Design, Synthesis and Anti-inflammatory Activity of Structurally Simple Anthranilic Acid Congeners Devoid of Ulcerogenic Side Effects

Amal Abdel Haleem Mohamed Eissa,*[a] Gamal Abd El-Hakeem Soliman,[b] and Moayad Hussein Khataibeh[b]

[a] *Pharmaceutical Chemistry Department, Faculty of Pharmacy, Cairo University; Kasr El-Aini Street, POB 11562, Cairo, Egypt: and* [b] *Pharmacology Department, Faculty of Pharmacy; Salman Bin Abdulaziz University; Al-Kharj, Saudi Arabia.* Received June 11, 2012; accepted July 16, 2012; advance publication released online August 2, 2012

Simple, three classes of new anthranilic acid derivatives were aimed at, synthesized and tested for their toxicity, anti-inflammatory, analgesic, antipyretic activity. Also, their potential protective role against ulcerative colitis in rats was performed. Furthermore, their effect on liver and kidney functions was detected through measurement of the serum level of alanine transaminase (ALT), aspartate aminotransferase (AST), urea, creatinine and other parameters. Compounds 4, 5, 6b, 6c, 7c and 7e showed significant anti-inflammatory activity. From those 6b and 7e best improved the inflammatory indices even producing better reduction in the intensity of lesion score, ulcer area and wet weight/length ratio and showed good analgesic activity. Fortunately, none of the tested compounds showed any hepatotoxicity or nephrotoxicity. None of the tested compounds showed any antipyretic activity. Conclusively, presence of a phenyl ring in the substituent added is a must, since any alteration in its nature led to decrease in activity. Also, the presence of an extra halogen in addition to the one already embedded in the main structure was detrimental to activity.

Key words anthranilic acid derivative; anti-inflammatory activity; ulcerative colitis; hepatotoxicity evaluation; nephrotoxicity evaluation

Non steroidal anti-inflammatory drugs (NSAID) continue to be one of the more widely used groups of therapeutic agents and useful tools in treatment of acute and chronic inflammation states, pain and fever. The therapeutic anti-inflammatory action of NSAID is produced by inhibition of cyclooxygenase-2 (COX-2), while unwanted side effects arise from inhibition of cyclooxygenase-1 (COX-1) activity. These two distinct isoforms of COXs were a constitutive form COX-1 and an inducible form COX-2.[1)] The COX-1 known as a housekeeping enzyme and expressed in resting cells of most tissues involved in protection of gastric mucosa, platelet aggregation and renal blood flow. On the other hand, the COX-2 was rapidly induced in response to a variety of pro-inflammatory stimuli such as tumor necrosis factor, interleukins and growth factors. It played an important role in pain, oncogenesis, and various acute and chronic inflammatory symptoms.[2–7)]

NSAIDs inhibited COX-1 and COX-2 with a varying degree of selectivity.[8)] Despite the known benefits of NSAID; chronic use of these agents might elicit appreciable gastrointestinal ulceration, bleeding and other complications.[9)] The incidence of clinically significant gastro-intestinal side effects due to NSAIDs was over 30% which led some patients to abandon their NSAIDs therapy.[10,11)] Since the early 1990s, it was reported that cyclooxygenases (COXs) were responsible for the production of prostaglandins H_2,[12,13)] which was a precursor for the biosynthesis of prostaglandins, thromboxanes and prostacyclins.[14)]

Inflammatory bowl diseases (IBDs) are chronic relapsing conditions with a high morbidity and remain largely incurable. Ulcerative colitis (UC) is characterized by chronic mucosal inflammation of the large intestine and the rectum and is limited to the first two layers of the intestinal lining; the mucosa and submucosa. This inflammatory process led to the development of ulcerations, resulting in diarrhea, abdominal pain and fecal blood loss. Over the disease course, these symptoms gave way to more serious complications such as hemorrhage, obstruction, perforation or cancer.[15)] NSAIDs could induce forms of acute kidney and liver injury or malfunction[16)] which is directly related to the reduction of prostaglandin synthesis induced by NSAID. The release of prostaglandin E2 and prostacyclin was increased by underlying glomerular disease, renal insufficiency and the vasoconstrictors angiotensin II and norepinephrin (NE).[17,18)] COX-2 inhibitors might have several adverse effects on the cardiovascular system including an increase in cardiovascular events and exacerbation of heart failure. Recent work suggested that COX-2 is equally important to human renal function and significant nephrotoxicity of selective COX-2 inhibitors was reported in salt-depleted patients.[19,20)] Renal side effects of nonselective cyclooxygenase inhibitors included hyperkalemia, edema, interstitial nephritis, minimal change glomerular lesions and transient impairment of glomerular perfusion.[21)] Congestive heart failure, cirrhosis and intrinsic renal disease such as the nephrotic syndrome were other well-established risk factors for nephrotoxicity due to non selective COX inhibitors.[22)]

N-Aryl anthranilic acid derivatives are a valued class of compounds that has been used for their pain killing, antipyretic and anti-inflammatory properties. Amongst these are mefenamic acid (MA), flufenamic acid (Fig. 1) and others. The main side effects of mefenamic acid included GIT disturbances, peptic ulceration and gastric bleeding. These gastroenteropathies were generally believed to be the result of direct contact effect which could be attributed to the combination of local irritation produced by the free carboxylic acid group present in its structure and/or the local blockage of prostaglandin biosynthesis in the GIT.[23)] Literature survey revealed the many efforts to synthesize prodrugs or derivatives of many NSAIDs[24,25)] *via* temporary masking the carboxylic group through ester or amide formation to give some relief to the patient from GI irritation.[25–27)]

The authors declare no conflict of interest.

* To whom correspondence should be addressed. e-mail: phd_amalabdelhaleem@hotmail.com

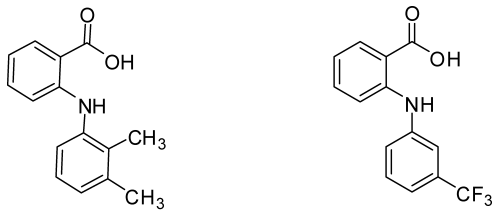

Fig. 1. Drugs with Anthranilic Acid Moiety

Also, the use of several drugs to treat inflammatory conditions especially in case of patients with problems associated with liver or kidney function was always a challenge.[28)]

In spite of this class of compounds, not being extensively studied; several anthranilic acid derivatives were synthesized and evaluated for their antipyretic, analgesic, anti-inflammatory and cytotoxic[29)] (I and II) and hypotensive[30)] (III and IV) efficacy (Fig. 2).

In continuation of previous work; some diphenylamines derived from anthranilic acid were synthesized, evaluated and found active as anti-human immunodeficiency virus (HIV)[31)] and anticancer,[31,32)] respectively (compounds V, VI and VII) (Fig. 3).

Focusing on modifying the carboxylic acid function with the aim of improving the pharmaceutical profile in mind; and since one drug out of three appeared to be a halogenated derivative for example chloro which increased lipophilicity, activity and decreased metabolism and discovering new activities for this type of compounds; new series of chlor-*N*-arylanthranilic acid derivatives were synthesized through formation of the corresponding chloro-diester (CDE-**4**) and chloro-dihydrazide (CDH-**5**) analogues. This was followed by reaction of this chloro dihydrazide (CDH-**5**) with chosen aldehydes, acetophenones and isothiocyanates to yield **6a**–**h**, **7a**–**g** and **8a**–**c**, respectively.

Chemistry The designed compounds were synthesized following the general procedure as depicted in Charts 1 and 2. The key starting compound 4-chloro-2-(2-(hydrazinecarbonyl)phenylamino)benzohydrazide (CDH; **5**) was prepared *via* the reaction of its precursor; the chlorodimethyl ester (CDE; **4**) with hydrazine hydrate. This chlorodimethyl ester[33)] was obtained in turn through the reaction of the corresponding 2-(2-carboxyphenylamino)-4-chlorobenzoic acid (**3**) with methanol in presence of thionyl chloride. This chlorodiacid (**3**) was formed from reaction of anthranilic acid with 2,4-dichloro benzoic acid under Ullmann reaction conditions.[34)] The chlorodihydrazide congener (CDH; **5**) was the building block for the preparation of the forthcoming derivatives (Chart 1).

Condensation of CDH (**5**) with various aldehydes; afforded the corresponding chloro-hydrazones (**6a**–**h**) while reaction of **5** with different acetophenones gave the disubstituted chloro-hydrazones (**7a**–**g**). On the other hand, reaction of some chosen isothiocyanates with CDH (**5**) produced the relative chloro-thiosemicarbazide derivatives (**8a**–**c**) (Chart 2).

Results and Discussion

Pharmacological Activities. Acute Toxicity Tests The tested new compounds were characterized by a low degree of toxicity. Oral administration of the new compounds in doses up to 200 mg/kg failed to kill any mouse within 24 h of observation. The calculated LD_{50} of the tested new derivatives in mice was found to be above 360 mg/kg. LD_{50} of compounds **6f**, **6h**, **7c** and **8a** was 370 mg/kg. LD_{50} of compounds **5**, **6c**, **7g** and **8b** was 380 mg/kg. LD_{50} of compounds **4**, **6a**, **6e**, **7a**, **7b**, **7d**, **7e**, and **8c** was 400 mg/kg. Also, LD_{50} for compounds **6b**, **6d** and **7f** was 420 mg/kg. Accordingly, the new synthesized compounds are considered safe since substances possessing LD_{50} higher than 50 mg/kg are non toxic.[35)]

Anti-inflammatory Activity Anti-inflammatory activity of the tested compounds was measured against acute paw edema induced by carrageenan (Table 1). Carrageenan-induced inflammation in the rat paw represents a classical model of acute inflammation that was used for evaluation of anti-inflammatory activity of drugs. Sub plantar injection of 1.0% carrageenan induced edema in the foot pad of rat hind paw.[36)] The data shown in Table 1 are the mean changes in paw volume at 3 h after carrageenan administration and the results were presented as percentage reduction in paw swelling.

