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Full Length Research Paper

Pharmacological activities evaluation of some new pyrazolo-pyrimidino-pyridazine derivatives

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Pyrazolo-pyrimidino-pyridazine derivatives are potent compounds with pharmacological activities like anti-inflammatory, analgesic and anti-microbial. Pharmacological screenings proved that the compounds 1 to 6 had high activities comparable with reference drugs. Anti-inflammatory evaluation of the tested compounds comparable with Indomethacin showed that edema inhibition increased with time. All the tested compounds had no gastric ulcerogenic effect on mice. Also, analgesic activity of the tested compounds comparable with valdecoxib showed that these compounds had activities. Analgesic activities of the tested compounds increased with time. The tested compounds were able to inhibit the growth of the Gram-positive, Gram-negative bacteria and fungi.

Key words: New hetero cyclic compounds, pharmacological screening, anti-inflammatory, analgesic, anti-microbial.

INTRODUCTION

In previous works we reported that certain substituted pyrazoles, pyridazines and their derivatives had antibacterial, antifungal (Fayed et al., 2009; Nagawade et al., 2009; Bahashwan et al., 2010) and anti-inflammatory (Al-Harbi et al., 2010) activities. Also, pyrazolo, pyrimidino and pyridazino derivatives have been reported to possess a variety of other pharmacological activities such as central nervous system (CNS) depressant (Julino et al., 1998; Abdou et al., 2004), neuroleptic, turberculostatic (Ali, 2009; Ghorab et al., 2004) and anti-parkinsonism (Bahashwan, 2011). Pyrazolo derivatives were reported as inhibitors of glycogen synthase kinase-3(GSK-3) (Witheringron et al., 2003) and potent antitumor agents (Lin et al., 2007). In view of these observations, we have synthesized some new poly heterocyclic fused ring systems containing pyridazine nuclei, and tested them for analgesic, anti-inflammatory and anti-microbial activities, in comparison to some reference drugs.

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MATERIALS AND METHODS

Animals

Female albino Sprague Dawley mice 16 to 18 g were purchased from Theodor Bilharz Research Institute (TBRI), Egypt. Approval of the institutional animal ethical committee for the animals' studies was obtained from the Office of Environmental Health and Radiation Safety, ACUC Protocol # 1096-5. The animals were maintained according to accepted standards of animal care

(Mahmoud, 1984).

Anti-inflammatory activity

Compounds 1 to 6 (Figure 1) (Amer et al., 2011) were dissolved in 0.5% carboxymethyl cellulose (CMC) as a homogeneous solution. One hundred and eight mice were divided into eighteen groups (six animals each). Anti-inflammatory activity of the compounds was studied in mice using carrageenan paw oedema method. A suspension of the tested compound and the reference drug, Indomethacin in aqueous solution, was administered orally at a dose 5 mg/kg. Control animals were treated with 0.5 % CMC only. After 30 min., 0.01ml of freshly prepared 1.0% carrageenan solution (in formol saline) was injected into the sub-plantar region of the right hind paw according to Hernandez-Perez et al. (1995). The right paw volume was measured using a digital plethysmometer (Model 7150) directly before and after 1, 2, 3 h intervals after administration of the tested compounds.

Ulcerogenic activity

Seventy-two mice were divided into twelve groups. Ulcerogenic activity was evaluated after oral administration of the tested compounds or indomethacin at doses of 10, 50, and 100 mg/kg. Control mice received 0.5% CMC. Food but not water was removed 24 h before administration of the test compounds. After 6 h, the mice were sacrificed; the stomach was removed and opened along the greater curvature, washed with distilled water and cleaned gently by dipping in saline. The mucosa damage for each stomach was examined using a stereoscopic microscope and compared with the reference drug indomethacin according to reported procedure (Ikuta et al., 1987).

Acute toxicity

The median lethal doses (LD_{50}) of the most active compounds 5 to 6 were determined in mice (Sztaricskai et al., 1999). Groups of male adult mice, each of six animals, were injected Intraperitoneally (i.p.) with graded doses of each of the test compounds. The percentage of mortality in each group of animals was determined 24 h after injection. Computation of LD_{50} was processed by a graphical method.

