Analgesic and anti-inflammatory activities of certain 6-aryl-9-substituted-6,9-diazaspiro-[4,5]decane-8,10-diones in mice

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Background and objective

2,6-Diketopiperazines are promising bioactive chemical entities in drug discovery. They could be considered a versatile template for the synthesis of a variety of scaffolds because of the presence of the reactive endocyclic nucleophilic imide group. It is worth mentioning that the 2,6-diketopiperazine system is embedded in the chemical skeleton of 6,9-diazaspiro-[4,5] decane-8,10-diones. The aim of the present work was to study both peripheral and central analgesic activities as well as the anti-inflammatory activity of 6-aryl-9-substituted-6,9-diazaspiro-[4,5]decane-8,10-diones 1–9 in different in-vivo experimental models.

Materials and methods

The test compounds 1–9 were evaluated for their analgesic activity in adult male Swiss albino mice using the writhing (12.5 and 25 mg/kg) and hot-plate (25 mg/kg) tests. Moreover, the anti-inflammatory activity was assessed for compounds 1–9 at dose 25 mg/kg (0.066–0.079 mmol/kg) by intraperitoneal administration using carrageenan-induced hind-paw edema assay in mice at 1, 2, and 3 h after carrageenan challenge and compared with diclofenac sodium at dose 10 mg/kg (0.031 mmol/kg) as the reference drug.

Results and conclusion

All compounds 1–9 showed significant inhibition of acetic acid induced writhing in the writhing test. Their percentage inhibition of abdominal writhing induced by acetic acid (peripheral effect) at doses of 12.5 mg/kg (0.033–0.039 mmol/kg) and 25 mg/kg (0.066–0.079 mmol/kg) ranged from 68.55 to 17.74% and from 88.71 to 53.23%, respectively. Compound 7 (R_1 = H, R_2 = CH_2CH_2Ph) demonstrated the highest writhing inhibition percentage at both dose levels (88.71 and 68.55%, respectively) and exhibited significantly lower number of writhes compared with diclofenac sodium as the reference standard. In the hot-plate test (central effect), compounds 2 (R_1 = CH_3, R_2 = CH_2COOH_3) and 3 (R_1 = 4-OCH_3, R_2 = CH_2COOCH_3) at doses of 0.076 and 0.072 mmol/kg, respectively (equivalent to 25 mg/kg), significantly raised the pain threshold and exhibited the best analgesic activity at 30 min. Both of them displayed nonsignificant difference from tramadol hydrochloride (0.095 mmol/kg, 25 mg/kg) at 30 min. In contrast, compounds 1 (R_1 = H, R_2 = CH_2COOH_3) at dose 0.079 mmol/kg and 5 (R_1 = CH_3, R_2 = CH_2Ph) at dose 0.068 mmol/kg showed slight lower analgesic potency compared with 2 and 3 with significant difference from the reference drug. The most powerful anti-inflammatory effect in this series was demonstrated in 9-*N*-methyl acetate derivatives (1–3), where compound 1 (R_1 = H, R_2 = CH_2COOH_3) at dose 0.079 mmol/kg exhibited 53.93% maximum protection (inhibition of edema size) at 3 h, compared with diclofenac sodium (0.0314 mmol/kg), which reached 60.40%. On the other hand, compounds 4–9 displayed 36.90–26.77% protection against carrageenan-induced edema.

Keywords:

analgesic, anti-inflammatory, 2,6-diketopiperazines, 6,9-diazaspiro-[4,5]decane-8,10-diones, hot-plate, writhing

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Introduction

Diketopiperazines (DKPs), also known as dioxopiperazines or piperazinediones, are a class of organic molecules in which the two nitrogen atoms of the piperazine six-membered ring are part of the amide linkages. There are three possible regioisomers of this class of compounds depending on the different locations of the two carbonyl groups around the ring – namely, 2,3-DKPs, 2,5-DKPs, and 2,6-DKPs (3-aza-glutarimides).

DKPs could be considered the least known peptides in cyclic form. They are promised bioactive chemical entities for drug discovery because of the interesting pharmacophoric-like peptide moieties in their rigid skeleton [1]. Certain DKPs are reported to display various biological effects such as analgesic [2], anticonvulsant [3–5], anti-inflammatory [6], antiviral [7], and anticancer [8].

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The 2,6-DKP system could be considered a versatile template for the synthesis of diversity of scaffolds, because of the presence of the reactive endocyclic nucleophilic imide group [1].

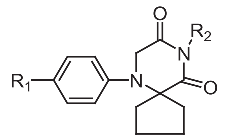
The analgesic and anticonvulsant profile of certain 2,6-DKPs – namely, 1-alkyl-1,4-diazaspiro[4,5]decane, and [5,5]undecane-3,5-diones – was reported [9]. Recently, the synthesis and the potent anticonvulsant effect of 6-aryl-9-substituted-6,9-diazaspiro[4,5]decane-8,10-diones 1–9 were disclosed in mice [5]. The structure skeletons of these 2,6-DKP derivatives 1–9 included several lipophilic bioactive groups, which may facilitate the development of new biologically active candidates.

