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Original Article

Synthesis of Substituted 7-Methyl-2,8-Dihydropyrazolo-[1,5-a][1,3,5]-Triazine Derivatives for Anti-Inflammatory and Anti-Microbial Screening

Niraj V. Patel* and Dr. Dhrubo Jyoti Sen

Department of Pharmaceutical Chemistry, Shri Sarvajanik Pharmacy College, Gujarat Technological University, Near Arvind Baug, Mehsana-384 001, Gujarat, India

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Address for Correspondence
Department of Pharmaceutical Chemistry, Shri Sarvajanik Pharmacy College, Gujarat Technological University, Near Arvind Baug, Mehsana-384 001, Gujarat, India
E-mail: ppnv123@yahoo.com

ABSTRACT

A series of condensed pyrazolo and triazine derivatives have been synthesized to their physical property melting point, % yield, retention factor (R_f) have been evaluated. Structure is confirmed by UV, IR, NMR and Mass. All synthesized compounds are screened for two biological activities (Anti-inflammatory and Anti-microbial). Anti-inflammatory activity has been carried out by rat paw edema method while anti-microbial activity has been carried out by filter paper disc method. MIC (minimum inhibitory concentration) for anti-microbial screening has been carried out by tube dilution method.

Keywords: Pyrazolo-triazine, Retention factor, MIC, Anti-inflammatory, Anti-microbial, Zone of inhibition, SEM (standard error mean).

INTRODUCTION

Bacterial infections often produce pain & inflammation. In normal practice, two groups of agents (chemotherapeutics & NSAIDs) are prescribed simultaneously. Unfortunately, none of the drugs possesses these activities in a single component. Therefore, our aim is to find a compound having dual effect both analgesic-anti-inflammatory & anti-microbial activities.

It has been found from the literature survey pyrazole ring condensed derivative show different activity like antifungal, protein kinase inhibition, potential estrogen receptor ligand activity, analgesic, anticancer, antimicrobial, anti-inflammatory.

It has been found from the literature survey triazine ring condensed derivative show different activity like anti-coagulant, antibacterial, and insecticidal activity.

It has been planned to attach pyrazole ring with triazine and their condensed derivatives

to screen for various biological activities like anti-inflammatory, antimicrobial, etc.

SYNTHETIC PROCEDURE

Compound No-1 (5-methyl-2,4-dihydro-3*H*-pyrazol-3-one)

13ml of ethylacetoacetate has been mixed with 5ml hydrazine hydrate in a conical flask and heated on water bath for some time. It was cooled in ice bath for solidification of liquid mixture. It was then filtered and recrystallized with water.[1]

Compound No-2 (2-benzoyl-5-methyl-2,4-dihydro-3*H*-pyrazol-3-one)

5gm of 5-methyl-2,4-dihydro-3*H*-pyrazol-3-one product has been dissolve in 5% NaOH solution then 12ml benzoyl chloride has been added and heated on water bath to produce semisolid like product then after cooling the reaction mixture to produced white colour solid product which has been filtered and recrystallized with methanol. [1]

Compound No-3a (7-methyl-4-phenylpyrazolo[1,5-α][1,3,5]triazine-2(8*H*)-thione)

5gm of 2-benzoyl-5-methyl-2,4-dihydro-3*H*-pyrazol-3-one and 2gm urea has been dissolved in methanol and refluxed for 9 hours [in microwave 30minute at 215 watt] then cooled the reaction mixture to produce white solid product which has been filtered and recrystallized with methanol. [1]

Compound No-3b (7-methyl-4-phenylpyrazolo[1,5-α][1,3,5]triazine-2(8*H*)-thione)

5gm of 2-benzoyl-5-methyl-2,4-dihydro-3*H*-pyrazol-3-one and 2gm thiourea has been dissolved in methanol and refluxed for 12 hours [microwave 50 minute at 215 watt] then cooled the reaction mixture to produce white solid product which has been filtered and recrystallized with methanol. [1]

Compound No-3c (7-methyl-4-phenylpyrazolo[1,5-α][1,3,5]triazin-2(8*H*)-imine)

5gm of 2-benzoyl-5-methyl-2,4-dihydro-3*H*-pyrazol-3-one and 2gm guanidine which has been dissolved in methanol and refluxed for 16 hours [in microwave 60 minute at 215 watt] then cooled the reaction mixture to produce white solid product which has been filtered and recrystallized with methanol. [1]

Compound No-4 (2-(chloroacetyl)-5-methyl-2,4-dihydro-3*H*-pyrazol-3-one)

10 gm of 5-methyl-2,4-dihydro-3*H*-pyrazol-3-one has been dissolved in glacial acetic acid (if not dissolved then heated on water bath) then added 10ml chloroacetyl chloride which has been heated at 80-90^{o}C under stirring condition for 4 to 5 hours then triethylamine has been added in reaction mixture under cooled condition to produce solid product which has been filtered and washed with ethyl acetate.[2]

Compound No-5 (5-methyl-2-(morpholin-4-ylacetyl)-2,4-dihydro-3*H*-pyrazol-3-one)

5 gm of 2-(chloroacetyl)-5-methyl-2,4-dihydro-3*H*-pyrazol-3-one has been dissolved in acetonitrile then added 2.5 ml morpholine and 1gm K_2CO_3 (as a catalyst amount) then heated on water bath for 10min after the reaction undissolved K_2CO_3 is removed by filtration then filtrate cooled on the ice bath to produced solid product and it washed with ethylacetate.[3]

