema induction. The efficacy of diclofenac sodium against egg white-induced edema was significantly greater ($p < 0.001$) than that of the normal control and the naproxen analogue. When compared to the untreated group, NS12 resulted in a 39.75% reduction in edema in the sub plantar region, followed by NS11 (33.73%), NS10 (30.12%), and NS9 (18.07%) after 4 h of treatment with the naproxen analogue. Meanwhile, diclofenac sodium inhibited edema by 27.94%.

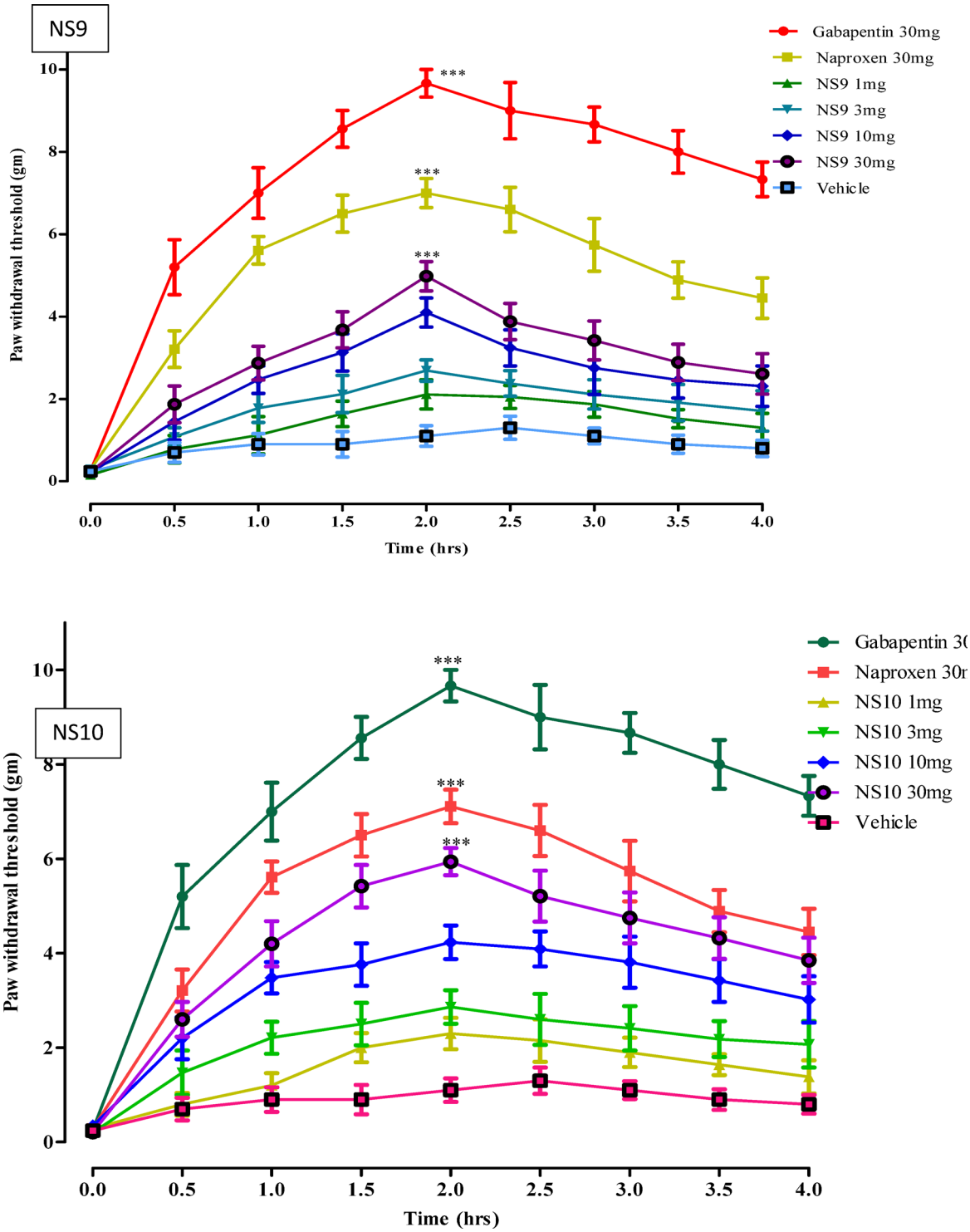


Fig. 3. Acute postoperative anti-nociceptive study of naproxen derivatives in animal model.

Acute anti-nociceptive effect of naproxen derivatives

Anti-nociceptive effects of naproxen derivatives were expressed as mean± SEM. Paw withdrawal threshold was calculated by using Von Frey filaments. All derivatives showed significant (p<0.001) attenuation in pain suppression in a dose-dependent manner with peak responses occurred 2 h after receiving derivatives and gabapentin. NS9 showed a significant effect on paw withdrawal threshold (2.110 ± 0.35, 2.690 ± 0.26, 4.10 ± 0.32, and 4.980± 0.38 at respective doses of 1, 3, 10 and 30 mg/kg (b.wt). Maximum pain suppression with compound NS9 was observed at dose 10 and 30 mg/kg (b.wt) 4.10 ± 0.32, and 4.98±0.02 as shown in Figure 3. Similar post-operative pain suppression effects with NS10 at a dose of 1, 3, 10, and 30 mg/kg (b.wt). Paw