Pain is a feeling that reflects a health concern. Pain management is one of the ultimate concerns for researchers. Analgesics or pain killers have been in existence for ages; however, their side effects have been related to gastric ulceration, hormonal imbalance, addiction, and dependence [10,11]. Therefore, seeking new molecules that target the nervous system either centrally and/or peripherally is of major interest.

Inflammation is a localized body nonspecific immune response/reaction toward harmful stimuli. The fundamental signs of inflammation involve swelling, redness, warmth, and pain as a result of increased blood flow, elevated cellular metabolism, vasodilatation, release of soluble mediators, extravasation of fluids, and cellular influx, which is a consequence of infection, irritation, or injury [12]. Inflammation could be acute (rapid onset) or chronic (long term). Chronic inflammation would subsequently result in some diseases such as cancers, rheumatoid arthritis, heart diseases, periodontitis, and hay fever. Anti-inflammatory drugs exhibit noteworthy therapeutic benefits in the treatment of pain and inflammation. However, their administration has been linked with an increased risk for serious side effects [13]. Therefore, further studies are needed to develop new bioactive candidates.

Therefore, the aim of this study was to evaluate certain 6-aryl-9-substituted-6,9-diazaspiro[4,5]decane-8,10-diones 1–9 for their peripheral and central analgesic potential using the writhing test and hot-plate assay in mice, respectively. In addition, the present study investigated the in-vivo anti-inflammatory effect of compounds 1–9 (Fig. 1).

Figure 1



Compound number	R_1	R_2
1	H	$-CH_2COOCH_3$
2	$4-CH_3$	$-CH_2COOCH_3$
3	$4-OCH_3$	$-CH_2COOCH_3$
4	H	$-CH_2Ph$
5	$4-CH_3$	$-CH_2Ph$
6	$4-OCH_3$	$-CH_2Ph$
7	H	$-CH_2CH_2Ph$
8	$4-CH_3$	$-CH_2CH_2Ph$
9	$4-OCH_3$	$-CH_2CH_2Ph$

The chemical structures of compounds 1–9.

Materials and methods

Animals

Adult male Swiss albino mice (20–25 g) were used for the evaluation of the analgesic and anti-inflammatory activities of the tested compounds (1–9). Animals were obtained from the Animal-Breeding Unit of the National Research Centre (Cairo, Egypt). Animals were housed under standardized conditions of light (12 h light/dark cycles) and temperature (23 ± 2°C) and relative humidity (55 ± 5%), and received standard rat chow and tap water *ad libitum*. All animal procedures were performed according to the guidelines of the ethical committee of the National Research Centre for experimental animal use.

Compounds 1–9

Series of 6-aryl-9-substituted-6,9-diazaspiro[4,5]decane-8,10-diones (1–9) were arranged according to their N-9 substitution in the spiro heterocyclic system. These are 9-*N*-methyl acetate derivatives (1–3) – namely, methyl-2-(8,10-dioxo-6-phenyl-6,9-diazaspiro[4,5]decane-9-yl)acetate (1), methyl-2-(8,10-dioxo-6-(4-methylphenyl)-6,9-diazaspiro[4,5]decane-9-yl)acetate (2), methyl-2-(6-(4-methoxyphenyl)-8,10-dioxo-6,9-diazaspiro[4,5]decan-9-yl)acetate (3); 9-*N*-benzyl derivatives (4–6) – namely, 9-benzyl-6-phenyl-6,9-diazaspiro[4,5]decane-8,10-dione (4), 9-benzyl-6-(4-methylphenyl)-6,9-diazaspiro[4,5]decane-8,10-dione (5), 9-benzyl-6-(4-methoxyphenyl)-6,9-diazaspiro[4,5]decane-8,10-dione (6); 9-*N*-phenethyl derivatives (7–9) – namely, 9-phenethyl-6-phenyl-6,9-diazaspiro[4,5]decane-8,10-dione (7), 6-(4-methylphenyl)-9-phenethyl-6,9-diazaspiro[4,5]decane-8,10-dione (8), and 6-(4-methoxyphenyl)-9-phenethyl-6,9-diazaspiro[4,5]decane-8,10-dione (9). Compounds 1–9 were synthesized by adopting the reported procedure by Aboul-Enein *et al.* [5]. All compounds were recognized through IR, NMR, and mass spectroscopy.

Drugs and chemicals

Tween-80 and carrageenan were obtained from Sigma-Aldrich (St. Louis, Missouri, USA). Tramadol hydrochloride was obtained from October Pharma (6 October City, Cairo, Egypt). Diclofenac sodium was obtained from Pharco (Al Amereyah, Alexandria, Egypt).

Biological evaluation

Analgesic activity assays

The analgesic profile of the target compounds 1–9 was evaluated in mice (n = 6) by adopting acetic acid-induced writhing test to determine the peripheral analgesic effect at doses of 12.5 and 25 mg/kg body weight, in addition to the hot-plate technique to determine the central analgesic effect at a dose level of 25 mg/kg body weight.

