

## Original article

Synthesis of some novel heterocyclic compounds derived from diflunisal hydrazide as potential anti-infective and anti-inflammatory agents<sup>☆</sup>Ş. Güniz Küçükgülzel<sup>a,\*</sup>, İlkay Küçükgülzel<sup>a</sup>, Esra Tatar<sup>a</sup>, Sevim Rollas<sup>a</sup>,  
Fikrettin Şahin<sup>b,c</sup>, Medine Güllüce<sup>b</sup>, Erik De Clercq<sup>d</sup>, Levent Kabasakal<sup>e</sup><sup>a</sup> Marmara University, Faculty of Pharmacy, Department of Pharmaceutical Chemistry, Tıbbiye Cad. No. 49, Haydarpasa, İstanbul 34668, Turkey<sup>b</sup> Ataturk University, Biotechnology Application and Research Center, Erzurum 25240, Turkey<sup>c</sup> Yeditepe University, Faculty of Engineering and Architecture, Department of Genetic and Bioengineering, İstanbul 34755, Turkey<sup>d</sup> Rega Institute for Medical Research, Katholieke Universiteit Leuven, 10 Minderbroedersstraat, B-3000 Leuven, Belgium<sup>e</sup> Marmara University, Faculty of Pharmacy, Department of Pharmacology, Tıbbiye Cad. No. 49, Haydarpasa, İstanbul 34668, Turkey

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## Abstract

Three novel series of 2',4'-difluoro-4-hydroxybiphenyl-3-carboxylic acid derivatives namely 4-substituted-1,2,4-triazoline-3-thiones (**4a–g**); 2-substituted-1,3,4-thiadiazoles (**5a–g**) and 2-substituted-1,3,4-oxadiazoles (**6a–g**) have been synthesized. Twenty-one of the newly synthesized compounds were tested against various bacteria, fungi, yeast species and virus. In addition, we have replaced the carboxylic acid group of diflunisal with heterocycles and the anti-inflammatory activity of heterocycles reported here. Compound (**5d**) showed activity against *Escherichia coli* A1 and *Streptococcus pyogenes* ATCC-176 at a concentration of 31.25 µg/mL, whereas cefepime, the drug used as standard, has been found less active against the bacteria mentioned above. Compound (**4b**) has exhibited activity against *Aspergillus variecolor* and *Trichophyton rubrum* at a concentration of 31.25 and 15.25 µg/mL, whereas Amphotericin B, the drug used as standard, has been found less active against the yeast and fungi. The highest antiviral activity was found in the 1,3,4-thiadiazole derivative (**5a**) having a methyl group at 2nd position against Sindbis virus at 9.6 µg/mL. Compound (**4c**) exhibited the highest anti-inflammatory activity (73.03%) whereas diflunisal, the drug used as standard, has been found less active (24.16%). Compound (**5f**) presented similar antinociceptive activity with the standard drug (paw withdrawal latency was 19.21 s compared to that of diflunisal which was 19.14 s, in hot plate test).

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Keywords: 1,2,4-Triazoline-3-thione; 1,3,4-Thiadiazole; 1,3,4-Oxadiazole; Diflunisal; Anti-inflammatory agents

## 1. Introduction

As resistance to antimicrobial drugs is widespread, there is an increasing need for identification of novel structure leads that may be of use in designing new, potent and less

toxic antimicrobial agents. Ribavirin, fluconazole and cefazolin are antiviral, antifungal and antibacterial drugs which contain 1,2,4-triazole and 1,3,4-thiadiazole rings. Moreover, owing to the increasing biological importance of 1,3,4-oxadiazoles, particularly in the field of chemotherapy and the existing antimicrobial activity of 1,2,4-triazoles [1,2] and 1,3,4-thiadiazoles [3,4], it was considered of interest to link the 2',4'-difluoro-4-hydroxybiphenyl-3-carboxylic acid (*Diflunisal*) backbone to some 1,3,4-oxadiazoles, 1,2,4-triazoles and 1,3,4-thiadiazoles in order to investigate the effect of such structural variation on the anticipated antimicrobial and antiviral

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\* Corresponding author. Tel.: +90216 349 12 16; fax: +90216 345 29 52.

E-mail address: [gkucukgulzel@marmara.edu.tr](mailto:gkucukgulzel@marmara.edu.tr) (Ş.G. Küçükgülzel).

activities. The use of several drugs to treat inflammatory conditions associated with infection is a problem especially in case of patients with impaired liver or kidney functions or to avoid drug–drug interaction. Moreover, studies describe the derivatization of the carboxylic acid function of representative NSAIDs, which resulted in an increased anti-inflammatory activity with reduced ulcerogenic effect [5]. In addition, 1,3,4-oxadiazole [6], 1,2,4-triazole [7,8] and 1,3,4-thiadiazole [9,10] derivatives were reported to possess anti-inflammatory activities. In view of the observations, we aimed investigating anti-infective and anti-inflammatory activities of these novel compounds related to diflunisal.

## 2. Chemistry

2',4'-Difluoro-4-hydroxybiphenyl-3-carboxylic acid (analgesic and anti-inflammatory drug, diflunisal) and methanol in the presence of a few drops of concentrated sulfuric acid were heated and 2',4'-difluoro-4-hydroxybiphenyl-3-carboxylic acid methyl ester (**1**) was isolated. By heating this methyl ester and hydrazine-hydrate in methanol 2',4'-difluoro-4-hydroxybiphenyl-3-carboxylic acid hydrazide (**2**) [11] was obtained. Compound (**2**) and alkyl/aryl isothiocyanates were heated in ethanol to yield original 1-(2',4'-difluoro-4-hydroxybiphenyl-3-carbonyl)-4-alkyl/arylthiosemicarbazides (**3a–g**) [12]. Alkaline cyclisation of the compounds (**3a–g**) using sodium hydroxide afforded the corresponding 5-(2',4'-difluoro-4-hydroxybiphenyl-5-yl)-4-substituted-1,2,4-triazoline-3-thiones (**4a–g**). Reacting thiosemicarbazides (**3a–g**) with concentrated sulfuric acid at room temperature resulted in the formation of the corresponding 5-(2',4'-difluoro-4-hydroxybiphenyl-5-yl)-2-alkyl/arylamino-1,3,4-thiadiazole (**5a–g**). Cyclisation of (**3a–g**) using iodine and alkali gave the desired products 5-(2',4'-difluoro-4-hydroxybiphenyl-5-yl)-2-alkyl/arylamino-1,3,4-oxadiazole (**6a–g**). Synthetic route to compounds (**4a–g**), (**5a–g**) and (**6a–g**) are shown in Scheme 1. However, in one of our previous studies [12], formation of desired 4-thiazolidinones from 1-(2',4'-difluoro-4-hydroxybiphenyl-3-carbonyl)-4-cyclohexylthiosemicarbazide failed and instead 5-(2',4'-difluoro-4-hydroxybiphenyl-5-yl)-2-cyclohexylamino-1,3,4-oxadiazole, which corresponds to compound **6g** in the present study, was obtained (Scheme 2). Structures of these compounds were characterized using <sup>1</sup>H NMR and HREI-MS spectral data. The physical and spectral data of the synthesized compounds in this study are given in Tables 1 and 2.

## 3. Biological studies

### 3.1. Antimicrobial activity

The antimicrobial activity of the synthesized compounds (**4a–g**), (**5a–g**) and (**6a–g**) against laboratory strains totally 54 microbial cultures' isolates of 35 bacteria and 19 fungi and yeast species, were used in this study by using microwell dilution assay [13,14] and MIC agar dilution assay [15]. The list of microorganisms used is given in Tables 3 and 4.