The starting compound CDE (**4**) and the new intermediate

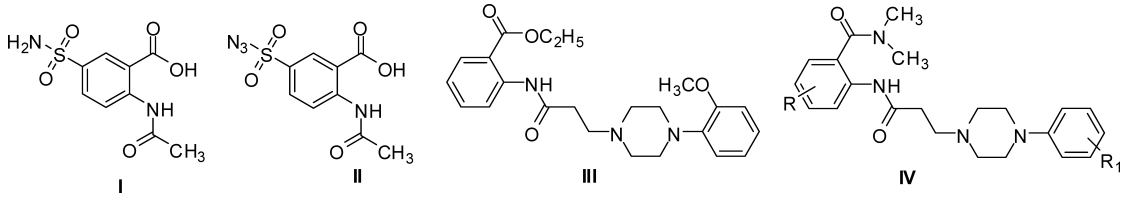

Fig. 2. Examples of Some Anthranilic Acid Derivatives

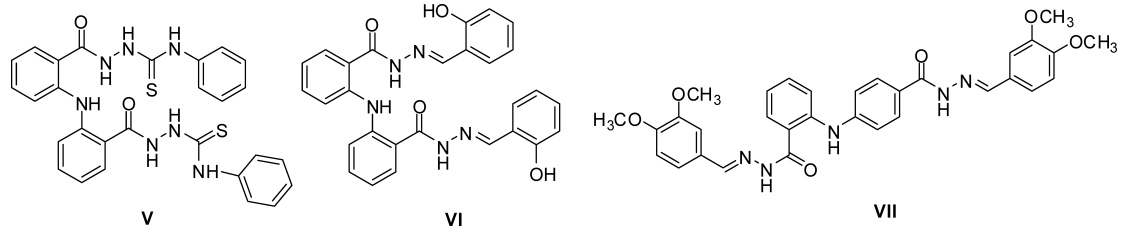

Fig. 3. Examples of Some Diphenylamines Derived from Anthranilic Acid

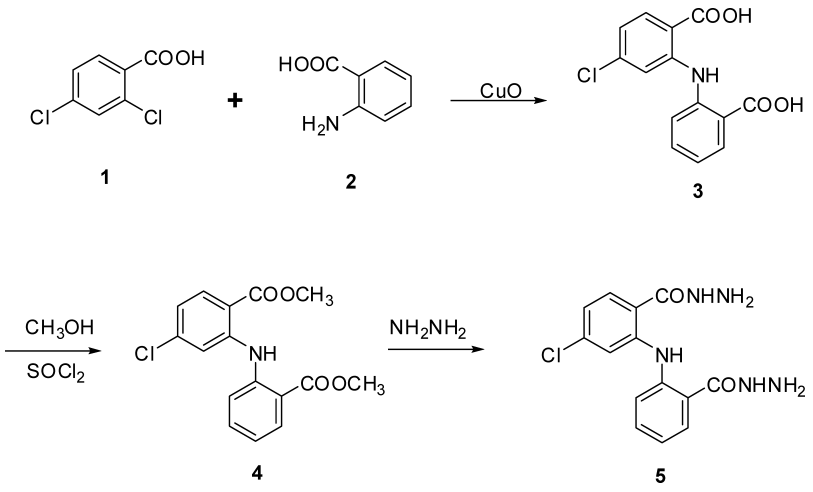

Chart 1

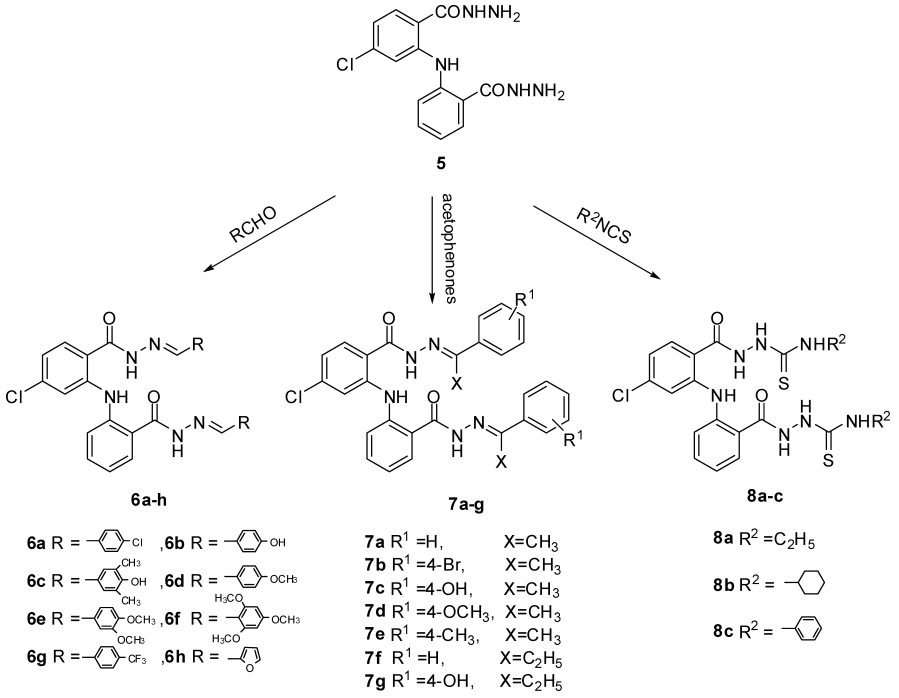

Chart 2

CDH (**5**) showed almost equal good anti-inflammatory activity affecting 38.46 and 35.90% reduction in paw swelling respectively; they both show significant difference from both the control and mefenamic acid. This might throw the light on lipophilicity/hydrophilicity of both compounds. The CDE is more lipophilic while the CDH is more hydrophilic.

Concerning the first group of compounds (**6a**–**h**); the hydrazones; all the compounds of this series bore phenyl ring variously substituted except **6h** which had a heterocyclic ring namely furan. The most active was **6b** (48.72% reduction in paw swelling) featuring a hydroxyl group attached to the phenyl ring. When two methyl groups were added to this hydroxy (**6c**); the activity decreased to 29.74% which might be attributed to the increase in bulkiness or size and the orientation of the final compound affected by the hydroxy and the two methyls. Putting a *p*-methoxy group (**6d**); the activity was almost as **6c** (28% reduction in rat paw swelling) but when the number was increased to two (**6e**) and three methoxy groups (**6f**) there was progressive decrease in the anti-inflammatory activity to 20 and 10% respectively. Presence of halogen as *p*-chloro (**6a**) dropped the anti-inflammatory activity to 4.10%. Replacement of the phenyl moiety with a heterocyclic furan ring (**6f**) also decreased the activity to 7.18% only. The above results strengthened the assumptions that the size, number in addition to the nature of the substituent—whether lipophilic or hydrophilic, electron withdrawing or donating—is of importance in this group of derivatives to presence and intensity of the activity. Also, there is significant difference between compounds **6b**, **6c** and **6d** and the control.

The second batch of derivatives is what could be presumed as disubstituted hydrazones; the story was different. Here two parameters could affect the activity; the alkyl side chain and the substituted phenyl portion. The alkyl side chain was restricted to either methyl or ethyl groups. The phenyl, on

Table 1. Anti-inflammatory Activity of the New Compounds (40 mg/kg) against Carrageenan-Induced Paw Edema in Rats (n=6)

Groups	Mean changes in paw volume (mL) 3 h after carrageenan administration	Reduction of paw swelling (%)
Control (DMSO)	1.95±0.13‡	00.00
Mefenamic acid	0.79±0.05*	59.49
4	1.27±0.10*,‡	38.46
5	1.25±0.10*,‡	35.90
6a	1.87±0.14‡	04.10
6b	1.20±0.11*,‡	48.72
6c	1.37±0.13*,‡	29.74
6d	1.40±0.13*,‡	28.20
6e	1.56±0.14‡	20.00
6f	1.76±0.14‡	09.74
6h	1.81±0.15‡	07.18
7a	1.63±0.13‡	16.41
7b	1.80±0.14‡	07.69
7c	1.41±0.13*,‡	29.69
7d	1.88±0.15‡	03.59
7e	1.16±0.13*,‡	40.51
7f	1.38±0.14*,‡	29.23
7g	1.86±0.16‡	04.61
8a	1.86±0.14‡	04.61
8b	1.81±0.15‡	07.18
8c	1.36±0.12*,‡	30.25

The results were expressed as means±S.E.M. *Significantly different *versus* control group at $p \leq 0.05$. ‡Significantly different *versus* mefenamic acid group at $p \leq 0.05$.

Table 2. Analgesic Efficacy of the New Compounds (40 mg/kg) Using the Hot-Plate Test in Mice (n=6)

Groups	Hot plate reaction time (s)	Increase in pain threshold (%)
Control (DMSO)	5.85±0.46‡	00.00
Mefenamic acid	9.06±0.75*	54.87
4	6.94±0.49‡	18.63
5	6.82±0.48‡	16.58
6a	6.25±0.55‡	06.83
6b	7.20±0.51	23.07
6c	6.54±0.45‡	11.79
6d	6.23±0.47‡	06.49
6e	6.12±0.49‡	04.61
6f	6.15±0.42‡	05.12
6h	6.15±0.57‡	05.12
7a	6.22±0.51‡	06.32
7b	6.27±0.46‡	07.17
7c	6.62±0.53‡	13.16
7d	6.21±0.39‡	06.15
7e	7.21±0.60	23.24
7f	6.43±0.52‡	09.91
7g	6.27±0.47‡	07.17
8a	6.28±0.47‡	07.35
8b	6.31±0.48‡	07.86
8c	6.55±0.38‡	11.96

The results were expressed as means±S.E.M. *Significantly different *versus* control group at $p \leq 0.05$. ‡Significantly different *versus* mefenamic acid group at $p \leq 0.05$.

the other hand was variably substituted or unsubstituted with electron donating or withdrawing groups of variable size and nature. Both parameters collectively affected the activity reaching a sensitive balance between hydrophilicity and lipophilicity; the steric effect exemplified by the size and nature of the substituents had an impact on activity as well. The most active was that bearing two methyl groups; one on phenyl and the other as side chain (**7e**), causing 40.51% reduction in paw swelling. The activity decreased to 29.69% when *p*-tolyl was exchanged by *p*-hydroxyphenyl retaining the methyl as a side chain (**7c**) or 29.23% for **7f** with unsubstituted phenyl and an ethyl side chain. These above mentioned three derivatives showed significant difference from the control. When one methyl from the side chain of **7f** was removed to afford **7a**; the anti-inflammatory activity decreased further to 16.41% reduction in paw swelling. As in the first type (**6a**–**h**); the appearance of a halogen- here a bromo- even in the presence of the methyl side chain (**7b**); led to reduction in activity to 7.69%. In this group of derivatives the hydroxy decreased the anti-inflammatory activity comparatively **7e** *versus* **7c** and **7f** *versus* **7g** and the least active was the one featuring a methoxy group with methyl side chain (**7d**, 4.61%). It was apparent from the results of the second type that also lipophilicity, size and nature of the substituents embedded and consequently orientation of the whole compound affected the anti-inflammatory activity.

