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Experimental Research Article

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Evaluation of the antinociceptive effects of a selection of triazine derivatives in mice

Valiollah Hajhashemi[1], Ghadamali Khodarahmi[2], Parvin Asadi[2], and Hamed Rajabi[1]

[1]*Department of Pharmacology and Toxicology, School of Pharmacy and Pharmaceutical Sciences, Isfahan University of Medical Sciences, Isfahan, Iran*
[2]*Department of Medicinal Chemistry, School of Pharmacy and Pharmaceutical Sciences, Isfahan University of Medical Sciences, Isfahan, Iran*

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Correspondence
Valiollah Hajhashemi
Department of Pharmacology and Toxicology, School of Pharmacy and Pharmaceutical Sciences, Isfahan University of Medical Sciences, Isfahan 81746-73461, Iran
Tel: +98-31-37927080
Fax: +98-31-36680011
E-mail: vhajhashemi@gmail.com

Background: The authors showed in a previous study that some novel triazine derivatives had an anti-inflammatory effect. The present study was designed to evaluate the antinociceptive effect of five out of nine compounds including two vanillin-triazine (5c and 5d) and three phenylpyrazole-triazine (10a, 10b, 10e) derivatives which showed the best anti-inflammatory effect.
Methods: Male Swiss mice (25–30 g) were used. To assess the antinociceptive effect, acetic acid-writhing, formalin, and hot plate tests were used after intraperitoneal injection of each compound.
Results: All compounds significantly ($P < 0.001$) reduced acetic acid-induced writhing at tested doses (50, 100, and 200 mg/kg). Also, the percent inhibition of writhing in the acetic acid test showed that at the maximum tested dose of these compounds (200 mg/kg), the order of potencies is as follows: 10b > 10a > 10e > 5d > 5c. In the formalin test, compounds 5d, 10a, and 10e showed an antinociceptive effect in the acute phase and all compounds were effective in the chronic phase. In the hot plate test, compounds 5c, 5d, and 10a demonstrated an antinociceptive effect.
Conclusions: The results clearly showed that both vanillin-triazine and phenylpyrazole-triazine derivatives had an antinociceptive effect. Also, some compounds which showed activity in the early phase of formalin test as well as in the hot plate test could control acute pain in addition to chronic or inflammatory pain.

Key Words: Acute Pain; Analgesics; Anti-Inflammatory Agents; Heterocyclic Compounds; Mice; Pain Measurement; Triazines; Vanillin.

INTRODUCTION

Currently available drugs to treat inflammatory pain include corticosteroids and non-steroidal anti-inflammatory drugs (NSAIDs) [1]. The mechanism of action of NSAIDs is mainly the inhibition of cyclooxygenase (COX), which is the key enzyme in the conversion of arachidonic acid to prostaglandins [2].

In 1991, it was discovered that in mammals the two isoforms of this enzyme, COX-1 and COX-2, have independent genes and different expression patterns [3]. Following this classification, extensive research conducted for the synthesis of selective COX-2 inhibitors and several drugs including celecoxib and rofecoxib were marketed [4,5]. Compared to traditional NSAIDs, these drugs had fewer gastrointestinal side effects [6–8], but after a short time, it

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was reported that they caused cardiovascular side effects such as thrombosis, myocardial infarction, and cardiac dysfunction [9–13]. The proposed mechanism for these effects is the inhibition of prostacyclin production in vessels which mediate platelet activation and atherogenesis [14]. Except celecoxib, other selective COX-2 inhibitors were withdrawn from the market due to these cardiovascular complications [15]. Therefore, the search for novel drugs devoid of the mentioned side effects is still an ongoing need for treatment of pain and inflammation.

Heterocyclic compounds have played an important role in the structure of anti-inflammatory drugs. Numerous synthesis methods have been developed to improve the NSAID structures through chemical modification of heterocyclic rings. Celecoxib and the other coxibs are diaryl heterocycles [16]. Studies on the structure-activity relationship of diaryl heterocycles have shown that the presence of a group of SO2NH2 or SO2Me in the para position of one of the aryl rings often provides the optimum activity for activity and specificity against COX-2 [17]. Compounds in which the pyrazole ring has been substituted with the pyrazoline, oxazolone, oxadiazolone, maleimide, and even quinazoline alternatives have also shown potent and specific COX-2 inhibition [18]. In a large number of biologically active heterocyclic compounds, the triazine ring plays an important role in the observed activity. Triazine derivatives in the form of 1, 2, 4-triazine have also shown analgesic activity [19,20].