Analgesic activity

Sixty mice of both sexes were divided into 10 groups. One group was kept as control (received formol saline), the second group received vehicle (gum acacia) and the third one received valdecoxib as a reference drug, whereas the other groups received the test compounds subcutaneously (S.C.). Mice were dropped gently in a dry glass beaker of 1 Liter capacity maintained at 55 to 55.5°C. Normal reaction time in seconds for all animals was determined at time intervals of 10, 30, 60 and 120 min. This is the interval extending from the instant the mouse reaches the hot beaker till the animals licks its feet or jumps out of the beaker (dose 5mg/kg) (Tjolsen et al., 1991). The relative potencies to valdecoxib were determined (Table 3).

Anti-microbial screening

All the compounds were evaluated *in vitro* for their anti-microbial activities. The anti-microbial activity was evaluated against three bacterial strains *Staphylococcus. aureus, S. epidermidis* and *Escherichia coli* and three fungal strains *Aspergillus fumigates, A. niger* and *A. alternate*, employing the nutrient agar disc diffusion method (Arthington et al., 2000) at 100 µg/ml concentration. Dimethyl sulphoxide (DMSO) was used as blank and it exhibited no activity against any of the used organisms. The anti-microbial activity was determined by measuring the inhibition zone (Table 4), after 16 to 20 h of incubation at 37°C for bacterial strains and 3 to 4 days at 37°C for fungal strains. Tetracycline and Ketoconazole were used as standard drugs bacterial and fungal strains, respectively at 30 µg/ml concentration.

The minimum inhibitory concentration (MIC)

A current definition of MIC is "the lowest concentration which results in maintenance or reduction of inoculums viability". The determination of the MIC involves a semi-quantitative test procedure which gives an approximation to the least concentration of antimicrobial agent needed to prevent microbial growth. The method displays tubes of growth broth containing a test level of pre-servatives, into which inoculums of microbes was added. The end result of the test was the minimum concentration of anti-microbial.

The serial dilution technique (Mostahar et al., 2006) was applied for the determination of MIC of the tested compounds 1 to 6 against two species of bacterial strains (*S. aureus and E. coli*) and two species of fungal strains (*A. niger* and *A. alternata*). Dilution series were set up with 6.25, 12.5, 25, 50 and 100 µg/ml of nutrient broth medium to each tube; 100 µl of standardized suspension of the test microbes (10^7 cells/ ml) were added and incubated at 37°C for 24 h (Table 5).

Cytotoxicity bioassay

Brine shrimp lethality bioassay (Jaki et al., 1999; Mayer et al., 1982) is a recent development in the assay which indicates cytotoxicity as well as a wide range of pharmacological activities (for example, antimicrobial, anticancer, antiviral, insecticidal, pesticidal, acquired immunodeficiency syndrome (AIDS), etc). In this method, the eggs of the brine shrimp, *Artemia salina* (Leach), were hatched for 48 h to mice shrimp, 38 g of sea salt was weighed, dissolved in one liter of distilled water, filtered off and was kept in a small tank. The eggs were then added to the divided tank. Constant oxygen supply was provided and temperature of 37°C was maintained for 48 h to hatch the shrimp known as nauplii (Larvae). The solutions of compounds 1 to 6 were prepared by dissolving 10 mg of each compound in 2 ml of DMSO. From this stock, a series of solution 5, 10, 20, 40 and 80 µg/ml were transferred to fifteen vials (three for each dilutions were used for each test sample and LC_{50} is the mean of three values) and one vial was kept as control having 2 ml of DMSO. Then, about 10 brine shrimp nauplii were applied to each of all experimental vials and control vial. The number of the nauplii that died after 24 h was counted. The resulting data were transformed to the probit analysis for the determination of LC_{50} values for the five tested compounds (Table 6).

Statistical analysis

Assay results are shown as mean ± standard error (SE). Statistical analyses were carried out with Sigma Plot software (SPSS Inc., Chicago, USA). One-way analysis of variance (ANOVA) followed by Tukey's posthoc test was used to assess the presence of significant differences. Differences were considered statistically significant at $p \leq 0.05$.

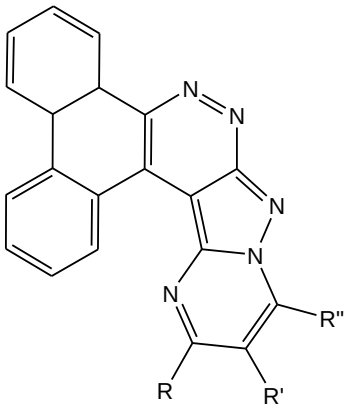

Figure 1. Structure of the tested compounds 1 to 6.