The third group was of substituted thiosemicarbazides where only the phenyl substituted derivative (**8c**) affected 30.25% reduction in paw swelling compared to the control and had significant difference *versus* control, after which came the cyclohexyl (**8b**) derivative whose activity dropped severely to 7.18%. The ethyl substitution (**8a**) further decreased the activity to 4.61% and this confirmed the assumption that aromaticity rather than alkyl chains were favored since by hydrogenation of the phenyl ring; the activity apparently decreased and when the cyclohexyl was substituted by an ethyl further decrease took place.

Analgesic Activity The analgesic activity of the new compounds was tested in mice using two different techniques; the hot plate method and the acetic acid-induced writhing reaction. The hot plate test in mice is an efficient analgesic model for the screening of centrally acting compounds against acute noxious thermal stimulation[37] while the acetic acid-induced writhing test in mice is an efficient analgesic model for the screening of analgesic compounds against visceral inflammatory pain.[38] The reference drug, mefenamic acid produced 54.87% increase in pain threshold in hot plate test model and 62.78% as percentage inhibition of induced abdominal contractions in acetic acid model (Tables 2, 3).

Concerning the hot plate test; all tested new compounds showed varying degrees of activity compared to reference mefenamic acid but not as significant (Table 2). CDE and CDH (**4** and **5**) showed about 19 and 17% increase in pain threshold relative to the control. It could be observed that the most potent anti-inflammatory compounds proved to be effective analgesic agents where the hydrazone derivatives **6b** and **6c** were the most active in the first type of compounds showing 23.07 and 11.79% inhibition respectively; **7e** and **7c** in the second (23.24 and 13.16%, respectively) and **8c** in the third (11.96%).

The results of analgesic activity by the acetic acid induced abdominal writhing method (Table 3) differed slightly. Still compound **6b** was the most active (22.27%) in the first group followed by **6f** (13%) indicating the importance of the size and nature of the substituent. The most active in second group was also **7e** as the rest showed very weak activity. In the third

group, **8b** and **8c** (10.50, 10.25%, respectively) were active while the least was **8a** (4.55%).

These results might give an indication that these new compounds may be acting centrally rather than against visceral inflammatory pain.

Antipyretic Activity The antipyretic activity of the newly synthesized compounds was screened in male Sprague-Dawley rats by using yeast-induced hyperpyrexia method. Rectal temperature was recorded after subcutaneous injection of an aqueous suspension of brewer's yeast.[39] Unfortunately, all tested compounds hadn't shown any significant effect on pyrexia induced by yeast in rats, while mefenamic acid (reference drug) reversed yeast induced fever (Table 4).

Effect on Ulcerative Colitis The model of acetic acid induced colitis shares many of the histologic features of UC in human beings including mucosal edema and submucosal ulceration.[40]

The start (**4**) and key intermediate (**5**) compounds improved inflammatory indices not as mefenamic acid but definitely better than the control colitis rats (Table 5).

Same pattern for activity was observed with few additions. **6b** was the best from the first set of derivatives (**6a**–**h**); it showed better reduction in intensity of lesion score, ulcer area, ulcer index and wet weight/length ratio compared to the control group. **6c** came next followed by **6d**. The other compounds **6a**, **6e**, **6f** and **6h** were the least but still better than the control.

The second set of compounds (**7a**–**g**); **7e** was best producing reduction in the lesion score intensity, ulcer area and wet weight/length ratio compared to the control group. This was followed by **7c**, **7f**, then **7a**, **7d**, **7b** and **7g** respectively.

Third set was better than the control and lower than mefenamic acid with **8c** showing highest and **8a** lowest (Table 5).

The compounds could be described as non-ulcer inducers as they did not increase the severity of the lesions.

Effect on Liver and Kidney Function The non toxic nature of the new compounds in acute toxicity study was well supported by the biochemical data following 5-d treatment period in rats by oral dosing of the new synthesized compounds to rats in a dose of 40 mg/kg. Compounds **6d**, **6e**, **7b**, **7g** and

Table 3. Antinociceptive Effect of the New Compounds (40 mg/kg) Using Acetic Acid-Induced Abdominal Writhing Test in Mice ($n=6$)

Groups	Number of writhing (mean±S.E.M.)	Inhibition (%)
Control (DMSO)	31.60±1.97‡	00.00
Mefenamic acid	11.76±0.87*	62.78
4	27.57±1.85‡	12.75
5	27.80±1.60‡	12.02
6a	29.87±1.88‡	05.47
6b	24.56±1.57*,‡	22.27
6c	29.37±1.77‡	07.05
6d	28.28±1.65‡	10.50
6e	30.12±1.32‡	04.68
6f	27.49±1.78‡	13.00
6h	30.16±1.92‡	04.55
7a	30.63±1.37‡	03.06
7b	30.55±1.31‡	03.32
7c	29.41±1.18‡	06.93
7d	29.50±1.37‡	06.64
7e	25.49±1.24*,‡	19.33
7f	29.38±1.26‡	07.02
7g	30.17±1.15‡	04.52
8a	30.16±1.81‡	04.55
8b	28.28±1.75‡	10.50
8c	28.36±1.25‡	10.25

The results were expressed as means±S.E.M. *Significantly different *versus* control group at $p\leq0.05$. ‡Significantly different *versus* mefenamic acid group at $p\leq0.05$.

Table 4. Antipyretic Activity of the New Compounds (40 mg/kg) in Hyperthermic Rats ($n=6$)

Groups	Mean rectal temperature (°C) after			
	1 h	2 h	3 h	4 h
Control (DMSO)	39.6±0.53‡	39.0±0.54‡	38.6±0.52‡	39.1±0.37
Mefenamic acid	37.5±0.65*	37.2±0.41*	37.3±0.35*	37.9±0.58
4	39.1±0.74	39.1±0.60‡	38.5±0.53	38.5±0.64
5	39.2±0.59	38.4±0.92	38.4±0.36	38.7±0.38
6a	39.0±0.77	38.9±0.96	38.5±0.61	38.7±0.50
6b	39.0±0.73	38.9±0.76	38.2±0.50	38.9±0.51
6c	39.5±0.78	38.8±0.51	38.1±0.36	38.6±0.64
6d	39.1±0.50	38.4±0.49	38.5±0.75	38.8±0.47
6e	38.6±0.59	39.1±0.50‡	38.5±0.32‡	38.5±0.85
6f	39.1±0.41	38.5±0.64	38.3±0.37	38.9±0.58
6h	39.7±0.74	39.0±0.89	38.6±0.44	38.6±0.83
7a	39.1±0.85	38.5±0.37	38.7±0.49	38.8±0.47
7b	38.5±0.38	39.2±0.58‡	38.5±0.44	38.4±0.85
7c	39.0±0.87	38.8±0.66	38.2±0.37	38.5±0.61
7d	39.6±0.74	39.2±0.45‡	38.4±0.69	38.4±0.70
7e	39.2±0.73	38.9±0.55	38.5±0.74	38.3±0.64
7f	39.4±0.50	39.1±0.61	38.1±0.62	38.8±0.73
7g	38.7±0.57	38.7±0.60	38.4±0.50	38.7±0.60
8a	39.5±0.58	39.0±0.75	38.3±0.58	38.8±0.70
8b	39.3±0.60	38.4±0.55	38.5±0.83	38.6±0.55
8c	39.2±0.59	38.6±0.45	38.1±0.50	38.7±0.40

The results were expressed as means±S.E.M. *Significantly different *versus* control group at $p\leq0.05$. ‡Significantly different *versus* mefenamic acid group at $p\leq0.05$.

Table 5. Effect of Mefenamic Acid (25 mg/kg) and the New Compounds (40 mg/kg) on the Macroscopic Parameters of Ulcerative Colitis Induced by Acetic Acid in Rats ($n=6$)

Groups	Lesion score (0–5)	Ulcer area (cm^2)	Wet W/L (g/cm)
Sham	0.0±0.0	0.0±0.0	0.33±0.02
Control colitis	4.8±0.24‡	5.5±0.37‡	0.95±0.05‡
Mefenamic acid	2.7±0.18*	2.9±0.19*	0.52±0.03*
4	3.5±0.25*,‡	3.8±0.29*,‡	0.67±0.04*,‡
5	3.6±0.25*,‡	3.9±0.27*,‡	0.69±0.04*,‡
6a	4.5±0.25‡	4.9±0.28‡	0.87±0.04‡
6b	3.1±0.20*	3.3±0.25*	0.61±0.03*
6c	3.7±0.27*,‡	4.0±0.33*,‡	0.69±0.04*,‡
6d	3.8±0.21*,‡	4.1±0.26*,‡	0.74±0.05*,‡
6e	4.5±0.28‡	5.1±0.36‡	0.88±0.04‡
6f	4.2±0.25‡	4.8±0.30‡	0.83±0.05‡
6h	4.3±0.27‡	4.7±0.29‡	0.83±0.05‡
7a	4.3±0.26‡	4.8±0.30‡	0.83±0.05‡
7b	4.4±0.25‡	5.0±0.43‡	0.86±0.04‡
7c	3.9±0.22*,‡	4.1±0.27*,‡	0.73±0.05*,‡
7d	4.4±0.27‡	4.9±0.31‡	0.85±0.06‡
7e	3.2±0.21*	3.5±0.26*	0.62±0.04*
7f	4.0±0.25*,‡	4.2±0.28*,‡	0.76±0.05*,‡
7g	4.5±0.28‡	5.0±0.35‡	0.87±0.06‡
8a	4.5±0.26‡	5.3±0.32‡	0.90±0.05‡
8b	4.3±0.24‡	5.0±0.36‡	0.85±0.06‡
8c	4.0±0.26*,‡	4.2±0.27*,‡	0.75±0.06*,‡

The results were expressed as means±S.E.M. *Significantly different *versus* control colitis group at $p\leq0.05$. ‡Significantly different *versus* mefenamic acid group at $p\leq0.05$.

