Research article

A novel and efficient synthesis of 1, 2, 3,4-tetrahydropyrimidine carboxamide derivatives by Biginelli reaction and their *in vitro* antibacterial activity

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ABSTRACT

Introduction and Aim: An efficient aspect of two step synthesis of tetrahydro pyrimidine carboxamide derivatives were developed by Biginelli reaction. The two-step synthesized 1,2,3,4-tetrahydropyrimidinecarboxamide compounds were evaluated by *In-vitro* studies like antifungal and bacterial activities. Few compounds were shown excellent zone of inhibition against fungal and Microbial activities. In this study showed significant effect of antifungal and bacterial action against tetrahydro pyrimidine derivatives.

Materials and Methods: In this procedure to synthesize 1,2,3,4-tetrahydropyrimidinecarboxamide derivatives, ethyl acetoacetate, cytosine, *para*-toluene sulphonic acid and ethanol were used.

Results: The synthesized compounds of 1,2,3,4-tetrahydropyrimidine carboxamide derivatives through Biginelli reaction used an efficient catalyst such as *para*-toluene sulphonic acid and solvent ethanol. In this study tested compounds have developed into two step protocol for synthesis of diversely substituted novel pyrimidines.

Conclusion: In this study the synthesized 1,2,3,4-tetrahydropyrimidine carboxamide (THPC) shows significant inhibiting action against Gram +ve, Gram –ve bacterial and fungal growth.

Keywords: Tetrahydro pyrimidine carboxamide derivatives; 2, 2-diphenyl-1-picrylhydrazyl (DPPH); nitrous oxide; antibacterial activity; antifungal activity.

INTRODUCTION

A recent development of synthetic organic compounds from readily available reagents is one of the major tasks in organic synthesis. 1,2,3,4-tetrahydro pyrimidine carboxamide derivatives, named Biginelli compounds, represent a heterocyclic system of remarkable pharmacological interest. Now a days, it exhibits a varied range of biological effects including antiviral, antitumor, antibacterial, and anti-inflammatory activities (1-3). The one step method is most widely used method as compared to multistep methods since they require time consumption reaction and gives sophisticated yield with easy workup. Pyrimidine moiety is an important class of N-containing heterocycles widely used as key building blocks for Pharmaceutical agents. Also, Biological and synthetic significance at a prestigious position in the medicinal chemistry research and preclinical data from literature survey indicates that polysubstituted pyrimidine as potential anti-tumor agents (4). The scope of the original Biginelli reaction was gradually extended by variation of all three building blocks, allowing access to many multifunctionalized tetrahydropyrimidines. The several protocols and different reaction conditions have been employed to improve yield of Biginelli reaction (5, 6).

The *in vitro* biological activity of these compounds was evaluated by their growth inhibitory potency against Bacterial and fungal. In this work we have screened some of the synthesized tetrahydro pyrimidine derivatives exhibits wide range of biologically effective antitumor, antifungal, and antibacterial activities. The pyrimidine derivative as antitumor agents has led to the preparation and anticancer activity evaluation of hundreds of such molecules.

They also play a significant role of combustion, plasma chemistry and many other chemical processes. Free radicals may generate different kinds of chemical and biological reactions of the body cells. Heterocyclic compounds have been synthesized and their wide range of biologically active such as antimicrobial, anti-inflammatory, analgesic, antiviral and anticancer (7,8). Development of free radicals to involve the improve various degenerative diseases, huge generation of free radicals particularly reactive oxygen species and their high activity may lead to progression of several pathological disturbances such as inflammation, atherosclerosis, cancer, Parkinson's disease, and Alzheimer's disease. This phenomenon of excessive production of free radicals is termed as oxidative stress. Mondal *et al.,* (9) synthesized a series of 1-(2-mercapto-6-indoline-2-one and 1-(2-amino-6-(substituted phenyl) pyrimidine-4-yl)-3-(2-subsitutedphenylimino) indolin-2-one from different