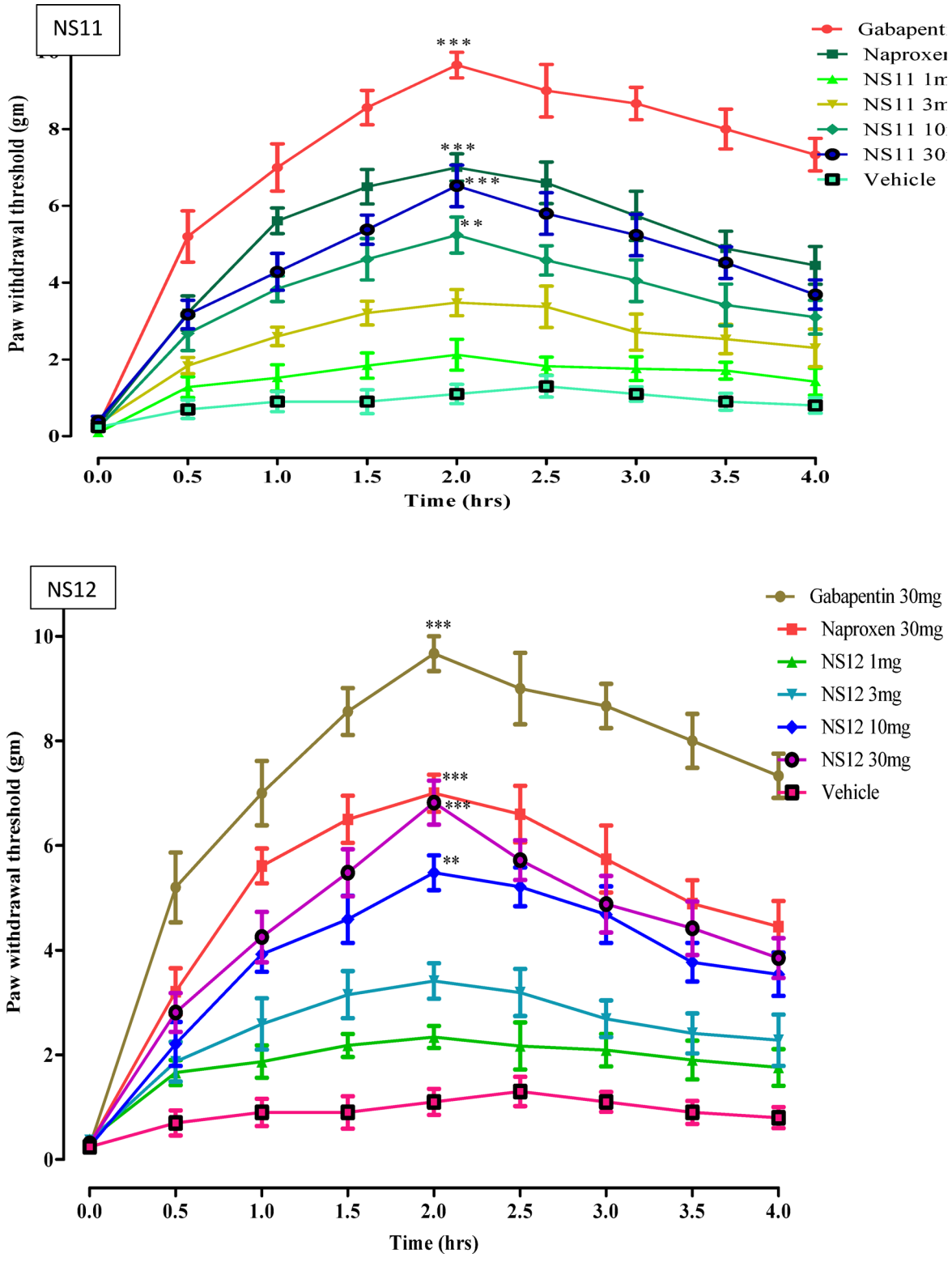


Fig. 3. (continued)

withdrawal thresholds with compound NS10 were (2.30 ± 0.33, 2.86 ± 0.35, 4.23 ± 0.35, 5.94 ± 0.29). It was found that the pain suppressant effect of NS12 was more significant as compared among all derivatives. The observed paw withdrawal thresholds of compounds NS11 were (2.12 ± 0.40, 3.480 ± 0.34, 5.24 ± 0.47 and 6.52 ± 0.54) and NS12 were (2.34 ± 0.21, 3.41 ± 0.34, 5.48 ± 0.33 and 6.82 ± 0.42. Among these four derivatives NS12 was found to be the most efficacious and exerted significant ($P<0.001$) pain suppression effect at dose 30 mg/kg (b.wt), having paw withdrawal threshold 6.82 ± 0.4 when compared to the normal saline group that was almost same as

Days	Gabapentin (30 mg)	Naproxen (30 mg)	NS9 (30 mg)	NS10 (30 mg)	NS11 (30 mg)	NS12 (30 mg)	Vehicle
1	9.66 ± 0.3***	7.00 ± 0.35***	4.98 ± 0.35***	5.94 ± 0.29***	6.50 ± 0.54***	6.82 ± 0.42***	0.17 ± 0.08
2	10.01 ± 0.3***	7.12 ± 0.51***	4.85 ± 0.36***	5.86 ± 0.27***	6.58 ± 0.41***	6.75 ± 0.43***	0.64 ± 0.08
3	10.22 ± 0.26***	7.38 ± 0.41***	5.49 ± 0.35***	6.35 ± 0.31***	6.78 ± 0.22***	6.94 ± 0.25***	0.69 ± 0.14
4	10.45 ± 0.26***	7.97 ± 0.22***	5.87 ± 0.32***	6.78 ± 0.37***	7.09 ± 0.38	7.52 ± 0.24***	0.90 ± 0.04
5	11.34 ± 0.48***	8.59 ± 0.40***	6.20 ± 0.34***	7.32 ± 0.40***	7.66 ± 0.36***	8.82 ± 0.41***	0.70 ± 0.14
6	11.85 ± 0.34	9.12 ± 0.35***	7.21 ± 0.32***	8.25 ± 0.42***	8.48 ± 0.35***	9.75 ± 0.22***	0.69 ± 0.18
7	12.20 ± 0.47***	10.15 ± 0.28***	7.65 ± 0.34***	8.67 ± 0.31***	9.27 ± 0.28***	10.68 ± 0.34***	0.87 ± 0.07

Table 4. Repeated dose postoperative antinociceptive effect of Naproxen analogues. Data are expressed as mean ± SE of five for each group, N = 5, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ significant compared with control. Statistical analysis was performed by Two-way ANNOVA with Bonferroni post hoc tests for multiple comparison.

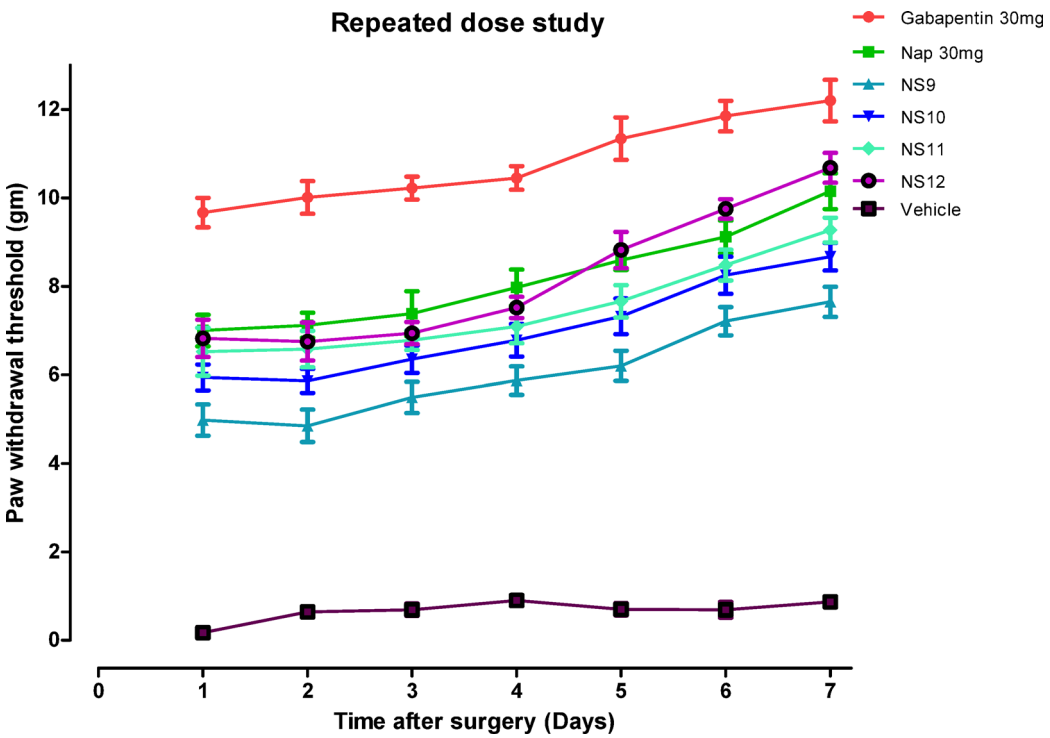


Fig. 4. Repeated dose Postoperative anti-nociceptive study of naproxen analogues.

