ISSN: 2349-9818
Nov. - Dec. 2020, Vol. 7 (6), 08-12

TROPICAL JOURNAL OF PHARMACEUTICAL AND LIFE SCIENCES
(An International Peer Reviewed Journal)
Journal homepage: http://informativejournals.com/journal/index.php/tjpls

Research Paper

SYNTHESIS AND EVALUATION OF ANTIFUNGAL ACTIVITY OF 4, 6-DIPHENYL-3, 4- DIHYDROPYRIMIDINE-2-(1H)-ONE DERIVATIVES

Vishal Garg[1]*, Dinesh Jindal[2] and Rambir Singh[3]

[1]Jaipur School of Pharmacy, MVGU, Jaipur, Rajasthan, India
[2]Department of Pharmaceutical Chemistry, Jaipur School of Pharmacy, MVGU, Jaipur, Rajasthan, India
[2]Department of Pharmacy, Maharishi Arvind University, Jaipur, Rajasthan, India

ARTICLE INFO:
Received: 15th Nov. 2020; **Received** in revised form: 5th Dec. 2020; **Accepted:** 19th Dec. 2020; **Available online:** 27th Dec. 2020

ABSTRACT

In the present work was designed to synthesize and evaluate the antifungal activity of 4, 6-diphenyl-3, 4-dihydropyrimidine-2-(1H)-one derivatives. The 3, 4- dihydropyrimidin 2 (1H)-one was used as nucleus for the synthesis of N-mannich bases of pyrimidine derivatives. All synthesized intermediates and products were identified on the basis of melting point range, Rf value, Elemental analysis, and IR spectral analysis. The synthesized compounds were screened for their antifungal activity against *Candida albicans* (MTCC 227) and *Aspergillus niger* (MTCC 282) in Sabouraud's dextrose agar medium using fluconazole as a standard drug.

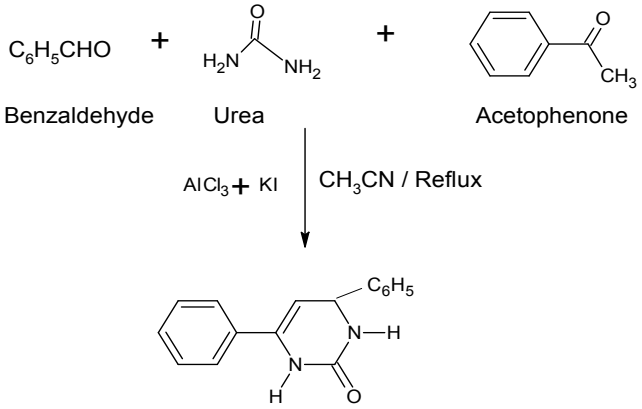
Keywords: Pyrimidine derivative, Antifungal activity, *Aspergillus niger*, *Candida albicans.*