substituted chalconised indole-2, 3-dione. These compounds show more potential antioxidant activity due to the substitution of the SH and NH_2 groups at the second position of pyrimidine ring. The newly synthesized compounds were subjected to antioxidant activity. Novel 1,2,3,4-Teterahydrocarboxamide derivatives incorporated with Indole, Urea Thiourea and other moieties possess potential antioxidant activities (10). Gressler *et al.,* (11) synthesized a series of 4-trifluoromethyl-2-(5-aryl-3-styryl-1 H-pyrazol-1yl)-pyrimidine derivatives and was screened for *in vitro* antioxidant activity and was evaluated using DPPH and HRP/luminol/H_2O_2 chemiluminescence assay method. The projected investigation of THPC in antioxidant screening, have shown more promising antioxidant activity as compared with the standard drug 5-Flurouracil, while other derivatives showed moderate activity.

Abu-Hashem *et al.,* (10) synthesized a series of pyrimidine derivatives 6-amino-2-thiouracil with ethyl bromoacetate yielded ethyl 2-(7-amino-2,5-dioxo-3,5-dihydro-2-H-thiazolo [3,2-a] pyrimidine-6-yl) acetate and 7-amino-6-[(5-thioxo-4,5-dihydro-1,3,4-oxadiazol-2-yl)-methyl]-5 thiazolo pyrimidine-3,5 (2H) - dione. Novel tetrahydro carboxamide derivatives incorporated with indole, urea, thiourea and other moieties possess potential antioxidant activities. Gressler *et al.,* (11) synthesized a series of 4-trifluoromethyl-2-(5-aryl-3-styryl-1 H-pyrazol-1yl)-pyrimidine derivatives and were screened for their *In-vitro* antioxidant activity. The antioxidant activity was evaluated using the DPPH and HRP/luminol/H_2O_2 chemiluminescence assay method. In the present study, the 3- (4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and neutral red (NR) assays for quantitative evaluation which have been adapted for human inactivated allografts are presented (12). The first group of assay measures the ability of viable cells to reduce a water-soluble yellow dye, MTT, to a water-insoluble purple formazan product. The DPPH assay measures the hydrogen-donating of the molecules in the sample. On other hand, chemiluminescence method is based on the light emission produced by a chemical reaction. The projected investigation of THPC's few compounds show more promising antioxidant activity as compared with the standard drug 5-Flurouracil.

Microbial studies

The synthesis of tetrahydro pyrimidines represent an important class of heterocycles and their structural framework is not only a key constituent of nucleic bases, alkaloids, and numerous pharmacophores with variety of potent biological activities, which enable rapid access to pyrimidines, that are desirable. The bis-pyrimidine derivatives potent activity against certain pathogenic strains of Gram-positive and Gram-negative bacteria and moderate activity against *Candida albicans* (13) exhibit potent inhibitory activity against the Gram-positive bacteria *Staphylococcus aureus* and *Bacillus subtilis* and the pathogenic fungi *Candida albicans* and *Aspergillus niger*, compared to trimethoprim, streptomycin and griseofulvin (14). The 2-oxo-1, 2-dihydropyrimidine-4yl)-1,2,3,4-Tetrahydropyrimidine carboxamide has excellent activity against pathogenic strains of the Gram-positive bacteria *Staphylococcus aureus* and the Gram-negative bacteria *Escherichia coli*. Meanwhile, the 4-chloro-5-cyanopyrimidine derivatives showed potent activity against six pathogenic bacteria including virulent and non-virulent strains of *Mycobacterium tuberculosis*, the Gram-negative bacteria *Pseudomonas aeruginosa* and *Escherichia coli* (15). Another derivative of pyrimidine like 5-substituted- 4-oxo-2-thioxo-1, 2, 3, 4-tetrahydropyrimidine (2- thiouracils) has anticancer activity. Tetrahydro pyrimidine derivatives with substituted amines in fifth position leads to anticancer activity (16). According to Selvam *et al.,* (17) pyrimidine with substituted amine in fifth position leads to anticancer activity. These findings led us to enlarge our investigations and to continue working on the synthesis of biologically active compounds. In this connection our aim of this research work, focused to develop newer synthetic formulation and methodology for widely used heterocyclic compounds, inspired us, to synthesize Tetrahydro pyrimidine carboxamide derivatives for the treatment of antibacterial and antifungal activities.