produced by standard naproxen. Previous study has shown that naproxen derivatives are effective in moderate to severe acute postoperative pain in different doses, enhancing the latency time after 1 h of injection[10] (Figure 3).

Repeated dose anti-nociceptive effect of naproxen derivatives

Parent naproxen and its derivatives for repeated dose study were administered for 7 days and the pain suppression effect was measured after 2 h of administration for the acute study. Maximum response was observed after 2 h of receiving the respective derivatives. Among all compounds, NS11 and NS12 showed a significant effect (P<0.01) with paw withdrawal threshold of 6.58 ±0.75 and 6.75 ±0.43 respectively. Maximum response (p<0.001) was observed with NS12, as compared to NS11 as shown in Table 4 and Figure 4. The paw withdrawal responses with NS9 and NS10 were 4.85± 0.36 and 5.86± 0.46 respectively. All naproxen analogue showed significant anti-allodynic effect in the repeated dose study. A previously reported study showed that naproxen in repeated doses directly caused gastrointestinal bleeding[27]. This showed that all derivatives are effective and safe when in the repeated treatment study along with the acute study. The results are summarized in Table 4 and Figure 4.

Acute anti-inflammatory study of new naproxen analogues

The anti-inflammatory effect of naproxen derivatives was calculated as mean ± SEM. The paw withdrawal threshold was calculated by using von Frey filaments. Dose-dependent pain suppressant effect was found with all naproxen analogues. NS9 showed an effect (2.110 ± 0.22, 2.880 ± 0.26, 4.50 ± 0.42, and 5.700 ± 0.51) at doses

of 1, 3, 10 and 30 mg/kg (b.wt). Maximum response was observed at 10 and 30 mg/kg doses. NS9 and NS10 showed almost similar inflammatory pain suppression. Peak effect was observed after 2 h of dose administration. Paw withdrawal threshold observed with NS10 were 2.310 ± 0.25, 2.950 ± 0.47, 4.62 ± 0.77 and 6.50 ± 0.27. NS11 and NS12 also showed significant effect with NS12 having higher efficacy than NS11. The results observed for NS11 and NS12 were (2.150 ± 0.71, 3.850 ± 0.34, 5.86 ± 0.66, and 7.320 ± 0.54) and (2.480 ± 0.22, 4.120 ± 0.44, 6.16 ± 0.77, and 7.55 ± 0.25. Among these NS12 was found to be the most efficacious derivative, as shown in Fig. 5. Previously the anti-inflammatory effect of naproxen derivatives was established by the replacement of the carboxyl group with bicyclic propionic acid derivative *S*–naproxen[28].

Repeated dose anti-inflammatory effect of naproxen analogues

Naproxen and its synthesized analogues were evaluated for their inflammatory pain suppression effect for 7 days according to a repeated dose study. Pain suppression effect was measured after 2 h of dose administration, as in case of acute study maximum effect was perceived after 2 h of dose administration. Substantial inflammatory pain suppression response was perceived compared to the control group (vehicle). Among all analogues, NS11 and NS12 showed remarkable effects with Paw withdrawal of 9.27±0.55 and 10.24 ±0.65. Maximum effect was seen in case of NS12 as compared to NS11 as presented in Table 5 and Figure 6. The observed responses with NS9 and NS10 were 9.65± 0.33 and 8.67± 0.73 respectively. Previous reported study showed that prolonged use of naproxen inhibits the secretion of gastric prostaglandins[23]. All the derivatives showed significant pain suppressive effect with repeated dose study where 30 mg of dose on daily basis was administered to each animal and effect was observed after 2hr of dose administration. This showed that all derivatives were effective while use in case of repeated treatment study along with the acute study.

Protection against ulcerogenecity of naproxen derivatives

Significant effect in lesion score ($F_{10,55}$ = 16.67, $p < 0.001$) was observed in the case of aspirin and the parent compound naproxen at a dose of 150 mg/kg. NS9 and NS10 showed no significant change in the lesion score compared to aspirin and the parent drug. NS11 and NS12 showed no significant change in lesion score. This revealed that synthesized derivatives are comparatively safe (Fig. 7).

Substantial decrease in the gastric juice pH ($F_{10, 55} = 13.02$, $p < 0.001$) up to the level of acidity was seen with aspirin and naproxen. The synthesized compounds NS9 to NS12 showed less decrease in gastric juice pH ($pP < 0.01$).

Remarkable increase ($F_{10, 55} = 14.58$, $p < 0.001$) in the gastric juice volume was observed in the case of aspirin. Lesser increase in the volume of gastric juice ($p < 0.01$) was observed with NS11 and NS12. Comparatively less increase in gastric juice volume ($p < 0.05$) with NS9 and NS10. Significant increase ($F_{10, 55} = 12.65$, $p < 0.001$) in free acidity was observed with aspirin and naproxen however, less significant effect ($p < 0.05$) with NS9 and NS10 and comparatively increased effect ($p < 0.01$) in case of NS11 and NS12. The total acidity was also considerably ($F_{10,55} = 10.16$, $p < 0.001$) increased with aspirin and the parent compound. A decrease in total acidity ($p < 0.01$) was observed with NS9 and NS10. NS11 and NS12 also showed a significant reduction in total acidity ($p < 0.01$). There was no protruding effect on pepsin concentration in with the parent compound and the synthesized derivatives at respective doses, when compared with the saline treated animals. Previous research study has shown that the use of naproxen to treat arthritis led to sever gastrointestinal disturbances[29]. No effect on the ulcerogenecity was observed at all tested doses of the synthesized compounds, and over all the gross appearance of mucosa in case of synthesized derivatives was similar to that of saline treated animals (Fig. 7) while gastric ulcer was observed in case of aspirin and naproxen. (Fig. 8).