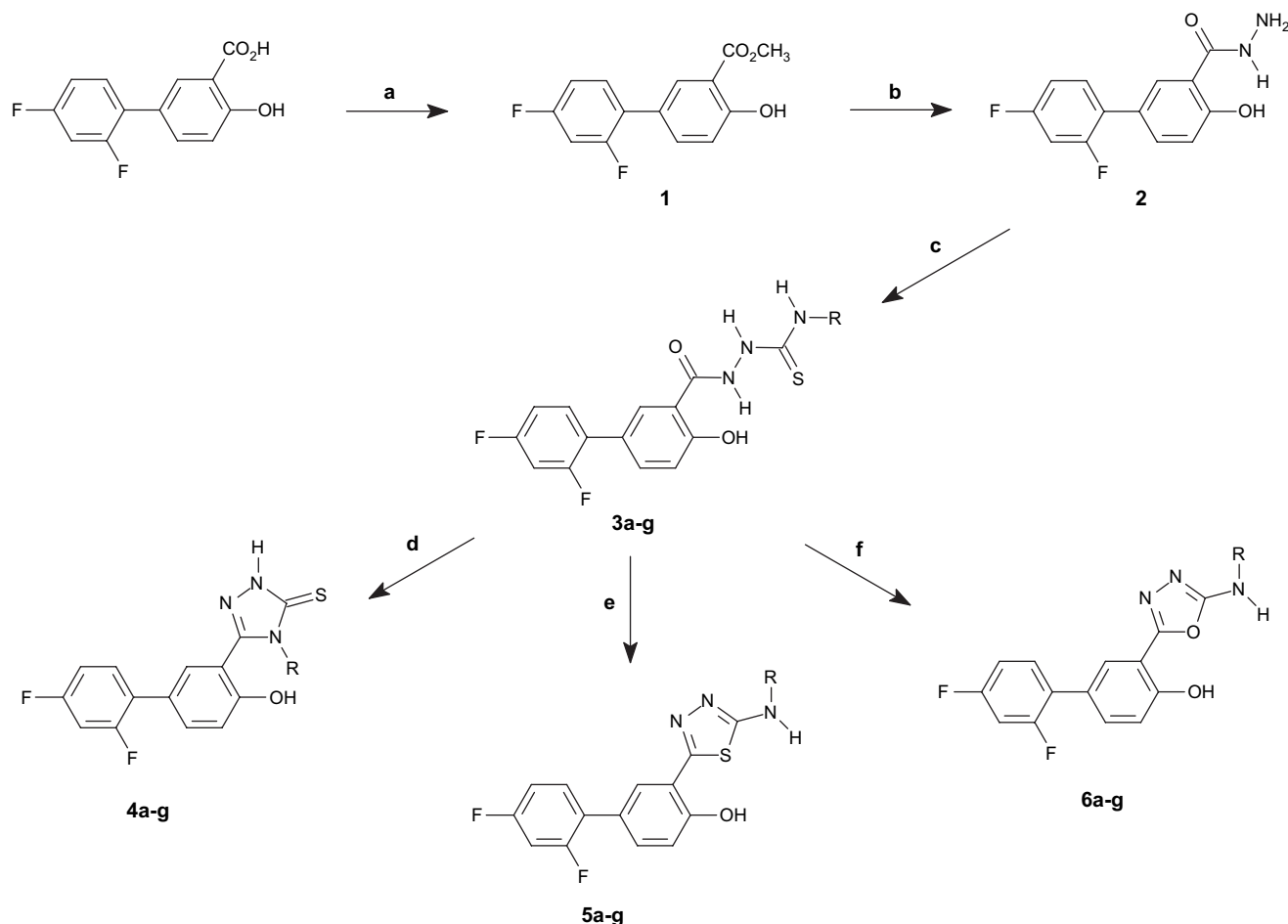
Microorganisms were provided by the Department of Clinical Microbiology, Faculty of Medicine; and Plant Diagnostic Laboratory, Faculty of Agriculture at Atatürk University, Erzurum, Turkey. Identity of the microorganisms used in this study was confirmed by Microbial Identification System in Biotechnology Application and Research Center at Atatürk University. The minimal inhibition concentration (MIC) values were also studied for the microorganisms which were determined as sensitive to (**4a–c**), (**4g**) and (**5d**) in disc diffusion assay. 5-(2',4'-Difluoro-4-hydroxybiphenyl-5-yl)-2-phenylamino-1,3,4-thiadiazole (**5d**) has shown activity against *Escherichia coli* A1 and *Streptococcus pyogenes* ATCC-176 at a concentration of 31.25 µg/mL, whereas Cefepime, the drug used as standard, has been found less active against the bacteria mentioned above. 5-(2',4'-Difluoro-4-hydroxybiphenyl-5-yl)-4-ethyl-1,2,4-triazoline-3-thiones (**4b**) has exhibited activity against *Aspergillus varicolor* and *Trichophyton rubrum* at a concentration of 31.25 and 15.25 µg/mL, respectively, whereas Amphotericin B, the drug used as standard, has been found less active against the yeast and fungi mentioned above. Some synthesized compounds demonstrated antifungal activity, whereas anticandidal activity was never observed.

### 3.2. Antiviral activity

Compounds (**4a–g**), (**5a–g**) and (**6a–g**) were tested for antiviral activity and cytotoxicity in various viral test systems, according to previously published procedures [16–20]. The synthesized compounds were tested against vesicular stomatitis virus, coxsackie virus B4, respiratory syncytial virus, parainfluenza-3 virus, reo virus, Sindbis virus, Punto Toro virus, herpes simplex virus type 1 and 2 and vaccinia virus-induced cytopathicity at subtoxic concentrations in HeLa, Vero or Hel cell culture. Compounds (**4b**), (**4d**), (**4e**) and (**4g**) which are the 1,2,4-triazole derivatives of diflunisal exhibited antiviral inhibitions against coxsackie virus B4, herpes simplex virus-1 TK-KOS, vaccinia virus and Sindbis virus at 16 µg/mL, respectively. Compounds (**5a**) and (**5c**) which are the 1,3,4-thiadiazole derivatives of diflunisal having methyl and allyl groups, respectively, at 2nd position exhibited antiviral inhibition against herpes simplex virus-1 TK-KOS and herpes simplex virus-1 TK-KOS ACV, Sindbis virus, Coxsackie virus B4 and Punto Toro virus at 16 µg/mL, respectively. 5-(2',4'-Difluoro-4-hydroxybiphenyl-5-yl)-2-allylamino-1,3,4-oxadiazole (**6c**) exhibited antiviral inhibition against herpes simplex virus-2 at 16 µg/mL. The highest antiviral activity was found in the 1,3,4-thiadiazole derivative (**5a**) having a methyl group at 2nd position against Sindbis virus at 9.6 µg/mL.