MATERIALS AND METHODS

Chemistry

All reagents and solvents were obtained from commercial suppliers and were used without further purification. Melting points (^{0}C) were measured in open glass capillaries using Branstead 9100 UK electrothermal melting point apparatus and are uncorrected. Infrared (IR) spectra were recorded on a FT/IR 6000, Fourier transform, infrared spectrometer (Japan) using the KBr disc technique and expressed in wave number υ(cm^{-1}). The ^{1}H-NMR spectra were recorded at 300MHz with a Bruker instrument and deuteriodimethyl sulphoxide (DMSO-d_6) used as solvents, TMS (tetramethylsilane) as internal standard and for ^{13}C-NMR spectral analysis were obtained at 75MHz, the chemical shifts are expressed in δ(ppm) downfield. The splitting patterns were designated as: s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet) and br. s (broad singlet). The mass spectra (MS) were measured with a JEOL GC mate mass spectrometer. Monitoring the reactions and checking the purity of the final products were carried out by thin layer chromatography (TLC) using silica gel precoated aluminum sheets (Type 60 F254, Merck, Darmstadt, Germany) and the spots were detected by using UV lamp at λ_{254} nanometer for few seconds.

General procedure for synthesis of tetrahydro-pyrimidine carboxamide derivatives

Using one-pot multicomponent Biginelli reaction, the tetrahydro pyrimidine carboxamides were synthesized using *para*-toluene sulphonic acid (PTSA) as an efficient catalyst and does not require any anhydrous conditions unlike the other Lewis acid catalysts.[130] A mixture of 3-oxo-N-(2-oxo-1,2-dihydropyrimidin-4-yl)butanamide (0.005 M), urea or thiourea (0.0075 M), and appropriate arylaldehyde (0.005M) with catalytic amount of *para*-toluene sulphonic acid (PTSA) (0.025 M) were taken in a two neck round bottom flask containing 15 ml of ethanol as a solvent. The round bottom flask was stirred well with magnetic stirrer for about 2 minutes to dissolve the reactants in the solvent. Then the reaction mixture was heated at reflux temperature for required time for the compounds. The reactions were monitored through TLC after the completion of reaction, the reaction mixtures were allowed to cool. The solid products formed were filtered, washed with water 2-3 times to remove the unreacted urea, thiourea and including PTSA and the solid product was dried. The collected products were further purified by recrystallization with ethanol to get pure tetrahydropyrimidine-5-carboxamide derivatives.

Antifungal screening

The antifungal activity, spread plate method was employed. Mueller Hinton agar plates were prepared aseptically to get a thickness of 5-6 mm. The plates were allowed to solidify and inverted to prevent the condensate falling on the agar surface with the help of sterile glass borer of 8 mm diameter in such a way that there was no overlapping of zone of inhibition. The plates were dried at 37°C before inoculation for half an hour for diffusion of the sample into agar media. The organisms were inoculated in the plates prepared earlier, by dipping a sterile swab in the previously standardized inoculums, removing the excess of inoculum by pressing and rotating the swab firmly against the sides of the culture tube above the level of the liquid and finally streaking the swab all over the surface of the medium three times, rotating the plates through an angle of 60° after each application. After the incubation period was over, the zones of inhibition produced by the sample in different plates were measured and recorded immediately. The same procedure was done in triplicate.

Antibacterial activity

The anti-bacterial activity was studied by selecting Gram-positive and Gram-negative bacteria via agar well diffusion method. The solutions were prepared in five different concentrations: (a) 750 ppm (b) 500 ppm (c) 250 ppm (d) 100 ppm and (e) 50 ppm.

Antibacterial activity sterilization

First step to sterilize the glass apparatus and other equipment. Glass apparatus are sterilized using hot air oven while cotton gloves are sterilized by using moist air sterilization in autoclave.

Preparation of culture medium

Then the culture medium is prepared. The culture medium used was nutrient agar medium. Composition of nutrient agar is as follows:

Beef Extract	- 10.0g
Peptone	- 10.0g
Sodium Chloride	- 5.0g
Distilled Water	- 1000 ml
Agar	- 15.0g

The ingredients were weighed and dissolve them with the aid of heat till a homogeneous solution is obtained (pH adjusted to 8.0 to 8.5 using 5M sodium hydroxide) followed by sterilization at 115°C for 30 minutes.