INTRODUCTION

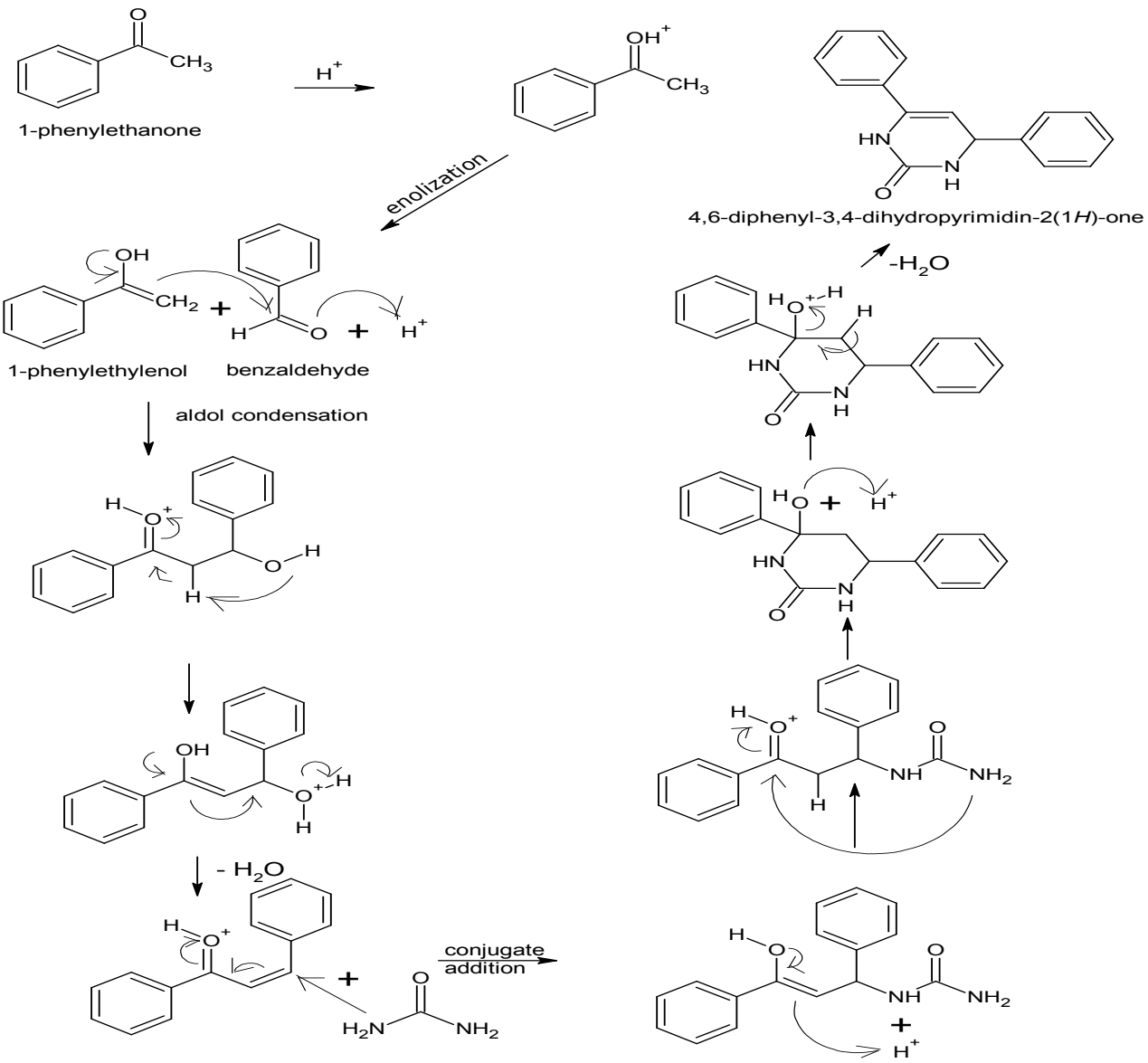
The pyrimidine is a heterocyclic ring possess a wide spectrum of biological activities. Pyrimidine is a six-member ring containing two nitrogen atoms at positions 1 and 3. Due to diverse biological activities, the pyrimidine skeleton is of great importance to biochemists as it is available in a large variety of naturally occurring compounds and also in clinically useful molecule. The pyrimidine ring system account for their biological properties especially as anti-infective agents 4-Aryldihydropyrimidinons and their derivatives. Fungal infections of the skin are caused by dermatophytes, such as *Trichophyton rubrum, Trichophyton tonsurans, Trichophyton mentagrophytes, Microsporum canis, Epidermophyton floccosum, Microsporum gypseum*, and *Trichophyton verrucosum.*

MATERIAL AND METHODS

Scheme Synthesize of 4, 6-Diphenyl-3, 4-dihydropyrimidine-2-(1H)-one (Parent-PYN): The scheme for the synthesis of 4, 6-diphenyl-3, 4- dihydropyrimidine-2-(1H)-one (parent-PYN) from reaction of benzaldehyde, urea and acetophenone in presence of almunium chloride, potassium iodide is outlined below:



Mechanism for the synthesis 4, 6-Diphenyl-3, 4- dihydropyrimidine-2-(1H)-one (Parent-PYN)



Synthesis of 1, 3-Bis (N, N-dialkyl-aminomethyl)-4, 6-diphenyl- 3, 4- dihydropyrimidine- 2 (1H)-one (PY-1D): m The scheme for the synthesis of N-mannich bases of 4,6-diphenyl-3,4**-** dihydropyrimidine-2-(1H)-one is depicted below and the derivatives to be synthesized are depicted in **Table 1**