RESULTS AND DISCUSSION

Three pharmacological activities namely; anti-inflammatory, analgesic and anti-microbial activities were tested despite their different biological receptors. Six representative compounds 1 to 6 (Figure 1), were studied with respect to their anti-inflammatory, analgesic and anti-microbial activities. The activities of these compounds are different according to the structure and function groups (Tables 1 to 7).

Anti-inflammatory activity

The results of anti-inflammatory activity after 1 h showing the percent inhibition of edema obtained by the reference drug and tested compounds, respectively is shown in Table 1. Results show that compounds 3 and 5 possess weak anti-inflammatory activity (7.3 ± 1.2, 14.3 ± 1.7% of inhibition), respectively in comparison to that of indomethacin (44.7 ± 1.8 %). Compounds 1, 2 and 6 possess intermediate activity (34.3 ± 1.3, 28.4 ± 1.3, 31.3 ± 1.4% of inhibition). Compound 4 showed activity of 41.7 ± 1.6%, nearly equal to that of indomethacin. The results of anti-inflammatory screening after 2 h showed the percent inhibition of edema obtained by the reference drug and tested compounds, respectively. Results show that compounds 3 and 5 possess weak anti-inflammatory activity (13.5 ± 1.1, 16.7 ± 1.6% of inhibition), respectively in comparison to that of indomethacin (52.4 ± 1.7%). Compounds 1, 2 and 6 possess intermediate activity (37.0 ± 1.4, 24.3 ± 1.8, 35.4 ± 1.7% of inhibition). Compound 4 showed activity of 45.1 ± 1.3%, nearly equal to that of indomethacin. The results of anti-inflammatory screening after 3 h showed the percent inhibition of edema obtained by the reference drug and tested compounds, respectively. Results show that compounds 3 and 5 possess weak anti-inflammatory activity (14.3 ± 1.8, 15.3 ± 1.1% of inhibition), respectively in comparison to that of indomethacin (61.2 ± 1.4%). Compounds 1, 2 and 6 possess intermediate activity (39.5 ± 1.8, 29.6 ± 1.3, 36.7 ± 1.7% of inhibition). Compound 4 showed activity of 49.1 ± 1.9%, nearly equal to that of indomethacin (Table 1).

Ulcerogenicity and acute toxicity

Compounds 1 and 4 were screened for their ulcerogenic activity at dose levels of 10, 50 and 100 mg/kg (Table 2). The tested compounds 1and 4 showed no ulcerogenic activity of 1.4 to 2.1 mm.

Acute toxicity

LD_{50} of compounds 1 and 4 was found to be 145, 155 and 165 mg/kg, respectively, whereas, LD_{50} of indomethacin was 50 mg/kg.

Analgesic activity

All tested compounds exhibited analgesic activity in the hot plate assay. Ten min after administration, compounds 3 and 5 exhibited weak analgesic activities (0.31 ± 0.01, 0.41 ± 0.03), respectively in comparison to that of valdecoxib (1.00 ± 0.01). Compounds 1 and 2 were found to possess intermediate activities (0.64 ± 0.01, 0.53 ± 0.02), respectively. Compounds 4 and 6 exhibited strong activities (0.72 ± 0.03, 0.78 ± 0.02), respectively. Comparison of analgesic potency of test compounds to valdecoxib after 30 min showed that compound 3 to possessed weak analgesic activities (0.37 ± 0.01) in comparison to that of valdecoxib (1.00 ± 0.01). Compounds 2 and 5 possess intermediate activities (0.61 ± 0.04, 0.47 ± 0.03), respectively. Compounds 1 and 6 possess strong activities (0.77 ± 0.03, 0.89 ± 0.02), respectively. Compound 4 possesed very strong activities (0.91 ± 0.08). Comparison of analgesic potency of test compounds to valdecoxib after 60 min showed that compound 3 to possessed weak analgesic activities (0.42± 0.04) in comparison to that of valdecoxib (1.00 ± 0.01). Compounds 2 and 5 possess intermediate activities (0.67 ± 0.01, 0.53 ± 0.03), respectively. Compounds 1 and 6 possess strong activities (0.83 ± 0.02, 0.97 ± 0.02), respectively. Compound 4 possessed very strong activity which was more than the activity of valdecoxib (1.12±0.01).