DPPH

Free radical scavenging activity of the test compounds were determined by DPPH assay method and compared with (EC_{50}) of Ascorbic acid as standard. Drug stock solutions (1µg/ml) were diluted to final concentrations of 2,4,6,8 and 10µg/ml in methanol. Minimum amount of dimethyl sulphoxide was used to solubilize the samples. One ml of 0.3 mM (12 gm in 100 ml) DPPH methanol solution was added to 2.5 ml of drug solution of different concentrations and allowed to react at room temperature. After 30 minutes the absorbance values were measured at 518 nm and converted to percentage antioxidant activity (AA %). It was calculated by the following formulae:

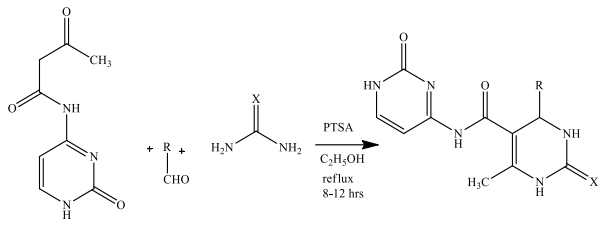
$$\% \text{ Reduction in Absorbance} = \frac{100 - (\text{Abs sample} - \text{Abs blank})}{\text{Abs control}} \text{ X } 100$$

Methanol (1ml) and drug solution (2.5 ml) was used as a blank. DPPH solution (1ml, 0.3 mM) and methanol (2.5 ml) was used as a control. Ascorbic acid was the standard solution. The EC_{50} values were calculated by linear regression of plots where the abscissa represented the concentration of the compounds (µg/ml) and the ordinate, the average percentage of antioxidant activity.

Scavenging activity (%) = $[1 - (A_1 - A_2) / A_0] \times 100\%$

RESULTS

All the products were characterized by ^{1}H-NMR, ^{13}C-NMR and Mass spectra.



Compound No.	R	X	Yield (%)	Time (Hours)
1	Furan-2-yl	S	72	10
2	Indole-5-yl	S	83	11
3	Indole-3-yl	S	73	11
4	Indole-3-yl	O	75	11

Scheme: Synthesis of tetrahydro pyrimidine carboxamide derivatives

The results of *in vitro* antifungal activities (Table 2) showed that the title compounds were active against nearly all fungi tested to some extent. Compounds 3 and 4 have exhibited substantial inhibitory activity against fungal growth when compared with Fluconazole.

Table 1: Antifungal activity of the pyrimidine compounds

Compound No	*C. albicans*		*A. niger*	
	50 µg/mL	100 µg/mL	50 µg/mL	100 µg/mL
1	2.9	4.2*	1.3**	3.4**
2	7.2	3.1	3.1	5.2
3	5.7*	5.9*	4.1*	6.8**
4	4.3*	5.2*	3.1*	5.7**
Standard (Fluconazole)	6.1*	6.9*	7.1*	6.8**

Values are expressed as mean ± S.E.M followed by One way ANOVA – Newman-Keuls multiple comparison tests. Abbreviation: THPC -1,2,3,4-Tetrahydropyrimidine carboxamide. Symbols represent statistical significance: ** - P < 0.01, * - 0.05, ns: non-significant.

The synthesized compounds 1,2,3 and 4 have been screened for their *in vitro* antimicrobial activity against Gram +ve bacteria (*S. aureus, Pseudomonas, B. subtilis and K. pneumoniae*) and Gram -ve bacteria (*Pseudomonas aeruginosa, Escherichia coli*) by measuring zone of inhibition at minimum inhibitory concentration 100 µl/mL (mm), revealed in Table (2).