8c showed some partial damaging effect on the liver tissue indicated by significant elevation ($p<0.05$) in serum alanine transaminase (ALT) and aspartate transaminase (AST) activities although showed insignificant change in total protein, total bilirubin and albumin levels. Fortunate enough, the rest of the new compounds had not any significant effect on the levels of ALT, AST, total bilirubin, total proteins and albumin (Table 6).

Urea and creatinine are the most sensitive biochemical markers employed in the diagnosis of renal damage. In kidney damage, there will be retention of urea and creatinine in the blood,[41] therefore marked increase in serum urea and creatinine are indications of functional damage to the kidney.[42] None of the tested compounds showed significant change in the mean values of urea and creatinine in sera of rats following five days of administration at 40 mg/kg dose when compared with the control rats. According to these indicators, all the new compounds are therefore, not nephrotoxic in rats. It is worthy to mention that most of the novel compounds were non toxic especially the most active ones (**6b**, **6c**, **7c** and **7e**) while the rest were slightly toxic.

Conclusion

In summary, three series of chloro-containing anthranilic acid derivatives were synthesized. Their anti-inflammatory, analgesic, antipyretic activities were evaluated. Effect on ulcerative colitis activities in addition to their effect on liver and kidney function was performed. Most of the compounds had moderate anti-inflammatory, analgesic activities with the exception of compounds **6b**, **6c**, **7c**, **7e** and **8c** which showed significantly promising good activity. Most of the compounds affected positively UC improving the inflammatory indexes. Fortunately, most of the new derivatives showed no damaging effect on the liver tissue and were not nephrotoxic. These results established the idea that in the first type of derivatives; the most active compounds were those bearing hydroxy moiety (**6b**) or hydroxy and methyl groups (**6c**) in the phenyl substituent. Also in the second group; the activity of the hydroxy phenyl substituted compound (**7c**) was apparent as well as the tolyl (**7e**) and both had methyl group as the second substituent. The third group had a sulphur atom incorporated in its structure which changed the nature of the resulting derivatives,

Table 6. Effect of Oral Administration of the New Compounds (40 mg/kg) for 5 d on Liver Function Parameters in Rats ($n=6$)

Groups	ALT (U/L)	AST (U/L)	Total bilirubin (mg/dL)	Total protein (g/dL)	Albumin (g/dL)
Control (DMSO)	68.8±3.28	141.4±6.62	1.71±0.07	8.4±0.25	3.5±0.19
4	74.3±3.66	152.8±6.57	1.86±0.08	8.4±0.32	3.3±0.18
5	66.6±2.78	140.9±5.34	1.80±0.07	8.5±0.33	3.6±0.20
6a	62.3±2.94	148.5±6.23	1.66±0.08	8.5±0.25	3.5±0.12
6b	64.8±2.88	154.4±6.84	1.80±0.06	8.7±0.33	3.4±0.20
6c	71.2±3.16	146.9±5.34	1.66±0.08	8.3±0.32	3.4±0.12
6d	81.5±3.64*	171.8±7.56*	1.83±0.11	8.6±0.26	3.4±0.14
6e	82.2±4.16*	175.0±8.74*	1.75±0.17	8.2±0.38	3.1±0.12
6f	66.5±2.75	132.4±5.42	1.83±0.07	8.8±0.27	3.5±0.15
6h	70.3±3.23	162.6±8.34	1.74±0.07	8.4±0.35	3.6±0.17
7a	74.9±3.11	136.1±6.62	1.86±0.12	8.8±0.33	3.1±0.11
7b	85.9±4.25*	172.3±8.11*	1.80±0.08	8.7±0.30	3.7±0.17
7c	72.4±3.10	142.0±6.33	1.84±0.13	8.1±0.40	3.2±0.15
7d	75.4±3.57	156.6±6.84	1.84±0.12	8.1±0.27	3.9±0.11
7e	69.3±2.73	160.4±6.54	1.66±0.10	8.3±0.32	3.2±0.12
7f	78.6±3.34	150.7±6.87	1.90±0.08	8.0±0.39	3.2±0.16
7g	82.5±4.14*	174.5±8.36*	1.74±0.17	8.7±0.29	3.3±0.16
8a	70.5±3.38	152.4±5.77	1.82±0.05	8.4±0.34	3.3±0.22
8b	74.3±3.23	164.8±6.45	1.66±0.09	8.3±0.27	3.2±0.20
8c	85.8±4.50*	174.5±8.98*	1.82±0.11	7.9±0.30	3.2±0.11

The results were expressed as means±S.E.M. *Significantly different *versus* control group at $p\leq0.05$.

hence the simplicity of the substituents added. The most active was the phenyl substituted (**8c**). It was obvious from the different tests results that the presence of phenyl ring is important for any activity. Any deviation caused drop like phenyl saturation, switching to heterocyclic ring or aliphatic chains even addition of halogens to this phenyl affected the activity negatively. Also, a certain balance between lipophilicity and hydrophilicity was essential for the compounds to show activity. Hence, the various activities tested gave a clue of the trend how these simple compounds act especially for future work.

From comprehensive examination of data, it was interesting to note that minor alteration in the molecular configuration of the investigated compounds exemplified by variation in the size and nature of the substituents added; might have a pronounced effect on the different pharmacological screening techniques applied.

Experimental

Chemistry Unless otherwise noted, all materials were obtained from commercial suppliers and used without further purification. TLC was monitored on FLUKA silica gel TLC aluminium cards (0.2 mm thickness) with fluorescent indicator 254 nm using chloroform–ethanol (9 : 1) as eluent and visualized by UV lamp. Melting points were performed on Schorpp Geratetechnik Schmelznunktbestimmer SMP II melting point apparatus and were uncorrected. Elemental microanalyses were performed at the Microanalytical Center, Cairo University and the Regional Center for Mycology and Biotechnology, Al-Azhar University. NMR spectra were recorded on Varian mercury 300BB at 300 MHz, Gemini-200 at 200 MHz and Jeol FX 90 Q 90 MHz Fourier Transform NMR spectrometer for ^{1}H-NMR, and at 75.45 MHz for ^{13}C-NMR; using tetramethylsilane (TMS) as internal reference. Chemical shift values were given in ppm. Mass spectra were performed on Fennigan MAT, SSQ 7000 mass spectrophotometer at 70 eV. IR spectra were recorded on Bruker FT-IR spectrophotometer as potassium bromide disc.

2-(2-Carboxyphenylamino)-4-chlorobenzoic Acid 3 The compound was prepared according to the literature procedure[34] (mp 286–287°C as reported).

Methyl-4-chloro-2-[2-(methoxycarbonyl)phenylamino]-benzoate 4[33] To a cold solution of 2-(2-carboxyphenylamino)-4-chlorobenzoic acid **3** (5.00 g; 17.15 mmol) in 200 mL methanol, 15 mL thionyl chloride was added drop wise with stirring. Stirring was continued for another 20 min at room temperature. The reaction mixture was then heated under reflux conditions with stirring for 4 h. The resulting solution was concentrated under vacuum and left to cool. The precipitate formed was collected, left to dry to give 4.85 g (88%) and then crystallized from ethanol to give cottony white solid.

mp 151–152°C; ^{1}H-NMR (DMSO-d_6) δ: 3.85 (6H, s, 2C$\underline{H}_3$), 6.98–7.12 (2H, m), 7.39 (1H, s), 7.40–7.57 (2H, m), 7.91–7.95 (2H, m), 10.80 (1H, s, N$\underline{H}$ exch. D_2O). IR (KBr) cm^{-1}: 3275, 2975, 1700, 1580, 1520, 750. *Anal.* Calcd for $C_{16}H_{14}ClNO_4$ (319.75): C, 60.10; H, 4.41; N, 4.38. Found: C, 60.30; H, 4.00; N, 4.40.

4-Chloro-2-[2-(hydrazinecarbonyl)phenylamino]benzohydrazide 5 Methyl-4-chloro-2-(2-(methoxycarbonyl)-phenylamino)benzoate **4** (1.00 g; 3.13 mmol) was heated in 40 mL butanol till solution then hydrazine hydrate 99% was added (0.35 g; 6.99 mmol). The reaction mixture was heated under reflux with stirring for 25 h and concentrated under vacuum. Enough ethanol was added to affect complete precipitation, precipitate filtered and dried to yield 0.77 g (77% yield). The product was recrystallized from DMF–H_2O to give faint beige light powder.

mp 262–263°C; ^{1}H-NMR (DMSO-d_6) δ: 4.48 (4H, s, 2N$\underline{H}_2$ exch. D_2O), 6.86–7.02 (2H, m), 7.19 (1H, s), 7.31–7.42 (2H, m), 7.48–7.51 (2H, m), 9.69 (1H, s, CON$\underline{H}$ exch. D_2O), 9.75 (1H, s, CON$\underline{H}$ exch. D_2O), 10.38 (1H, s, N$\underline{H}$ exch. D_2O). IR (KBr) cm^{-1}: 3350, 3300, 1660, 1640, 1615, 1580, 1560, 1510, 770. *Anal.* Calcd for $C_{14}H_{14}ClN_5O_2$ (319.76): C, 52.59; H, 4.41; N, 21.90. Found: C, 52.74; H, 4.26; N, 22.00.

General Procedure for 4-Chloro-*N*′-(substituted)-2-{2-[2(substituted)hydrazinecarbonyl]phenylamino}benzohydrazide 6a–h 4-Chloro-2-[2-(hydrazinecarbonyl)phenylamino]benzohydrazide **5** (0.60 g; 1.88 mmol) and the appropriate aldehyde (3.80 mmol) in 30 mL absolute ethanol were heated under reflux for 20–30 h with stirring in presence of 1 mL glacial acetic acid. Precipitate formed was filtered, left to dry then crystallized from the suitable solvent.

4-Chloro-*N*′-(4-chlorobenzylidene)-2-{2-[2-(4-chlorobenzylidene)hydrazinecarbonyl]phenylamino}benzohydrazide **6a**: mp 165–166°C; yield: 98%. Crystallization solvent: *N*,*N*-dimethylformamide (DMF)–ethanol. Yellowish white powder. ^{1}H-NMR (DMSO-d_6) δ: 7.07–7.16 (2H, m), 7.31 (1H, s), 7.51–7.55 (6H, m), 7.75–7.76 (6H, m), 8.40 (2H, s, 2N=C$\underline{H}$), 10.37 (1H, s, N$\underline{H}$ exch. D_2O), 12.04 (2H, s, 2 CON$\underline{H}$ exch. D_2O). IR (KBr) cm^{-1}: 3225, 2900, 1670, 1590, 1580, 750. MS, *m*/*z*: 565.15 [M$^+$] (Calcd for $C_{28}H_{20}Cl_3N_5O_2$: 564.87). *Anal.* Calcd for $C_{28}H_{20}Cl_3N_5O_2$: C, 59.54; H, 3.57; N, 12.39. Found: C, 59.64; H, 3.69; N, 12.32.