Compound No-6a (7-methyl-4-(morpholin-4-ylmethyl)pyrazolo[1,5-α][1,3,5]triazin-2(8*H*)-one)

5 gm of 5-methyl-2-(morpholin-4-ylacetyl)-2,4-dihydro-3*H*-pyrazol-3-one has been dissolved in methanol then added 2gm urea which has been refluxed in microwave for 25 min at 215 watt which has been heated on water bath to evaporated the methanol,

then cooled the reaction mixture to get product and recrystallized with methanol.[1]

Compound No-6(b) (7-methyl-4-(morpholin-4-ylmethyl)pyrazolo[1,5-α][1,3,5]triazine-2(8*H*)-thione)

5 gm of 5-methyl-2-(morpholin-4-ylacetyl)-2,4-dihydro-3*H*-pyrazol-3-one has been dissolved in methanol then added 2gm thiourea which has been refluxed in microwave for 35 min at 215 watt which has been heated on water bath to evaporated the methanol then cooled the reaction mixture to get product and recrystallized with methanol.[1]

Compound No-6(c) (7-methyl-4-(morpholin-4-ylmethyl)pyrazolo[1,5-α][1,3,5]triazin-2(8*H*)-imine)

5 gm of 5-methyl-2-(morpholin-4-ylacetyl)-2,4-dihydro-3*H*-pyrazol-3-one has been dissolved in methanol then added 2gm guanidine which has been refluxed in microwave for 42 min at 215 watt which has been heated on water bath to evaporated the methanol Then cooled the reaction mixture to get product and recrystallized with methanol.[1]

Biological Evaluation

Anti-Inflammatory Screening Method Principle

Inflammation is a tissue-reaction to infection, irritation or any foreign substances. It is a part of host defense mechanism. The inflammatory reaction is readily produced in mice in the form of paw edema with the help of irritants. Substances such as carrageenan, formalin, bradykinin, histamine, mustard. When injected to the dorsum of the foot of mice, they produce acute paw edema within few min. of injection. Carrageenan is a sulphated polysaccharide obtained from sea weed (Rhodophyceae) causing the releasing of histamine, 5-HT, bradykinin and prostaglandins. It produces inflammation and edema.[4]

Anti-inflammatory activity

The anti-inflammatory activity of newly synthesized substituted condensed pyrazolone and Triazine derivatives was carried out using Carrageenan induced rat hind paw edema method.

Method: Inhibition of carrageenan induced inflammation in rat paw

Animals used: Swiss Albino rat

No. of animals used: 3 (in each group)

Dose of compound: 100mg/kg

Dose of std. drug: 100mg/kg (Phenylbutazone)

Route of administration: Oral (suspended in 1% tween-80 solution)

Requirements

- Instruments: fluid displacement plethysmometer.
- Inflammation inducer: carrageenan solution (1%w/v) in saline solution was prepared and injected (0.1ml) in sub planter region to induce paw edema.
- Chemicals: tween-80
- Standard drug: Phenylbutazone (100mg/kg) aq. suspension was prepared using solution of tween-80 as a suspending agent.
- Test compounds: suspension of compounds was prepared and administered orally similar to that of standard drug.
- Apparatus: feeding needles (for oral dosing), syringes (1ml, 2ml) and sample tubes (to prepare suspension of test compounds).

Experimental design and procedure

- Rats were assigned into 8 groups of 3 animals each. They were marked with picric acid for individual animal identification. The animals were starved overnight with water and libitum prior to the day of experiment.

- First 0.1ml of 1%w/v of carrageenan in normal saline was injected in to the subplanter region of the left hind paw of rat.
- Synthetic compounds & std. compounds were administered after 1hr of the injection of carrageenan. Dose volume not exceeding 0.5ml/100gm orally administered.
  - Group I: The solvent control received vehicle orally.
  - Group II: Positive control received phenylbutazone (100mg/kg).
  - Group III: Received test compound-3a at a dose of 100mg/kg suspended in 1%w/v tween-80.
  - Group IV: Received test compound-3b at a dose of 100mg/kg suspended in 1%w/v tween-80.
  - Group V: Received test compound-3c at a dose of 100mg/kg suspended in 1%w/v tween-80.
  - Group VI: Received test compound-6a at a dose of 100mg/kg suspended in 1%w/v tween-80.
  - Group VII: Received test compound-6b at a dose of 100mg/kg suspended in 1%w/v tween-80.
  - Group VIII: Received test compound-6c at a dose of 100mg/kg suspended in 1%w/v tween-80.
- Immediately after administered the test compounds & Std. compounds, the volume of its displacement was measured using plethysmometer. The reading was recorded at 0, 1, 2, 3 hrs.
- The percentage inhibition calculates by the following equation.[5]

$$\% \text{ Inhibition} = \frac{\text{Control} - \text{test}}{\text{control}} \times 100$$

Antimicrobial Screening Method

Anti-Bacterial Screening (filter paper disc method)

Anti-Bacterial Screening

Anti-Bacterial screening of all the derivatives was done by using filter paper disc method. It is based upon a comparison of inhibition of growth of micro-organisms by measured concentrations of test compounds with that produced by known concentration of a standard antibiotic.[6]

Name of microorganism:-

- **Gram +ve microorganisms**

Staphylococcus aureus (MTCC No. 96)

- **Gram -Ve microorganisms**

Escherichia coli (MTCC No. 521).

Preparation of medium:-

- Nutrient agar 2%
- Peptone 1%
- Beef extract 1%
- Sodium chloride 0.5%
- Distilled water up to 100ml

All the ingredients have to be weighed and add to water. This solution has been heated on water bath for about one and half-hour till it became clear. This nutrient media was sterilized by autoclave.