Table 2: Preliminary antibacterial screening test for the tested compounds

Compounds	*E. coli*	*P. aeruginosa*	*S. aureus*	*Pseudomonas*	*B. subtilis*	*K. pneumoniae*
1	3.8**	5.3**	6.1**	5.2*	6.3**	7.2**
2	9.3	4.1	1.4	13.2	4.1	3.6
3	7.1**	6.2**	6.9**	4.7*	6.13**	5.7*
4	8.2**	8.6**	8.1**	9.5**	6.7**	7.9**
Ciprofloxacin (10µg/ml)	11.4	16.2	17.1	15.7	18.4	21.3
DMSO	1.4	-	-	2.4	-	2.3

Values are expressed as mean ± S.E.M followed by ONE Way ANOVA – Newman-Keuls multiple comparison tests. Abbreviation: THPC - 1,2,3,4-Tetrahydropyrimidine carboxamide; STD – Ciprofloxacin. Symbols represent at statistical significance: ** - P < 0.01, * - 0.05, ns – non-significant.

Table 3: Quantitative assay of antioxidant activity by DPPH Method

Compound No.	Absorbance at 516 nm					% µg/ml
	5 µg/ml	10 µg/ml	25 µg/ml	50 µg/ml	100 µg/ml	
Standard	0.79	0.85	0.86	0.88	0.95	4.1
1	0.56	0.47	0.45	0.41	0.23	3.8**
2	0.51	0.44	0.36	0.28	0.22	4.4**
3	0.42	0.36	0.29	0.21	0.19	3.5**
4	0.45	0.35	0.27	0.23	0.17	3.8**

Values are expressed as mean ± S.E.M followed by ONE way ANOVA – Newman-Keuls multiple comparison tests. Abbreviation: THPC - 1,2,3,4-Tetrahydropyrimidine carboxamide; STD: Vitamin C. Symbols represent at statistical significance: ** - P < 0.01, * - 0.05, ns – non-significant.

Table 4: *In vitro* nitrous oxide scavenging effect of pyrimidine derivatives

Compound No.	Pyrimidine Compounds conc. µg/ml			
	10 µg/ml	**25 µg/ml**	**50 µg/ml**	**100 µg/ml**
1	4.31 ± 0.06	5.66 ± 0.04*	8.41 ± 0.05*	3.27 ± 0.07*
2	15.10 ± 0.61	19.37± 0.46	24.17± 0.47	29.31± 0.51
3	4.1± 0.01	5.23± 0.03*	6.62± 0.06*	7.21± 0.09*
4	5.26 ± 0.04	4.17 ± 0.03*	6.31± 0.03*	8.52 ± 0.09*
STD (BHA)	4.42± 0.03	6.37± 0.04	10.76± 0.04	15.34 ± 0.07

Values are expressed as mean ± S.E.M followed by ONE Way ANOVA – Newman-Keuls multiple comparison tests. Abbreviation: THPC - 1,2,3,4-Tetrahydropyrimidine carboxamide; STD – BHA. Symbols represent at statistical significance: ** - $P < 0.01$, * - 0.05, ns: non-significant.

In the present study 1, 4-tetrahydropyrimidine carboxamide derivatives (4, 3, 1 and 2**)** have shown better antioxidant activity due to the presence of 3-indole, furan moiety at fourth position of tetrahydro pyrimidine carboxamide ring (Table 3).

The results have shown that synthesized compounds 4 and 3 exhibited promising effect of nitrous oxide released and acted as scavenger compared with standard. The sequential order of Nitric oxide is BHA (Butylated hydroxy anisole) ≥ compound 4 ≥ compound 3 ≥ compound 1 ≥ compound 2 values of 3.27 ± 0.07, 8.14 ± 0.21, 7.21 ± 0.09, 8.52 ± 0.09µg/ml, respectively (Table 4).

DISCUSSION

Antifungal activities

The antifungal activity of compounds 1-4 on *C. albicans* and *A.niger* growth and was evaluated. Many of the pharmacological relevant substitution patterns on the aromatic ring could be introduced with high efficiency. Aromatic aldehydes carrying electron-donating substituent afforded high yields of products in high purity (18). The newly synthesized compounds were subjected for their potent antioxidant activity. Novel 1,2,3,4-teterahydrocarboxamide derivatives incorporated with Indole, Urea Thiourea and other moieties possess that could be possible to inhibit the fungal growth and free radical generation. The compounds (1 and 2) have showed moderate function against *C. albicans* and *A. niger*. The inhibitor function has been attributed, due to substitution of indole-3-yl at the fourth position of 1,2,3,4-Tetrahydropyrimidine ring that may improve the biological property.