Considering the structural properties of COX-2 inhibitors, Asadi et al. [21] synthesized new compounds with a heterocyclic ring based on triazine and evaluated their anti-inflammatory effect. In the continuation of this research, the present study aimed to evaluate the antinociceptive effects of selected triazine derivatives in mice.

MATERIALS AND METHODS

1. Drugs

Five compounds derived from triazine that showed considerable anti-inflammatory activity in the previous study were synthesized and provided by Asadi et al. [21] for evaluation of their possible antinociceptive effects. They include:

4-(4-(4-formyl-3-methoxyphenoxy)–6–chloro-1,3,5-triazin-2-ylamino)benzonitrile (5c)

4-(4-(p-tolylamino)–6–chloro-1,3,5-triazin-2-yloxy)–2-methoxybenzaldehyde (5d)

4–chloro-N-methyl-6-(4-phenyl-1H-pyrazol-1-yl)–1,3,5-triazin-2-amine (10a)

4–chloro-N-ethyl-6-(4-phenyl-1H-pyrazol-1-yl)–1,3,5-triazin-2-amine (10b)

2,4-dichloro-6-(4-phenyl-1H-pyrazol-1-yl)–1,3,5-triazine (10e)

2. Animals

In this study, healthy male Swiss mice weighing 25 to 30 grams were provided by the animal house of the School of Pharmacy (Isfahan University of Medical Sciences, Isfahan, Iran). The animals were kept in standard conditions of light, humidity, and temperature, and were fed with standard pellets. Six mice were housed in each cage and all the animal experiments were performed according to guidelines for the care and use of laboratory animals provided by The National Ethical Committee (Iran) (Ethics code: IR.MUI.RESEARCH.REC.1400.189). All experiments were performed with the least annoyance to the animals. For adaptation, the animals were transferred from the animal house to the laboratory one week before the experiments.

3. Experimental design

Acetic acid, formalin, and hot plate tests were used to evaluate the antinociceptive effect. In the first two tests, three doses of each compound (50, 100, and 200 mg/kg) were used. In the hot plate test only the maximum dose (200 mg/kg) was tested. A total number of 246 healthy male mice (41 groups with six mice in each group) were used in this study (**Fig. 1**). In all experiments, the animals were randomly divided into groups.

4. Acetic acid test

The acetic acid test is a chemical method used to assess the pain. Extension of hind limbs and contraction of the abdominal musculature (writhing) were considered to be

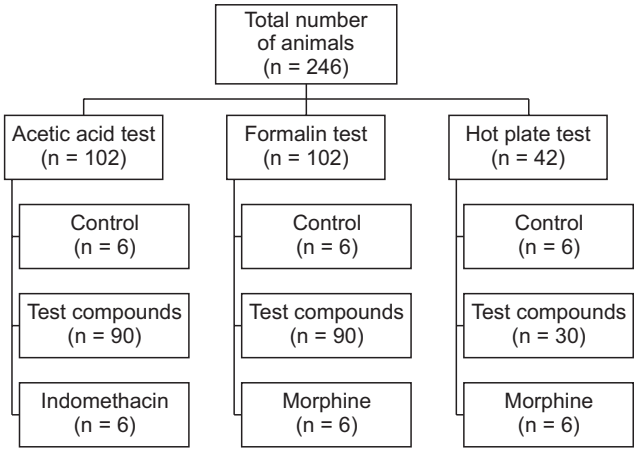

Fig. 1. Flowchart of animals grouping.

signs of pain. In the acetic acid test, drugs were administered intraperitoneally half an hour before injection of 1% acetic acid (10 mL/kg, intraperitoneal [i.p.]), and 10 minutes after acetic acid injection, the number of abdominal contractions was counted over a 10 minutes period and compared [22]. In this test, indomethacin at a dose of 10 mg/kg used as the standard drug.

5. Formalin test

The formalin test is a common chemical test to assess the antinociceptive effects of drugs in mice. Three doses of each test compound (50, 100, and 200 mg/kg) was injected i.p. and thirty minutes later formalin (20 μL of a 2.5% solution (v/v)) was injected into the hind paw of mice. The acute phase of this test was evaluated from 0–5 minutes and the chronic phase of the test from 20–40 minutes after formalin injection. The duration of paw licking was considered an indicator of pain behavior. In the formalin test, morphine (10 mg/kg) was used as a standard drug [23,24].