Preparation of test compounds:-

Specified quantity (100mg) of the compound was accurately weighed and dissolved in 100ml of DMSO to get the 1000µg/ml stock solution. Further dilution was made to obtain the concentration in the range 100µg/ml, 200µg/ml, and 300µg/ml.

Apparatus:-

All the apparatus like Petri dishes, pipettes, glass rods, test-tubes etc. to be properly wrap with papers and sterilize in hot air oven.

Procedure:-

- All the Petri dishes were sterilized in oven at 160°C for 1 hour.
- Agar media, Adsorbent paper and test solutions were sterilized in autoclave at 121°C at 15psi for 15min.
- Molten sterile agar to be poured in sterile Petri dishes asceptically.

- The agar was allowed to cool and the bacterial suspension was poured into the petridishes asceptically.
- Impregnated absorbent paper with solution of the drugs (test and standard) in the agar plate petridishes aseptically.
- Incubated the Petridishes at 37°C for 24hrs and observed the zone of inhibition.

Determination of MIC by tube dilution method

Preparation of Nutrient Broth medium

- Peptone 10 g
- Beef extract 10 g
- Sodium chloride 5 g
- Distilled water up to 1000 ml

All the ingredients were weighed and dissolved in water. This nutrient media was sterilized by autoclaving at 121° C (15 psi) for 15 minutes.

Procedure

Different dilutions of testcompounds 1000, 800, 500, 350, 200, 100, 50µg/ml has been prepared. 1ml of each dilution of all test compound solution was aseptically transferred to the sterile nutrient broth medium and made up to 10 ml with sterile nutrient media. The tubes were mixed well after each addition. The process was repeated with different test organism. The tubes were incubated at 37° C for 48 hours. The presence or absence of growth of organisms was observed after incubation.[7-9]

Screening of Antifungal activity (by filter paper disc method)[10,11]

Antifungal screening of all the derivatives was done by using filter disc method. Activity of the compounds were recorded by measuring the zone of inhibition in mm, and compared with the standard zone of inhibition produced by Anilazine. This determination indicates whether the organism is sensitive or resistant to the compound.[10]

- **Test organisms:** *Candida albicans* was used for the determination of the activity.
- **Growth Media:** The activity was conducted on the Sabouraud dextrose agar media.

Composition of Sabouraud dextrose agar media.

Sabouraud agar typically contains:
40 g/L dextrose
10 g/L peptone
20 g/L agar
pH 5.6

Apparatus:

- Petri plate: Glass plate, which was previously sterilized by Dry Heat Sterilization was used.
- Pipette: Micropipette was used for adding the required concentration of the analogues to the plates.
- Glass wares: 500ml conical flask and test tubes were used.
- Compounds screened: all the synthesized derivatives.
- Solvent used: Dimethyl sulfoxide (DMSO)
- Standard used: Anilazine

Preparation of test compounds

Specified quantity (100mg) of the compound was accurately weighed and dissolved in 100ml of DMSO to get the 1000µg/ml stock solution. Further dilution was made to obtain the concentration in the range 100µg/ml, 200µg/ml, and 300µg/ml.

Procedure:-

- All the Petri dishes were sterilized in oven at 160°C for 1 hour.
- Sabouraud dextrose agar media, Adsorbent paper and test solutions were sterilized in autoclave at 121°C at 15psi for 15 min.
- Molten sterile agar to be pour in sterile Petri dishes asceptically.
- The agar was allowed to cool and the bacterial suspension was poured into the petridishes asceptically.

- Impregnated absorbent paper with solution of the drugs (test and standard) in the agar plate petridishes aseptically.
- Incubated the Petridishes at 24°C for 24 hrs and observed the zone of inhibition.

Determination of MIC by tube dilution method

Preparation of Sabouraud Dextrose medium

- Peptone 10 g
- Dextrose 20 g
- Distilled water up to 1000 ml

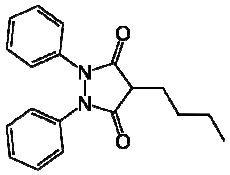
All the ingredients were weighed and dissolved in water. This nutrient media was sterilized by autoclaving at 121°C (15 psig) for 15 minutes.

Procedure

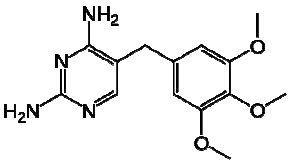
Prepare a different dilution of test compound 1000, 800, 500, 350, 200, 100, 50 µg/ml. 1ml of each dilution of all test compound solution was aseptically transferred to the sterile nutrient broth medium and made up to 10 ml with sterile nutrient media. The tubes were mixed well after each addition. The process was repeated with different test organism. The tubes were incubated at 24°C for 48 hours. The presence or absence of growth of organisms was observed after incubation.[11]

CONCLUSION

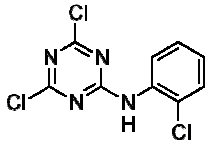
All the synthesized compounds were characterized by IR, Mass and H^1-NMR Spectroscopy and were screened for **Anti-inflammatory activity** and **Anti-microbial (anti-bacterial & anti-fungal) activity.** Phenylbutazone was used as standard reference drug for Anti-inflammatory screening. Trimethoprim was used as a standard reference drug for anti-bacterial activity and anilazine was used as a standard reference drug for anti-fungal activity.