Writhing test

Screening for the in-vivo analgesic effect of 6-aryl-9-substituted-6,9-diazaspiro-[4,5]decane-8,10-diones, 1–9, was carried out by performing the writhing test [14]. Adult male Swiss albino mice were classified into XXI groups. Group I served as the control group and received the vehicle. Groups II and III were injected with the reference drug (diclofenac sodium: 12.5 and 25 mg/kg, respectively), whereas groups IV–XXI were administered with two different concentrations of test compound 1–9 (12.5 and 25 mg/kg). The animals were injected with freshly prepared acetic acid [2% (w/v) in saline, 10 ml/kg body weight] as analgesic agent 30 min after intraperitoneal injection of the vehicle, reference drug, or the test compound. The mice were individually separated and observed for a period of 30 min. The observation included counting the number of writhes produced by each mouse, which is a response consisting of abdominal muscle contraction and pelvic rotations followed by hind limb extension:

$$\%\,\text{Inhibition of abdominal writhing} = \left(\frac{N_c - N_t}{N_c}\right) \times 100,$$

where N_c is the number of writhes of the control group and N_t is the number of writhes of the treated group.

Hot-plate test

The hot-plate method [15] involves observing the normal response to pain stimulus in untreated mice and comparing it with the response to the same stimulus after administration of the drug or test compound at definite time intervals. The animals were classified into XI groups of mice. Group I served as the control group and received saline. Group II received tramadol hydrochloride at a dose of 25 mg/kg, and groups III–XI each received one of the test compounds by intraperitoneal injection at a dose of 25 mg/kg for each compound. The mice were dropped gently into a 1-l dry glass beaker, and the temperature was adjusted to 55–56°C. The mice were pretested, and those having a latency time greater than 15 s were excluded from the testing. The reaction time was measured and it is considered as the time interval (s) starting when the mouse reaches the hot beaker until paw licking or jumping occurs. The normal reaction time was determined three times at 5-min intervals and the average was calculated for all animals before injection of the vehicle, reference drug, or test compounds. The reaction time was determined at 10-, 20-, 30-, 45-, 60-, 90-, and 120-min intervals after vehicle, reference drug, or test compound injection.

Anti-inflammatory activity

The anti-inflammatory activity was evaluated in an acute model using the carrageenan-induced paw edema assay [16,17]. Adult male Swiss albino mice (n = 6) were classified into XI groups. Group I served as the control group and received the vehicle. Group II received the reference drug (diclofenac sodium: 10 mg/kg), whereas groups III–XI were intraperitoneally dosed with 25 mg/kg of the test compounds dissolved in Tween-80 (2% aqueous solution) 1 h before carrageenan challenge. The mouse paw edema was induced with subplantar injection of 0.05 ml of 0.5% suspension of carrageenan in saline into the plantar tissue of one hind paw in all groups. An equal volume of saline was injected into the other hind paw and served as control. The thickness of the mouse hind paws was measured using a Vernier Caliper (SMEC, Shanghai, China) 1, 2, and 3 h after carrageenan challenge.

The percentage swelling of the paw was calculated using the following equation:

$$\%\,\text{Swelling} = \left(\frac{V_c - V_s}{V_s}\right) \times 100,$$

where V_c is the carrageenan paw thickness and V_s is the saline paw thickness at each time interval.

The average paw swelling in the test compound-treated and diclofenac sodium-treated mice was compared with that of the untreated mice. The percentage inhibition of edema was determined using the following equation:

$$\%\,\text{Inhibition} = \left[1 - \left(\frac{\%\,\text{swelling of treated group}}{\%\,\text{swelling of carrageenan group}}\right)\right] \times 100.$$

Statistical analysis

Results were expressed as mean ± SEM. Statistical analysis of the obtained data was performed using one-way analysis of variance followed by the Student–Newman–Keuls post-hoc comparison. A result was considered statistically significant when P values were less than 0.05.