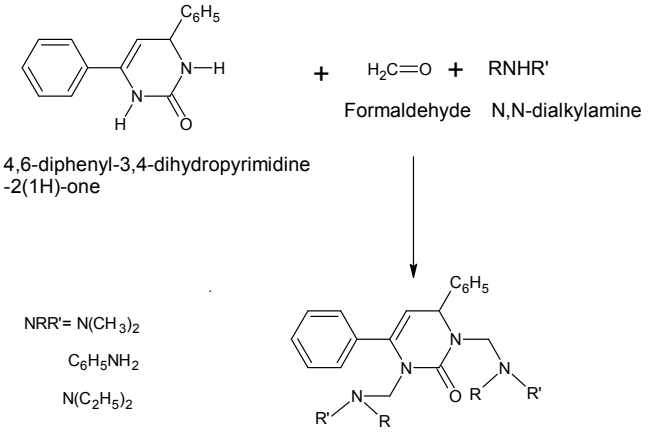
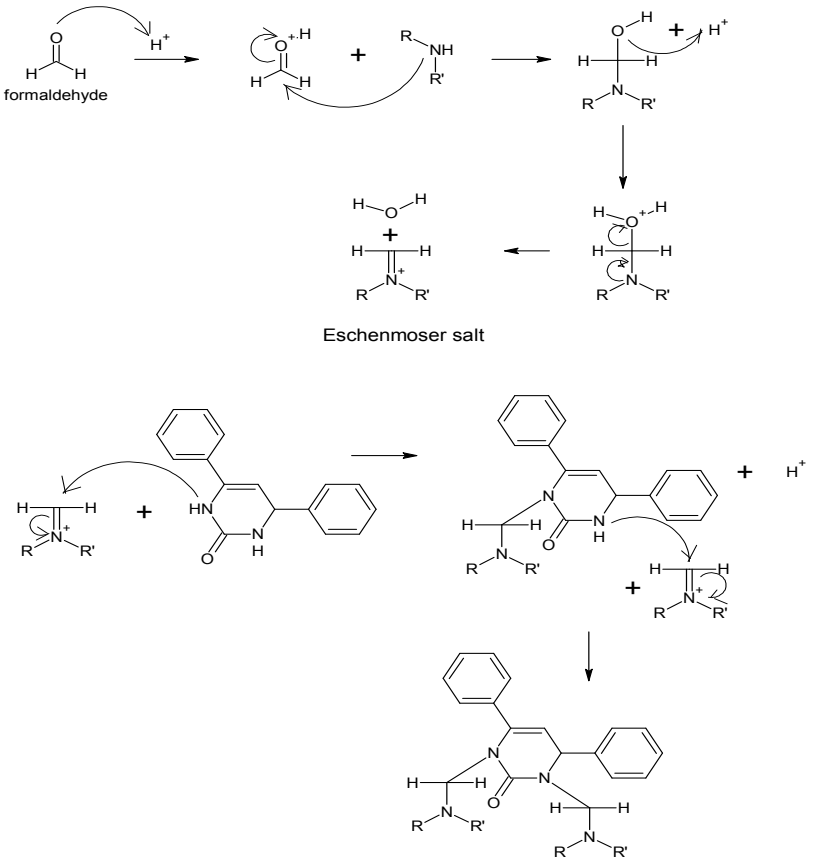


Table 1: Derivatives:

S.No.	R	R’	Derivative code
1.	CH_3	CH_3	PY-1-1D
2.	C_2H_5	C_2H_5	PY-2-1D
3.	C_6H_5	H	PY-3-1D

<

Mechanism for the synthesis of n-mannich bases of pyrimidine derivative (PY-1 D)



Synthesis of 5-(ethoxycarbonyl)-4-alkyl-6-methyl-3, 4-dihydropyrimidin-2(1H)-one: Biginelli reported this reaction shown in Scheme below. A variety of Lewis acid catalysts are employed eg. LiBr, $ZrCl_4$, $CeCl_3.7H_2O$, $LaCl_3.7H_2O$, $SnCl_2.2H_2O$ etc. These conditions were applied to enolisable ketones, urea and benzaldehyde to obtain 3, 4- dihydropyrimidine –2- (1H)-one in 82-90% yield.

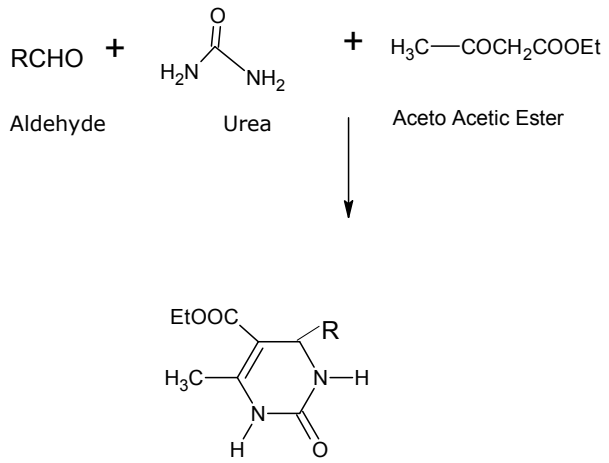
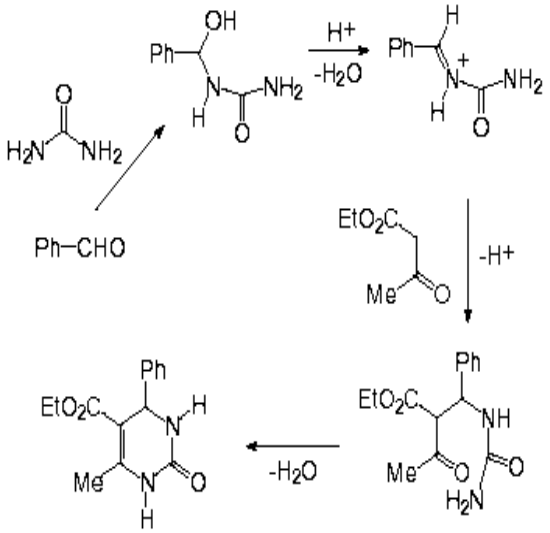


Table 2: Intermediate

S. No.	R	Compound code
1.	p-C6H4OCH3	PY-ANI
2.	o-C6H4Cl	PY-CHLOR

Mechanism:

The first step in the mechanism is believed to be the condensation between the aldehyde and urea, with some similarities to the Mannich Condensation. The iminium intermediate generated acts as an electrophile for the nucleophilic addition of the ketoester enol, and the ketone carbonyl of the resulting adduct undergoes condensation to give the cyclized product.