6. Hot plate

A hot plate apparatus (Borj Sanat, Tehran, Iran) was used in this test. The temperature of the hot plate was set at 52°C and the latency time to paw licking was recorded three times at 5 minute intervals and the mean of the three measurements was considered the control latency for each mouse. Then vehicle, the test drugs (200 mg/kg), or morphine (10 mg/kg) were injected i.p. into the animals of different groups and the reaction time was again measured at 30, 60, 90, and 120 minutes [25]. The percent of maximum possible antinociceptive effect (MPE%) was calculated for each mouse at different time intervals using the following formula:

MPE% = [(test latency (s) – control latency (s)) / (cut-off time (s) – control latency (s))] × 100

Cut-off time was 30 seconds in the authors' experiments.

7. Statistical analysis

Mean ± SEM of the time spent paw licking in the acute and chronic phases were calculated for each group. The one-way analysis of variance (ANOVA) test was used to analyze the data, followed by the Scheffe post hoc test. *P* values less than 0.05 indicated significant results. The software programs used for data analysis and making graphs were SPSS version 16 (SPSS Inc., Chicago, IL) and Excel 2020 (Microsoft, Redmond, WA), respectively.

RESULTS

1. Acetic acid test

In the acetic acid test, the number of writhings counted in a 10 minutes period and the results have been shown in **Table 1**. As can be seen in the table, there is a significant difference between all 5 triazine-derived substances in all 3 doses of 50, 100, and 200 mg/kg with the control group in terms of the number of writhings of the mouse. Compared with the control group, only the 200 mg/kg dose of 10a and 10b showed better antinociceptive effect (95% and 98% respectively). Indomethacin also showed a statistically significant difference ($P < 0.001$) with the control group.

2. Antinociceptive effect of compounds 5c and 5d at different doses in the formalin test

As it is seen in **Fig. 2**, compound 5c in all 3 doses only showed a significant ($P < 0.001$) antinociceptive effect in the chronic phase of the formalin test when compared with the control group. There is also a significant difference between the morphine group as the standard drug and the control group in both phases of the formalin test ($P < 0.001$).

Compound 5d reduced the paw licking time in both

Table 1. Antinociceptive effect of triazine derivatives in acetic acid-induced writhing test in mice

Compound	Dose (mg/kg)	Number of writhings	Percent inhibition
Control	–	69.9 ± 3.5	–
5c	50	27.8 ± 3.1***	60%
	100	19.7 ± 7.6***	71%
	200	15.5 ± 6.6***	77%
5d	50	18.5 ± 6.2***	73%
	100	6.4 ± 5.5***	90%
	200	6.0 ± 3.9***	91%
10a	50	7.7 ± 3.5***	88%
	100	7.5 ± 4.4***	89%
	200	2.8 ± 1.0***	95%
10b	50	16.8 ± 3.9***	75%
	100	13.4 ± 3.9***	80%
	200	1.0 ± 0.8***	98%
10e	50	31.7 ± 3.0***	54%
	100	7.5 ± 0.4***	89%
	200	4.7 ± 2.2***	93%
Indomethacin	10	3.3 ± 1.3***	95%

Data shows mean ± SEM of six mice in each group.
5c: 4-(4-(4-formyl-3-methoxyphenoxy)−6−chloro-1,3,5-triazin-2-ylamino) benzonitrile, 5d: 4-(4-(p-tolylamino)−6−chloro-1,3,5-triazin-2-yloxy)−2-methoxybenzaldehyde, 10a: 4−chloro-N-methyl-6-(4-phenyl-1H-pyrazol-1-yl)−1,3,5-triazin-2-amine, 10b: 4−chloro-N-ethyl-6-(4-phenyl-1H-pyrazol-1-yl)−1,3,5-triazin-2-amine, 10e: 2,4-dichloro-6-(4-phenyl-1H-pyrazol-1-yl)−1,3,5-triazine, SEM: standard error of the mean.
***$P < 0.001$ compared to control group.

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phases of the formalin test. In the chronic phase, the compound at doses of 100 and 200 mg/kg showed comparable effect with morphine so that the means of these two doses were not significant from morphine group.