Results and discussion

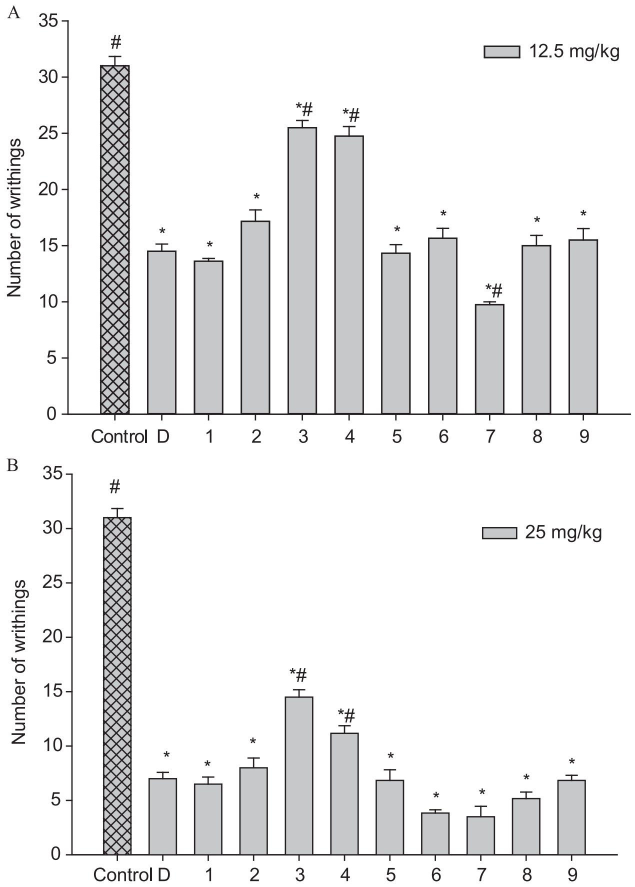
Writhing test

Compounds 1–9 were evaluated for their analgesic activity by using the writhing test and diclofenac sodium as the reference drug. The induction of abdominal writhing through injection with acetic acid is a well-established and sensitive in-vivo model that is widely used to evaluate peripheral analgesic activity [18]. Moreover, acetic acid does not bind to the peritoneal nociceptive receptors directly. Mainly, the mechanism of induction of pain with acetic acid involves the liberation of endogenous substances such as prostaglandins, which excite nerve endings and produce acute inflammation in the peritoneal area [19]. It has been reported that compounds that inhibit acetic acid-induced writhes and possess analgesic effect may act through central mechanisms that involve receptor systems or peripherally through inhibition of synthesis and release of mediators involved in pain and inflammation, such as prostaglandins, leukotrienes, and other endogenous substances [20].

Diclofenac sodium is a NSAID that is commonly used in the market for its analgesic, antipyretic, and anti-inflammatory activity. Diclofenac sodium is a nonselective, reversible, and competitive inhibitor of cyclooxygenase. It blocks the conversion of arachidonic acid into prostaglandin precursors, thus leading to the inhibition of the formation of prostaglandins that are involved in pain, inflammation, and fever.

All tested compounds 1–9 were assessed for the number of abdominal writhes induced by acetic acid in mice. The compounds were administered intraperitoneally at doses of 12.5 mg/kg (Fig. 2a) and 25 mg/kg (Fig. 2b). All compounds showed significantly lower number of writhes compared with the control group at both doses. Compound 7 demonstrated a significantly lower number of writhes compared with diclofenac sodium as the reference standard and compared with all compounds in the series at a dose of 12.5 mg/kg, whereas at a dose of 25 mg/kg it maintained its ranking but was not significant from all the compounds in the series except compounds 2, 3, and 4. Remarkably, compounds 3 and 4 showed significantly higher writhing values compared with diclofenac sodium and other tested compounds at both dose levels (Fig. 2a and b).

Figure 2



(a, b) Effect of compounds 1–9 (12.5 and 25 mg/kg, intraperitoneal) on acetic acid-induced writhing. Swiss albino mice were injected with compounds 1–9 (12.5 mg/kg, intraperitoneal) and diclofenac sodium (12.5 mg/kg, intraperitoneal), which was used as the reference standard (D), or vehicle (control) (a), or with compounds 1–9 (25 mg/kg, intraperitoneal) and diclofenac sodium (25 mg/kg, intraperitoneal) or vehicle (b), 30 min before the intraperitoneal injection of freshly prepared acetic acid [2% (w/v) in saline] at 10 ml/kg body weight. The mice were individually separated and were observed for 30 min. Each value represents the mean of the number of writhes ± SEM. *Significant difference from control at $P < 0.05$; #significant different from diclofenac sodium at $P < 0.05$.

Figure 3 demonstrates that all tested compounds and diclofenac sodium used as a reference standard exhibited significant analgesic potency from control at a dose of 12.5 mg/kg (0.033–0.039 mmol/kg) with writhing inhibition percentage of 17.74–68.55%.

At a dose of 25 mg/kg (0.066–0.079 mmol/kg), compounds 1–9 demonstrated percentage inhibition of writhing of 53.23–88.71%. The most active compound was 7 (R_1 = H, R_2 = CH_2CH_2Ph), which demonstrated the highest writhing inhibition percentage at both dose levels. The percentage inhibition of writhing of compound 7 was 88.71% at dose 25 mg/kg (0.0717 mmol/kg) and 68.55% at dose of 12.5 mg/kg (0.036 mmol/kg) (Fig. 3). In addition, compound 6 (R_1 = 4-OCH_3, R_2 = CH_2Ph) at dose 25 mg/kg (0.069 mmol/kg) exhibited almost similar high percentage of inhibition of writhing of 87.63% to compound 7. Diclofenac sodium showed 53.23 and 77.42% inhibition of writhing at doses of 12.5 and 25 mg/kg (0.039 and 0.079 mmol/kg), respectively. Moreover, it was noticeable that compound 8 (R_1 = CH_3, R_2 = CH_2CH_2Ph), which constitutes a phenethyl moiety at the N-9 position of the spiro heterocyclic system, possessed writhing inhibition value of 83.33% at 25 mg/kg (0.069 mmol/kg). Interestingly, compounds 1 (R_1 = H, R_2 = CH_2COOH_3), 5 (R_1 = CH_3, R_2 = CH_2Ph), and 9 (R_1 = OCH_3, R_2 = CH_2CH_2Ph) exerted comparable percentage of writhing inhibition that was in the range of 77.96–79.03%, whereas 2 (R_1 = CH_3, R_2 = CH_2COOH_3) possessed nonsignificant lower writhing percentage of inhibition (74.19%) at doses of 25 mg/kg (0.079, 0.068, 0.066, and 0.076 mmol/kg, respectively).