Comparison of analgesic potency of test compounds to valdecoxib after 120 min showed compound 3 to possess weak analgesic activities (0.49 ± 0.03) in comparison to that of valdecoxib (1.00 ± 0.01). Compounds 2 and 5 possessed intermediate activities (0.69 ± 0.02, 0.59 ± 0.01), respectively. Compound 1 possessed strong

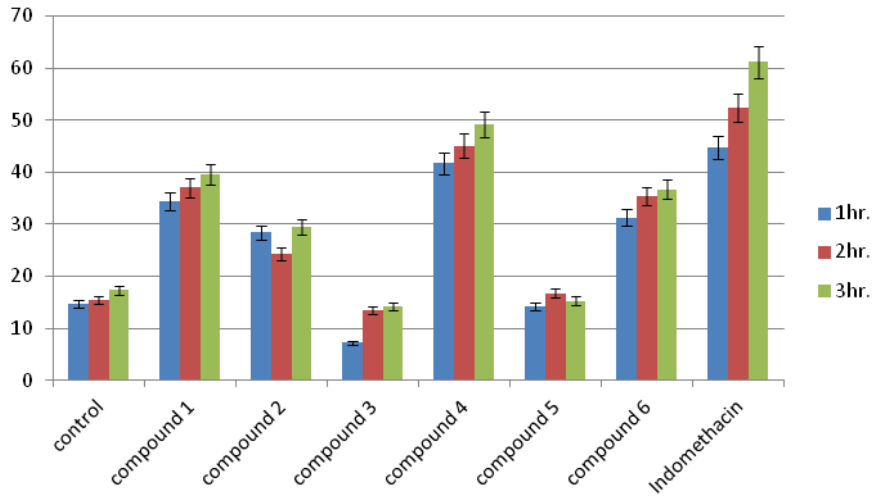

Figure 2. Anti-inflammatory activity of the tested compounds.

activities (1.02 ± 0.03) which was equal to the activity of valdecoxib. Compounds 4 and 6 possessed very strong activity which was more than the activity of valdecoxib (1.27 ± 0.04, 1.23 ± 0.04), respectively (Table 3).

Anti-microbial activity

The organic compounds 1 to 6 were evaluated *in vitro* for their anti-microbial activity. The antimicrobial activities were carried out against three bacterial strains (*S. aureus, S. epidermidis* and *E. coli*) and three fungal strains (*A. fumigates*, *A. niger* and *A. alternate*). The preliminary screening results indicated that most of the compounds showed moderate to good antimicrobial activity. From the inhibition zone diameter data analysis, It was found that compound 1 showed in general, moderate inhibition against *S. aureus* and *S. epidermidis*. However, compound 1 showed lower inhibition against *E. coli* and the three species of fungal strains. Compounds 2, 3 and 5 showed in general, good inhibitions against the species of bacterial and fungal strains but compound 2 showed only moderate inhibitions against *A. alternate*, compound 3 exhibited lower inhibitions against *A. niger* and *A. alternata*. Compound 4 showed moderate inhibitions against *S. aureus* and *A. fumigates*, but compound 4 showed lower inhibitions against *S. epidermidis*, *E. coli*, *A. niger* and *A. alternata*. Compound 6 showed moderate inhibitions against *E. coli*. However, compound 6 showed lower inhibitions against other species of bacterial and fungal strains (Table 4).

The minimum inhibitory concentration (MIC)

The MIC (µg/ml) of the most active compounds 2, 3 and 5 against two species of bacteria (*S. epidermidis and E. coli*) and also two species of fungi (*A. niger* and *A. alternate*) were determined (Table 5). Compounds 3, 4 and 5 demonstrated good inhibitions against the selected bacterial and fungal strains.

Cytotoxicity activity

The LC_{50} values of tested compounds 2, 3 and 5 were found to be 3.54, 6.49 and 2.31 µg/ml, respectively (Table 6). The standard drug Bleomycin has LC_{50} value at 0.41 g/ml. The lowest LC_{50} value was found in the case of compound 3, indicating higher cytotoxicity than the other compounds. Compounds 2 and 5 showed potent biocidal activity against brine shrimp

DISCUSSION

Three pharmacological activities namely; anti-inflammatory, analgesic and antimicrobial activities were tested despite their different biological receptors. Six representative compounds 1 to 6 (Figure 1) were studied for the above purpose. The activities of these compounds are different according to the structure and functional groups (Tables 1 to 6, Figures 2 and 3). Compounds 4 showed activity nearly equal to that of indomethacin. Evaluation results of anti-inflammatory activity of the test compound was comparable with indomethacin over time periods of 1, 2 and 3 h.

