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Research paper



Spectral characterization, microbial activities and toxicity study of some newly synthesized hydrazone derivatives

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Abstract

Hydrazone derivatives of 2-Thioxodihyropyrimidine-2-dione were synthesized by addition of thiobarbituric acid with phenyl hydrazine, dinitrophenyl hydrazine, semicarbazide, thiosemicarbazide and benzohydrazide respectively. Structures of these synthesized compounds are characterized by means of UV, IR, Proton-NMR, Carbon- NMR. Finally the hydrazone derivatives synthesized are screened for biological activities namely antibacterial and antifungal activities. Also in addition the toxicity studies of the compounds are also performed.

Keywords: *Thiobarbituric; IR; NMR; Antimicrobial; Toxicity.*

1. Introduction

Hydrazones are important class of organic compounds used in the field of development of new drugs. They play major role in synthetic chemistry hence researchers are interested to synthesis the hydrazone derivatives by using different substituents and to evaluate their biological activities. It plays an important role in improving the antitumour selectivity and toxicity profile of antitumour agents by forming drug carrier systems employing suitable carrier proteins (Kratz et al. 1988). They are reported to possess antimicrobial, antitubercular, anticonvulsant, analgesic, anti-inflammatory, antiplatelet, anticancer, antifungal, antiviral, antitumor, antibacterial and antimalarial activities (Imramovsky et al. 2007; Janin et al. 2007; Salgin-Goksen et al. 2007; Bhalla et al. 2006; Silva et al. 2004; Savini et al. 2004; Bijev et al. 2006; Abdel- Aal et al. 2006; El-Hawash et al. 2006; Cocco et al. 2005; Malhotra et al. 2014). In the present study some new derivatives of 2-thioxodihydropyrimidine derivatives have been synthesized by nucleophilic substitution elimination reaction and evaluated for structural and microbial activities.

2. Materials and method

The chemical used for this synthesize were purchased from sigma Aldrich and Merck chemical company. Melting points were determined on a Mettler FP51 melting point apparatus and are uncorrected. UV spectra are recorded using ELICO-BL222 spectrophotometer λmax nm using spectral grade methanol solvent. Infrared spectra KBr, 4000–400 cm^{-1} have been recorded on SHIMADZU Fourier transform spectrophotometer. NMR spectra recorded using Bruker 300 MHz spectrometer for ^{1}H-NMR and 100MHz spectrometer for ^{13}C-NMR, with DMSO-d^{6} as solvent. Synthesized compounds were evaluated for their in-vitro antibacterial and antifungal activity against pathogenic bacterial and fungal species by disc diffusion method. Toxicity study was carried out using MTT assay method.

3. Experimental

3.1. Synthesis of thiobarbituric acid (TBA)

About 6g (0.25mol) of sodium metal is dissolved in 200ml of ethanol. To this solution 15g (0.25mol) of thiourea and 40ml of diethyl malonate is added. The reaction mixture is refluxed for 6hrs in an oil bath and then vacuum distillated to make ethanol recovery. The clear solution thus obtained is filtered, cooled in ice bath overnight and the resulting solution is acidified with HCl. The crude product obtained is collected, washed with 50ml water and dried in oven at 105-110 °C for nearly 4 hrs. The obtained white coloured precipitate Thiobarbituric acid-TBA is purified by recrystallization with ethanol. (M.P - 243 °C, yield-80%).

3.2. Synthesis of 2-thioxodihydropyrimidine-2-dione derivatives

Equimolar mixture of synthesized thiobarbituric acid and substituents (phenyl hydrazine, dinitrophenyl hydrazine, semicarbazide, thiosemicarbazide, benzohydrazide) are dissolved in 25ml ethanol separately and the content is poured into a RB fixed with a condenser and refluxed for 4hrs in an oil bath, then the reaction mixture is stand overnight and the precipitate obtained is filtered washed with ethanol and dried to obtain the derivatives, the product thus obtained is recrystallized to obtain pure product. The schematic procedures for synthesis of thiobarbituric acid and 2-thioxodihydropyrimidine-2-dione derivatives are shown in Scheme. I & Scheme. II of the picture Fig.1. The mechanisms of formation are digrammatically presented in Fig. 2.
The symbol X in the linkage X-NH-NH_2 represented in Scheme-II of the Fig.1 is probably mentioned below.
X= i) $–C_6H_5$; ii) $–C_6H_3(NO_2)_2$; iii) $–CONH_2$; IV) –CSNH2; V)-COC_6H_5

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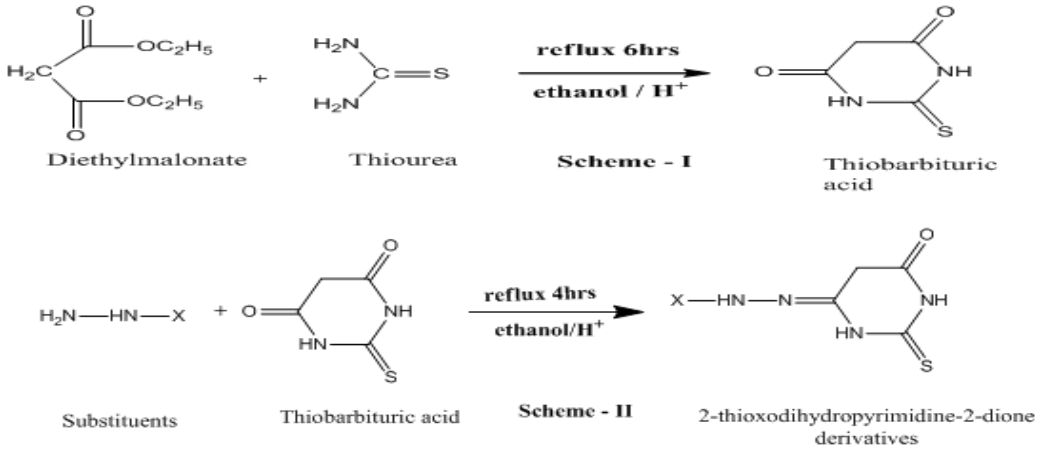


Fig. 1: Schematic Diagram of Synthesis.