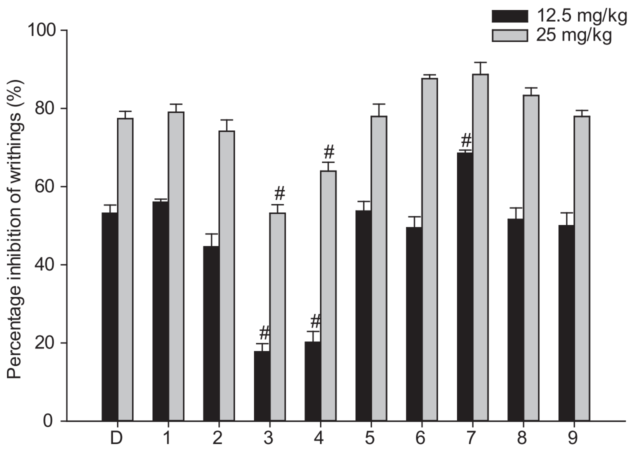
Only compounds 4 (R_1 = H, R_2 = CH_2Ph) and 3 (R_1= OCH_3, R_2 = $COOCH_3$) showed quite moderate inhibitory percentages of 63.98 and 53.23%, respectively, at dose 25 mg/kg (0.075 and 0.072 mmol). These results could consider the different structural scaffolds of 6-aryl-9-substituted-6,9-diazaspiro-[4,5]decane-8,10-diones 1–9, which are promising candidates for analgesic bioactive compounds.

Hot-plate technique

The hot-plate test is one of the well-established models for the evaluation of centrally acting analgesic activity. Moreover, it is one of the most commonly used models for neurologic pain. One of the postulated mechanisms involved in the elevation of the reaction time in the hot-plate test by centrally acting analgesics is their action on the spinal cord by acting on the opioid receptors [19,21].

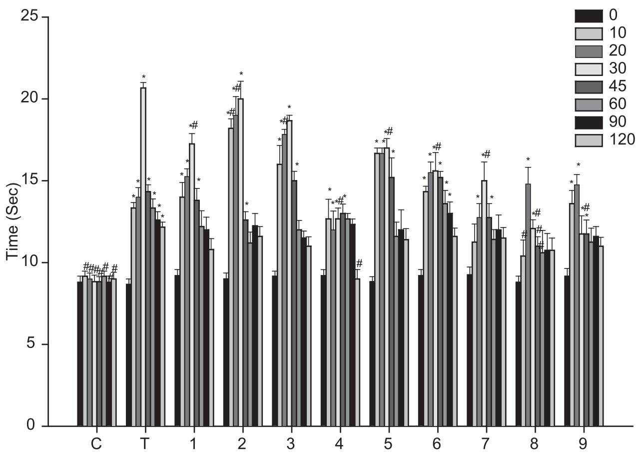
Figure 4 presents the analgesic activity at dose 0.066–0.079 mmol/kg equivalent to 25 mg/kg of the screened compounds 1–9 compared with that of tramadol hydrochloride (0.095 mmol/kg, 25 mg/kg) as a reference drug. The analgesic effect in this model assay was measured 10, 20, 30, 45, 60, 90, and 120 min after intraperitoneal administration of vehicle, tramadol hydrochloride, or test compound in mice. It was found that the highest analgesic peak of tramadol hydrochloride was observed at 30 min after drug administration. Compounds 2 (R_1 = CH_3, R_2 = CH_2COOH_3) and 3 (R_1 = 4-OCH_3, R_2 = CH_2COOCH_3) at doses 0.076 and 0.072 mmol/kg, respectively (equivalent to 25 mg/kg), exhibited the best analgesic activity in the screened test compounds 1–9 at 30 min. Both of them displayed their effect 10–30 min after intraperitoneal administration and presented a nonsignificant difference from tramadol hydrochloride (0.095 mmol/kg, 25 mg/kg) at 30 min. Compounds 2 and 3 exhibited significantly higher analgesic effect compared with compounds 4 and 7–9 at 20 and 30 min. Meanwhile, compound 1 (R_1 = H, R_2 = CH_2COOH_3) at dose 0.079 mmol/kg showed slightly

Figure 3



Percentage inhibition of compounds 1–9 (12.5 and 25 mg/kg, intraperitoneal) on acetic acid-induced writhing. Swiss albino mice were injected with compounds 1–9 (12.5 and 25 mg/kg, intraperitoneal) and diclofenac sodium (12.5 and 25 mg/kg, intraperitoneal), which was used as the reference standard (D), or vehicle (control) 30 min before intraperitoneal injection of freshly prepared acetic acid [2% (w/v) in saline] at 10 ml/kg body weight. The mice were individually separated and were observed for 30 min. The percentage inhibition of writhes was calculated relative to the control group (acetic acid group). Each value represents the mean of the percentage inhibition of writhes ± SEM. *Significant difference from control at $P < 0.05$; #Significant difference from diclofenac sodium at $P < 0.05$.