Synthesis of 1, 3-Bis (N, N-dialkyl-aminomethyl)-4, 6-diphenyl- 3, 4- dihydropyrimidine- 2 (1H)-one

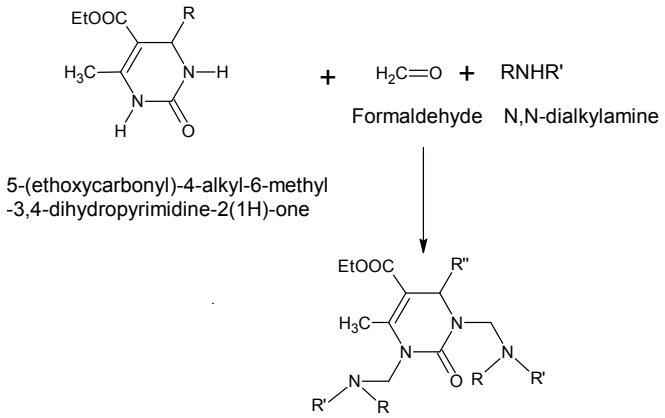
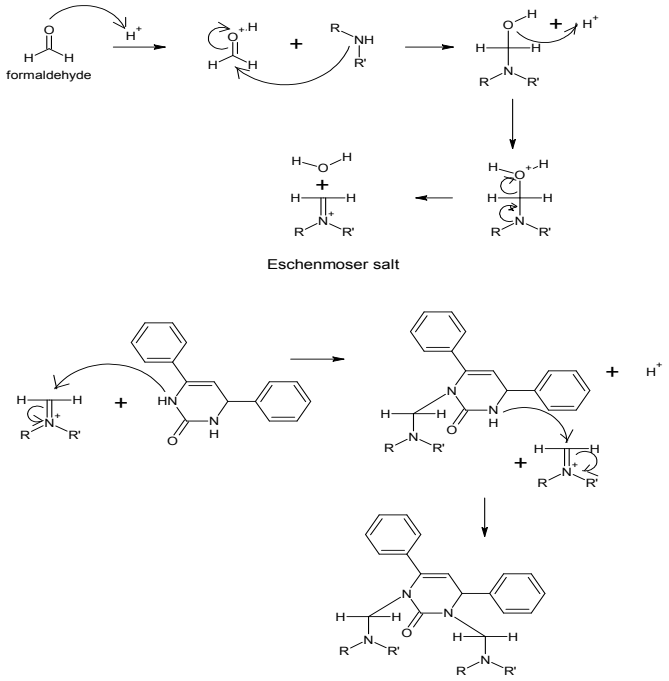


Table 3: Derivatives

S. No.	R	R’	R’’	Derivative code
1.	CH_3	CH_3	p-C6H4OCH3	PY-A-21D-4
2.	C_2H_5	C_2H_5	p-C6H4OCH3	PY-A-22D-5
3.	C_6H_5	H	p-C6H4OCH3	PY-A-23D-6
4.	CH_3	CH_3	o-C6H4Cl	PY-C-31D-7
5.	C_2H_5	C_2H_5	o-C6H4Cl	PY-C-32D-8
6.	C_6H_5	H	o-C6H4Cl	PY-C-33D-9

Mechanism for the synthesis of N-mannich bases of pyrimidine derivative



Synthesis of 4, 6-Diphenyl-3, 4-dihydropyrimidine-2-(1H)-one (parent-PYN)

Procedure:

In a 500 ml. RBF, fitted with a reflux condenser, added a mixture of 6.0 gm of (5.0 mmol) acetophenone, 0.66 gm (0.5 mmol) of aluminium chloride and 0.83 gm of (0.5 mmol) potasium iodide in 250 ml. methyl cynide. After this, 5.3 gm of (5.0 mmol) benzaldehyde and 4.5 gm (7.5 mmol) urea was added .Then the reaction mixture was refluxed for 6-7 hrs. After completion of reaction (monitored by TLC), the mixture was brought to room temp. and poured into ice cold water. The crude product thus obtained was filtered and recrystallized by using ethanol.