Antibacterial activities

The antibacterial activities of the newly synthesized THPC compounds were evaluated by microbial strains *P. aeruginosa* by microbroth dilution assay. All the microbial strains have been used are, non-invasive species of their genera and thus, applicable for analytical work. *S. aureus, Escherichia coli, P. aeruginosa, Pseudomonas, S. pneumonia* are the common causes for food poisoning. Therefore, the selected these organisms screened their antimicrobial activities have shown sensitive inhibitor function against strain of *E. coli* with standard of Ciprofloxacin. The compound 4 and 3 are more sensitive action and effective inhibitors due to the presence of Indole-3-yl substituent at fourth position of 1,4 tetrahydro pyrimidine ring. It has proved to be potent action (MIC), against Gram +ve and Gram -ve organism and rest of the compounds present moderate inhibition. Compounds (1 and 2) are found to be moderate action against strains of *S. aureus*, as compared to Ciprofloxacin used as standard (Table 3). The anti-bacterial study of synthesized compounds has shown more inhibitor action against tested Gram-+ve and Gram-ve bacteria. Among these, interestingly, new compound derivatives found to be more potent biological property against Gram + ve and Gram –ve microbial organism.

DPPH

The synthesized compounds were evaluated for their antioxidant activity by DPPH method and hydrogen peroxide free radical scavenging method of Gressler *et al.*, (11) using ascorbic acid as standard drug. Some of the synthesized tetrahydro pyrimidine carboxamide derivatives were screened for *In-vitro* antioxidant activity. The antioxidant activity was evaluated by chemiluminescence method using DPPH and H_2O_2. The DPPH antioxidant assay measures hydrogen-donating capacity of the molecules in the sample. On the other hand, the chemiluminescence method is based on light emission induced chemical reaction. In these tested compounds 4 and 3 have shown potent antioxidant activity as compared to standard. In this connection pyrimidine derivatives act as scavenger for free radical generation, due to presence of indole-3-yl at the fourth position of tetrahydro pyrimidine carboxamide derivative, this may be responsible for its antioxidant activity. The present study revealed that the tested compounds particularly 4 and 3 have performed more significant as compared with standard and rest of the compounds (1 and 2) was found to be in moderate activity of the antioxidant.

Nitric oxide

Formation of reactive oxygen species (ROS) characterized by aerobic condition includes superoxide radicals and hydroxyl radicals often generated as byproducts of biological reaction.[202] Some of them play a positive role in the energy production. Phagocytosis, regulation of cell growth or inter cellular signaling or synthesis of biologically important compounds are involved in ROS system. Antioxidants can terminate the chain reactions by removing radical intermediates and can inhibit other oxidation reaction by being oxidized themselves. In the present study, the followed procedure is of sodium nitroprusside in aqueous solution at physiological pH, which interacts with oxygen to produce nitrate ions that can be estimated by use of Griess reagent. Scavenger of nitric oxide complete with the oxygen, leading to reduced production of nitric oxide.

CONCLUSION

In conclusion, a series of synthesized 1,2,3,4-tetrahydropyrimidine carboxamide derivatives through Biginelli type reaction using an efficient catalyst *para*-toluene sulphonic acid and solvent ethanol. In these studies, tested compounds have developed into two step protocol for synthesis of diversely substituted novel pyrimidines. All new molecules revealed admirable *in vitro* antioxidant, antifungal, and antibacterial actions. The potency of most of the active compounds was better than standard drug fluconazole. In this study, it was observed correlation between antifungal and bacterial action against tetrahydro pyrimidines derivatives. This study has shown clear indication of antioxidant, antibacterial and antifungal activities of pyrimidine which governed the substituted indole-3-yl and furan attached at fourth position of pyrimidine ring responsible for significant effect of *in vitro* free radical scavenger, anti-fungal and antibacterial action.

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CONFLICT OF INTEREST

Authors declare that there is no conflict of interest.

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