Figure 4



Effect of 6-aryl-9-substituted-6,9-diazaspiro-[4,5]decane-8,10-diones series 1–9 in the hot-plate test in adult male albino mice. The animals were classified into XI groups of mice. Group I served as the control group and received saline. Group II received tramadol hydrochloride at dose 0.095 mmol/kg body weight (25 mg/kg) and groups III–XI each received one of the test compounds by intraperitoneal injection at dose 25 mg/kg (0.066–0.079 mmol/kg) for each compound. The animals were observed for their response toward pain stimulus after administration of the test compound at definite time intervals. Each value represents the mean reaction time (s) ± SEM. *Significant difference from control at $P < 0.05$; #significant difference from tramadol hydrochloride at $P < 0.05$.

lower analgesic potency than 2 and 3 in the 9-*N*-methyl acetate derivatives (1–3), and exerted significantly lower antinociceptive activity from the reference drug at 30 min post-administration. In addition, the analgesic potency of compound 5 (R_1 = CH_3, R_2 = CH_2Ph) at dose 0.068 mmol/kg exhibited almost similar effect to compound 1. Moreover, both compounds 6 (R_1 = OCH_3, R_2 = CH_2Ph) at dose 0.068 mmol/kg and 7 (R_1 = H, R_2 = CH_2CH_2Ph) at dose 0.072 mmol/kg were nearly of similar activity, and possessed significantly higher effect than compounds 4 (R_1 = H, R_2 = CH_2Ph), 8 (R_1 = CH_3, R_2 = CH_2CH_2Ph), and 9 (R_1 = OCH_3, R_2 = CH_2CH_2Ph) at dose 0.075, 0.069, and 0.066 mmol/kg, respectively. Accordingly, at 30 min post-administration, the least analgesic effect was observed in the 9-*N*-phenethyl derivatives 8 (R_1 = CH_3, R_2 = CH_2CH_2Ph) and 9 (R_1 = OCH_3, R_2 = CH_2CH_2Ph), whereas 7 (R_1 = H, R_2 = CH_2CH_2Ph) demonstrated the highest analgesic activity in the 9-*N*-phenethyl derivatives (7–9). It was found that the antinociceptive potential of the tested compounds 1–9 was arranged in the descending order of $2>3>1\simeq5>6\simeq7>4>8>9$ at 30 min post-administration (Table 1 and Fig. 4). It is worth noticing that unsubstituted-6,9-diazaspiro-[4,5] decane-8,10-diones exhibited remarkable central analgesic effect [2], compared with the substituted derivatives 1–9.

Anti-inflammatory activity

The carrageenan-induced paw edema model is a well-established animal model that is widely used to assess the anti-inflammatory activity of compounds acting on the mediators of acute inflammation [22]. Edema is a pathophysiological inflammatory condition that is commonly associated with swelling, erythema, hyperalgesia, and increase in body temperature [23]. According to Morris [22], inflammation produced upon carrageenan challenge is due to the release of proinflammatory agents such as bradykinin, histamine, tachykinins, complement and reactive oxygen, as well as nitrogen species that are generated *in situ* at the site of insult or by infiltrating cells. Also, neutrophils readily migrate to sites of inflammation and can generate proinflammatory reactive oxygen and other species [22]. Another mechanism postulates that edema formation due to injection of carrageenan is a biphasic event that involves an initial phase occurring at 1 or 1.5 h and is primarily a nonphagocytic edema. The first phase is associated with the release of histamine, serotonin, and bradykinin, and their effect on vascular permeability occurs in the first hour. The second phase is attributed to the overproduction of prostaglandins and release of inducible cyclooxygenase and lysosome enzymes for 2–3 h [24].

Table 1 Effect of 6-aryl-9-substituted-6,9-diazaspiro-[4,5]decane-8,10-diones series 1–9 in the hot-plate test in adult male albino mice

Treatments	Dose (mmol/kg)	Reaction time (s)							
		0 min	10 min	20 min	30 min	45 min	60 min	90 min	120 min
Control	—	0.37 ± 8.80	9.17 ± 0.31$^{\#}$	9.00 ± 0.37$^{\#}$	8.83 ± 0.40$^{\#}$	8.83 ± 0.31$^{\#}$	9.17 ± 0.31$^{\#}$	8.80 ± 0.37$^{\#}$	9.00 ± 0.41$^{\#}$
Tramadol	0.095	8.67 ± 0.33	13.33 ± 0.33*	14.00 ± 0.58*	20.67 ± 0.33*	14.33 ± 0.42*	13.33 ± 0.56*	12.60 ± 0.51*	12.17 ± 0.31*
1	0.079	9.20 ± 0.37	14.00 ± 0.89*	15.25 ± 0.48*	17.25 ± 0.63*$^{\#}$	13.80 ± 0.74*	12.20 ± 0.97	12.00 ± 1.12	10.80 ± 0.66
2	0.076	9.00 ± 0.37	18.20 ± 0.58*$^{\#}$	19.00 ± 1.14*$^{\#}$	20.00 ± 1.08*	12.60 ± 0.51*	11.20 ± 0.66	12.25 ± 0.75	11.60 ± 0.60
3	0.072	9.17 ± 0.31	16.00 ± 1.16*	17.83 ± 0.31*$^{\#}$	18.67 ± 0.33*	15.00 ± 0.58*	12.00 ± 0.58	11.50 ± 0.43	11.00 ± 0.58
4	0.075	9.20 ± 0.37	12.67 ± 1.20*	12.00 ± 1.15*	12.67 ± 0.67*$^{\#}$	13.00 ± 0.58*	12.67 ± 0.33	12.33 ± 0.33	9.00 ± 0.58$^{\#}$
5	0.069	8.83 ± 0.31	16.67 ± 0.33*	16.67 ± 0.33*	17.00 ± 0.58*$^{\#}$	15.20 ± 1.20*	11.60 ± 0.87	12.00 ± 1.23	11.40 ± 0.68
6	0.069	9.20 ± 0.37	14.33 ± 0.33*	15.50 ± 0.65*	15.60 ± 1.12*$^{\#}$	15.20 ± 0.37*	13.60 ± 0.81*	13.00 ± 0.71*	11.60 ± 0.51
7	0.072	9.25 ± 0.48	11.25 ± 1.11	12.75 ± 0.85*	15.00 ± 1.15*$^{\#}$	12.75 ± 0.85*	11.42 ± 0.59	12.00 ± 0.91	11.50 ± 0.65
8	0.069	8.80 ± 0.37	10.40 ± 0.98*	14.80 ± 1.02$^{\#}$	12.08 ± 0.53*$^{\#}$	11.00 ± 0.58$^{\#}$	10.60 ± 1.36	10.75 ± 1.03	10.75 ± 0.75
9	0.066	9.17 ± 0.48	13.60 ± 0.81*	14.75 ± 0.63*	11.75 ± 1.11*$^{\#}$	11.75 ± 0.85*	11.25 ± 0.85	11.60 ± 0.60	11.00 ± 0.55