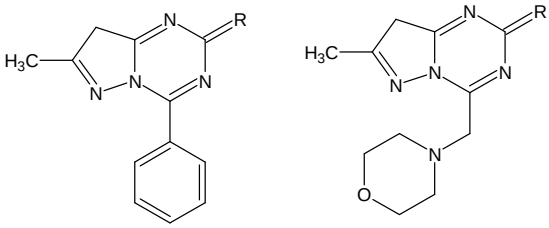
Phenylbutazone



Trimethoprim



Anilazine



Synthesized moiety

Compound **3b** found to have better Anti-inflammatory activity while Compounds **3c**, **6b**, and **6c** showed anti-inflammatory activity but less potent than compound **3b** and Phenylbutazone and Compound **3a** and **6a** were found to be least potent among the series. Synthesized Compounds showed more activity against *E.coli* than *S.aureus.* Compounds **6a** and **3a** shown highest zone of inhibition against *S.aureus* and Compounds **6a** and **6c** shown highest zone of inhibition against *E.coli* where compound **3b** shown least zone of inhibition against both *S.aureus & E.coli.* Synthesized Compounds showed more Antifungal activity than Anti-bacterial activity. Compound **6a** was highest anti-fungal activity near equal to anilazine. Compounds **3a**, **3c**, **6b** and **6c** shown anti-fungal but less active than **6a** and anilazine

where Compound **3b** shown least anti-fungal activity against *C.albicans* among series. The reference standard compounds phenylbutazone has pyrazolone moiety, trimethoprim has pyrimidine moiety (bioisosteres with triazine) and anilazine has triazine moiety and this both moieties are present in the designed nucleus of synthesised moiety, so the anti-inflammatory activity has been compared with respect to phenylbutazone and antimicrobial screening has been done with comparison with trimethoprim and antifungal screening has been performed against anilazine which showed promising activity.

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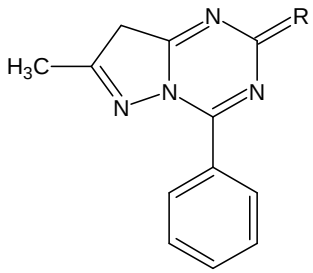
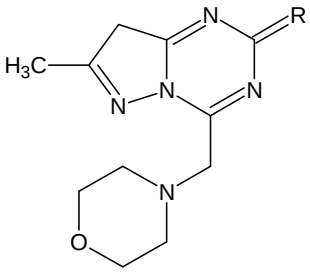
Physical Characteristics of Synthesized Compounds



R	Compound Code
O	3a
S	3b
NH	3c



R	Compound Code
O	6a
S	6b
NH	6c

Table 1. Physicochemical parameters

Compound Code	Molecular formula	Molecular weight (gm/mol)	Melting Point (°C)	Yield	R_f value & Mobile phase
1	$C_4H_6N_2O$	98	220-222 (222)	70% w/w	0.48
2	$C_{11}H_{10}N_2O_2$	189	122-124 (120-123)	85% w/w	0.85
3(a)	$C_{12}H_{10}N_4O$	226	110-112	69% w/w	0.74
3(b)	$C_{12}H_{10}N_4S$	242	114-116	78% w/w	0.60
3(c)	$C_{12}H_{11}N_5$	225	108-110	60% w/w	0.62
4	$C_6H_7N_2O_2Cl$	174	286-290	80% w/w	0.73
5	$C_{10}H_{15}N_3O_3$	225	306	45% w/w	0.61
6(a)	$C_{11}H_{15}N_5O_2$	249	>300	50% w/w	0.54
6(b)	$C_{11}H_{15}N_5OS$	265	>300	62% w/w	0.52
6(c)	$C_{11}H_{17}N_6OCl$	284	>300	46% w/w	0.53

Mobile phase: Compound-1 to Compound-4: Ethyl acetate : Hexane: 2:1
Compound-5 to Compound-6: Ethyl acetate : Hexane: Methanol: 1:1:2

Table 2. Spectral datas of UV, IR, NMR and Mass

Compound Code	UV λmax (nm)	IR(υ, cm^{-1})	Mass (m/z)	^{1}H NMR (δ, ppm) (DMSO)
1	257	-CONH (1685), =N- (1163)	---	---
2	255	=N- (1180), >C=O (1747), Aryl C=C (1598)	---	---
3(a)	225	=N- (1191), >C=O (1743), Aryl C=C (1585)	227.1 [M+1], 175.0, 84.3	0.87 (s, 3H,-C**H$_3$**), 1.23 (s, 2H,-C**H$_2$**), 7.35-7.40 (t, 5H, Ar-**H**)
3(b)	260	=N- (1180), Aryl -C=C(1598), -C=S (744)	242.6 [M+1], 244 [M+2], 175.2, 84.0	---
3(c)	210	=N- (1180), Aryl -C=C (1594), =NH (3419)	226.2 [M+1], 173.3, 85.1	---
4	248	=N- (1170), >C=O (1714), -C-Cl (808)	174.9 [M+1], 176 [M+2], 140.9, 97.0	---
5	203	=N- (1186), >C=O (1743), Cyclic C-O-C (1095.49)	226.3 [M+1], 140.2, 96.1	---
6(a)	227	=N-(1155), >C=O (1781), Cyclic C-O-C (1066.56)	249.8 [M+1], 162.6, 111.6, 84.0	0.87 (s, 3H, -C**H$_3$**), 1.23 (s, 2H, -C**H$_2$**), 2.26-2.33 (m, 6H, -C**H$_2$**N(C**H$_2$**)$_2$), 3.54-3.61 (t, 4H, C**H$_2$**-O-C**H$_2$**)
6(b)	271	=N- (1189), -C=S (745), Cyclic C-O-C (1069.19)	265.9 [M+1], 267 [M+2], 181.0, 112.6, 83.0	---
6(c)	206.5	=N- (1218), =NH (3496), Cyclic C-O-C (1056.85)	---	---