### 3.3. Anti-inflammatory activity

The anti-inflammatory activity of the synthesized compounds (**4a–g**), (**5a–g**) and (**6a–g**) was evaluated by carrageenan induced paw edema method of Kasahara et al. [21]. The compounds were tested at 50 mg/kg oral dose and were compared with the reference drug diflunisal. The tested compounds showed anti-inflammatory activity ranging from



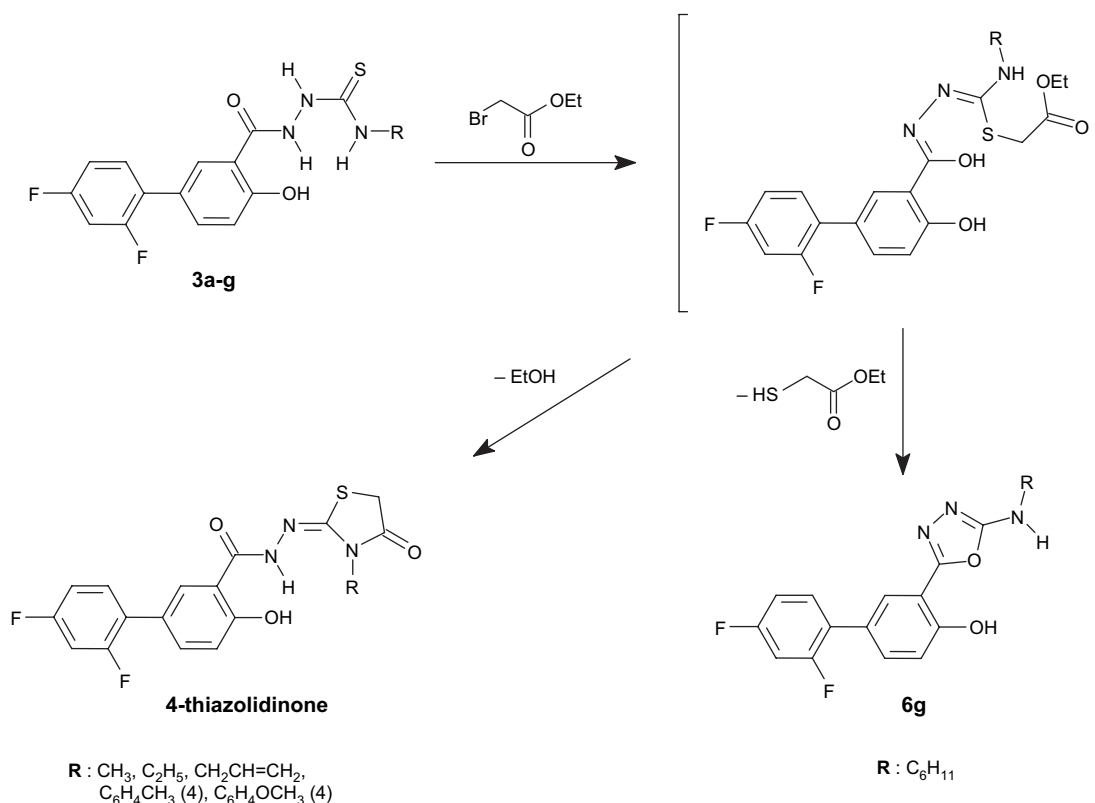
Scheme 1. Synthetic route to compounds **4a–g**, **5a–g** and **6a–g**. Reagents and conditions: (a) CH<sub>3</sub>OH/H<sub>2</sub>SO<sub>4</sub>, reflux; (b) NH<sub>2</sub>NH<sub>2</sub>·H<sub>2</sub>O, reflux; (c) R-NCS/EtOH, reflux; (d) NaOH (2 N), reflux; (e) H<sub>2</sub>SO<sub>4</sub>; (f) I<sub>2</sub>/KI–NaOH.

23.85 to 73.03% inhibition (Fig. 1 and Table 5) whereas the reference drug diflunisal showed 24.16% inhibition after 4 h. The anti-inflammatory activity of 1,2,4-triazole derivatives (**4a–g**), 1,3,4-thiadiazole derivatives (**5a–g**) and 1,3,4-oxadiazole derivatives (**6a–g**) was in the range from 36.34% to 73.03%, from 41.36% to 57.25% and from 23.85% to 41.36%, respectively. 5-(2',4'-Difluoro-4-hydroxybiphenyl-5-yl)-4-allyl-1,2,4-triazoline-3-thiones (**4c**) was found to possess highest anti-inflammatory activity by 73.03% inhibition of against the carrageenan induced paw edema. We have replaced the carboxylic acid group of diflunisal with additional heterocycles, which have been found to possess an interesting profile of anti-inflammatory activity.

For determination of antinociceptive activity conventional hot plate testing according to the method of Woolfe and McDonald [22] was used. The latency to jumping/licking of a hind paw was measured once before (control) and at 60 min after drug administration of the tested compounds. The tested compounds showed antinociceptive activity resulting in paw withdrawal latency ranging from 9.81 s to 19.21 s whereas the same values for the reference drug diflunisal remained at 19.14 s after 1 h of drug administration (Fig. 2 and Table 5).

The antinociceptive activity results using hot plate test model showed that 1,2,4-triazole derivatives (**4a–g**), 1,3,4-thiadiazole derivatives (**5a–g**) and 1,3,4-oxadiazole derivatives (**6a–g**) exhibited paw withdrawal latency values ranging from 13.25 s to 17.07 s, from 12.50 s to 19.21 s and from 9.81 s to 12.06 s, respectively. 5-(2',4'-Difluoro-4-hydroxybiphenyl-5-yl)-4-(4-methoxyphenyl)-1,3,4-thiadiazole (**5f**) presented similar antinociceptive activity with the standard drug (paw withdrawal latency was 19.21 s compared to that of diflunisal which was 19.14 s, in hot plate test).

As most of the designed compounds of the present study showed higher anti-inflammatory activity and some of them showed comparable antinociceptive properties compared to diflunisal, oral bioavailability was considered to play an important role for the development of bioactive molecules as therapeutic agents. Therefore, a computational study for prediction of ADME properties of the molecules was performed by determination of lipophilicity, topological polar surface area (TPSA), absorption (% ABS) and simple molecular descriptors used by Lipinski in formulating his “rule of five”. Calculations were performed using *Molinspiration online property calculation toolkit* (<http://www.molinspiration.com>) [24]. Table 6 represents a calculated percentage of absorption



Scheme 2. Mechanism of 4-thiazolidinone or 1,3,4-oxadiazole ring closure from 1-acyl-4-alkyl/aryl thiosemicarbazides.

(% ABS), topological polar surface area (TPSA) and Lipinski parameters of the synthesized compounds **4a–g**, **5a–g** and **6a–g**. Percentage of absorption (% ABS) was estimated using the equation: % ABS = 109 – 0.345 × TPSA, according to Zhao et al. [23]. TPSA was also calculated using Molinspiration

online property calculation toolkit [24] according to the fragment-based method of Ertl et al. [25]. Polar surface area, together with lipophilicity, is an important property of a molecule in transport across biological membranes. Too high TPSA values give rise to a poor bioavailability and absorption