Conclusion and future recommendations

In present study, four novel naproxen analogues (NS9–NS12) were synthesized from parent naproxen (NS8) by altering the carboxyl group. The process of esterification yielded NS9, while the conversion to hydrazide resulted in NS10 and the subsequent reaction with benzaldehyde and salicylaldehyde produced the imine derivatives NS11 and NS12. To the best of our knowledge, these analogues (NS9-NS12) have not been documented previously. Furthermore, these compounds were assessed for their analgesic and anti-inflammatory properties using von Frey filament models for post-operative pain inflammatory model and their potential for causing ulcerogenicity was evaluated through various gastric parameters (lesion score, pH, volume, and acidity) at dose 100 and 150 mg/kg (b.wt). Among all the derivatives, NS12 (the salicylaldehyde Schiff base) exhibited the most potent pain-suppressing effect in both post-operative and repeated dose studies without causing ulcerogenicity at doses of 1, 3, 10 and 30 mg/kg (b.wt). At dose 30 mg/kg (b.wt), NS12 achieved highest paw-withdrawal threshold ($p <$ 0.001) after 2h of receiving the sample. On the other hand, esterified derivatives NS9 and hydrazide NS10 on the same dosage resulted in significant decreases in paw-withdrawal threshold ($p<0.01$) respectively. Furthermore, NS11 showed significant reduction in paw withdrawal threshold that that was comparable to NS12 ($p< 0.001$). However, additional studies are required to find the mechanisms of action, investigate cytokine inhibition, and conduct molecular docking and simulation analyses against COX-1 and COX-2 to verify selectivity.

Material and methods

Chemicals and drugs

Various chemicals, reagents and solvents of analytical grade were used for the synthesis of novel naproxen analogues purchased from Sigma Aldrich. Naproxen, Paracetamol and Diclofenac sodium was purchased from local market of Pakistan. ^{1}H NMR (Bruker 400 MHz), ^{13}C NMR (Bruker 100 MHz) were used for structure confirmation and characterization.

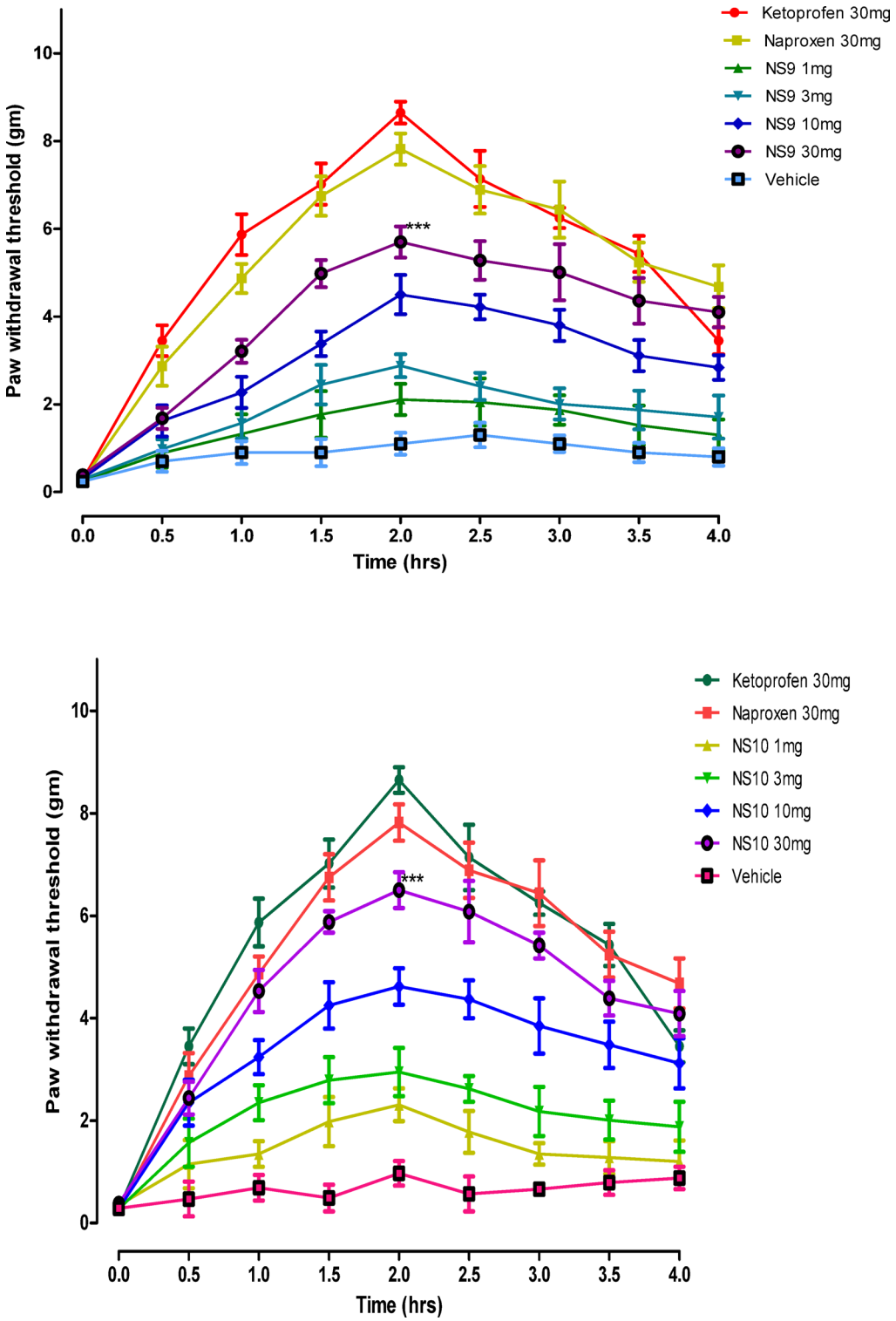


Fig. 5. Paw edema inhibition with different doses of naproxen analogues, Ketoprofen and Naproxen.

Test animals

Balb/c mice of both male and female having weight 23–30 g were used. Before the start of experiment, animals were placed in the animal's house under controlled condition 12 h/12 h light/dark cycle. The study was approved by the institutional ethical committee having an approval number of (DAEC/PHARM/2016/17). All the experimental protocols related to animals were performed according to the UK Animals (Scientific Procedures) Act 1986 and accordance with the ARRIVE guidelines.