Synthesis of 1, 3-Bis (N, N-dimethyl-aminomethyl)-4, 6-diphenyl- 3, 4-dihydropyrimidine- 2 (1H)-one (PY-1-1d)

Procedure:

Placed 0.96 gm (0.003 mole) of (parent-PYN), 5 ml. of ethanol and 1 ml. of formalin in a 250 ml. beaker. Stirred well till a slurry was obtained. Added 0.27 gm (0.006 mole) of N, N-dimethylamine drop wise with good stirring. The reaction mixture was cooled and allowed to stand at room temp. for one hr. with occasional stirring. It was warmed on a steam bath for 15 minutes, cooled and product was recovered. The crude product thus obtained was filtered and recrystallized by using ethanol.

5-(Ethoxycarbonyl)-4-(4-methoxyphenyl)-6-methyl-3, 4-dihydropyrimidine-2(1H)-one (PY-ANI)

Procedure:

In a 250 ml. RBF, fitted with a reflux condenser, added a mixture of 4.85 ml (5.0 gm, 38.0 mmol) of ethyl acetoacetate, 5.25 gm (38.0 mmol) of anisaldehyde and 2.75 gm (46.0 mmol) of urea in 100 ml ethanol was refluxed in presence of 0.84 gm (0.2mol) $Cacl_2$ for 6-7 hrs. After completion of reaction, the mixture was poured on to crushed ice. The crude product thus obtained was filtered and recrystallized from ethanol.

Synthesis of (5-ethoxycarbonyl)-4-(2-chlorophenyl)-6-methyl- 3, 4-dihydropyrimidine-2-(1H)-one (PY-Chlor)

Procedure:

In a 250 ml. RBF, fitted with a reflux condenser, added 4.85 ml (5.0 gm, 38.0 mmol) of ethyl acetoacetate, 4.27 ml (5.25 gm, 38.0 mmol) of 2-chlorobenzaldehyde, 2.75 gm (46.0 mmol) of urea and 0.84 gm (0.2 mol) of $Cacl_2$, stirred well then added 100 ml of ethanol. The resulting solution was refluxed for 6-7 hrs. After completion of reaction, the mixture was poured on to crushed ice. The crude product thus obtained was filtered and recrystallized from ethanol.

Synthesis of Derivatives:

Synthesis of 5-(Ethoxycarbonyl) -4-(4-methoxyphenyl)- 6-methyl- 3, 4- (N, N-dimetyl methamine) pyrimidine-2-(1H)-one (PY-A-21D-4)

Procedure:

Placed 0.78 gm (0.003 mole) of compound (PYN-ANI), 5 ml. of ethanol and 1 ml. of formalin in a 250 ml of RBF. Stirred well till a slurry was obtained. Added 0.14 gm (0.006 mole) of N, N- dimethylamine drop wise with good stirring. The reaction mixture was cooled and allowed to stand at room temp. for one hr with occasional stirring. It was refluxed on a steam bath for 5 to 6 hrs, cooled and product was recovered. The crude product thus obtained was filtered and recrystallized by using chloroform-ether (1:1)

Synthesis of 5-(Ethoxycarbonyl) - 4-(4-methoxyphenyl) - 6-methyl- 3, 4- (N, N-diethyl methamine)- pyrimidine-2-(1H)-one (PY-A-22 D-5)

Procedure:

Placed 0.78 gm (0.003 mole) of compound (PYN-ANI), 5 ml. of ethanol and 1 ml. of formalin in a 250 ml of RBF. Stirred well till a slurry was obtained. Added 0.44 gm (0.006 mole) of N, N- diethylamine drop wise with good stirring. The reaction mixture was cooled and allowed to stand at room temp. for one hr with

occasional stirring. It was refluxed on a steam bath for 5 to 6 hrs, cooled and product was recovered. The crude product thus obtained was filtered and recrystallized by using chloroform-ether (1:1)

Synthesis of 5-(ethoxycarbonyl)- 4-(4-methoxyphenyl)- 6-methyl- 3, 4- (N, N-diphenyl methamine)- pyrimidine-2-(1H)-one (PY-A-23D-6)

Procedure

Placed 0.78 gm (0.003 mole) of compound (PYN-ANI), 5 ml. of ethanol and 1 ml. of formalin in a 250 ml of RBF. Stirred well till a slurry was obtained. Added 0.55 ml (0.006 mole) of aniline drop wise with good stirring. The reaction mixture was cooled and allowed to stand at room temp. for one hr with occasional stirring. It was refluxed on a steam bath for 5 to 6 hrs, cooled and product was recovered. The crude product thus obtained was filtered and recrystallized by using chloroform-ether (1:1)

Table 4: Characteristic infrared absorptions of functional groups of compound **(PY-A-21D-4)**