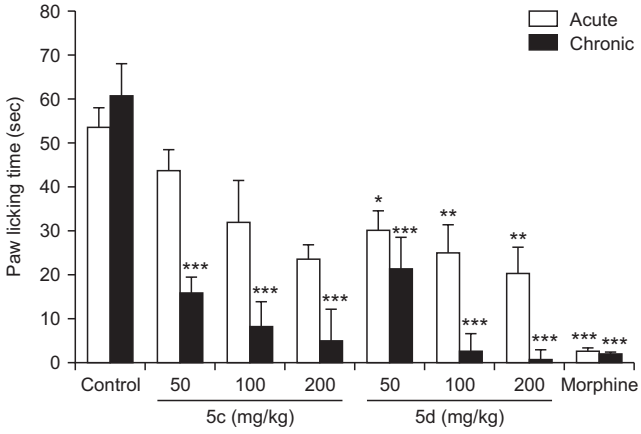

Fig. 2. Antinociceptive effect of three different doses of compound 5c and 5d in formalin test. Control animals received vehicle (10 mL/kg, i.p.). Compound 5c and 5d (50, 100, and 200 mg/kg) and morphine (10 mg/kg) were injected intraperitoneally. Thirty minutes later formalin (20 microliters, 2.5% v/v) was injected into the right hind paw of the animals. The time spent for paw licking was considered as an index of pain severity. Data shows mean ± SEM of 6 animals in each group. 5c: 4-(4-(4-formyl-3-methoxyphenoxy)−6−chloro-1,3,5-triazin-2-ylamino) benzonitrile, 5d: 4-(4-(p-tolylamino)−6−chloro-1,3,5-triazin-2-yloxy)−2-methoxybenzaldehyde, i.p.: intraperitoneal, SEM: standard error of the mean. *P < 0.05, **P < 0.01, and ***P < 0.001 compared to control group.

3. Antinociceptive effect of different doses of compounds 10a, 10b, and 10e in the formalin test

Compound 10a at a dose of 200 mg/kg significantly (P < 0.001) suppressed the acute phase, so that, compared with the control group, it reduced paw licking time by 80.6%. In the chronic phase, this compound at all three doses (50, 100, and 200 mg/kg) showed significant (P < 0.001) antinociceptive activity and compared with control group's 57.7%, 57.4%, and 99.2% reduction of the paw licking time, respectively. The percent reduction for morphine was 97% in the chronic phase and the effect of compound 10a (200 mg/kg) was greater than morphine (99.2% *vs.* 97%).

The duration of paw licking time for compound 10b is shown in **Fig. 3**. In the acute phase this compound did not exert any significant antinociceptive activity, while in the chronic phase, at doses of 100 and 200, showed a significant effect (P < 0.001) in comparison with the control group. As is seen in **Fig. 3**, all 3 tested doses of compound 10e showed a significant (P < 0.001) antinociceptive effect in the chronic phase of the formalin test when compared with the control group, and in the acute phase only the highest dose (200 mg/kg) could produce a significant (P = 0.002) suppression of the pain behavior. Morphine as the standard drug in both phases of the formalin test demonstrated significant (P < 0.001) antinociception.

4. Antinociceptive effect of triazine compounds in the hot plate test

The highest dose tested in the formalin and acetic acid tests (200 mg/kg) was used in this test. Morphine showed a significant antinociceptive effect at 30, 60, 90, and 120