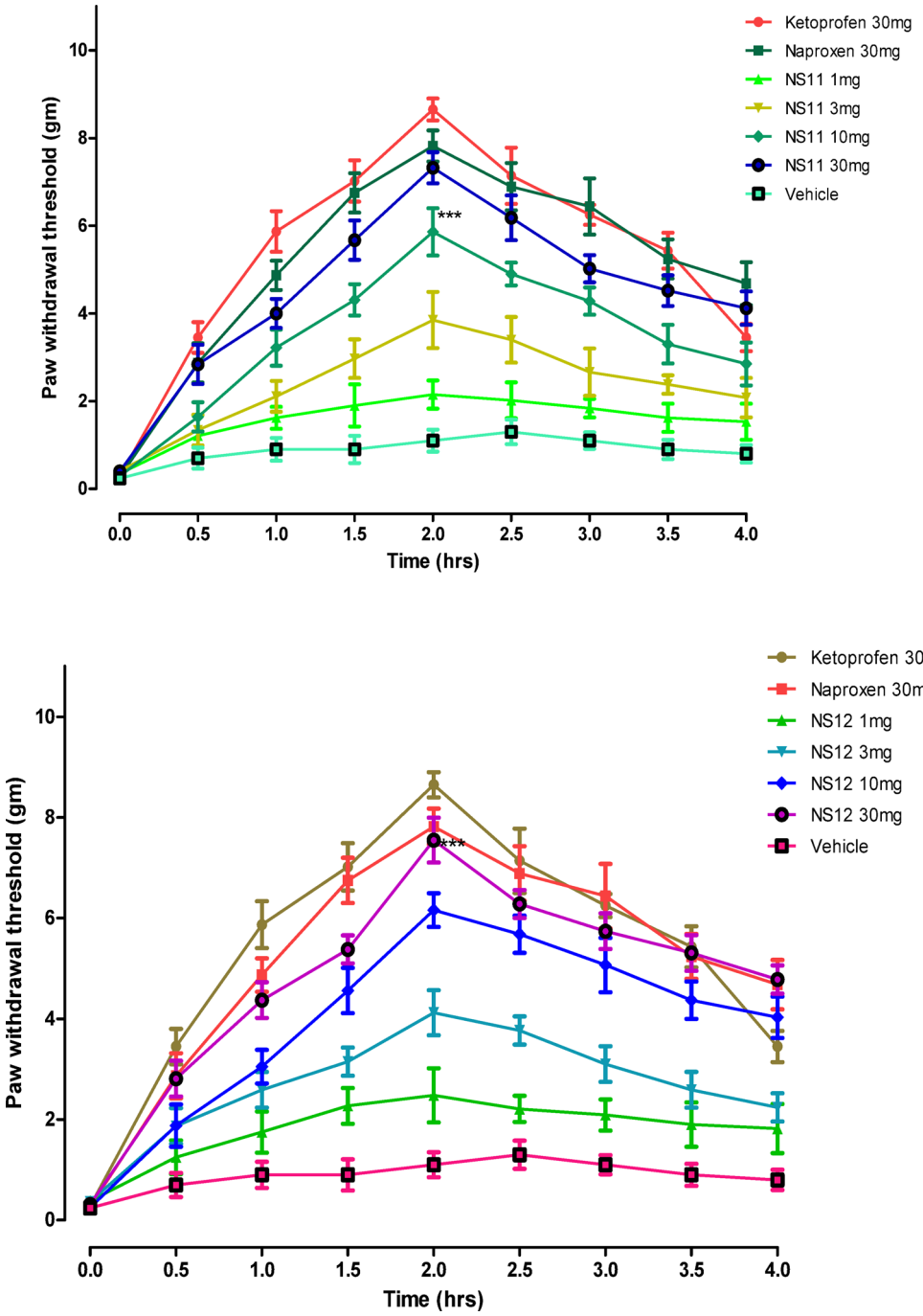


Fig. 5. (continued)

Experimental

All reactions were carried out under microwave irradiation, using the monomodal Emrys Creator microwave synthesiser (Biotage, Uppsala, Sweden) for 10 min. The reaction was then allowed to proceed at 80 °C under Microwave Irradiation (MWI) with a maximum power of 300W. After the reaction was completed, monitored by Thin Layer Chromatography (TLC), the combined organic layer underwent drying over anhydrous $MgSO_4$. Subsequently, it was concentrated under reduced pressure using a rotary evaporator to yield the crude product. It is reported in the literature that the synthesis of naproxen involves various intermediate pathways such as by halogenation, ketalization, rearrangement and hydrolysis by cupric halide to obtain naproxen that was purified by crystallization. Furthermore, naproxen purification is carried out through distillation, chromatography and solvent extraction techniques[12].

Days	Ketoprofen	Naproxen	NS9 30 mg	NS10 30 mg	NS11 30 mg	NS12 30 mg	Vehicle
1	8.65 ± 0.52***	7.82 ± 0.35***	5.700 ± 0.35***	6.50 ± 0.40***	7.32 ± 0.35***	7.55 ± 0.44***	0.290 ± 0.05
2	8.45 ± 0.37***	7.86 ± 0.28***	5.750 ± 0.27***	6.68 ± 0.35***	7.24 ± 0.41***	7.38 ± 0.43***	0.7800.09
3	9.34 ± 0.26***	7.62 ± 0.51***	5.89 ± 0.31***	6.58 ± 0.40***	7.49 ± 0.32***	7.67 ± 0.25***	0.640 ± 0.12
4	9.78 ± 0.40***	7.97 ± 0.41***	5.96 ± 0.37***	6.78 ± 0.32***	7.34 ± 0.38***	8.21 ± 0.24***	0.490 ± 0.17
5	9.50 ± 0.42***	8.59 ± 0.22***	6.20 ± 0.40***	7.32 ± 0.34***	7.66 ± 0.35***	8.820 ± 0.41***	0.710 ± 0.07
6	10.31 ± 0.31***	9.12 ± 0.37***	7.21 ± 0.42***	8.25 ± 0.0.34***	8.48 ± 0.35***	9.75 ± 0.22***	0.810 ± 0.03
7	10.16 ± 0.42***	10.15 ± 0.40***	7.65 ± 0.31***	8.67 ± 0.34***	9.27 ± 0.28***	10.24 ± 0.34***	0.640 ± 0.11

Table 5. Repeated dose anti-inflammatory effect of Naproxen analogues. Data are expressed as mean ± SE of five for each group, N = 5, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ significant compared with control. Statistical analysis was performed by Two-way ANNOVA with Bonferroni post hoc tests for multiple comparison.