Table 1  
Physical and spectral data for **4a–g**, **5a–g**, **6a–g**

Compound	R	Molecular formula	M.p (°C)	Yield (%)	HREI-MS (m/z) calculated/found
<b>4a</b>	CH <sub>3</sub>	C <sub>15</sub> H <sub>11</sub> F <sub>2</sub> N <sub>3</sub> OS	238–240	90.54	319.0524/319.0589
<b>4b</b>	C <sub>2</sub> H <sub>5</sub>	C <sub>16</sub> H <sub>13</sub> F <sub>2</sub> N <sub>3</sub> OS	180–182	71.06	333.0780/333.0730
<b>4c</b>	CH–CH=CH <sub>2</sub>	C <sub>17</sub> H <sub>13</sub> F <sub>2</sub> N <sub>3</sub> OS	192–194	82.79	345.0781/345.0752
<b>4d</b>	C <sub>6</sub> H <sub>5</sub>	C <sub>20</sub> H <sub>15</sub> F <sub>2</sub> N <sub>3</sub> OS	272–274	67.97	381.0781/381.0745
<b>4e</b>	C <sub>6</sub> H <sub>4</sub> CH <sub>3</sub> (4)	C <sub>21</sub> H <sub>15</sub> F <sub>2</sub> N <sub>3</sub> OS	258–262	83.62	395.0937/395.0911
<b>4f</b>	C <sub>6</sub> H <sub>4</sub> OCH <sub>3</sub> (4)	C <sub>21</sub> H <sub>15</sub> F <sub>2</sub> N <sub>3</sub> OS	244	72.43	411.0886/411.0858
<b>4g</b>	C <sub>6</sub> H <sub>11</sub>	C <sub>20</sub> H <sub>19</sub> F <sub>2</sub> N <sub>3</sub> OS	242–244	79.36	387.1251/387.1219
<b>5a</b>	CH <sub>3</sub>	C <sub>15</sub> H <sub>11</sub> F <sub>2</sub> N <sub>3</sub> O <sub>2</sub>	292 (dec.)	68.44	319.0624/319.0613
<b>5b</b>	C <sub>2</sub> H <sub>5</sub>	C <sub>16</sub> H <sub>13</sub> F <sub>2</sub> N <sub>3</sub> O <sub>2</sub>	222–224	84.07	333.0780/333.0736
<b>5c</b>	CH–CH=CH <sub>2</sub>	C <sub>17</sub> H <sub>13</sub> F <sub>2</sub> N <sub>3</sub> O <sub>2</sub>	171–180	61.50	345.0781/345.0749
<b>5d</b>	C <sub>6</sub> H <sub>5</sub>	C <sub>20</sub> H <sub>15</sub> F <sub>2</sub> N <sub>3</sub> O <sub>2</sub>	174	93.98	381.0781/381.0766
<b>5e</b>	C <sub>6</sub> H <sub>4</sub> CH <sub>3</sub> (4)	C <sub>21</sub> H <sub>15</sub> F <sub>2</sub> N <sub>3</sub> O <sub>2</sub>	246–260	55.72	395.0937/395.0885
<b>5f</b>	C <sub>6</sub> H <sub>4</sub> OCH <sub>3</sub> (4)	C <sub>21</sub> H <sub>15</sub> F <sub>2</sub> N <sub>3</sub> O <sub>2</sub>	224–226	68.90	411.0886/411.0869
<b>5g</b>	C <sub>6</sub> H <sub>11</sub>	C <sub>20</sub> H <sub>19</sub> F <sub>2</sub> N <sub>3</sub> O <sub>2</sub>	178–180	45.98	387.1250/387.1224
<b>6a</b>	CH <sub>3</sub>	C <sub>15</sub> H <sub>11</sub> F <sub>2</sub> N <sub>3</sub> O <sub>2</sub>	239–240	75.45	303.0819/303.0811
<b>6b</b>	C <sub>2</sub> H <sub>5</sub>	C <sub>16</sub> H <sub>13</sub> F <sub>2</sub> N <sub>3</sub> O <sub>2</sub>	176–177	56.43	317.0976/317.0974
<b>6c</b>	CH–CH=CH <sub>2</sub>	C <sub>17</sub> H <sub>13</sub> F <sub>2</sub> N <sub>3</sub> O <sub>2</sub>	142–144	43.65	329.0973/329.1008
<b>6d</b>	C <sub>6</sub> H <sub>5</sub>	C <sub>20</sub> H <sub>15</sub> F <sub>2</sub> N <sub>3</sub> O <sub>2</sub>	224–227	73.21	365.0975/365.0990
<b>6e</b>	C <sub>6</sub> H <sub>4</sub> CH <sub>3</sub> (4)	C <sub>21</sub> H <sub>15</sub> F <sub>2</sub> N <sub>3</sub> O <sub>2</sub>	238–240	67.41	379.1165/379.1140
<b>6f</b>	C <sub>6</sub> H <sub>4</sub> OCH <sub>3</sub> (4)	C <sub>21</sub> H <sub>15</sub> F <sub>2</sub> N <sub>3</sub> O <sub>3</sub>	248–250	62.15	395.1114/395.1096
<b>6g</b>	C <sub>6</sub> H <sub>11</sub>	C <sub>20</sub> H <sub>19</sub> F <sub>2</sub> N <sub>3</sub> O <sub>2</sub>	194–195	67.45	371.1478/371.1447

<sup>a</sup> Ref. [12].