Table 3. Anti-inflammatory screening

Compound Code	Inhibition of Inflammation (cm)±SEM				% Inhibition		
	0 hr	1 hr	2 hr	3 hr	1 hr	2 hr	3 hr
Control	0.72±0.02	0.71±0.04	0.71±0.04	0.70±0.03	-	-	-
Phenylbutazone	0.72±0.03	0.53±0.04	0.42±0.03	0.28±0.04	25.3	40.84	60
3a	0.71±0.02	0.64±0.02	0.56±0.01	0.46±0.03	9.8	21.12	34.28
3b	0.70±0.02	0.56±0.02	0.46±0.02	0.32±0.01	12.12	35.2	54.28
3c	0.72±0.02	0.62±0.02	0.51±0.01	0.40±0.02	12.6	28.16	42.85
6a	0.71±0.04	0.67±0.01	0.59±0.01	0.48±0.01	5.6	16.9	31.42
6b	0.72±0.02	0.63±0.01	0.52±0.03	0.35±0.02	11.26	26.76	50
6c	0.71±0.01	0.65±0.03	0.56±0.03	0.45±0.03	8.4	21.12	35.71

No. of animal used in each Group (n) =3, Values were expressed as Mean±SEM

Table 4. Zone of inhibition (Antibacterial screening)

Compound Code	Concentration (µg/ml)	Zone of Inhibition (mm)	
		Gram +ve	Gram -ve
		S.aureus	*E.coli*
Control	100	--	--
	200	--	--
	300	--	--
Trimethoprim	100	08	09
	200	10	12
	300	11	15
3a	100	02	05
	200	04	06
	300	06	07
3b	100	01	03
	200	02	04
	300	02	06
3c	100	02	03

	200	03	05
	300	05	07
6a	100	05	06
	200	06	09
	300	07	11
6b	100	02	05
	200	02	07
	300	04	08
6c	100	02	07
	200	03	08
	300	04	10

Table 5. MIC for antibacterial screening

Compound Code	Minimum Inhibitory Concentration (μg/ml)	
	S.aureus	*E.coli*
Trimethoprim	50	50
3a	200	200
3b	500	350
3c	350	350
6a	200	100
6b	500	350
6c	350	200

Table 6. Zone of inhibition (Antifungal screening)

Compound Code	Concentration (μg/ml)	Zone of Inhibition (mm)
		C.albicans
Control	100	00
	200	00
	300	00
Anilazine	100	14
	200	16

	300	16
3a	100	07
	200	09
	300	11
3b	100	06
	200	08
	300	09
3c	100	07
	200	08
	300	10
6a	100	08
	200	12
	300	15
6b	100	08
	200	09
	300	11
6c	100	07
	200	10
	300	12

Table 7. MIC of Antifungal Screening (tube dilution method)

Compound Code	Minimum Inhibitory Concentration (µg/ml)
	C.albicans
Anilazine	50
3a	200
3b	500
3c	350
6a	100
6b	350
6c	200

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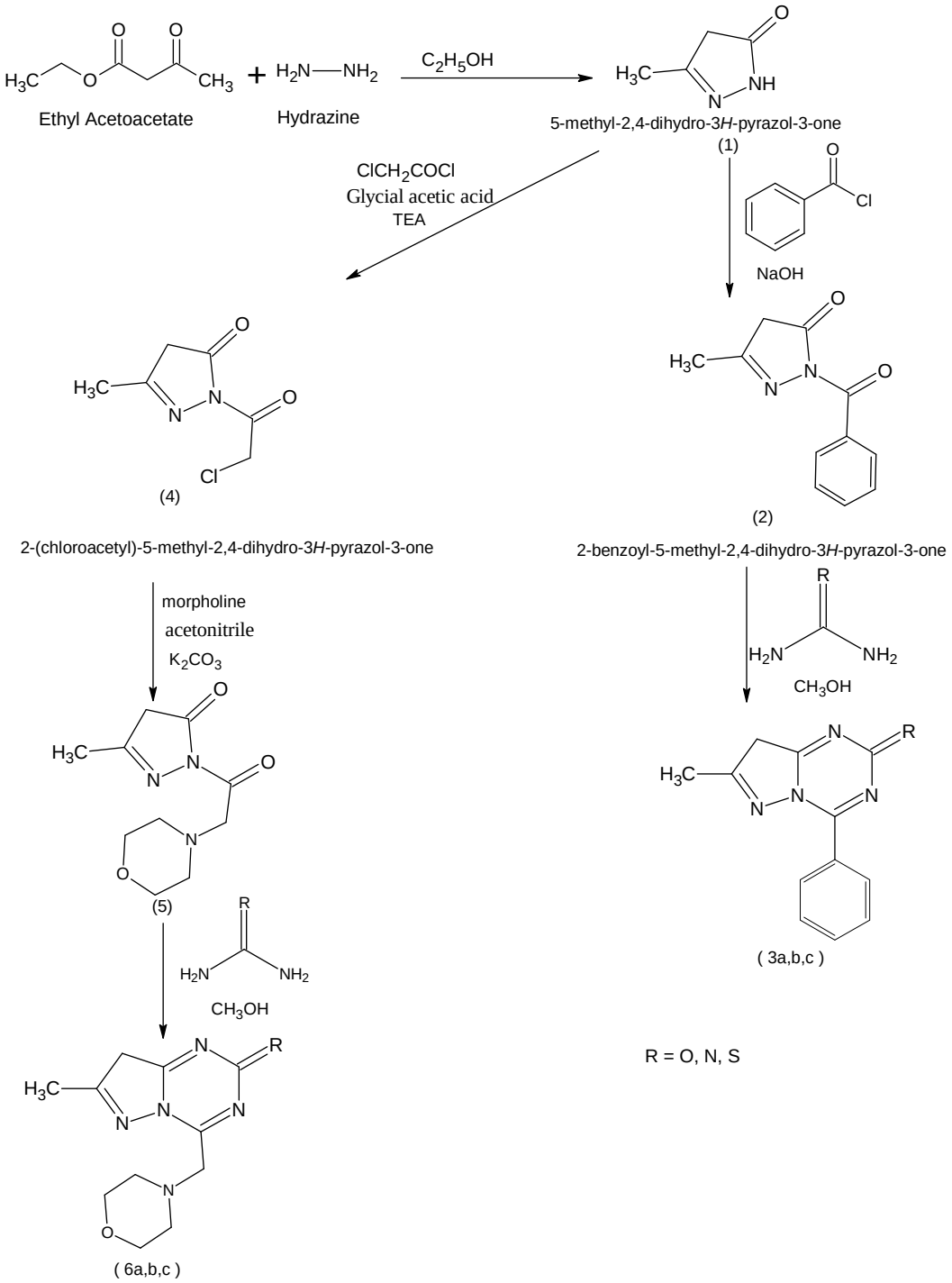