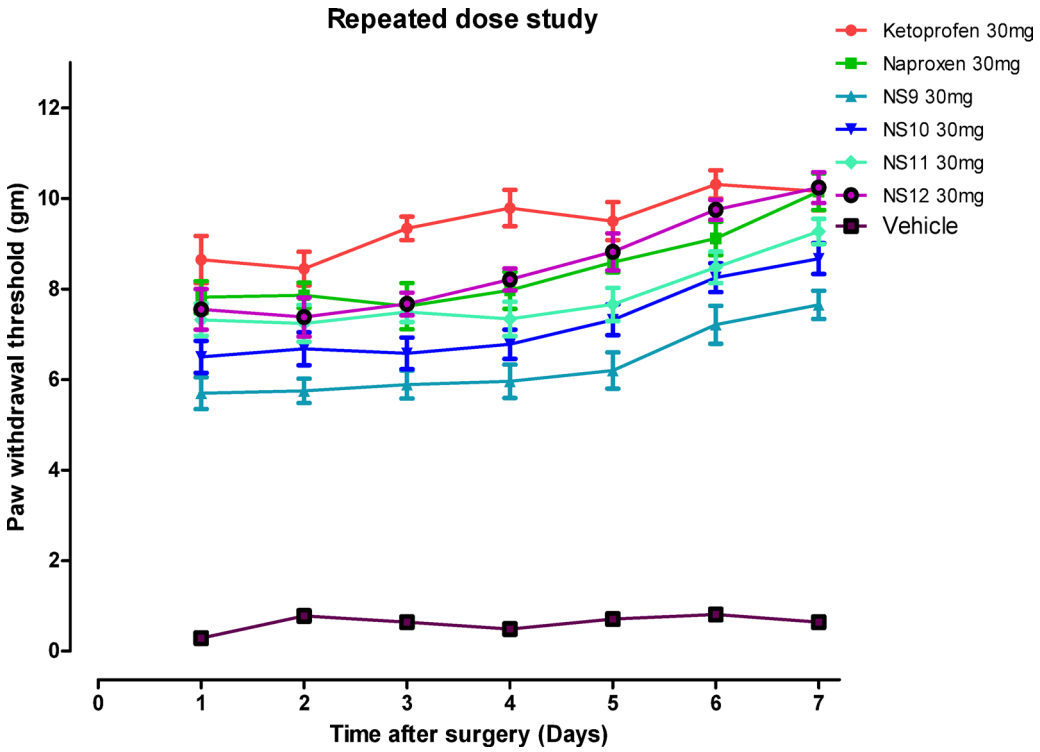


Fig. 6. Repeated dose anti-inflammatory study of Naproxen derivatives.

General procedure for the synthesis of naproxen esterified compound (NS9)

Naproxen esterified compound (NS9) was synthesized by using parent compound naproxen though monomodal Emrys Creator microwave synthesizer. The reaction was processed by using 20 mL methanol and 0.6 mL of Con. sulphuric acid (H_2SO_4). The reaction proceeded at 85 °C under Microwave-Irradiation (MWI) with a maximum power of 300W for 12 min. Completion of the reaction was indicated by formation of clear oily layer known as naproxen esterified compound (NS9) and the reaction process was stopped. For separation of oily layer, first it was neutralized by addition of calculated amount 1.2 g of sodium carbonate to the final product that was already dissolved in distilled water. For the separation, the entire product along with chloroform was transferred to the separating funnel and the oily layer was collected in chloroform and was further purified by addition of anhydrous $MgSO_4$ to remove excess of water. The crude product was then purified through flash column chromatography on silica gel with *n*-hexane/EtOAc (94:6 v/v) as an eluent, resulting in the isolation of pure naproxen esterified compound (NS9) in an excellent yield of 90%[12].

General procedure for the synthesis of naproxen hydrazide compound (NS10)

From purified naproxen esterified compound (NS9) to naproxen hydrazide compound (NS10) was synthesized by combining equimolar amounts of compound NS9 and hydrazine monohydrate and ethanol 20 mL was added to the reaction vessel of the monomodal Emrys Creator microwave synthesizer. The reaction mixture proceeded at 90 °C under Microwave-Irradiation (MWI) with a maximum power of 300 W. The appearance of clay-like solid indicated the formation of the product, which was purified by using distilled water and was

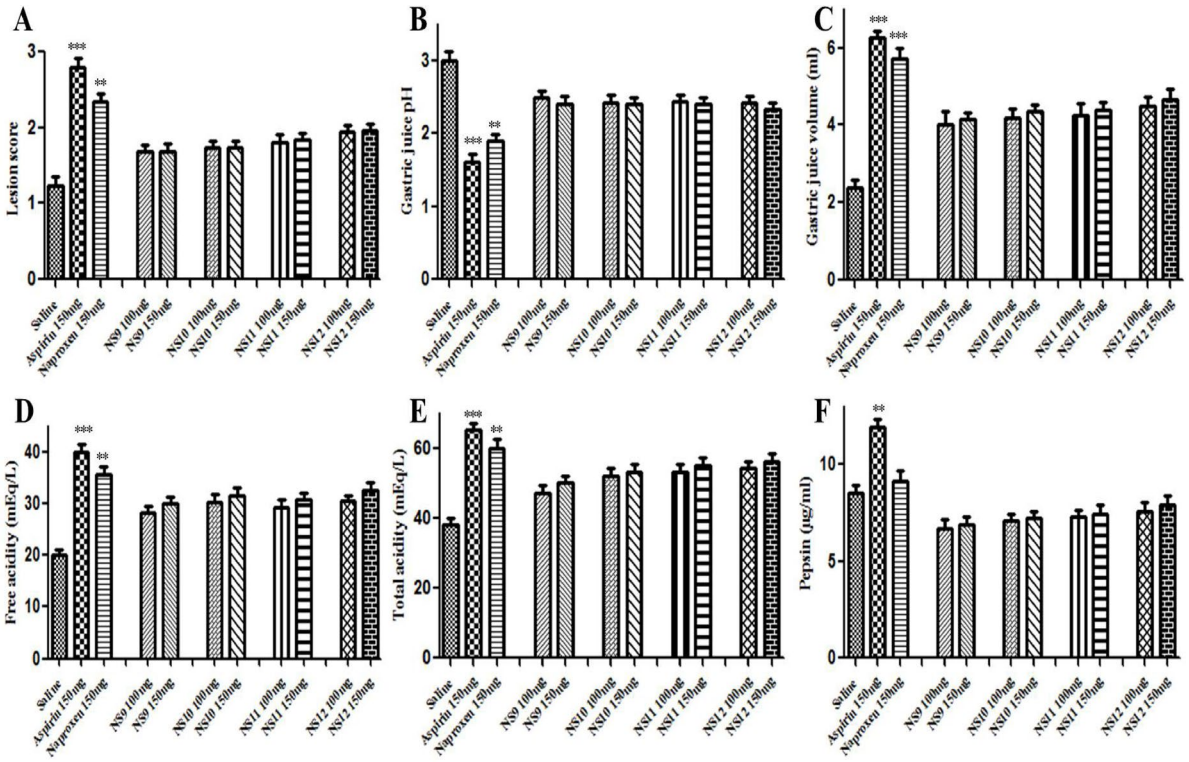


Fig. 7. Ulcerogenic potential of naproxen and its derivatives in mean ± SEM by evaluating lesion score (**A**), gastric juice pH (**B**), gastric juice volume (**C**), free acidity (**D**), total acidity (**E**), and pepsin concentration (**F**).