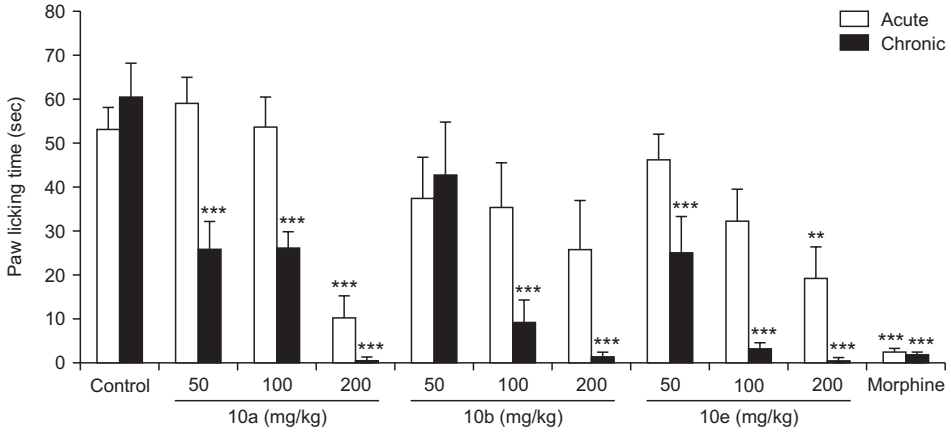

Fig. 3. Antinociceptive effect of different doses of compounds 10a, 10b, and 10e in formalin test. Control animals received vehicle (10 mL/kg, i.p.). Compounds 10a, 10b, and 10e (50, 100, and 200 mg/kg) and morphine (10 mg/kg) were injected intraperitoneally. Thirty minutes later formalin (20 microliters, 2.5% v/v) was injected into the right hind paw of the animals. The time spent for paw licking was considered as an index of pain severity. Data shows mean ± SEM of 6 animals in each group. 10a: 4−chloro-N-methyl-6-(4-phenyl-1H-pyrazol-1-yl)−1,3,5-triazin-2-amine, 10b: 4−chloro-N-ethyl-6-(4-phenyl-1H-pyrazol-1-yl)−1,3,5-triazin-2-amine, 10e: 2,4-dichloro-6-(4-phenyl-1H-pyrazol-1-yl)−1,3,5-triazine, i.p.: intraperitoneal, SEM: standard error of the mean. **P < 0.01, ***P < 0.001 compared to control group.

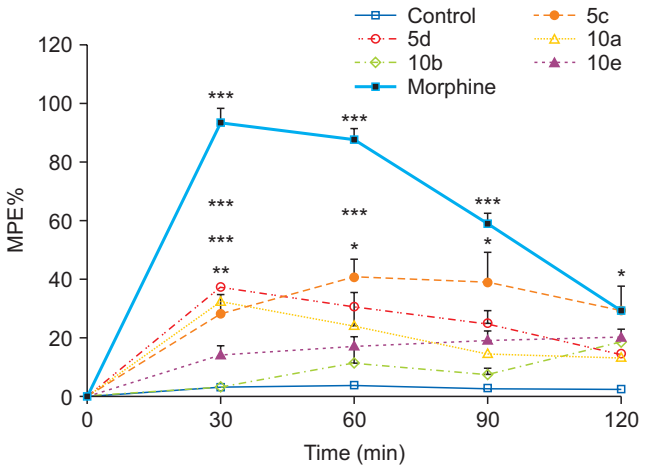

Fig. 4. The antinociceptive effect of triazine derivatives in hot plate test. Vehicle (10 mL/kg), test compounds (200 mg/kg) and morphine (10 mg/kg) were injected intraperitoneally to mice and the latency time was recorded at 0, 30, 60, 90, and 120 minutes. The percent of maximum possible antinociceptive effect (MPE%) was calculated for each time interval and compared. Data shows mean ± SEM of 6 animals in each group. 5c: 4-(4-(4-formyl-3-methoxyphenoxy)−6−chloro-1,3,5-triazin-2-ylamino)benzonitrile, 5d: 4-(4-(p-tolylamino)−6−chloro-1,3,5-triazin-2-yloxy)−2-methoxybenzaldehyde, 10a: 4−chloro-N-methyl-6-(4-phenyl-1H-pyrazol-1-yl)−1,3,5-triazin-2-amine, 10b: 4−chloro-N-ethyl-6-(4-phenyl-1H-pyrazol-1-yl)−1,3,5-triazin-2-amine, 10e: 2,4-dichloro-6-(4-phenyl-1H-pyrazol-1-yl)−1,3,5-triazine, SEM: standard error of the mean. *P < 0.05, **P < 0.01, and ***P < 0.001 compared to control group.

minutes after injection and the maximum effect was observed at 30 minutes. Compounds 5c, 5d, and 10a also showed significant antinociceptive activity when compared with the control group. Mice that were treated with compounds 10b and 10e had reaction latency times similar to vehicle-treated control animals (**Fig. 4**).

DISCUSSION

In the authors' previous work, five out of nine novel triazine compounds were found to have potent anti-inflammatory activity in a carrageenan-induced paw edema model in rats [21].

In this study, these five compounds including two vanillin-triazine and three phenylpyrazole-triazine derivatives, which were evaluated for their possible antinociceptive activity in animal models and all of them showed a significant antinociceptive effect. These findings are in agreement with previous reports on the analgesic activity of some other triazine derivatives [19,20]. In the present study three different animal models, including acetic acid-induced writhing, formalin, and hot plate tests were used to evaluate the antinociceptive effect of the triazine compounds.