The data generated showed that edema inhibition increased with time. Edema inhibition of compounds 4 and 6 by time from 31.3±1.4 to 49.1±1.9 respectively nearly equal the activity of Indomethacin (Table 1) and

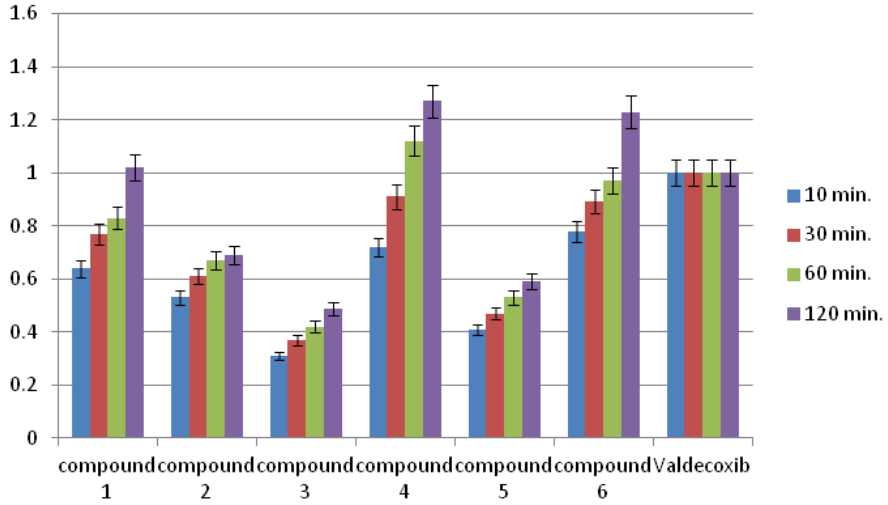

Figure 3. Analgesic activities of tested compounds 1-6.

Table 1. Anti-inflammatory activity of the tested compounds.

Compound number	Edema inhibition (mean ± SEM)[a,b] (%)		
	1 h	2 h	3 h
Control	14.6±1.2	15.5±1.2	17.3±1.6
1	34.3±1.3	37.0±1.4	39.5±1.8
2	28.4±1.3	24.3±1.8	29.6±1.3
3	7.3±1.2	13.5±1.1	14.3±1.8
4	41.7±1.6	45.1±1.3	49.1±1.9
5	14.3±1.7	16.7±1.6	15.3±1.1
6	31.3±1.4	35.4±1.7	36.7±1.7
Indomethacin	44.7±1.8	52.4±1.3	61.2±1.4

[a]Dose 5 mg/kg b.m (p.o.), [b]n = 6

Table 2. Gastric ulceration in mice.

Compound number	Dose(mg/kg)		
	10	50	100
Control	0/6	0/6	0/6
1	0/6	0/6	0/6
4	0/6	0/6	0/6
Indomethacin	3/6 (1.5±0.2)[b,c]	5/6 (1.9±0.2)[b,c]	6/6 (2.2±0.2)[b,c]

[a]number of rats lesions bigger than 0.5 mm in length per total no of rb, [b]mean ulcer lesions ± SEM (mm) (n = 6) in parentheses, [c]significant difference at $p < 0.05$ compared to the control.

the tested compound showed no ulcerogenic activity of 1.4 to 2.1 mm. All tested compounds exhibited analgesic activity in the hot plate assay. Interestingly, compound 4 showed more activity than valdecoxib after 1 h, and compound 6 showed equal activity of valdecoxib after 1 h. Also, compound 1 showed activity equal to valdecoxib after 2 h, but compounds 4 and 6 showed more activity than valdecoxib after 2 h. The analgesic activities of the tested compounds were comparable to valdecoxib over time 10, 30, 60 and 120 min. It can be observed that the

Table 3. Analgesic activities of tested compounds 1 to 6.