SCHEME OF SYNTHESIS

Figure-1: Plethysmometer Carrageenan induced inflammation

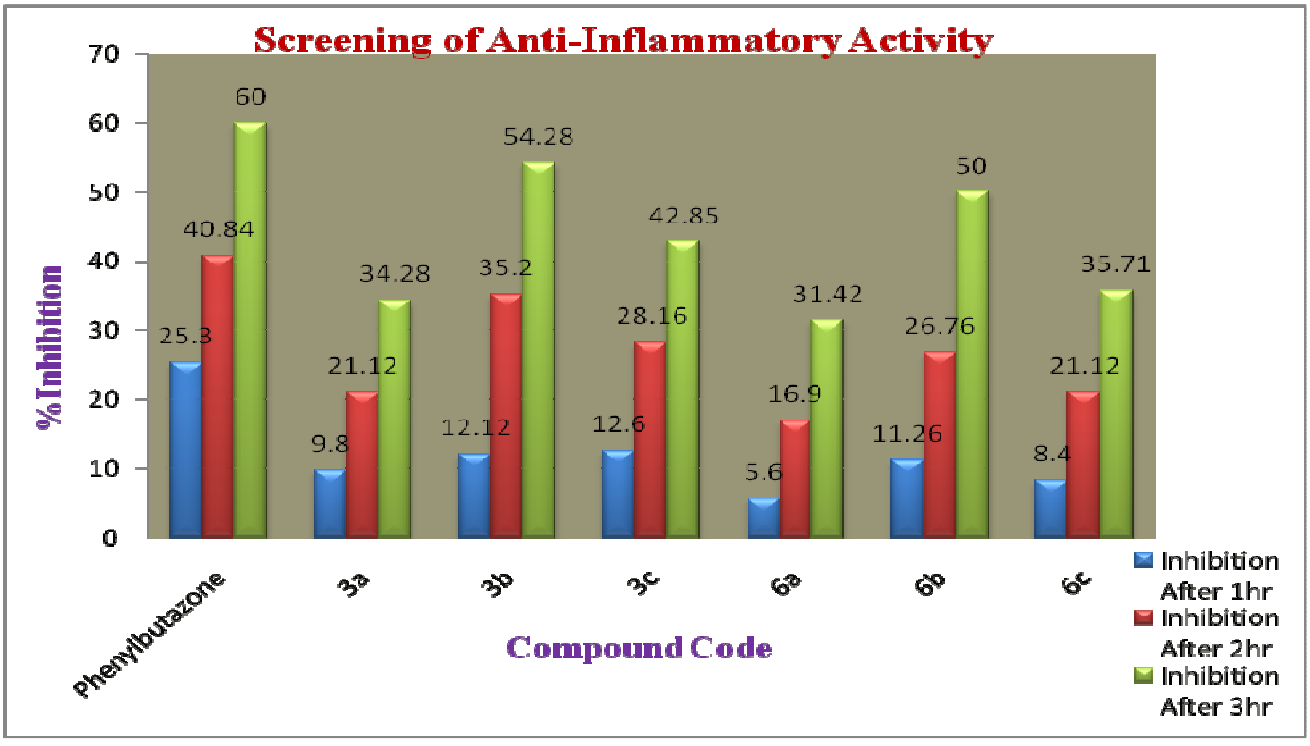

Figure-2: Histogram of Anti-inflammatory activity

Figure-3: Zone of inhibition

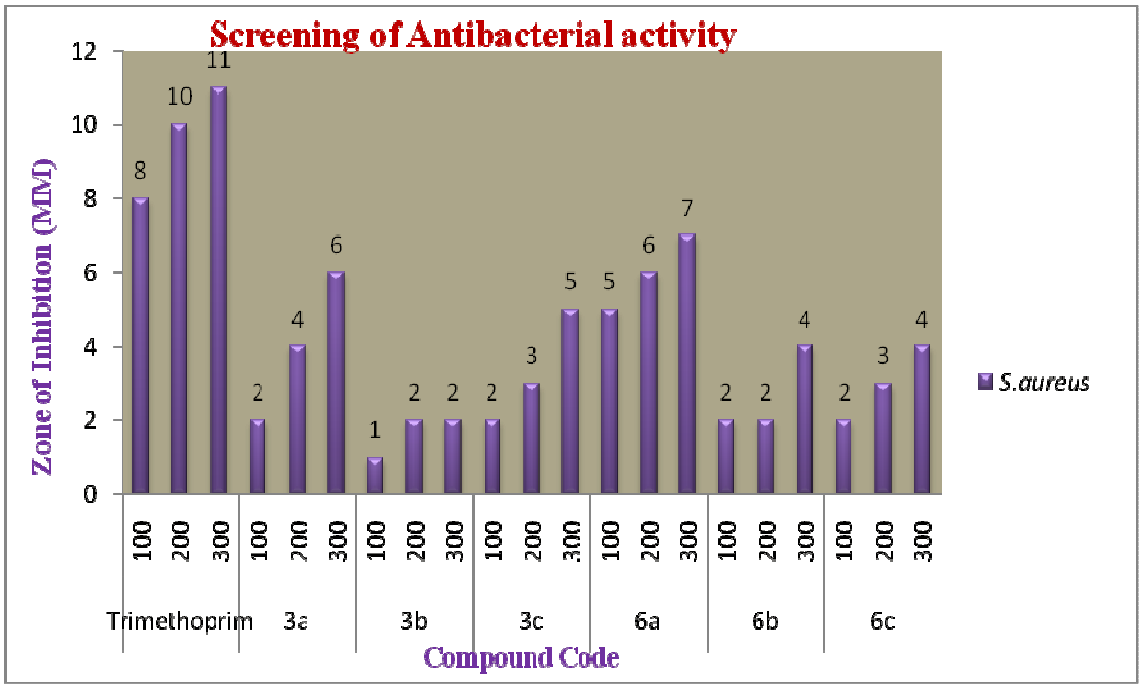