Table 2

<sup>1</sup>H NMR spectral data of **4a–g**, **5a–g**, **6a–g**

Compound	<sup>1</sup> H NMR
<b>4a</b>	3.25 (s, 3H, N–CH <sub>3</sub> ); 6.75–7.52 (m, 6H, Ar–H); 9.88, 10.61 (2s, 1H, Ar–OH); 13.78 (2s, 1H, NH).
<b>4b</b>	0.99 (t, 3H, N–CH <sub>2</sub> –CH <sub>3</sub> ); 3.78 (q, 2H, N–CH <sub>2</sub> –CH <sub>3</sub> ); 7.01–7.51 (m, 6H, Ar–H); 10.29–10.77 (br, 1H, Ar–OH); 13.54 (s, 1H, NH).
<b>4c</b>	4.47 (d, 2H, N–CH <sub>2</sub> –CH=); 4.65 (d, 1H, –CH=CH <sub>2</sub> , <i>J</i> = 17.2 Hz, <i>trans</i> ); 4.88 (d, 1H, –CH=CH <sub>2</sub> , <i>J</i> = 10.3 Hz, <i>cis</i> ); 5.49–5.68 (m, 1H, –CH=CH <sub>2</sub> ), 6.99–7.49 (m, 6H, Ar–H); 10.56 (s, 1H, Ar–OH); 13.84 (s, 1H, NH).
<b>4d</b>	6.89–8.12 (m, 11H, Ar–H); 10.30 (s, 1H, Ar–OH); 11.07 (s, 1H, NH).
<b>4e</b>	2.29 (s, 3H, Ar–CH <sub>3</sub> ); 6.83–7.46 (m, 10H, Ar–H); 9.48–11.35 (br, 1H, Ar–OH); 12.93–14.89 (br, 1H, NH).
<b>4f</b>	3.63 (s, 3H, OCH <sub>3</sub> ); 6.73–7.40 (m, 10H, Ar–H); 9.97 (s, 1H, Ar–OH); 13.66 (s, 1H, NH).
<b>4g</b>	0.80–1.66 (m, 10H, cyclohexyl CH <sub>2</sub> ); 2.06 (s, 1H, N–CH–cyclohexyl); 6.97–7.45 (m, 6H, Ar–H); 10.43 (s, 1H, Ar–OH); 13.71 (s, 1H, NH).
<b>5a</b>	2.89 (s, 3H, N–CH <sub>3</sub> ); 6.97–8.00 (m, 7H, Ar–H and NH–CH <sub>3</sub> ); 11.10 (s, 1H, Ar–OH).
<b>5b</b>	1.17 (t, 3H, N–CH <sub>2</sub> –CH <sub>3</sub> ); 3.31 (q, 2H, N–CH <sub>2</sub> –CH <sub>3</sub> ); 7.02–7.98 (m, 7H, Ar–H and NH–CH <sub>2</sub> –CH <sub>3</sub> ), 11.10 (s, 1H, Ar–OH).
<b>5c</b>	3.97 (d, 2H, N–CH <sub>2</sub> –CH=); 5.07 (d, 1H, –CH=CH <sub>2</sub> , <i>J</i> = 17.0 Hz, <i>trans</i> ); 5.25 (d, 1H, –CH=CH <sub>2</sub> , <i>J</i> = 10.2 Hz, <i>cis</i> ); 5.75–5.94 (m, 1H, –CH=CH <sub>2</sub> ), 7.01–8.34 (m, 7H, Ar–H and NH–Ar); 11.09, 12.02 (2 s, 1H, Ar–OH).
<b>5d</b>	6.97–8.18 (m, 11H, Ar–H); 9.85 (s, 1H, NH–C <sub>6</sub> H <sub>5</sub> ); 12.00 (s, 1H, Ar–OH).
<b>5e</b>	2.47 (s, 3H, Ar–CH <sub>3</sub> ); 7.08–8.17 (m, 11H, Ar–H); 10.26 (s, 1H, NH–C <sub>6</sub> H <sub>4</sub> –CH <sub>3</sub> ); 11.15 (s, 1H, Ar–OH).
<b>5f</b>	3.72 (s, 3H, OCH <sub>3</sub> ); 6.91–8.14 (m, 11H, Ar–H); 10.15 (s, 1H, Ar–OH).
<b>5g</b>	1.14–1.99 (m, 10H, cyclohexyl CH <sub>2</sub> ); 2.24 (s, 1H, N–CH–cyclohexyl); 7.11–7.78 (m, 6H, Ar–H); 11.12 (s, 1H, NH cyclohexyl);
<b>6a</b>	2.49 (s, 3H, N–CH <sub>3</sub> ); 7.10–7.78 (m, 7H, Ar–H and NH–CH <sub>3</sub> ).
<b>6b</b>	1.16 (t, 3H, N–CH <sub>2</sub> –CH <sub>3</sub> ); 3.25 (q, 2H, N–CH <sub>2</sub> –CH <sub>3</sub> ); 7.10–7.90 (m, 7H, Ar–H and NH–CH <sub>2</sub> –CH <sub>3</sub> ).
<b>6c</b>	3.86 (d, 2H, N–CH <sub>2</sub> –CH=); 5.12 (d, 1H, –CH=CH <sub>2</sub> , <i>J</i> = 17.3 Hz, <i>trans</i> ); 5.21 (d, 1H, –CH=CH <sub>2</sub> , <i>J</i> = 10.4 Hz, <i>cis</i> ); 5.84–5.96 (m, 1H, –CH=CH <sub>2</sub> ), 7.05–8.13 (m, 7H, Ar–H and NH); 10.28 (s, 1H, Ar–OH).
<b>6d</b>	6.97–7.79 (m, 11H, Ar–H); 10.41 (s, 1H, NH–C <sub>6</sub> H <sub>5</sub> ); 10.73 (s, 1H, Ar–OH).
<b>6e</b>	2.23 (s, 3H, Ar–CH <sub>3</sub> ); 7.05–7.78 (m, 10H, Ar–H); 10.38 (s, 1H, NH–C <sub>6</sub> H <sub>4</sub> –CH <sub>3</sub> ); 10.61 (s, 1H, Ar–OH).
<b>6f</b>	3.63 (s, 3H, OCH <sub>3</sub> ); 6.91–7.76 (m, 10H, Ar–H); 10.37 (s, 1H, NH–C <sub>6</sub> H <sub>4</sub> –OCH <sub>3</sub> ); 10.51 (s, 1H, Ar–OH).
<b>6g</b>	1.15–1.93 (m, 10H, cyclohexyl CH <sub>2</sub> ); 2.24 (s, 1H, N–CH–cyclohexyl); 7.10–7.88 (m, 6H, Ar–H); 10.33 (s, 1H, NH cyclohexyl).

<sup>a</sup> Ref. [12].

of a drug. According to the above criterions, calculated percentages of absorption for compounds **4a–g**, **5a–g** and **6a–g** ranged between 81 and 90%.

In conclusion, a functional group transformation in a well-known anti-inflammatory drug diflunisal was performed to observe whether replacement of carboxylic acid function with several heterocyclic rings (1,2,4-triazole; 1,3,4-thiadiazole and 1,3,4-oxadiazole) leads to an increase in biological activity, or a different pharmacological profile. There were no direct correlations observed between simple molecular properties such as log *P* and anti-inflammatory or antinociceptive activity. Nevertheless, it was notable that three most active derivatives in each

heterocyclic classes (**4c**, **5c** and **6a**) with allyl or methyl substituent at R position had zero violations to Lipinski rule of five.

According to these results, it has been understood that heterocyclic derivatives of diflunisal exhibited higher anti-inflammatory activity than diflunisal and compound **5f** presented similar antinociceptive activity.

#### 4. Experimental

All chemical compounds were purchased from Fluka. Diflunisal was provided by Sanovel Pharmaceuticals. Melting points were taken on Buchi-530 apparatus and are uncorrected.

Table 3

Antimicrobial activities of new synthesized compounds against the bacterial strains tested based on disc diffusion method and microwell dilution method

Microorganisms – Code (Source) <sup>a</sup>	<b>4a</b>		<b>4b</b>		<b>4c</b>		<b>4f</b>		<b>4g</b>		<b>5d</b>		Antibiotics <sup>b</sup>	
	DD	MIC	DD	MIC	DD	MIC	DD	MIC	DD	MIC	DD	MIC	DD <sup>c</sup>	MIC <sup>d</sup> (max)
<i>Enterococcus faecalis</i> – ATCC-29122 (1)	–	–	–	–	10	250	12	125	–	–	–	–	18 (SCF)	31.25
<i>Escherichia coli</i> – A1 (1)	–	–	–	–	12	62.50	–	–	–	–	16	31.25	–(OFX)	62.50
<i>Proteus vulgaris</i> – A161 (1)	10	125	9	125	10	125	–	–	10	125	14	62.50	12 (OFX)	125
<i>Proteus vulgaris</i> – KUKEM1329 (1)	8	125	9	125	9	250	10	125	12	125	16	62.50	13 (OFX)	125
<i>Streptococcus pyogenes</i> – ATCC-176 (1)	–	–	–	–	12	62.25	10	62.25	–	–	18	31.25	10 (OFX)	62.50
<i>S. pyogenes</i> – KUKEM-676 (1)	–	–	–	–	14	62.25	9	125	–	–	–	–	13 (OFX)	31.25

Bacteria: *Acinetobacter baumannii* A8 (1), *Acinetobacter lwoffii* F1 (3), *Bacillus macerans* A199 (1), *Bacillus megaterium* A59 (2), *Bacillus subtilis* ATCC 6633 (1), *B. subtilis* A57 (1), *Brucella abortus* A77 (1), *Burkholderia cepacia* A225 (2), *Clavibacter michiganensis* A227 (2), *Cedecea davisae* F2 (3), *Enterobacter cloacae* A135 (2), *Klebsiella pneumoniae* F3 (3), *K. pneumoniae* A137 (2), *Morganella morganii* F4 (3), *Pseudomonas aeruginosa* ATCC9027 (1), *P. aeruginosa* ATCC27859 (1), *P. aeruginosa* F5 (3), *Pseudomonas pseudoalkaligenes* F6 (3), *Pseudomonas syringae* pv. tomato A35 (2), *Salmonella choleraesuis arizonae* F7 (3), *Salmonella enteritidis* ATCC13076 (1), *Serratia plymuthica* F8 (3), *Shigella sonnei* F9 (3), *Staphylococcus aureus* A215 (1), *S. aureus* ATCC 29213 (1), *Staphylococcus epidermidis* A233 (1), *Staphylococcus hominis* F10 (3), *Xanthomonas campestris* A235 (2), *Yersinia enterocolitica* F11 (3).