The animals were classified into XI groups of mice. Group I served as the control group and received saline. Group II received tramadol hydrochloride at dose 0.095 mmol/kg body weight (25 mg/kg) and groups III–XI each received one of the test compounds by intraperitoneal injection at dose of 25 mg/kg (0.066–0.079 mmol/kg) for each compound. The animals were observed for their response toward pain stimulus after administration of the test compound at definite time intervals. Each value represents the mean reaction time in second ± SEM; *Significantly different from control at $P<0.05$; $^{\#}$Significantly different from tramadol hydrochloride at $P<0.05$.

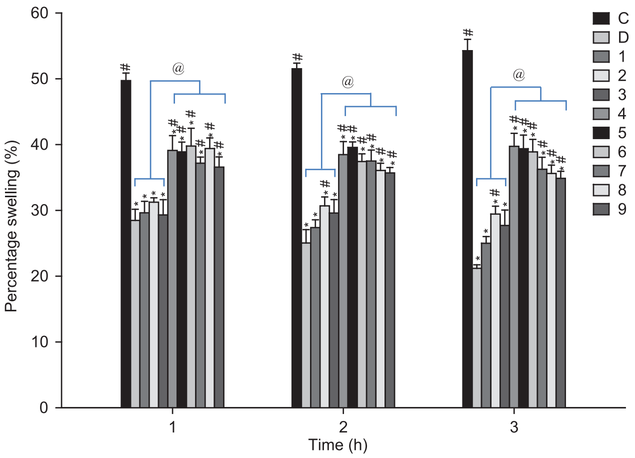
The in-vivo anti-inflammatory activity of 6-aryl-9-substituted-6,9-diazaspiro[4,5]decane-8,10-diones series (1–9) was examined at a dose level of 25 mg/kg (0.066–0.079 mmol/kg) by intraperitoneal administration using carrageenan-induced hind paw edema assay in mice at 1, 2, and 3 h after carrageenan challenge and compared with diclofenac sodium at dose 10 mg/kg (0.031 mmol/kg) as the reference drug (Figs 5 and 6).

Figure 5 illustrates that compounds 1–9 showed significantly lower percentage swelling in mouse paw challenged with carrageenan compared with control at 1, 2, and 3 h. In addition, compounds 1–3 and diclofenac sodium used as reference standard (10 mg/kg) demonstrated significantly lower percentage swelling compared with compounds 4–9 at 1, 2, and 3 h after carrageenan challenge. Interestingly, compound 2 demonstrated significantly higher percentage swelling compared with diclofenac only at the 2 and 3 h time points. Interestingly, Wang *et al.* [6] reported that DKPs demonstrated multiple biological effects including anti-inflammatory effect [6].

Figure 6 illustrates that the screened compounds at 3 h after drug administration demonstrated the best anti-inflammatory effect, where 9-*N*-methyl acetate derivative 1 (R_1 = H, R_2 = CH_2COOH_3) demonstrated the most powerful anti-inflammatory effect in this series. It showed 53.93% protection at 3 h (inhibition of edema size), whereas that of diclofenac sodium reached 60.95%. Concerning the protection against carrageenan-induced edema of compounds 3 (R_1 = 4-OCH_3, R_2 = CH_2COOCH_3) and 2 (R_1 = CH_3, R_2 = CH_2COOH_3) from 9-*N*-methyl acetate derivatives, they possessed lower percentage inhibition in paw edema (48.94 and 45.75%, respectively) compared with compound 1. In contrast, 9-*N*-phenethyl derivatives (7–9) exhibited 35.7% for compound 9 (R_1 = OCH_3, R_2 = CH_2CH_2Ph), 34.38% for 8 (R_1 = CH_3, R_2 = CH_2CH_2Ph), and 33.19% for 7 (R_1 = H, R_2 = CH_2CH_2Ph) at doses 0.066, 0.069, and 0.072 mmol/kg, respectively. Moreover, this pharmacological test disclosed that the anti-inflammatory activity of the 9-*N*-benzyl derivatives (4–6) displayed lower effect than both 9-*N*-methyl acetate derivatives (1–3) and 9-*N*-phenethyl derivatives (7–9). Their percentage protection reached 28.33, 27.44, and 26.77% for compounds 6 (R_1 = OCH_3, R_2 = CH_2Ph), 5 (R_1 = CH_3, R_2 = CH_2Ph), and 4 (R_1 = H, R_2 = CH_2Ph), respectively.