4-Chloro-*N*′-(4-hydroxybenzylidene)-2-{2-[2(4-hydroxybenzylidene)hydrazinecarbonyl]phenylamino}benzohydrazide **6b**: mp 206–207°C; yield: 88%. Crystallization solvent: ethanol–H_2O. Creamy powder. ^{1}H-NMR (DMSO-d_6) δ: 6.61–6.64 (2H, m), 6.87–7.11 (5H, m), 7.25–7.45 (6H, m), 7.58–7.62 (2H, m), 8.27 (2H, s, 2N=C$\underline{H}$), 9.97 (2H, s (br) 2OH exch. D_2O), 10.35 (1H, s, N$\underline{H}$ exch. D_2O), 11.72 (2H, s, 2CON$\underline{H}$ D_2O exch.). IR (KBr) cm^{-1}: 3464, 3334, 2916, 2849, 1644, 1570, 1500, 755. *Anal.* Calcd for $C_{28}H_{22}ClN_5O_4$ (527.97): C, 63.69; H, 4.19; N, 13.26. Found: C, 63.75; H, 4.45; N, 13.51.

4-Chloro-*N*′-(4-hydroxy-3,5-dimethylbenzylidene)-2-{2-[2(4-hydroxy-3,5-dimethylbenzylidene)hydrazinecarbonyl]-phenylamino}benzohydrazide **6c**: mp 202–203°C; yield: 98.5%. Crystallization solvent: ethanol–H_2O. Beige powder. ^{1}H-NMR (DMSO-d_6) δ: 3.43 (12 H, s, 4CH_3), 6.98–7.01 (6H, m), 7.28 (1H, s), 7.46–7.48 (2H, m), 7.67–7.70 (2H, m), 8.29 (2H, s, 2N=C$\underline{H}$), 8.91 (2H, s, 2 OH exch. D_2O), 10.32 (1H, s, N$\underline{H}$ exch. D_2O), 11.83 (2H, s, 2 CON$\underline{H}$ exch. D_2O). IR (KBr) cm^{-1}: 3300, 3264, 2910, 2845, 1640, 1584, 1509, 756. *Anal.* Calcd for $C_{32}H_{30}ClN_5O_4$ (584.08): C, 65.81; H, 5.18; N, 11.99. Found: C, 65.75; H, 5.34; N, 12.11.

4-Chloro-*N*′-(4-methoxybenzylidene)-2-{2-[2-(4-methoxybenzylidene)hydrazinecarbonyl]phenylamino}benzohydrazide **6d**: mp 159–160°C; yield: 99%. Crystallization solvent: DMF–H_2O. Yellow powder. ^{1}H-NMR (DMSO-d_6) δ: 3.81 (6H, s, 2OCH_3), 6.99–7.03 (6H, m), 7.26 (1H, s), 7.46–7.48 (2H, m), 7.66–7.69 (6H, m), 8.32 (2H, s, 2N=C$\underline{H}$), 10.29 (1H, s, N$\underline{H}$ exch. D_2O), 11.79 (2H, s, 2 CON$\underline{H}$ exch. D_2O). IR (KBr) cm^{-1}: 3200, 2900, 1640, 1600, 1550, 750. *Anal.* Calcd for $C_{30}H_{26}ClN_5O_4$ (556.03): C, 64.81; H, 4.71; N, 12.59. Found: C,

64.87; H, 4.58; N, 12.89.

4-Chloro-*N′*-(3,4-dimethoxybenzylidene)-2-{2-[2(3,4-dimethoxybenzylidene)hydrazinecarbonyl]phenylamino}-benzohydrazide **6e**: mp 164–165°C; yield: 97%. Crystallization solvent: DMF–ethanol–H_2O. Pale beige powder. ^{1}H-NMR (DMSO-d_6) δ: 3.82 (12H, s, 4OC$\underline{H}_3$), 6.99–7.02 (4H, m), 7.16–7.20 (2H, m), 7.28 (1H, s), 7.38 (2H, s (br)), 7.48 (2H, s (br)), 7.71–7.73 (2H, m), 8.37 (2H, s, 2N=C$\underline{H}$), 10.38 (1H, s, N$\underline{H}$ exch. D_2O), 11.96 (2H, s, 2CON$\underline{H}$ exch. D_2O). IR (KBr) cm^{-1}: 3227, 3158, 2910, 2840, 1652, 1576, 1500, 754. MS, *m/z*: 616.35 [M^+] (Calcd for $C_{32}H_{30}ClN_5O_6$: 616.08). *Anal.* Calcd for $C_{32}H_{30}ClN_5O_6$: C, 62.39; H, 4.91; N, 11.37. Found: C, 62.45; H, 4.70; N, 11.01.

4-Chloro-*N′*-(2,4,6-trimethoxybenzylidene)-2-{2-[2(2,4,6-trimethoxybenzylidene)hydrazinecarbonyl]-phenylamino}benzohydrazide **6f**: mp 130–132°C; yield: 92%. Crystallization solvent: ethanol. Light brown powder. ^{1}H-NMR (DMSO-d_6) δ: 3.8–3.86 (18H, d, 6OC$\underline{H}_3$), 6.17–6.3 (4H, m), 6.97–7.08 (2H, m), 7.16 (1H, s), 7.44 (2H, s (br)), 7.65–7.71 (2H, m), 8.54 (2H, s, 2N=C$\underline{H}$), 10.21 (1H, s, N$\underline{H}$ exch. D_2O), 11.56 (2H, s, 2CON$\underline{H}$ exch. D_2O). ^{13}C-NMR (DMSO-d_6) δ: 55.54 (OCH_3), 55.92 (OCH_3), 90.63–134.00 (aromatic Cs), 144.00 (CH=N), 159.89 (C–O), 163.30 (C=O). IR (KBr) cm^{-1}: 3253, 3159, 2916, 2843, 1643, 1601, 1570, 757. MS, *m/z*: 676.35 [M^+] (Calcd for $C_{34}H_{34}ClN_5O_8$: 676.13). *Anal.* Calcd for $C_{34}H_{34}ClN_5O_8$: C, 60.39; H, 5.07; N, 10.36. Found: C, 60.55; H, 5.20; N, 10.36.

4-Chloro-*N′*-[4-(trifluromethyl)benzylidene]-2-{2-[2(4-trifluromethylbenzylidene)hydrazinecarbonyl]phenyl-amino}benzohydrazide **6g**: mp 142–143°C; yield: 92%. Crystallization solvent: ethanol–H_2O. Buff powder. ^{1}H-NMR (DMSO-d_6) δ: 6.98–7.02 (2H, m), 7.36 (1H, s), 7.45–7.79 (6H, m, aromatic), 7.91–7.96 (6H, m), 8.41 (2H, s, 2N=C$\underline{H}$), 10.76 (1H, s, N$\underline{H}$ exch. D_2O), 12.05 (2H, s, 2CON$\underline{H}$ exch. D_2O). IR (KBr) cm^{-1}: 3248 br, 2924, 2850, 1651, 1578, 1508, 752. *Anal.* Calcd for $C_{30}H_{20}ClF_6N_5O_2$ (631.97): C, 57.02; H, 3.19; N, 11.08. Found: C, 57.24; H, 3.16; N, 11.27.

4-Chloro-*N′*-(furan-2-ylmethylene)-2-{2-[2(furan-2-ylmethylene)hydrazinecarbonyl]phenylamino}benzohydrazide **6h**: mp 179–180°C; yield: 95.5%. Crystallization solvent: ethanol–H_2O. Orange yellow powder. ^{1}H-NMR (DMSO-d_6) δ: 6.64 (2H, d, 2H-3 furan, J=1.90 Hz), 6.94–7.12 (4H, m, 2H and 2H-4 furan), 7.24 (s, 1H), 7.46 (2H, m), 7.65–7.68 (2H, m), 7.85 (2H, d, 2H-5 furan, J=2.10 Hz), 8.28 (2H, s, 2N=C$\underline{H}$), 10.21 (1H, s, N$\underline{H}$ exch. D_2O), 11.87 (2H, s, 2 CON$\underline{H}$ exch. D_2O). IR (KBr) cm^{-1}: 3252, 2915, 2847, 1650, 1570, 1509, 751. MS, *m/z*: 475.25 [M^+] (Calcd for $C_{24}H_{18}ClN_5O_4$: 475.89). *Anal.* Calcd for $C_{24}H_{18}ClN_5O_4$ (475.89): C, 60.57; H, 3.81; N, 14.72. Found: C, 60.68; H, 3.96; N, 14.43.

General Procedure for 4-Chloro-*N′*-[1-(4-un/substitutedphenyl)ethylidene]-2-{2-[2-(1-(4-un/substitutedphenyl-ethylidene)hydrazinecarbonyl]phenylamino}benzohydrazide 7a–g The chosen acetophenone (4.38 mmol), 4-chlorobenzohydrazide intermediate **5** (0.70 g; 2.19 mmol), glacial acetic acid (2 mL) and absolute ethanol (30 mL) were refluxed with stirring for 25 h. The reaction mixture was concentrated and poured over ice-water. The solid obtained was collected, dried and crystallized from the appropriate solvent.

4-Chloro-*N′*-(1-phenylethylidene)-2-{2-[2-(1-phenylethylidene)hydrazinecarbonyl]phenylamino}benzohydrazide **7a**: mp 137–138°C; yield: 98%. Crystallization solvent: ethanol. Yellow powder. ^{1}H-NMR (DMSO-d_6) δ: 2.31 (s, 6H, 2CH_3), 6.92–7.28 (m, 4H, aromatic), 7.41 (7H, s, (br)), 7.62–7.71 (4H, m), 9.90 (1H, s (br) N$\underline{H}$ exch. D_2O), 10.92 (2H, s, 2CON$\underline{H}$ exch. D_2O). IR (KBr) cm^{-1}: 3250, 2950, 2850, 1660, 1580, 1510, 760. MS, *m/z*: 524.25 [M^+] (Calcd for $C_{30}H_{26}ClN_5O_2$: 524.03). *Anal.* Calcd for $C_{30}H_{26}ClN_5O_2$: C, 68.76; H, 5.00; N, 13.36. Found: C, 68.64; H, 5.29; N, 13.25.