<sup>a</sup> Source of microorganisms: 1 = Clinic human pathogenic organism, 2 = Plant pathogenic, 3 = Food pathogenic.<sup>b</sup> OFX = Ofloxacin (10 µg/disc); SCF = sulbactam (30 µg) + cefoperazone (75 µg) (105 µg/disc) and NET = Netilmicin (30 µg/disc), were used as positive reference standards antibiotic discs (Oxoid); Maxipime (µg/mL) was used as reference antibiotic in microwell dilution assay (Sigma).<sup>c</sup> Inhibition zone in diameter (mm) around the discs impregnated with 10 µL of the synthesized compounds.<sup>d</sup> Minimal inhibitory concentrations are µg/mL.



Table 4

Anticandidal and antifungal activities of **4b** and **4c** against the yeast and fungi isolates tested based on disc diffusion method and MIC agar dilution assay

Test fungi (Source) <sup>a</sup>	<b>4b</b>		<b>4c</b>		Antibiotics <sup>b</sup>	
	DD	MIC	DD	MIC	DD	MIC
Fungi						
<i>Aspergillus flavus</i> (3)	16	31.25	14	31.25	—(NET)	15.62
<i>Aspergillus varicolor</i> (3)	14	31.25	10	62.50	—(NET)	62.50
<i>Trichophyton rubrum</i> (1)	16	15.25	—	—	—(NET)	15.62
<i>Trichophyton mentagrophytes</i> (1)	—	—	14	31.25	—(NET)	31.25

Yeast: *Candida albicans* A117 (1), \*Fungi: *Alternaria solani*, *Aspergillus niger*, *Fusarium acuminatum*, *Fusarium oxysporum*, *Fusarium solani*, *Fusarium tabacinum*, *Microsporum canis*, *Monilinia fruticola*, *Mortierella alpina*, *Penicillium* spp., *Rhizopus* spp., *Rhizoctonia solani*, *Sclerotinia minor*, *Sclerotinia sclerotiorum*. (\*These fungi were isolated from foodstuff).

<sup>a</sup> Source of microorganisms: 1 = Clinic human pathogenic organism, 2 = Plant pathogenic, 3 = Food pathogenic.

<sup>b</sup> DD = Inhibition zone in diameter (mm) around the discs impregnated with the synthesized compounds (300 µg/disc); NET = Netilmicin (30 µg/disc) was used as positive reference standard antibiotic discs (Oxoid); MIC = Minimal inhibitory concentrations (µg/mL) of standard antibiotic, AmpB = Amphotericin B (µg/mL) (Sigma).

<sup>1</sup>H NMR spectra were obtained on a Bruker AVANC-DPX 400 instrument. High resolution 70 eV EI mass spectra were obtained from a JEOL JMS-700 magnetic sector instrument.

#### 4.1. Chemistry

##### 4.1.1. 5-(2',4'-Difluoro-4-hydroxybiphenyl-5-yl)-4-substituted-1,2,4-triazoline-3-thiones (**4a–g**)

A solution of 0.01 mol (**3a–g**) in 2 N sodium hydroxide solution (25 mL) was heated under reflux for 4 h. After cooling to room temperature, concentrated hydrochloric acid was added. The precipitate was filtered and washed several times with distilled water. The compounds were recrystallized from ethanol [26].

##### 4.1.2. 5-(2',4'-Difluoro-4-hydroxybiphenyl-5-yl)-2-alkyl/arylamino-1,3,4-thiadiazole (**5a–g**)

Compounds (**3a–g**) (0.01 mol) were dissolved in concentrated sulfuric acid. The mixture was stirred at room temperature for 30 min. It was poured into ice-cold cooling water and washed with distilled water. The precipitate was recrystallized from ethanol [27].

##### 4.1.3. 5-(2',4'-Difluoro-4-hydroxybiphenyl-5-yl)-2-alkyl/arylamino-1,3,4-oxadiazole (**6a–g**)

The appropriate **3a–g** (0.01 mol) were suspended in ethanol (30 mL), aqueous NaOH (0.16 mol, 2 N) and iodine in KI (aqueous 5%) were added with shaking at room temperature until the color of iodine persisted. The excess solvent was removed under reduced pressure. The contents were cooled and acidified with acetic acid (10%) to get the corresponding free oxadiazoles. The solid was filtered, washed with water, dried and recrystallized from ethanol [6].

#### 4.2. Microbiological studies

##### 4.2.1. Antimicrobial activity

**4.2.1.1. Microbial strains.** New synthesized compounds were individually tested against laboratory strains totally 54 microbial culture isolates of 35 bacteria and 19 fungi and yeast species. Microorganisms were provided by the Department of Biology, Faculty of Art and Science, and at Ataturk University, Erzurum, Turkey. The identity of the microorganisms used in this study was confirmed by Microbial Identification System in Biotechnology Application and Research Center at Ataturk University.

**4.2.1.2. Disc-diffusion assay.** Antimicrobial tests were carried out by disc diffusion method using 100 µL of suspension

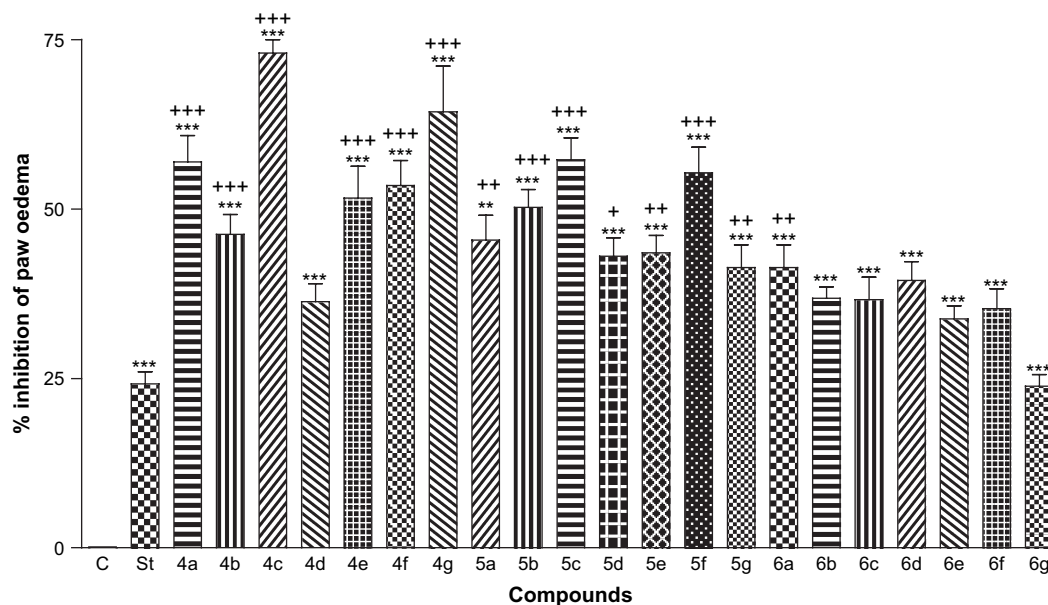


Fig. 1. Anti-inflammatory activity of compounds **4a–g**, **5a–g** and **6a–g**; \*\*\* $p < 0.001$ , \*\* $p < 0.01$ , \* $p < 0.05$ ; compared with vehicle (control); +++ $p < 0.001$ , ++ $p < 0.01$ , + $p < 0.05$ ; compared with diflunisal (as standard drug).