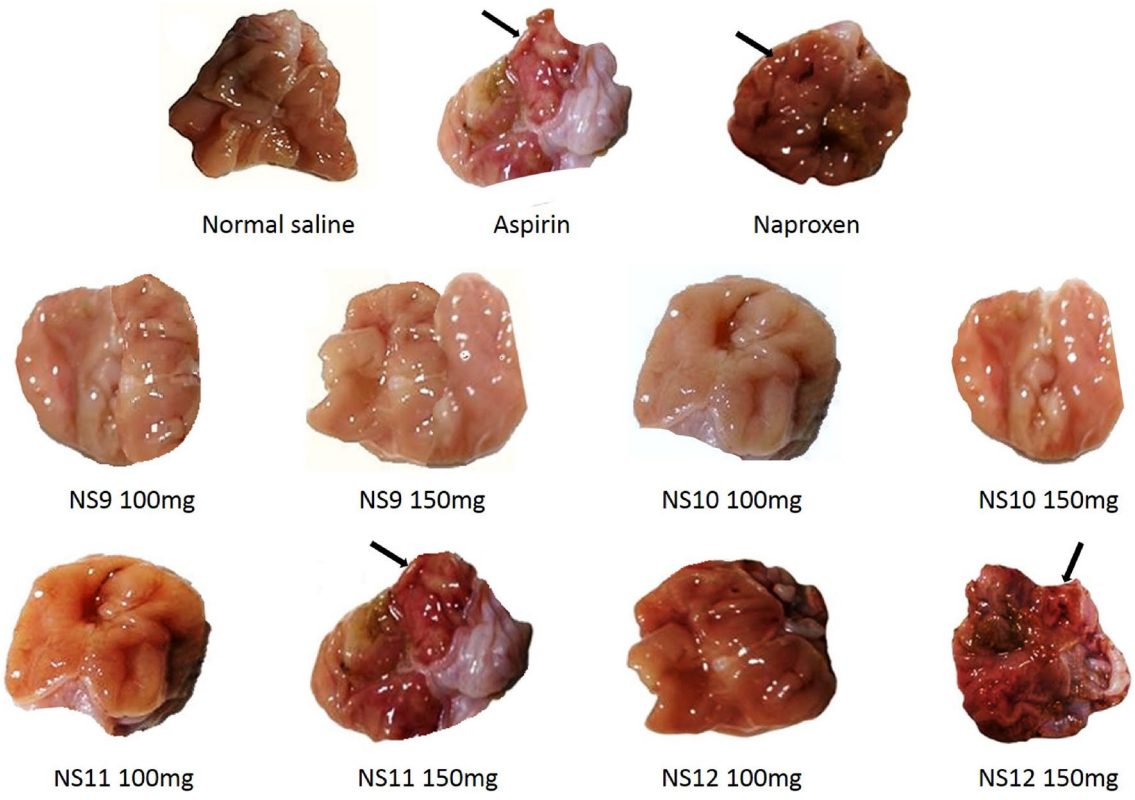


Fig. 8. Gross appearance of gastric mucosa in animal model exposed to Normal saline, aspirin, ibuprofen and synthesized compound (NS9 to NS12).

dried. Monitoring the reaction's progress was done by using TLC. The crude product was further purified by flash column chromatography on silica gel, utilizing *n*-hexane/EtOAc (90:10 v/v) as an eluent; yielding the pure desired naproxen hydrazide compound (NS10) with a high yield of 94%[13].

General procedure for the synthesis of naproxen imine with benzaldehyde (NS11) and naproxen imine with salicylaldehyde (NS12*)*

In the final step, equimolar amounts of naproxen hydrazide compound (NS10) and either benzaldehyde or salicylaldehyde were combined with 20 mL of ethanol in the reaction vessel of the monomodal Emrys Creator microwave synthesizer[14]. The reaction took place at 85 °C under Microwave-Irradiation (MWI) with a maximum power of 300W. The reactions were monitored with thin layer chromatography (TLC) until the completion of the reaction and to confirm the formation of the product. After completion of the reaction, the reaction mixture was diluted with EtOAc (30 mL), and 15 mL of aqueous saturated NH_4Cl solution was added, and the aqueous layer was extracted with EtOAc (3 × 30 mL). Then, the combined organic layer was washed with brine (1 × 20 mL), dried over anhydrous $MgSO_4$, filtered, and the filtrate was concentrated in vacuo. The crude residue was further purified by flash column chromatography (silica gel, *n*-hexane/EtOAc, 85:15 v/v) to furnish the desired pure novel products (NS11 & NS12). For crystallization, the product underwent a hot filtration process, and the resultant crystals were filtered, dried, and characterized. The desired novel naproxen imine with benzaldehyde (NS11) and naproxen imine with salicylaldehyde (NS12) target products obtained in high yields (NS11, 94% & NS12, 96%)[14].

Structure confirmation and characterization of compounds

Physical appearance, isolated yields of pure compound and R_f value was properly recorded and for structural confirmation the synthesized compounds were subjected to ^1H NMR and ^{13}C NMR to give an idea of carbon and hydrogen frame within the basic skeleton of the synthesized compounds.

Hydrolytic stability study of novel derivatives

The study was carried out at three pH levels 2, 7.4 and 9 mimicking gastric, plasma and intestinal pH. The pH solutions were prepared according to previously mentioned methods of European pharmacopoeia. For pH2.0 total of 6.57gm KCl was dissolved in distilled water followed by 119 ml HCl and 0.5gm of pepsin and volume was made to 1L with distilled water. Plasma 7.4pH media was obtained by dissolving 2.38gm Na_2HPO_4, 0.19 g KH_2PO_4, and 8.0 g NaCl in half liter distilled water. Further 100 mg trypsin was added to the solution and volume was made to 1L. Intestinal buffer solution of pH9 was obtained by dissolving 6.18gm H3BO3 in 0.1 M KCl making volume of solution-I to 1L. Second solution of 0.1 M NaOH is prepared. To get pH9 420 ml of solution –II was added to 1000 ml of solution-I. to 1L of mixed solution 100 mg trypsin was added to get 0.1 mg/ml concentration. Hydrolytic stability of novel compounds was determined using Perkin Elmer HPLC system (Norwalk, USA) connected with UV/V is detector (series 200). The samples were eluted via Athena C18-WP (100A, 4.6 mm × 150 mm, 5 µm) HPLC column at wavelength of 273 nm λ-max. The mobile phase was composed of acetonitrile and 0.1% trifluoro-acetic acid (TFA) in 05:95 ratio pumped at 1 mL min^{-1} isostatically[15].

Acute toxicity studies naproxen derivatives

The acute toxicity of naproxen derivatives was performed according to OECD guidelines 423, using a maximum single dose of 2000 mg/kg (b.wt). Animals were categorized into five groups, each consisting of six animals and derivatives were administered orally. First four groups were served as tested groups that received derivatives of naproxen NS9-NS12 at dose of 2000 mg/kg (b.wt). Animals that received derivatives were monitored for the first four hours, followed by next 72 h for mortality and then assessed daily for 14 days for any significant adverse effects[16].