N′-[1-(4-Bromophenyl)ethylidene]-2-{2-[2-(1-(4-bromophenyl)ethylidene)hydrazinecarbonyl]phenyl-amino}-4-chlorobenzohydrazide **7b**: mp 145–146°C; yield: 98%. Crystallization solvent: DMF–H_2O. Faint yellow powder. ^{1}H-NMR (DMSO-d_6) δ: 2.49 (6H, s, 2CH_3), 6.89–7.17 (3H, m), 7.42–7.44 (4H, m), 7.62–7.68 (8H, m), 9.90 (1H, s, N$\underline{H}$ exch. D_2O), 10.95 (2H, s, 2 CON$\underline{H}$ exch. D_2O). IR (KBr) cm^{-1}: 3300, 3264, 2916, 2849, 1662, 1571, 1509, 756, 600. *Anal.* Calcd for $C_{30}H_{24}Br_2ClN_5O_2$ (682.01): C, 52.83; H, 3.55; N, 10.27. Found: C, 53.07; H, 3.79; N, 10.20.

4-Chloro-*N′*-[1-(4-hydroxyphenyl)ethylidene]-2-{2-[2-(1-(4-hydroxyphenyl)ethylidene)hydrazinecarbonyl]phenyl-amino}benzohydrazide **7c**: mp 148–151°C; yield: 83%. Crystallization solvent: ethanol. Dark yellow powder. ^{1}H-NMR (DMSO-d_6) δ: 2.24 (6H, s, 2CH_3), 6.74–6.83 (4H, m), 6.94–7.05 (4H, m), 7.21 (1H, s), 7.42–7.47 (4H, m), 7.57–7.62 (2H, m), 9.81 (1H, s, N$\underline{H}$ exch. D_2O), 10.36 (2H, s, 2OH exch. D_2O), 10.78 (2H, s, 2 CON$\underline{H}$ exch. D_2O). IR (KBr) cm^{-1}: 3250 br, 2900, 2850, 1650, 1580, 1510, 750. MS, *m/z*: 555.50 [M^+] (Calcd for $C_{30}H_{26}ClN_5O_4$: 556.03). *Anal.* Calcd for $C_{30}H_{26}ClN_5O_4$: C, 64.81; H, 4.71; N, 12.59. Found: C, 64.65; H, 4.90; N, 12.50.

4-Chloro-*N′*-[1-(4-methoxyphenyl)ethylidene]-2-{2-[2-(1-(4-methoxyphenylethylidene)hydrazinecarbonyl]phenylamino}-benzohydrazide **7d**: mp 154–155°C; yield: 91%. Crystallization solvent: ethanol. Dark yellow powder. ^{1}H-NMR (DMSO-d_6) δ: 2.34 (6H, s, 2CH_3), 3.88 (6H, s, 2 OCH_3), 6.99–7.16 (7H, m), 7.26 (1H, s), 7.34–7.55 (3H, m), 7.74–7.83 (4H, m), 9.98 (1H, s, N$\underline{H}$ exch. D_2O), 10.89 (2H, s, 2 CON$\underline{H}$ exch. D_2O). IR (KBr) cm^{-1}: 3200, 2950, 2850, 1660, 1600, 1580, 760. MS, *m/z*: 584.25 [M^+] (Calcd for $C_{32}H_{30}ClN_5O_4$: 584.08). *Anal.* Calcd for $C_{32}H_{30}ClN_5O_4$: C, 65.81; H, 5.81; N, 11.99. Found: C, 66.09; H, 5.02; N, 12.09.

4-Chloro-*N′*-(1-*p*-tolylethylidene)-2-{2-[2-(1-*p*-tolylethylidene)hydrazinecarbonyl]phenylamino}benzohydrazide **7e**: mp 220–222°C; yield: 95%. Crystallization solvent: DMF–ethanol–H_2O. Dark buff powder. ^{1}H-NMR (DMSO-d_6) δ: 2.28 (6H, s, 2 C-CH_3), 3.42 (6H, s, 2 CH_3), 7.16–7.23 (7H, m), 7.40–7.44 (2H, m), 7.61–7.64 (6H, m), 9.60 (1H, s, N$\underline{H}$ exch. D_2O), 10.86 (2H, s, 2 CON$\underline{H}$ exch. D_2O). IR (KBr) cm^{-1}: 3300, 3200, 2900, 1650, 1580, 1510, 755. MS, *m/z*: 552.25 [M^+] (Calcd for $C_{32}H_{30}ClN_5O_2$: 552.08). *Anal.* Calcd for $C_{32}H_{30}ClN_5O_2$: C, 69.62; H, 5.48; N, 12.69. Found: C, 69.83; H, 5.56; N, 12.65.

4-Chloro-*N′*-(1-phenylpropylidene)-2-{2-[2-(1-phenylpropylidene)hydrazinecarbonyl]phenylamino}benzohydrazide **7f**: mp 109–111°C; yield: 88%. Crystallization solvent: ethanol–H_2O. Creamy white powder. ^{1}H-NMR (DMSO-d_6) δ: 1.01–1.17 (6H, t, 2$CH_2C\underline{H}_3$, J=7.10 Hz), 2.79–3.00 (4H, m, 2 $C\underline{H}_2CH_3$, overlapped), 6.95–7.04 (3H, m), 7.13–7.18 (2H, m), 7.39–7.42 (6H, m), 7.58–7.66 (6H, m), 9.89 (1H, s, N$\underline{H}$ exch. D_2O), 11.02 (2H, s, 2 CON$\underline{H}$ exch. D_2O). IR (KBr) cm^{-1}: 3250, 2950, 1660, 1580, 1510, 760. MS, *m/z*: 552.25 [M^+] (Calcd for $C_{32}H_{30}ClN_5O_2$: 552.08). *Anal.* Calcd for $C_{32}H_{30}ClN_5O_2$ (552.08): C, 69.62; H, 5.48; N, 12.69. Found: C, 69.39; H, 5.70; N, 12.75.

4-Chloro-*N*′-[1-(4-hydroxyphenyl)propylidene]-2-{2-[2-(1-(4-hydroxyphenyl)propylidene)hydrazinecarbonyl]-phenylamino}benzohydrazide **7g**: mp 100–102°C; yield: 81%. Crystallization solvent: ethanol. Yellow powder. ^{1}H-NMR (DMSO-d_6) δ: 0.99–1.15 (6H, t, $2CH_2C\underline{H}_3$, J=7.38 Hz), 2.97–3.05 (4H, m, $2C\underline{H}_2CH_3$), 6.81–6.90 (4H, d), 7.19 (1H, s), 7.37–7.47 (4H, m), 7.57 (2H, s (br)), 7.81–7.90 (4H, d), 9.90 (1H, s (br) N$\underline{H}$ exch. D_2O), 10.40 (2H, s, 2OH exch. D_2O), 10.90 (2H, s, 2CON$\underline{H}$ exch. D_2O). IR (KBr) cm^{-1}: 3200 br, 2900, 1655, 1570, 1510, 750. MS, *m*/*z*: 583.25 [M^+-1] (Calcd for $C_{32}H_{30}ClN_5O_4$: 584.08). *Anal.* Calcd for $C_{32}H_{30}ClN_5O_4$: C, 65.81; H, 5.18; N, 11.99. Found: C, 65.62; H, 5.53; N, 11.69.

General Procedure for 2-{4-Chloro-2-[2-(2-(substituted-carbamothioyl)hydrazine carbonyl)phenylamino]benzoyl}-*N*-substitutedhydrazinecarbthioamide 8a–c The 4-chlorobenzohydrazide derivative **5** (0.60 g; 1.88 mmol) and the chosen isothiocyanate (3.75 mmol) in 25 mL absolute ethanol were heated under reflux with stirring for more than 30 h. The reaction solution obtained was concentrated and poured over ice- water to precipitate the desired compounds. The precipitate was filtered, left to dry and crystallized from the appropriate solvent.

2-{4-Chloro-2-[2-(2-(ethylcarbamothioyl)hydrazine-carbonyl)phenylamino]benzoyl}-*N*-ethylhydrazine-carbthioamide **8a**: mp 134–136°C; yield: %. Crystallization solvent: ethanol–H_2O. Fluffy grey powder. ^{1}H-NMR (DMSO-d_6) δ: 1.03–1.29 (2H, m, $2C\underline{H}_2CH_3$), 3.87–3.88 (6H, m, $2CH_2C\underline{H}_3$), 6.98–7.15 (2H, m), 7.40 (1H, s), 7.56–7.57 (2H, m), 7.63–7.96 (2H, m), 9.19 (2H, s (br) 2N$\underline{H}$–C_2H_5 exch. D_2O), 10.30 (2H, s (br) 2N$\underline{H}$C=S exch. D_2O), 10.50 (1H, s, N$\underline{H}$ exch. D_2O), 10.76 (2H, s, 2 CON$\underline{H}$ exch. D_2O). IR (KBr) cm^{-1}: 3294 br, 2920, 2851, 1701, 1639, 1562, 1512, 1215, 748. *Anal.* Calcd for $C_{20}H_{24}ClN_7O_2S_2$ (494.04): C, 48.62; H, 4.89; N, 19.85. Found: C, 48.84; H, 4.98; N, 20.12.

2-{4-Chloro-2-[2-(2-(cyclohexylcarbamothioyl)hydrazine-carbonyl)phenylamino]benzoyl}-*N*-cyclohexylhydrazine-carbothioamide **8b**: mp 165–167°C; yield: 90%. Crystallization solvent: DMF–ethanol–H_2O. White fluffy powder. ^{1}H-NMR (DMSO-d_6) δ: 1.05–1.24 (10H, m), 1.56–1.79 (10H, m), 4.08 (2H, m, 2$\underline{H}$-1′ cyclohexyl), 6.96–7.07 (2H, m), 7.25 (1H, s), 7.41–7.50 (2H, m), 7.82–7.86 (2H, m), 9.19 (2H, s, 2N$\underline{H}$-cyclohexyl exch. D_2O), 10.23 (3H, s (br) 2N$\underline{H}$C=S and 1H, N$\underline{H}$ exch. D_2O), 10.49 (2H, s, 2 CON$\underline{H}$ exch. D_2O). ^{13}C-NMR (DMSO-d_6) δ: 24.67 (C-3′ and C-5′), 25.08 (C-4′), 31.75 (C-2′ and C-6′), 52.67 (C-1′), 115.50–144.71 (aromatic Cs), 162.21 (C=O), 166.95 (C=S). IR (KBr) cm^{-1}: 3264 br, 2930, 2853, 1661, 1577, 1536, 1509, 1216, 770. MS, *m*/*z*: 601.90 [M^+] (Calcd for $C_{28}H_{36}ClN_7O_2S_2$: 602.23). *Anal.* Calcd for $C_{28}H_{36}ClN_7O_2S_2$: C, 55.85; H, 6.03; N, 16.28. Found: C, 55.88; H, 6.01; N, 16.11.