Table 5  
Pharmacological data for **4a–g**, **5a–g**, **6a–g**

Compound	Anti-inflammatory activity (%inhibition $\pm$ S.E.M)	Antinociceptive activity (paw withdrawal latency in seconds $\pm$ S.E.M)
Control	—	9.6 $\pm$ 0.99
Diflunisal	24.2 $\pm$ 1.83***	19.1 $\pm$ 1.18***
<b>4a</b>	56.9 $\pm$ 3.92***	16.4 $\pm$ 1.44*
<b>4b</b>	46.2 $\pm$ 2.92***	17.1 $\pm$ 1.81**
<b>4c</b>	73.0 $\pm$ 1.97***	16.8 $\pm$ 1.34**
<b>4d</b>	36.3 $\pm$ 2.63***	16.0 $\pm$ 1.15*
<b>4e</b>	51.6 $\pm$ 4.69***	14.2 $\pm$ 1.02
<b>4f</b>	53.5 $\pm$ 3.65***	15.0 $\pm$ 1.47
<b>4g</b>	64.4 $\pm$ 6.72***	13.2 $\pm$ 0.29
<b>5a</b>	45.4 $\pm$ 3.63***	13.5 $\pm$ 0.38
<b>5b</b>	50.2 $\pm$ 2.63***	13.5 $\pm$ 0.38
<b>5c</b>	57.2 $\pm$ 3.24***	13.9 $\pm$ 0.42
<b>5d</b>	43.0 $\pm$ 2.71*	16.5 $\pm$ 1.30**
<b>5e</b>	43.5 $\pm$ 2.57**	14.5 $\pm$ 0.89
<b>5f</b>	55.3 $\pm$ 3.76***	19.2 $\pm$ 0.91***
<b>5g</b>	41.4 $\pm$ 3.35*	12.5 $\pm$ 0.54 <sup>+</sup>
<b>6a</b>	41.4 $\pm$ 3.35*	10.9 $\pm$ 0.97 <sup>+++</sup>
<b>6b</b>	36.8 $\pm$ 1.66	11.0 $\pm$ 0.76 <sup>+++</sup>
<b>6c</b>	36.6 $\pm$ 3.33	9.8 $\pm$ 1.07 <sup>+++</sup>
<b>6d</b>	39.5 $\pm$ 2.73	12.0 $\pm$ 1.30 <sup>++</sup>
<b>6e</b>	33.8 $\pm$ 1.93	11.1 $\pm$ 0.94 <sup>+++</sup>
<b>6f</b>	35.3 $\pm$ 2.94	12.0 $\pm$ 1.79 <sup>++</sup>
<b>6g</b>	23.8 $\pm$ 1.72	12.0 $\pm$ 1.79 <sup>++</sup>

The results were given as means  $\pm$  SEM. Analysis of variance ANOVA, followed by Turkey's multiple comparisons test.

\*\*\* $p$  < 0.001, \*\* $p$  < 0.01, \* $p$  < 0.05: compared with vehicle (control); +++ $p$  < 0.001, ++ $p$  < 0.01, + $p$  < 0.05: compared with diflunisal (as standard drug).

containing  $10^8$  CFU/mL of bacteria,  $10^6$  CFU/mL of yeast and  $10^4$  spore/mL of fungi spread on Mueller–Hinton agar, sabourand dextrose agar (SDA), and potato dextrose agar (PDA) medium, respectively. The discs (6 mm in diameter) were

impregnated with 15  $\mu$ L of each compounds (300  $\mu$ g/disc) at the concentration of 20 mg/mL and placed on the inoculated agar. DMSO impregnated discs were used as negative controls. Ofloxacin (10  $\mu$ g/disc), sulbactam (30  $\mu$ g) + cefoperazon (75  $\mu$ g) (105  $\mu$ g/disc) and/or netilmicin (30  $\mu$ g/disc) were used as positive reference standards to determine the sensitivity of one strain/isolate in each microbial species tested. The inoculated plates were incubated at 37 °C for 24 h for clinical bacterial strains, 48 h for yeast and 72 h for fungi isolates. Plant associated microorganisms were incubated at 27 °C. Antimicrobial activity was evaluated by measuring the zone of inhibition against the test organisms. Each assay in this experiment was repeated twice.

**4.2.1.3. Microwell dilution assay.** The minimal inhibition concentration (MIC) values were also studied for the microorganisms which were determined as sensitive to (**4a–c**), (**4f–g**) and (**5d**) in disc diffusion assay. The inocula of microorganisms were prepared from 12 h broth cultures and suspensions were adjusted to 0.5 McFarland standard turbidity. Compounds (**4a–c**), (**4f–g**) and (**5d**) dissolved in 10% dimethylsulfoxide (DMSO) were first diluted to the highest concentration (500  $\mu$ g/mL) to be tested, and then serial two-fold dilutions were made in a concentration range from 7.8 to 500  $\mu$ g/mL in 10 mL sterile test tubes containing nutrient broth. MIC values of (**4a–c**), (**4f–g**) and (**5d**) against bacterial strains and *Candida albicans* isolates were determined based on a microwell dilution method [13,14].

The 96-well plates were prepared by dispensing into each well 95  $\mu$ L of nutrient broth and 5  $\mu$ L of the inoculum. A 100  $\mu$ L (**4a–c**), (**4f–g**) and (**5d**) initially prepared at the concentration of 600  $\mu$ g/mL was added into the first wells. Then, 100  $\mu$ L from their serial dilutions was transferred into six consecutive wells. The last well containing 195  $\mu$ L of

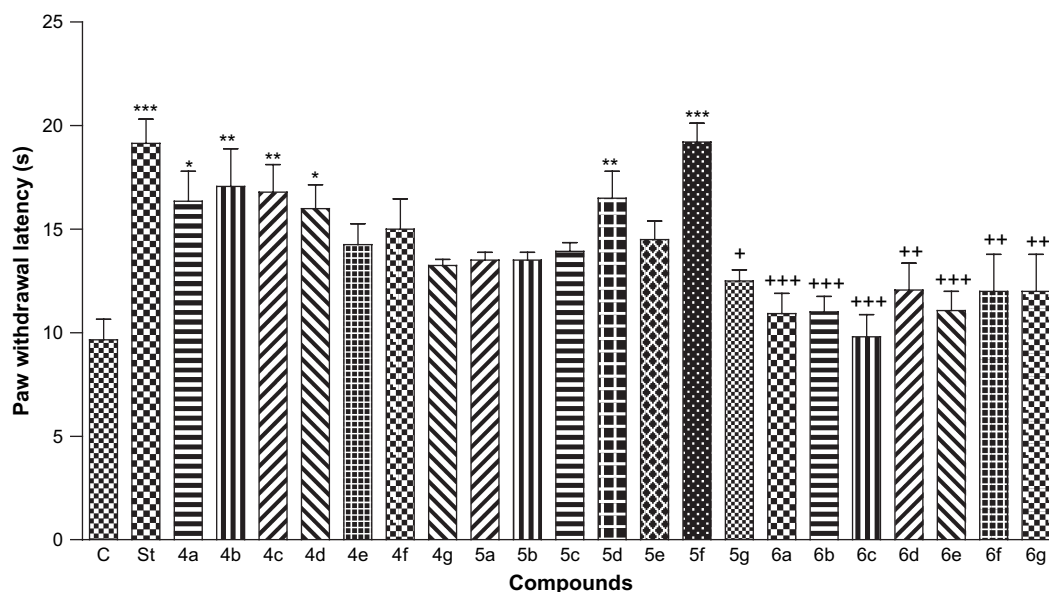


Fig. 2. Antinociceptive activity of compounds **4a–g**, **5a–g** and **6a–g**; \*\*\* $p$  < 0.001, \*\* $p$  < 0.01, \* $p$  < 0.05: compared with vehicle (control); +++ $p$  < 0.001, ++ $p$  < 0.01, + $p$  < 0.05: compared with diflunisal (as standard drug: St).