S.No.	Functional group	IR Absorbance (cm^{-1}) (Observed)	Inferences
1.	C-H str (in aromatic)	3111	Indicate aromatic ring
2.	C=C str (in aromatic)	1513-1440	Indicate aromatic ring
3.	N-H str	3240	Indicate N-H linkage
4.	C-N str (in aromatic)	1307-1176	Indicate NH_2 group
5.	C-H bending (in aromatic)	838-791	Indicate aromatic ring
6.	C=O str	1724	Indicate C=O linkage
7.	C-H str (in aliphatic)	2930-2834	Indicate CH_3, CH_2 group
8.	C-N str (in aliphatic)	1221-1087	Indicate Aliphatic amine

Table 4: Characteristic infrared absorptions of functional groups of compound **(PY-A-22D-5)**

S.No.	Functional group	IR Absorbance (cm^{-1}) (Observed)	Inferences
1.	C-H str (in aromatic)	3089	Indicate aromatic ring
2.	C=C str (in aromatic)	1641	Indicate aromatic ring
3.	N-CH_3 str	2808	Indicate 3^0 amine
4.	C-N str (in aromatic)	1307-1186	Indicate NH_2 group
5.	C-H bending (in aromatic)	870-700	Indicate aromatic ring
6.	C=O str	1704	Indicate C=O linkage
7.	C-H str (in aliphatic)	2935-2808	Indicate CH_3, CH_2 group
8.	C-N str (in aliphatic)	1232-1083	Indicate Aliphatic amine

Table 5: Characteristic infrared absorptions of functional groups of compound **(PY-A-23D-6)**

S.No.	Functional group	IR Absorbance (cm^{-1}) (Observed)	Inferences
1.	C-H str (in aromatic)	3111	Indicate aromatic ring
2.	C=C str (in aromatic)	1651-1460	Indicate aromatic ring
3.	N-CH_3 str	2834	Indicate 3^0 amine
4.	C-N str (in aromatic)	1307-1110	Indicate NH_2 group
5.	C-H bending (in aromatic)	865-752	Indicate aromatic ring
6.	C=O str	1705	Indicate C=O linkage
7.	C-H str (in aliphatic)	2955	Indicate CH_3, CH_2 group
8.	C-N str (in aliphatic)	1222-1110	Indicate Aliphatic amine

CONCLUSION

In the present work, the compound, 3, 4-dihydropyrimidin 2 (1H)-one (as per scheme 1) was used as nucleus for the synthesis of N-mannich bases of pyrimidine derivative. All synthesized intermediates and products were identified on the basis of melting point range, R_f value, Elemental analysis, and IR spectral analysis. IR spectral data confirmed the identity of synthesized compounds. The summary of observed values are given in Table 6.

Table 6: Parents, Intermediate compounds along with identification parameters in the synthesis of final products

S.No.	Compound code & M. Formula	Mol.Wt.	Yield %	M.P. Range	R_f value	Solvent system (T.L.C)	Element present
1.	(Parent-PYN) $C_{16}H_{14}N_2O$	322	70%	234-236^{O}C	0.77	$CHCl_3$: EtOH (95:5)	Nitrogen
2.	PY-1-1D $C_{22}H_{28}N_4O$	432	77.34%	188-190^{O}C	0.70	$CHCl_3$	Nitrogen
3.	PY-2-1D $C_{26}H_{36}N_4O$	488	64.8%	198-200^{O}C .	0.79	$CHCl_3$	Nitrogen
4.	PY-3-1D $C_{30}H_{28}N_4O$	528	57.9%	150-152^{O}C	0.65	$CHCl_3$: EtOH (95:5)	Nitrogen

5.	PY-ANI $C_{15}H_{18}N_2O_3$	264	26.05%	188-190^{0}C	0.4	$CHCl_3$: EtOH (4:1)	Nitrogen
6.	PY-CHLOR $C_{14}H_{15}N_2O_2Cl$	281.5	47.38%	192-194^{0}C	0.52	$CHCl_3$	Nitrogen
7.	PY-A-21D-4 $C_{21}H_{32}N_4O_3$	378	20.5%	208-210^{0}C	0.81	$CHCl_3$: EtOH (9:1)	Nitrogen
8.	PY-A-22D-5 $C_{25}H_{40}N_4O_3$	434	58.33%	178-180^{0}C	0.62	$CHCl_3$	Nitrogen
9.	PY-A-23D-6 $C_{29}H_{30}N_4O_3$	472	29.49%	180-182^{0}C	0.67	$CHCl_3$: EtOH (4:1)	Nitrogen

ANTIFUNGAL ACTIVITY

Procedure for antifungal activity:

Preparation of stock solution of synthesized compounds

25 mg of each compound was accurately weighed and transferred to different 12 volumetric flasks of 25 ml. Each compound was then dissolved in 25 ml. of DMF taken in 25 ml of volumetic flask and these solutions (each having conc. 1000 µg/ml) were used as stock solutions. From these stock solutions, further dilutions were made to get concentrations of of 25 µg /ml, 50µg/ml and 100 µg /ml of each compound.