2-{4-Chloro-2-[2-(2-(phenylcarbamothioyl)hydrazine-carbonyl)phenylamino]benzoyl}-*N*-phenylhydrazinecarbothioamide **8c**: mp 154–156°C; yield: 90%. Crystallization solvent: ethanol–H_2O. Off-white powder. ^{1}H-NMR (DMSO-d_6) δ: 7.02–7.19 (3H, m), 7.33–7.37 (8H, m), 7.48–7.50 (4H, m), 7.94–7.96 (2H, m), 9.83 (2H, s (br) 2N$\underline{H}$-phenyl D_2O exch.), 10.58 (2H, s (br) 2N$\underline{H}$C=S exch. D_2O), 10.75 (1H, s, N$\underline{H}$ exch. D_2O), 11.07 (2H, s, 2CON$\underline{H}$ exch. D_2O). IR (KBr) cm^{-1}: 3254 br, 1652, 1625, 1591, 1558, 1220, 748. *Anal.* Calcd for $C_{28}H_{24}ClN_7O_2S_2$ (590.13): C, 56.99; H, 4.09; N, 16.61. Found: C, 57.00; H, 3.80; N, 16.59.

Pharmacological Activities. Animals Swiss albino mice of both sex (27–30 g body weight (b.wt)) and male Sprague-Dawley rats (200–220 g b.wt) were obtained from the Animal Center of National Research Center, Cairo. Animals were maintained under standard conditions of temperature (23±1.0°C), humidity (55±10%), and 12 h light/12 h dark cycle and fed with a standard pellet diet with water *ad libitum*. All the rats were housed in standard polypropylene cages with wire mesh top. Animals were allowed to adapt to the laboratory environment for one week before experimentation. The experimental tests on animals have been performed in accordance with the Institutional Ethical Committee approval.

Preparation of the Tested Chemical Compounds All the tested chemicals and the reference drug (mefenamic acid) were dissolved in 2% v/v dimethylsulfoxide (DMSO) before oral administration to the experimental animals.

Acute Toxicity (LD_{50}) Test The oral median lethal doses (LD_{50}) of the tested compounds (**4**–**8**) were determined in Swiss albino mice as described by Lorke.[43] In a preliminary test, mice of either sex in groups of three, received one of 10, 100, or 1000 mg/kg of the tested compounds. Animals were observed for 24 h for signs of toxicity and mortality. From the results of the preliminary test, doses of 200, 400, 600, 800 and 1000 mg/kg of the tested compounds were administered to fresh groups, each of 5 mice. Control animals received the solvent and kept under the same conditions without any treatment. Signs of toxicity and number of deaths per dose during 24 h were recorded and the LD_{50} was calculated as the geometric mean of the minimum dose that resulted in 100% mortality and the maximum dose that caused no lethality at all.

Doses In this investigation, an experimental dose of 40 mg/kg that's equal to $^1/_{10}$ LD_{50} of most of the tested compounds (**4**, **6a**, **6e**, **7a**, **7b**, **7d**, **7e** and **8c**) were selected to be given orally to rats. The reference drug; mefenamic acid was given orally at a dose of 25 mg/kg. The doses were calculated by converting the therapeutic dose used in humans to rat's dose according to the Table of Paget and Barnes.[44] Acetic acid was diluted in saline to be 4% and infused into the colon of rats through a rubber catheter at the dose of 5 mL/kg.

Anti-inflammatory Activity The anti-inflammatory activity was evaluated in male Sprague-Dawley rats using a carrageenan-induced paw edema test.[36] The rats were divided into 21 groups (n=6). Rats of the 1st (normal control) and 2nd (standard) groups were treated orally with the vehicle and mefenamic acid (25 mg/kg), respectively. Animals of the 3rd–21st groups were orally given the tested compounds (**4**–**8**), respectively in a dose of 40 mg/kg. Acute inflammation was induced by the subplantar injection of 0.1 mL of 1% carrageenan in normal saline in the right hind paw of all rats after 30 min of administration of the tested compounds. The volume of the injected paws was measured in mL using a plethysmometer immediately before and 3 h following carrageenan injection. The mean change in the volume of injected paw with respect to the initial paw volume was calculated and the percentage inhibition of paw edema with respect to control group was estimated using the formula:

$$\%\ \text{inhibition of paw edema} = (1 - V_t / V_c) \times 100$$

Where: V_t and V_c are the mean change in paw volume of treated and control rats, respectively.

Analgesic Activity Analgesic activity of the newly

synthesized compounds was carried out in Swiss albino mice (27–30 g b.wt) by using two different models.

Hot Plate Method The hot plate test[45] was used with few modifications. Mice (25–30 g) were divided into 21 groups, each containing six animals. Mice of the 1st (normal control) and 2nd (reference) groups were treated orally with the vehicle and mefenamic acid (25 mg/kg), respectively. Animals of the 3rd–21st groups were orally given the tested compounds (**4**–**8**), respectively in a dose of 40 mg/kg. After one h of medication, mice were placed individually on a hot plate maintained at 55±1°C. The time taken by the animals to lick the fore or hind paw or jump out of the place was taken as the reaction time.

Acetic Acid-Induced Writhing Test The writhing test was carried out as described by Collier *et al.*[46] Mice were assigned into 21 groups, each containing 6 animals and medicated as those in the hot plate method. Writhing was induced by an intraperitoneal injection of a 0.6% acetic acid solution (0.25 mL/animal) after 30 min of medication with the tested compounds. Immediately after administration of acetic acid, each animal was isolated in an individual cage to be observed for 15 min. The number of writhing per animal was recorded and expressed as the percentage of protection.

Antipyretic Activity The antipyretic activity of the newly synthesized compounds was screened in male Sprague-Dawley rats by using yeast-induced hyperpyrexia method.[39] After measuring rectal temperature of each rat by introducing 1.5 cm of digital thermometer into rectum, pyrexia was induced by subcutaneous injection of 1 mL of 15% brewer's yeast suspension in saline solution. After 18 h of yeast injection, rats which showed a rise in temperature of at least 0.6°C were taken for the study. One hundred thirty two hyperthermic rats were divided into 21 equal groups. Rats of the 1st (hyperthermic control) and 2nd (reference) groups were treated orally with the vehicle (5 mL/kg) and mefenamic acid (25 mg/kg), respectively. Animals of the 3rd–21st groups were orally given the tested compounds (**4**–**8**), respectively in a dose of 40 mg/kg. Rectal temperature of each rat was then recorded at 1 h interval after administration for 4 h. The antipyretic efficacy was decided on the basis of the difference in the mean temperature between the control and the tested compounds.

Effect on Ulcerative Colitis Male Sprague-Dawley rats were divided into 22 experimental groups, each of 6 animals. Rats of groups 1 and 2 (Sham and Control colitis groups, respectively) were given the solvent only (2%, v/v, DMSO) in a dose of 5 mL/kg. Group 3 (reference group) was given mefenamic acid in a dose of 25 mg/kg. Rats of groups 4–22 received the test compounds **4**–**8**, respectively in a dose of 40 mg/kg. All medications were administered orally, once daily *via* the aid of an orogastric cannula for 5 successive days and the last dose was administered 2 h before colitis induction.

In rats of sham group, no abnormal changes were observed suggesting that handling procedure had no interference with the experimental outputs. Macroscopic damage parameters of the colon of control colitis rats, 2 d after rectal infusion of acetic acid revealed dark brown lesions, mucosal hyperemia, edema, erosion, and ulceration. Control colitis rats showed lesion score, ulcer area and values of 4.8±0.24 and 5.5±0.37 cm^2 respectively (Table 5). The inflammatory changes of the intestinal tract were associated with a significant increase of wet weight/length of the colon specimens as an indicator of inflammation. These inflammatory indices were significantly improved by oral dosing of mefenamic acid.

Induction of Ulcerative Colitis All animals were fasted overnight, with access to water *ad libitum*, and then anesthetized with ether inhalation before induction of the ulcerative colitis. A polyethylene catheter with 2 mm diameter was inserted into the lumen of the colon *via* the anus to a distance of 8 cm.[47] A solution of 2 mL (4%, v/v) acetic acid in saline was slowly infused into the colon through the catheter. The rats were then maintained in a supine Trendelenburg position for 30 s to prevent early leakage of the acetic acid.[48] In sham group, equivolume of normal saline was infused into the colon instead of acetic acid. The acetic acid was then aspirated and 2 mL of phosphate buffer solution (pH=7) was instilled into the rectum of each rat.[49] The catheter was left in place for a few seconds then gently removed. Two days after the induction of colitis, each animal was sacrificed using ether anesthesia and laparatomy was performed. Colonic segments (8 cm in length and 3 cm proximal to the anus) were excised, opened along its mesenteric border, washed with saline, and were used for macroscopic scoring.

Assessment of Colonic Lesions The colon specimens were weighted and wet weight/length ratio was calculated for all the rats. The specimens were examined under a dissecting microscope and the mucosal lesions were quantified by the scoring system (0–5) given by Millar *et al.*,[48] after some modifications.

The Lesion Scores were: 0=no damage, 1=local edema and inflammation without ulcers; 2=one ulcer without inflammation; 3=one to two ulcers with inflammation and lesion diameter <1 cm; 4=more than two ulcers with lesion diameter 1–2 cm; 5=severe ulceration with lesion diameter >2 cm.

Ulcer Area was measured using plane glass square. Each cell on the glass square was 1 mm^2 in area and the number of cells was counted and the ulcer area was determined for each colon.