Selection of naproxen derivative dose

The acute study indicates that naproxen derivatives were safe at doses up to 2000 mg/kg (b.wt) during acute toxicity evaluations. In light of the safety findings, particular doses of derivatives 1, 3, 10, and 30 mg/kg (b.wt) and 150 mg/kg (b.wt) were chosen for subsequent pharmacological investigations to evaluate their efficacy and possible therapeutic advantages.

Central and peripheral analgesic activities of naproxen derivatives

Central and peripheral analgesic activities for naproxen derivatives were evaluated by using Eddy hot plate and Acetic acid induced writing method[17]. Animals were categorized into two primary groups (A and B). Each group were further subdivided into six group of five animals each. For central analgesic activity, each animal was placed on a metal plate heated to 55 °C. Group I received saline solution, while Group II received 100 mg/kg (b.wt) of paracetamol. Naproxen analogues 30 mg/kg (b.wt) were administered to groups III, IV, V, and VI through intraperitoneal route. The latency response of each animal group was monitored for three hours, with observations recorded at 30 min intervals and cut-off time of 30 s to prevent tissue damage. The duration from placement to jumping or paw licking was recorded as the measure of response latency. The peripheral analgesic activity was evaluated using the same grouping and one hr after receiving naproxen analogues, 0.2 ml of 0.6% v/v acetic acid was injected, as it is a widely recognized model for studying visceral pain. The percentage of inhibition of writhing was calculated using a specific equation and the total number of writhing was counted for twenty minutes just 05 min of receiving acetic acid solution. Percentage of inhibition of writhing was determined by equation[17].

$$\%\text{ age of inhibition } = \frac{\text{Nc} - \text{Nt}}{\text{Nc}} \times 100$$

Nc stand for number of writhing for control group, while Nt stand for number of writhing for tested group.

Anti-inflammatory Activity of naproxen derivatives
Egg-induced paw edema method was used to assess the anti-inflammatory effects of Naproxen derivatives that was previously describe with some modifications to the procedure[18]. Balb/C mice (25 to 35 g) were divided into six group per five animals. Group I received saline solution while Group II was received diclofenac sodium at dose 100 mg/kg (b.wt). Naproxen analogues were administered to groups III, IV, V, and VI through intraperitoneal route 1 h before the induction of edema. Egg albumin (0.1 ml, 1%) was injected into the subplantar tissue of the right hind paw for induction of inflammation. Edema (inflammation) was determined as the difference in paw circumference between the control and 0.5, 1, 2, 3, 4 and 5 h. Mean edema was assessed by measuring with vernier calipers [18].

$$\%Inhibition \frac{\text{Means edema of Control} - \text{Means edema of Sample}}{\text{Mean paw edema of Control}} x100$$

Evaluation of post-operative pain
Naproxen derivatives at doses of 1, 3, 10 and 30 mg/kg (b.wt) and gabapentin 30 mg/kg (b.wt) were used for evaluation of post-operative pain in animal model[19]. Animals were divided into 7 groups with 8 animals in each group. 1st group was kept as control, 2nd group received gabapentin, while 3rd group received naproxen analogue. Groups 4th, 5th, 6th and 7th were kept as tested groups that received the derivatives of naproxen at dose 1, 3, 10 and 30 mg/kg (b.wt). The paw withdrawal threshold was observed by using a series of von frey filaments apparatus (0.008g/1.65 -300g/6.65).

On the planter surface of the right hind paw of each animal 1cm longitudinal incision cut was made and the entire doses were administered by intraperitoneal route 24 h of post-operative condition and studied both acute and repeated dose conditions. For acute study paw withdrawal was measured for every 30 min for 4 h after administration of naproxen analogue. For repeated dose study all test samples were administered for 7 days daily and paw withdrawal was measured 2 h after administration of drugs[20].

Evaluation of post operative inflammatory pain
Naproxen, along with their derivatives and standard drug was evaluated for *in-vivo* anti-inflammatory activity in animals' model according to previously procedure with some changes in protocols[21]. Balb/C mice were divided into different groups as specified for the Post-Operative Pain model. Standard laboratory conditions were followed before administration of complete freund's' adjuvant (CFA) and base line study was performed. Series of calibrated von frey filaments ranging from (0.008g/1.65 -300g/6.65) were used. To induce inflammatory pain in the experimental animals, CFA was injected, while normal saline was given to the control group one day post CFA injection and paw withdrawal to mechanical stimuli was measured after every 30 min for 4 h after administration of test samples. For repeated dose study all test samples were administered for 7 days each daily and paw withdrawal was measured 2 h after administration of the drugs[22].

Gastro-protective activity of naproxen derivatives
Derivatives of naproxen were evaluated for gastro protective potential using previously protocols with some changes in procedure[23]. Normal saline, standard drug and naproxen derivatives (100 and 150 mg/kg) were orally administered to various groups for six days. After 1 hr from the last dose of 12 hr, the pyloric ligation procedure was carried out under ketamine induced anesthesia. Food and water were withheld for 4 hrs to accumulate gastric juice. The stomach was then opened and gastric contents and tissue were examined for any ulcerogenecity. The gastroprotective effect was evaluated by examining free and total acidity and pepsin concentration[24]. The gross morphologic changes were recorded by examining photomicrographs of gastric mucosa and the degree of ulceration was graded according to previously reported ulcer scoring method[25].

Statistical analysis
Data are expressed as mean ± SEM. Significant at $^{*}P < 0.05$, $^{**}P < 0.01$ and*** $P < 0.001$ when compared to Control. One way or two-way ANOVA followed by either Dunnett's or Bonferroni test.

Data availability
No datasets were generated or analyzed during the current study.

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Author contributions

Nisar Zamin Shah: Methodology, Writing—original draft, Satya Kumar Avula: Methodology, Writing—original draft, Nasiara Karim: Conceptualization, Supervision, Project administration, Writing—review and editing. Muhammad Kifayatullah: Methodology, Formal analysis, Imran Khan: Software, Validation, Writing—original draft. Sadia Azeem: Methodology, Formal analysis, Data curation. Abeer Abdelhalim: Methodology, Validation, Software. Magda H. Abdellattif: Software, Formal analysis and Writing—review and editing. Mohammed Elnibras: Methodology, Data curation. Abdalla Ali Abdalla Mohamed: Methodology, Data curation. Ajmal Khan: writing—review and editing, Supervision and Project administration. Ahmed Al-Harrasi: writing—review and editing, Supervision and Project administration.

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Declarations

Competing interests
There is no conflict of interest.

Ethical approval
All animal procedures were approved by the Departmental Animal Ethical Committee, University of Malakand, KPK, and Pakistan (DAEC/PHARM/2016/17) and were conducted according to the UK animal scientific procedure act, 1986 and accordance with the ARRIVE guidelines.

Additional information
Supplementary Information The online version contains supplementary material available at https://doi.org/10.1038/s41598-025-34034-y.

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