Figure-4: Histogram of Zone of inhibition (Antibacterial screening for S.aureus)

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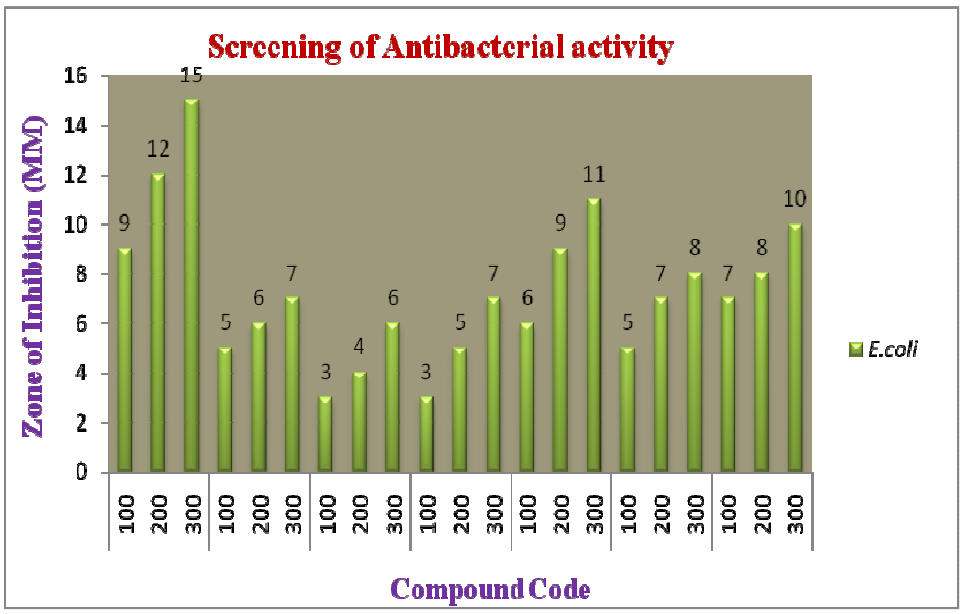

Figure-5: Histogram of Zone of inhibition (Antibacterial screening for E.coli)