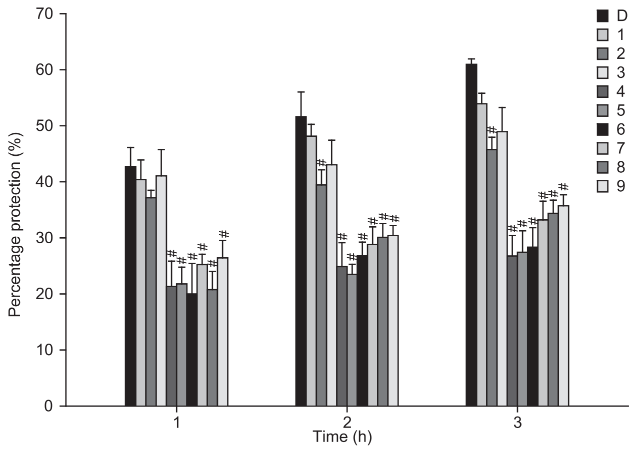
Conclusively, these pharmacological data demonstrated that R_2 substitution in the 2,6-DKPs system with methyl acetate moiety obviously enhanced the anti-inflammatory activity (Figs 5 and 6).

Figure 5



Effect of 6-aryl-9-substituted-6,9-diazaspiro-[4,5]decane-8,10-diones series 1–9 on percentage swelling in carrageenan-induced paw edema. Mice were injected with compounds 1–9 (25 mg/kg, intraperitoneal) and diclofenac sodium (D) (10 mg/kg, intraperitoneal) or vehicle (C) 1 h before carrageenan challenge. The mouse hind paw was injected with 0.05 ml of 0.5% suspension of carrageenan in saline into the plantar tissue. The other hind paw was injected with an equal volume of saline and served as control. The thickness of the mouse hind paws was measured with a Vernier Caliper 1, 2, and 3 h after carrageenan challenge. The percentage swelling was calculated by measuring the difference between the thicknesses of the two paws. The data represent the mean percentage swelling ± SEM. *Significant difference from control at P < 0.05; #Significant difference from diclofenac sodium at P < 0.05; @significant difference from compounds 1, 2, and 3 at P < 0.05.

Figure 6



Effect of 6-aryl-9-substituted-6,9-diazaspiro-[4,5]decane-8,10-diones series 1–9 on percentage protection in carrageenan-induced paw edema. Mice were injected with compounds 1–9 (25 mg/kg, intraperitoneal) or diclofenac sodium (D) (10 mg/kg, intraperitoneal) or vehicle (C) 1 h before carrageenan challenge. The mouse hind paw was injected with 0.05 ml of 0.5% suspension of carrageenan in saline into the plantar tissue. The other hind paw was injected with an equal volume of saline and served as control. The thickness of the mouse hind paws was measured with a Vernier Caliper 1, 2, and 3 h after carrageenan challenge. The percentage inhibition of the test compounds as well as diclofenac sodium, used as reference, was calculated relative to carrageenan. The data represent the mean percentage protection ± SEM. *Significant difference from controls at P < 0.05; #significant difference from diclofenac sodium at P < 0.05.

Conclusion

We performed the pharmacological evaluation of 6-aryl-9-substituted-6,9-diazaspiro[4,5]decane-8,10-diones 1–9 for their analgesic (peripheral and central) and anti-inflammatory potential. Regarding the peripheral analgesic effect (writhing test) all the tested compounds showed significant inhibition of abdominal writhings. The percentage inhibition of the number of writhes induced by acetic acid at doses 12.5 mg/kg (0.033–0.039 mmol/kg) and 25 mg/kg (0.066–0.079) ranged from 68.55 to 17.74% and from 88.71 to 53.23%, respectively. To examine the potential central effect of compounds 1–9, the hot-plate test was performed, wherein 9-*N*-methyl acetate derivatives 2 and 3 at doses 0.076 and 0.072 mmol/kg, respectively, significantly raised the pain threshold and exhibited the best analgesic activity at 30 min. It is worth mentioning that 9-*N*-methyl acetate derivatives 1–3 exhibited the best anti-inflammatory effect in this series, where compound 1 at dose 0.079 mmol/kg exhibited 53.93% maximum protection (inhibition of edema size) at 3 h, compared with 60.40% for diclofenac sodium at 0.0314 mmol/kg dose level. These results could consider the different structural scaffolds of 6-aryl-9-substituted-6,9-diazaspiro[4,5]decane-8,10-diones (1–9) as promising candidates for peripheral analgesia, in addition to 9-*N*-methyl acetate derivatives 1–3 for central analgesia and as anti-inflammatory bioactive compounds. This augments the numerous biological activities of 2,6-DKPs in the field of medicinal chemistry.

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Conflicts of interest

There are no conflicts of interest.

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