Blood Sampling for Liver and Kidney Functions One hundred twenty six male Sprague-Dawley rats were randomly divided into 20 equal groups. Rats of the 1st group were given the solvent (DMSO) in a dose of 5 mL/kg and left as normal control. Rats of the 2nd–20th groups were administered the test compounds **4**–**8**, respectively in a dose of 40 mg/kg b.wt. All medications were administered orally *via* the aid of an orogastric cannula for 5 d. The animals were observed for signs of abnormalities throughout the experiment. At the end of the experimental period, blood samples (2 mL) were collected from the retro-orbital venous plexus of each rat (under ether anesthesia) and kept for 30 min at 4°C. Serum was separated by centrifugation at 3000 rpm for 10 min and used for the biochemical estimations.

Biochemical Estimation of Liver and Kidney Functions Liver functions were evaluated by measuring the serum activity of ALT and AST.[50] Serum levels of total bilirubin,[51] total proteins,[52] and albumin[53] were also assayed. Serum concentrations of urea[54] and creatinine[55] were determined colormetrically as measures of kidney functions.

Statistical Analysis Statistical analysis was performed using SPSS 10.0 statistical software. Differences among groups were examined using parametric one-way analysis of variance (ANOVA) with Tukey HSD as *post hoc* test. Non-parametric data were analyzed by Kruskal–Wallis

followed by Mann–Whitney *U* test. Results are expressed as the mean±S.E.M. Statistical significance was set up at a value of $p \leq 0.05$.

References

1) Sano H., Noguchi T., Tanatani A., Miyachi H., Hashimoto Y., *Chem. Pharm. Bull.*, **52**, 1021–1022 (2004).
2) Herschman H. R., *Biochim. Biophys. Acta*, **1299**, 125–140 (1996).
3) Xu X. C., *Anticancer Drugs*, **13**, 127–137 (2002).
4) Jiménez P., García A., Santander S., Piazuelo E., *Curr. Pharm. Des.*, **13**, 2261–2273 (2007).
5) Wuest F., Kniess T., Bergmann R., Pietzsch J., *Bioorg. Med. Chem.*, **16**, 7662–7670 (2008).
6) Ghodsi R., Zarghi A., Daraei B., Hedayati M., *Bioorg. Med. Chem.*, **18**, 1029–1033 (2010).
7) Lu X., Zhang H., Li X., Chen G., Li Q. S., Luo Y., Ruan B. F., Chen X. W., Zhu H. L., *Bioorg. Med. Chem.*, **19**, 6827–6832 (2011).
8) Gadad A. K., Palkar M. B., Anand K., Noolvi M. N., Boreddy T. S., Wagwade J., *Bioorg. Med. Chem.*, **16**, 276–283 (2008).
9) Borhade N., Pathan A. R., Halder S., Karwa M., Dhiman M., Pamidiboina V., Gund M., Deshattiwar J. J., Mali S. V., Deshmukh N. J., Senthilkumar S. P., Gaikwad P., Tipparam S. G., Mudgal J., Dutta M. C., Burhan A. U., Thakre G., Sharma A., Deshpande S., Desai D. C., Dubash N. P., Jain A. K., Sharma S., Nemmani K. V., Satyam A., *Chem. Pharm. Bull.*, **60**, 465–481 (2012).
10) Abouzid K., Frohberg P., Lehmann J., Decker M., *Med. Chem.*, **3**, 433–438 (2007).
11) Abouzid M. A. K., Youssef M. K., Ahmed E. A. A., Al-Zuhair H. H., *Med. Chem. Res.*, **14**, 26–39 (2005).
12) Suizu M., Muroya Y., Kakuta H., Kagechika H., Tanatani A., Nagasawa K., Hashimoto Y., *Chem. Pharm. Bull.*, **51**, 1098–1102 (2003).
13) Joo Y., Kim H.-S., Woo R.-S., Park C. H., Shin K.-Y., Lee J.-P., Chang K.-A., Kim S., Suh Y.-H., *Mol. Pharmacol.*, **69**, 76–84 (2006).
14) Reddy M. V. R., Billa V. K., Pallela V. R., Mallireddigari M. R., Boominathan R., Gabriel J. L., *Bioorg. Med. Chem.*, **16**, 3907–3916 (2008).
15) Dhaneshwar S. S., Gairola N., Kandpal M., Vadnerkar G., Bhatt L., Rathi B., Kadam S. S., *Eur. J. Med. Chem.*, **44**, 3922–3929 (2009).
16) Ragab F. A., Eid N. M., Hassan G. S., Nissan Y. M., *Chem. Pharm. Bull.*, **60**, 110–120 (2012).
17) Patrono C., Dunn M. J., *Kidney Int.*, **32**, 1–12 (1987).
18) Schneider V., Lévesque L. E., Zhang B., Hutchinson T., Brophy J. M., *Am. J. Epidemiol.*, **164**, 881–889 (2006).
19) Rossat J., Maillard M., Nussberger J., Brunner H. R., Burnier M., *Clin. Pharmacol. Ther.*, **66**, 76–84 (1999).
20) Swan S. K., Rudy D. W., Lasseter K. C., Ryan C. F., Buechel K. L., Lambrecht L. J., Pinto M. B., Dilzer S. C., Obrda O., Sundblad K. J., Gumbs C. P., Ebel D. L., Quan H., Larson P. J., Schwartz J. I., Musliner T. A., Gertz B. J., Brater D. C., Yao S. L., *Ann. Intern. Med.*, **133**, 1–9 (2000).
21) Schlondorff D., *Kidney Int.*, **44**, 643–653 (1993).
22) Woywodt A., Schwarz A., Mengel M., Haller H., Zeidler H., Köhler L., *J. Rheumatol.*, **28**, 2133–2135 (2001).
23) Rasheed A., Theja I., Ashok Kumar K. C., Lavanya Y., Ravindra Royal P., Vamsee Krishna S., *Der Pharma Chemica*, **1**, 59–71 (2009).
24) Makhija T. D., Somani R. R., *Der Pharmacia Lettre*, **2**, 300–309 (2010).
25) Rasheed A., Ashok Kumar K. C., *Int. J. Curr. Pharm. Res.*, **1**, 47–55 (2009).
26) Mishra A., Veerasamy R., Jain P. K., Dixit V. K., Agrawal R. K., *Eur. J. Med. Chem.*, **43**, 2464–2472 (2008).
27) Akhter M., Husain A., Azad B., Ajmal M., *Eur. J. Med. Chem.*, **44**, 2372–2378 (2009).
28) Bekhit A. A., El-Sayed O. A., Aboulmagd E., Park J. Y., *Eur. J. Med. Chem.*, **39**, 249–255 (2004).
29) Fadeyi O. O., Obafemi A. C., Adewunmi O. C., Iwalewa O. E., *Afr. J. Biotechnol.*, **3**, 426–431 (2004).
30) Abou El Ella A. D., Taha A. R., *Bull. Fac. Pharm. Cairo Univ.*, **45**, 267–281 (2007).
31) Eissa M. A. A., *Bull. Fac. Pharm. Cairo Univ.*, **36**, 99–105 (1998).
32) Abou-Seri S. M., *Eur. J. Med. Chem.*, **45**, 4113–4121 (2010).
33) Novoa de Armas H., Paneque Quevedo A., Henao Martinez J. A., *Powder Diffraction*, **13**, 175–177 (1998).
34) Ebeid Y. M., Mikhael N. A., El-Moghazy M. S., Eissa A.-H. A., *Bull. Fac. Pharm. Cairo Univ.*, **32**, 361–368 (1994).
35) Buck W. B., Osweiter G. D., Van Gelder A., "Clinical and Diagnostic Veterinary Toxicology," 2nd ed., Kendall, Hunt Publishing Co., Iowa, 1976, p. 5211.
36) Winter C. A., Risley E. A., Nuss G. W., *Proc. Soc. Exp. Biol. Med.*, **111**, 544–547 (1962).
37) Abbott F. V., Melzack R., *J. Brain Res.*, **251**, 149–155 (1982).
38) Tjølsen A., Hole K., "Animal Models of Analgesia in the Pharmacology of Pain," Vol. 130, ed. by Dickenson A., Besson J., Springer, Berlin, 1997, pp. 1–20.
39) Loux J. J., DePalma P. D., Yankell S. L., *Toxicol. Appl. Pharmacol.*, **22**, 672–675 (1972).
40) Sharon P., Stenson W. F., *Gastroenterology*, **88**, 55–63 (1985).
41) Nwanjo H. U., Okafor M. C., Oze G. O., *J. Clin. Sci.*, **23**, 10–12 (2005).
42) Panda N. C., "Kidney in: Textbook of Biochemistry and Human Biology," 2nd ed., Prentise Hall India, 1999, pp. 290–296.
43) Lorke D., *Arch. Toxicol.*, **54**, 251–287 (1983).
44) Paget G. E., Barnes J. M., Laurence D. R., Bacharach A. L., "Evaluation of Drug Activities: Pharmacometrics," Academic Press, London, 1964, pp. 135–166.
45) Eddy N. B., Leimbach D., *J. Pharmacol. Exp. Ther.*, **107**, 385–393 (1953).
46) Collier H. O., Dinneen L. C., Johnson C. A., Schneider C., *Br. Pharmacol. Chemother.*, **32**, 295–310 (1968).
47) Mascolo N., Izzo A. A., Autore G., Maiello F. M., Di Carlo G., Capasso F., *J. Pharmacol. Exp. Ther.*, **272**, 469–475 (1995).
48) Miller S., Hubble M., Duncan B., "Handbook of Solution-Focused Brief Therapy," Jossey-Bass, San Francisco, 1996.
49) Empey L. R., Jewell L. D., Garg M. L., Thomson A. B., Cladinin M. T., Fedorak R. N., *Can. J. Physiol. Pharmacol.*, **69**, 480–487 (1991).
50) Reitman S., Frankel S., *Am. J. Clin. Pathol.*, **28**, 55–60 (1957).
51) Walter M., Gerarade H., *Microchem. J.*, **15**, 231–243 (1970).
52) Henary R. J., Cannon D. C., Winkleman J. W., "Clinical Chemistry Principles and Techniques," 2nd ed., Harper & Roe, New York, 1974.
53) Doumas B. T., Watson W. A., Biggs H. G., *Clin. Chim. Acta*, **31**, 87–96 (1971).
54) Wills M. R., Savory J., *Ann. Clin. Lab. Sci.*, **11**, 292–299 (1981).
55) Kroll M. H., Roach N. A., Poe B., Elin R. J., *Clin. Chem.*, **33**, 1129–1132 (1987).