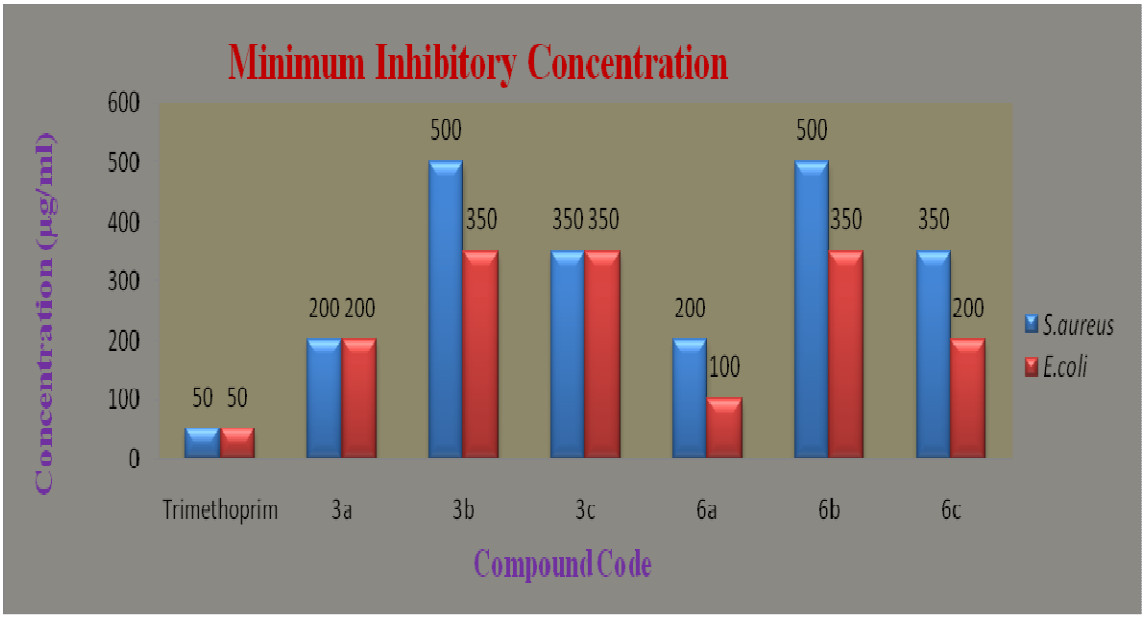

Figure-6: Histogram of MIC (Antibacterial Screening)

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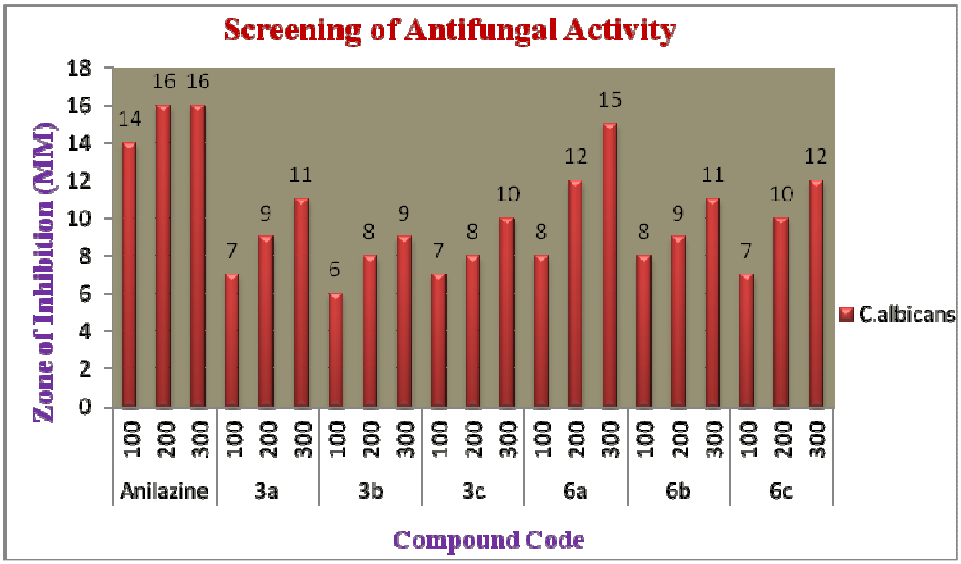

Figure-7: Histogram of Zone of inhibition (Antifungal screening for *C.albicans)*

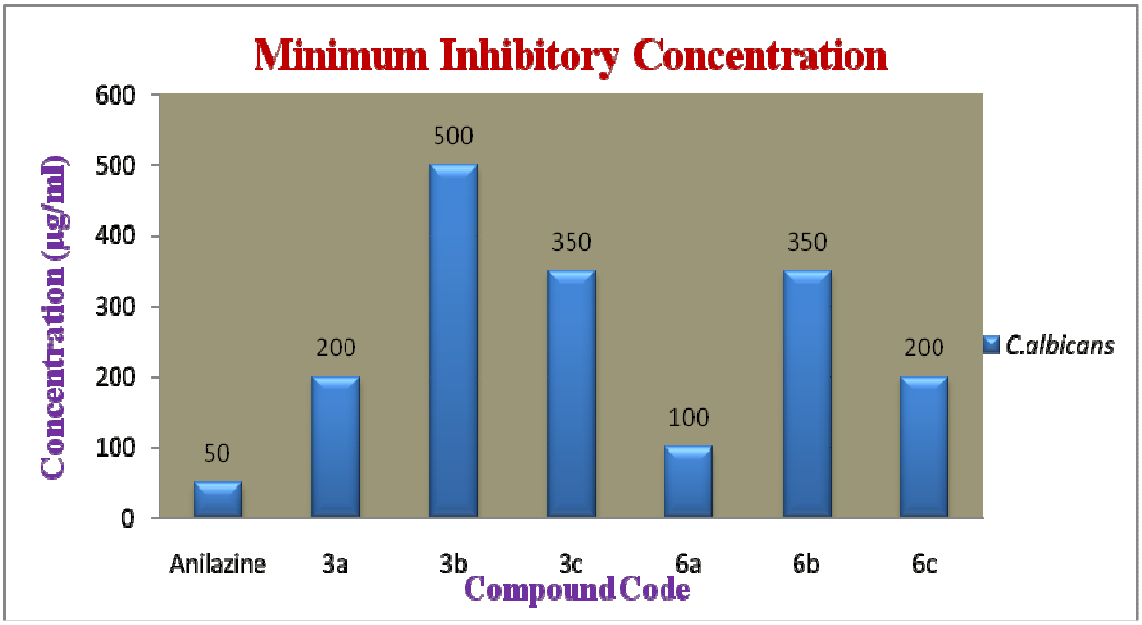

Figure-8: Histogram of MIC (Antifungal screening for *C.albicans)*