The acetic acid-induced writhing test has been widely used as a screening test for assessing pain killers or anti-inflammatory drugs. Following injection of acetic acid into the peritoneal cavity of mice, the animals show a stretching behavior also called writhing. This chemical agent induces both pain and inflammation by the stimulation of nociceptors and release of inflammatory mediators, respectively. Acetic acid test is considered a non-selective model because NSAIDs, opioids, calcium channel blockers, antispasmodic drugs, and some other drugs are able to reduce the frequency of writhing and show antinociceptive effect in this model. Despite the non-selectivity of the test, it has been known as the most frequently used screening test for antinociceptive compounds [22,26].

In the acetic acid test, all five triazine derivatives significantly reduced the number of writhings and, as expected, indomethacin as the reference drug also significantly controlled the pain behavior. The percentage of the inhibition of abdominal contractions showed that at the maximum tested dose of these compounds (200 mg/kg), the order of their potencies is as follows:

10b > 10a > 10e > 5d > 5c. Indomethacin at a dose of 10 mg/kg exerted 95% inhibition of the acetic acid-induced writhes and the effect of the 10b, 10a, and 10e compounds were comparable with that of indomethicin.

In the formalin test, this study's results clearly showed that an acute 2.5% formalin injection produced two phases of the nociceptive process in mice. The formalin test is known as a useful model for screening novel compounds because it simultaneously reveals the inflammatory, neurogenic, and central mechanisms of pain behavior. Intraplantar injection of formalin into the hind paw produced a typical pain characterized by two phases. In the early phase which began just after the formalin injection, the licking of the injected paw was recorded for 5 minutes and in the second phase, also called the late phase or chronic phase, the licking behavior was recorded in a 20 minutes period which started 20 minutes after formalin administration.

According to previous reports, prostaglandins do not play an important role during the early phase and therefor NSAIDs as inhibitors of prostaglandin synthesis could not suppress this phase [23,27,28]. Based on this study's results, compound 5d at all the tested doses (50, 100, and 200 mg/kg) and compounds 10a and 10e at a dose of 200 mg/kg significantly inhibited the early phase, and this means that these compounds might have additional mechanisms of action. Also, the molecular docking performed in the previous study predicted that the best compounds for inhibition of COX-2 were 5c and 10c while, surprisingly, the pharmacological study showed that compounds 5c and 5d among the vanillin derivatives and 10a, 10b, and

10e among the phenylpyrazole-triazine derivatives had the best anti-inflammatory activity [21], and the present study also emphasized the potent antinociceptive effect of these compounds. Therefore, it might be concluded that mechanisms other than inhibition of COX-2 might also be involved, but further studies are needed for determining a definite mechanism. Consistent with this viewpoint, Choi et al. [29] reported that DUP-697 as a selective COX-2 inhibitor reduced the pain response evoked by formalin injection during both phases and, using some opioid receptor antagonists, they concluded that the endogenous opioid system is involved in its antinociceptive effect.

Compounds 5c, 5d, and 10a also showed significant antinociceptive effect in the hot plate test. The hot plate test has an advantage over other thermal models in that repeated placement of the same animal on the plate and at different time intervals over a short period of time (2–3 hours) does not cause tissue injury [30,31]. In this test, foot-licking and jumping are the two parameters commonly measured as indexes of pain severity. Analgesics increase the latency to licking/jumping. NSAIDs like aspirin and ibuprofen, and also paracetamol, demonstrate little antinociceptive effect in this test while centrally-acting drugs like opioids show potent effect and therefor the hot plate model has been considered a very useful method to detect centrally-acting analgesics [29–32]. According to the authors' findings that compounds 5c, 5d, and 10a showed significant antinociceptive effect in the hot plate model, it might be concluded that in addition to peripheral activity which was confirmed in the acetic acid test and in the chronic phase of the formalin test, central mechanisms are also involved.

In conclusion, the results clearly showed that both vanillin-triazine and phenylpyrazole-triazine derivatives had an antinociceptive effect. Also, some compounds which showed activity in the early phase of the formalin test as well as in the hot plate test could control acute pain in addition to chronic or inflammatory pain.

DATA AVAILABILITY

Data files are available from Harvard Dataverse: https://doi.org/10.7910/DVN/EMQHIO.

CONFLICT OF INTEREST

No potential conflict of interest relevant to this article was reported.

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ORCID

Valiollah hajhashemi, https://orcid.org/0000-0001-5783-2324
Ghadamali Khodarahmi, https://orcid.org/0000-0003-3054-6963
Parvin Asadi, https://orcid.org/0000-0002-5388-1548
Hamed Rajabi, https://orcid.org/0000-0003-3747-8852

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