Table 6

Predicted ADME, Lipinski parameters and molecular properties of the synthesized compounds **4a–g**, **5a–g** and **6a–g**<sup>a</sup>

Compound	% ABS	TPSA	<i>n</i> -ROTB	<i>n</i> -ON acceptors	<i>n</i> -OHNH donors	mi log <i>P</i>	Formula weight	<i>n</i> violations
<b>4a</b>	90	53.85	2	4	2	3.32	319.34	0
<b>4b</b>	90	53.85	3	4	2	3.70	333.36	0
<b>4c</b>	90	53.85	4	4	2	3.97	345.37	0
<b>4d</b>	90	53.85	3	4	2	4.60	381.41	0
<b>4e</b>	90	53.85	3	4	2	5.04	395.43	1
<b>4f</b>	87	63.08	4	5	2	4.65	411.43	0
<b>4g</b>	90	53.85	3	4	2	5.23	387.46	1
<b>5a</b>	89	58.04	3	4	2	4.19	319.34	0
<b>5b</b>	89	58.04	4	4	2	4.57	333.36	0
<b>5c</b>	89	58.04	5	4	2	4.83	345.37	0
<b>5d</b>	89	58.04	4	4	2	6.32	381.41	1
<b>5e</b>	89	58.04	4	4	2	6.77	395.43	1
<b>5f</b>	86	67.27	5	5	2	6.38	411.43	1
<b>5g</b>	89	58.04	4	4	2	6.10	387.46	1
<b>6a</b>	84	71.18	3	5	2	3.55	303.27	0
<b>6b</b>	84	71.18	4	5	2	3.92	333.36	0
<b>6c</b>	84	71.18	5	5	2	4.19	329.31	0
<b>6d</b>	84	71.18	4	5	2	5.68	365.34	1
<b>6e</b>	84	71.18	4	5	2	6.13	379.37	1
<b>6f</b>	81	80.41	5	6	2	5.74	395.37	1
<b>6g</b>	84	71.18	4	5	2	5.45	371.39	1
Diflunisal	89	57.53	2	3	2	3.90	250.20	0

<sup>a</sup> % ABS: Percentage of absorption, TPSA: topological polar surface area, *n*-ON: number of hydrogen bond acceptors, *n*-OHNH: number of hydrogen bond donors, *n*-ROTB: number of rotatable bonds. Calculations were performed using *Molinspiration online property calculation toolkit* (<http://www.molinspiration.com>) [24].

nutrient broth without compound and 5 µL of the inoculum on each strip was used as negative control. The final volume in each well was 200 µL. Maxipime (Bristol–Myers Squibb) at the concentration range of 500–7.8 µg/mL was prepared in nutrient broth and used as standard drug for positive control. The plate was covered with a sterile plate sealer. Contents of each well were mixed on plate shaker at 300 rpm for 20 s and then incubated at appropriate temperatures for 24 h. Microbial growth was determined by plating 5 µL samples from clear wells on nutrient agar medium. The compounds tested in this study was screened two times against each organism. The MIC was defined as the lowest concentration of the compounds to inhibit the growth of microorganisms.

**4.2.1.4. MIC agar dilution assay.** MIC values were studied for the fungi isolates which were determined as sensitive to (**4b**) and (**4c**) in disc diffusion assay, as described previously [15]. Compounds (**4b**) and (**4c**) dissolved in 10% dimethylsulfoxide (DMSO) were added aseptically to sterile PDA medium, containing Tween 20 (Sigma 0.5%, v/v), at the appropriate volume to produce the concentration range of 7.8–500 µg/mL. The resulting PDA solutions were immediately poured into Petri plates after vortexing. The plates were spot inoculated with 5 µL (10<sup>4</sup> spore/mL) of each fungal isolate. Amphotericin B (Sigma A 4888) was used as a reference antifungal drug. The inoculated plates were incubated at 27 °C and 37 °C for 72 h for plant and clinical fungi isolates, respectively. At the end of incubation period, the plates were evaluated for the presence or absence of growth. MIC values were determined as the lowest concentration of the

compounds (**4b**) and (**4c**) where absence of growth was recorded. Each test repeated at least twice.

#### 4.2.2. Antiviral activity

Compounds (**4a–g**), (**5a–g**) and (**6a–g**) were tested for antiviral activity and cytotoxicity in various viral test systems, according to previously published procedures [16–20]. The synthesized compounds tested vesicular stomatitis virus, Coxsackie virus B4, respiratory syncytial virus, parainfluenza-3 virus, reo virus, Sindbis virus, Punto Toro virus, herpes simplex virus type 1 and 2 and vaccinia virus-induced cytopathicity at subtoxic concentrations in HeLa, Vero or Hel cell culture.

#### 4.3. Pharmacological activity

##### 4.3.1. Anti-inflammatory activity

Balb/c mice of both sexes weighing approximately 20–25 g were used. All the animals were left two days in the laboratory conditions for acclimatization and maintained on standard pellet diet and water ad libitum before the day of the experiment. The last day food was withdrawn and they were given water only. Test samples and reference compound were suspended in 0.5% methyl cellulose and administered to each mouse by using gastric gavage needle, in 50 mg/kg. The control group animals, however, received the same volume of dosing vehicle, each group consisted of six mice. The method of Kasahara et al. was employed for anti-inflammatory activity testing with some modifications. Diflunisal was used as reference compound. Test samples were administered orally 60 min before the injection on 25 µL of freshly prepared solution of γ



carrageenan (0.5 mg/mL) in physiological saline into subplantar tissue of the right hind paw of each mouse. The same volume of saline solution was injected into that of the left hind paw as internal control. The differences in foot pa thickness between the right and left foot were measured with microcaliper (Mitutoyo, Japan). Foot thickness of each mouse was measured 4 h after administration of drugs or vehicle. Percent inhibitory effect were estimated according to the following equation, where  $n$  was the average differences in thickness between the left and right hind paw of control group and  $n'$  was that of the test group of animals.

$$\text{Inhibition\%} = [(n - n')/n] \times 100$$

#### 4.3.2. Hot plate test

For conventional hot plate testing, the method of Woolfe and Mc Donald was used. Analgesia meter (Armed, Turkey) was set to a temperature of 55 °C. The latency to jumping/licking of a hind paw was measured once before (control) and at 60 min after drug administration of the tested compounds. A cutoff latency of 30 s is employed to prevent tissue injury. Groups of at least eight mice each received an orally of test compounds (50 mg/kg) or an equivalent volume of vehicle, and testing was performed 60 min after administration of drugs or vehicle. Determination of antinociceptive was assessed between 2:00 and 4:00pm.

#### 4.3.3. Statistical analysis

All experimental results were given as means  $\pm$  SEM. Analysis of variance ANOVA, followed by Tukey's multiple comparisons test. Data were analyzed with the GraphPad Prism 4 software. A significance level ( $\alpha$ ) less than 0.05 was considered significant.

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