Preparation of stock solution of standard drug (Fluconazole)

100 mg of fluconazole was accurately weighed and transferred to 100 ml of volumetric flask. This was dissolved in sufficient amount of distilled water and then diluted up to 100 ml with distilled water. This final solution having conc. of 1000 µg/ml. of the drug was used as stock solution. From this stock solution, further dilutions were made to get concentrations of of 25 µg /ml, 50µg/ml and 100 µg /ml of the drug.

Preparation of glassware

Petri dishes, pipettes, culture tubes were washed with distilled water, dried in oven, packed and sterilized at 160^o C for 2 hours in hot air oven.

Preparation of reagent and media

All reagent and media were prepared in distilled water.

Preparation of nutrient broth (pH 5.6 ±0.2)

The broth used for the microorganism was the Sabouraud's dextrose broth.

Table 7: Composition

S. No.	Reagents	Amount taken
1.	Dextrose	40.0 g
2.	Peptone	10.0 g
3.	Distilled water	Up to 1000 ml

Procedure for the preparation of nutrient broth

Dextrose (6.0 gm) and peptone (1.5 gm) were weighed and transferred to 250 ml. of conical flask. The contents were then dissolved in 150 ml. of distilled water with heating, cooled and the pH was adjusted to 5.6 with lactic acid and filtered. Total matrix was sterilized at 121^{0}C for 15 minutes.

Preparation of Sabouraud's dextrose agar media (pH 5.6 ±0.2)

9.75 gm of Sabouraud's dextrose agar was suspended in 150 ml. of distilled water taken in 250 ml. of conical flask, heated to boiling to dissolve the medium completely. The medium

was then sterilized by autoclaving at 15 lb/inch^2 pressure (121^0 C) for 15 minutes.

Test microorganisms

The fungal strains were obtained from the institute of microbial technology sector 39-A, Chandigarh, India. The fungal strains studied were *Candida albicans MTCC-227* and *Aspergillus niger MTCC- 282*

Preparation of inoculum:

Vial containing lactose dilution (dehydrated powder) of *Aspergillus niger* was broken using sterile scalpul knife in aseptic condition in flask containing 100 ml of nutrient broth. This flask was incubated for 48 hours at 28^0 C in Biological Oxygen Demand (BOD) incubator. After 48 hours, turbidity was observed in the flask.

Standardization of *Aspergillus niger* in inoculum:

48 hours old culture of *Aspergillus niger* in 100 ml of nutrient broth was serially diluted in 10 folds. Dilution was made according to the following steps:

- 1.0 ml. of culture was transferred aseptically into a sterile test tube containing 9.0 ml. of sterile water. The test tube was shaken vigorously for proper mixing.
- 1.0 ml. of this fungal suspension from the first test tube was transferred aseptically into second test tube containing 9.0 ml. of sterile water. The test tube was shaken vigorously for proper mixing.
- This procedure was repeated 10 times to get dilution of fungal suspension ranging from 10^{-1} (first test tube) to 10^{-10} (10^{th} test tube)
- 0.1 ml. of the culture from the test tube 6,7,8,9 & 10 was transferred separately in triplicate on the surface of solidified Sabouraud's dextrose agar media placed in sterile petri dishes.
- These petri dishes were incubated at 28^{o}C for 48 hours. Subsequently the number of colony forming units (C.F.U) were counted on each petri dish and results are shown in **Table8.**

Table 8: No. of colony forming unit (C.F.U.) for *A. niger*

S. No.	Test tube No.	Petri dish No.	No. of C.F.U.
1.	6th	1	2
		2	1
		3	2
2.	7th	1	4
		2	8
		3	6
3.	8th	1	7
		2	9
		3	9
4.	9th	1	11
		2	12
		3	14
5.	10th	1	25
		2	31
		3	29

Following the above table, it was concluded that dilution of 7th test tube was suitable for calculation of C.F.U. present in stoke solution.

Calculation of C.F.U.

Average no. of C.F.U. on 7th plate: (4+8+6)/3 = (18/3) = 6 C.F.U.