Compound number	Comparative analgesic potency to valdecoxib after time			
	10 min	30 min	60 min	120 min
1	0.64±0.01	0.77±0.03	0.83±0.02	1.02±0.03
2	0.53±0.02	0.61±0.04	0.67±0.01	0.69±0.02
3	0.31±0.01	0.37± 0.01	0.42±0.04	0.49±0.03
4	0.72±0.03	0.91± 0.08	1.12±0.01	1.27±0.04
5	0.41±0.01	0.47 ± 0.03	0.53±0.03	0.59±0.01
6	0.78±0.03	0.89 ± 0.02	0.97±0.02	1.23±0.04
Valdecoxib	1.00±0.01	1.00 ±0.01	1.00±0.01	1.00±0.01

All results were significantly different from the standard and control value at $p \leq 0.05$.*

Table 4. The Antimicrobial activity of tested compounds.

Compound number	Diameter of the inhibition zonea (mm)					
	Bacteria			Fungi		
	S. aureus	*S. epidermidis*	*E. coli*	*A.fumigatus*	*A. niger*	*A. althernata*
1	16	13	9	9	10	5
2	24	22	20	20	18	11
3	27	27	26	17	12	8
4	14	11	5	17	9	10
5	26	23	20	21	19	19
6	9	11	13	11	7	2
Tertacyclineb	30	25	28	-	-	-
Ketoconazoleb	-	-	-	18	20	21

[a]15 mm or less: resistant or no inhibition, 16 to 20 mm: moderate inhibition, 20 mm or more: maximum inhibition. [b]The concentration of used standard drugs was 30 µg/ml

Table 5. The minimum inhibitory concentration (MIC, µg/ml) of tested compounds 2,3 and 5.

Organism	The minimum inhiditory conectration (MIC)			
	2	3	5	Standard[a]
S. aureus	50	50	50	6.25
E. coli	25	12.5	>100	12.5
A. niger	50	2.5	>100	6.25
A. althernata	50	>100	25	6.25

[a]Tetracycline and ketoconazole were used as standard drugs against bacterial and fungal strains, respectively.

Table 6. Cytotoxicity activity of tested compounds 2, 3 and 5.

Sample	95% confidence limite ppm			Regression equation	X^2 (df)
	LC_{50}	Lower	Upper		
2	3.54	2.08	6.02	Y = 3.98 + 1.85X	3.38 (2)
3	6.49	4.15	10.15	Y = 3.17 + 2.27X	0.35 (2)
5	2.31	1.3	4.1	Y = 4.36 + 1.78X	0.32 (2)
Bleomycin[a]	0.41	0.27	0.62	Y = 3.16 + 1.98X	0.62 (2)
Gallic acid[a]	4.53	3.33	6.15	Y = 3.93 + 1.62X	1.25 (2)

[a]Bleomycin and gallic acid were used as standard drugs in cytotoxicity activity.

Table 7. Various functional groups present in compounds 1 to 6.

Compound	R	R'	R''
1	$-C_6H_5$	-H	$-NH_2$
2	$-C_6H_5$	-H	$-C_6H_5$
3	$-C_6H_5$	-H	$-p(OCH_3)C_6H_4$
4	-H	$-Coo\ C_2H_5$	$-NH_2$
5	$-C_6H_5$	$-Co\ C_6H_5$	$-NH_2$
6	$-C_6H_5$	$-Coo\ C_2H_5$	$-NH_2$

Conclusion

The objective of the present study was to investigate the anti-inflammatory, analgesic and anti-microbial activities of new pyrazolo-pyridazino-pyrimidino derivatives 1 to 6. The newly tested compounds 1 to 6 and the standard drug Indomethacin were found to exhibit essentially equipotent anti-inflammatory activity (7.3 ± 1.2 to 49.1 ± 1.9%). Also, the newly tested synthesized compounds 1 to 6 and the standard drug valdecoxib were found to exhibit essentially equipotent analgesic activity (0.31 ± 0.01 to 1.23 ± 0.01). Further newly synthesized 1 to 6 were able to inhibit the growth of the Gram-positive, Gram-negative bacteria and fungi. Compound 5 could be identified as most biologically active as anti-inflammatory compound, compound 6 as analgesic and compound 5 as antimicrobial. Activity of compound 5 may be due to the existence of aromatic carbonyl ($-COC_6H_5$) and amino group (NH_2) and also the activity of compound 6 due to ester group ($-COOC_2H_5$).

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