Therefore no. of C.F.U. in main stock would be = (6*10^7*100)

=

(6*10^9)

0.1 ml standard culture was used for antifungal screening therefore:

100 ml---------- $6*10^9$

0.1 ml---------- $(6*10^9/100)*0.1 = 6*10^6$

The same procedure was applied for the standardization of *Candida albicans.*

Antifungal assay:

All the compounds were screened for antifungal activity using cup-plate agar diffusion method by measuring the zone of inhibition in mm. Following steps were followed while performing antifungal assay:

- Laminar airflow bench was swapped with 70 % alcohol and UV was switched on. After 30 min, the UV was switched off.
- All the reagents, media, inoculum and glassware were transferred to laminar airflow bench taking all aseptic conditions.
- About 20-25 ml. of sterilized Sabouraud's dextrose agar medium (while it was still hot) was placed into each sterilized petri dish. The petri dishes were then left for some time to solidify the agar media.
- When the dextrose agar media of each petri dish was solidified, 0.1 ml of inoculum was added on the surface of agar media using sterile pipette. The inoculum was then spread with the help of sterilized L-shaped glass rod. After this, made quadrate well on the surface of agar media using a stainless steel cylinder of 8 mm diameter. The petri dishes were suitably marked for name of organism, date of incubation, name of plate and drug concentration.
- Two wells were filled by same concentration (duplicate method) of each drug and other two wells with same concentration of standard drug. After diffusion of drug, the petri dishes were incubated at 28^0C for 48 hours. Simultaneously negative control (medium without drug and without inoculum) and positive control (medium without drug but with inoculum) were prepared and kept in incubator at 28^0 C for 48 hours.
- After 48 hours, the plates were observed for fungal growth. The results are summarized in **Table 9**

Table 9: Antifungal activity of synthesized compounds:

	Zone of inhibition (mm)					
Compounds Code	*C. albicans*			*A. niger*		
	25 ppm	50 ppm	100 ppm	25 ppm	50 ppm	100 ppm
(Parent-PYN)	11	12	13	08	09	11
PY-1-1D	08	10	11	11	12	13
PY-2-1D	08	10	11	09	10	13
PY-3-1D	08	09	11	11	12	13
PY-ANI	11	12	13	08	10	11
PY-Chlor	11	12	13	09	10	11
PY-A-21D-4	08	09	11	13	14	15
PY-A-22D-5	08	10	11	11	12	13
PY-A-23D-6	11	12	13	15	16	17
Fluconazole	26	27	28	25	26	27

RESULT AND DISCUSSION

All the newly synthesized compounds were screened for their antifungal activity against *Candida albicans* (MTCC 227) and *Aspergillus niger* (MTCC 282) in Sabouraud's dextrose agar medium using fluconazole as a standard. The zone of inhibition were measured in mm (09-12 mm, 13-15 mm, 16-19 mm for weak, moderate and highly active zones respectively) Fluconazole exhibited a zone of inhibition 28 mm for *C. albicans* and 27 mm for *Aspergillus niger* at 1000 ppm concentration. The results of antifungal activity of the test compounds were found to be

somewhat different from their antibacterial activity. All the test compounds showed activity ranging from 08 to 17 mm. Compounds (PY-A-23D-6) exhibited very good activity towards *A. niger*. Compound (PY-A-23D-6) showed moderate activity towards *C. albicans* and the compound (PY-A-21D-4) showed moderate activity towards *A. niger* while the compound (PY-A-22D-5) was found to be weakly active towards C. *albicans* and *A. niger*.

REFERENCES:

1. Saini A., Kumar S. and Sandhu S.,(2006), ***Indian J of Chem***, Vol.45B, 684-688.
2. Biginelli P., and Gazz,(1893)**,** ***Chem. Ital***, 23, 360.
3. Atwal S., Swanson N., Unger E., Floyd M. and Moreland S.,(1991), ***J Med Chem***, 34, 806.
4. Bussolari C., and Donnell P., J. (2000),***Org. Chem***, 65.
5. Yadav S., Reddy S., Reddy J., and Ramalingam T., J. (2000),***Chem Res***, 354.
6. Ranu B., Hajra A. and Jana U., (2000), ***J Org Chem***, 65, 6270.
7. Lu J., Ma H. and Li W.,(2000), ***J Org Chem***, 20, 815.
8. Karube I., and Suzuki.,(1990), ***M Biosensors***, 155, 170.
9. Papariello G., Mukherji A . and Shcarer C., (1973), ***Anal. Chem***., 45, 792.
10. Gerhard H.and Vogel (2002) , ***Drug Discovery And Evaluation Pharmacological Assays***, II Edition,1345-1346.
11. Metwally MA, El-Hussiny MS, El-Ablak FZ. and Khalil AM, (1989), "Synthesis of Some Heterocycles of Pharmaceutical Interest", ***Pharmazie***, Vol. 44(4), 261-5.
12. Gupta RR, Kumar M and Gupta V(1999), ***Heterocyclic Chemistry***: ***Five Membered Heterocycles,*** Vol.2, 421-422.
13. (1996), ***Pharmacopoeia of India*****,** Vol-II, Controller of Publication, Delhi, A-9.
14. Ananthanarayan R., (1990), ***A Text Book of Microbiology*****,** Orient Longman Ltd, 160 Anna, Salai, Madras; 4th Edition, 591-592.

Published by:
Informative Journals
Jadoun Science Publishing Group India