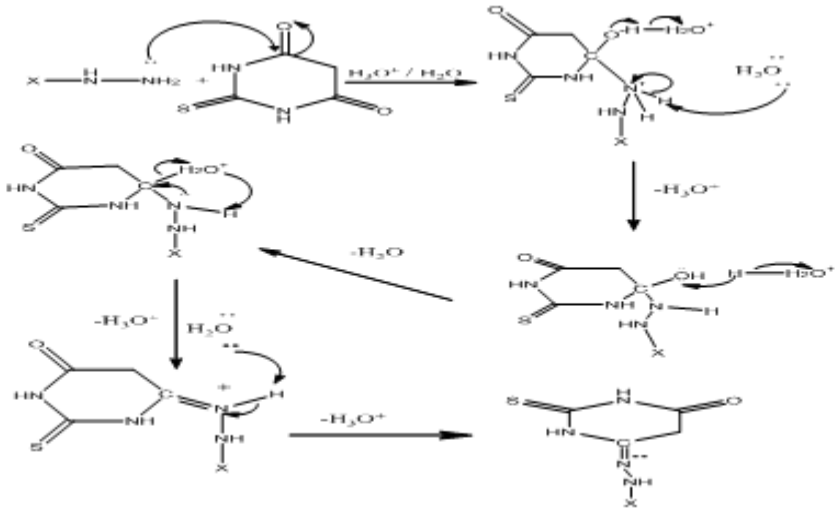


Fig. 2: Mechanism of Synthesis.

4. Result and discussion

The physical parameters such as melting point, yield and colour of the compound are noted. Structures of the compound were elucidated by IR, Raman and NMR as assigned below according to the literature (Long 2004, Wong 2015). The spectral assignments of the synthesized parent compound and their substituted derivatives are discussed below.

4.1. Structural elucidation of the synthesized compound by spectral assignments

4.1.1. 2-Thioxodihyropyrimidine-2-dione (TBA)

^{1}HNMR spectra (δ value) - NH $_{(RING)}$- 12.1-12.2, CH_2 methylene- 4.979; ^{13}C-NMR spectra (ppm) – 163, 175.08, 30.53; Yield - 82%; M.P. - 243℃; Colour – white powder.

4.1.2. 6 - 2 – phenylhydrazono – 2 – thioxotetrahydropyrimidin – 4 (1H) – one (PHTTO)

UV spectra (nm) - 345, 302, 238; IR Spectra (Cm^{-1}) - 1697(C=O), 3186(N-H), 1163(C=S), 1625(C=N), 3014(Ar-H), 2870, 2667($CH_{2\text{-methylene}}$), 1537(Ar-C), 1105(N-N), 1386(C-N); Raman Spectra (Cm^{-1}) -1709(C=O), 3107(N-H), 1156(C=S), 1680(C=N), 3022(Ar-H), 2924, 2869($CH_{2\text{-methylene}}$), 1535(Ar-C), 1093(N-N), 1391(C-N); ^{1}HNMR spectra (δ value)- NH $_{(Hydrazide)}$ –5.0, NH $_{(RING)}$- 9.946, 10.922, CH_2 methylene- 1.163, Ar-H- 8.75, 8.17-8.20, 7.60-7.62; ^{13}C-NMR spectra ppm - 155.74,163.45,149.14,44.21,134.20,123.45,127.48, 129.53,115.55. Yield - 78%; M.P. - 180℃; Colour - light yellow powder.

4.1.3. 6-(2-(2, 4-dinitrophenyl) hydrazono)-2-thioxotetrahydropyrimidin-4(1H)-one

UV spectra (nm)- 343.5, 306.5; IR Spectra (Cm^{-1}) - 1653(C=O), 3292(N-H), 1122(C=S), 1593(C=N), 3118(Ar-H), 2924, 2835($CH_{2\text{-}}$ methylene), 1421(C-N), 1292(NO_2); Raman Spectra (Cm^{-1}) - 3320(N-H), 1124(C=S), 1600(C=N), 3268(Ar-H), 3086, 2972($CH_{2\text{-}}$ methylene), 1427(C-N), 1259(NO_2); ^{1}HNMR spectra (δ value)- NH $_{(Hydrazide)}$ –8.541, NH $_{(RING)}$- 8.775, 11.087, CH_2 methylene- 1.321, Ar-H- 7.25-7.30, 7.42, 7.55; ^{13}C-NMR spectra (ppm) -172.67, 175.67,168.38, 71.84, 150.0,144.54,131.97, 163.20. Yield - 72%; M.P. - 170℃; Colour - Dark reddish brown.

4.1.4. 2-(6-oxo-2-thioxotetrahydropyrimidin-4(1H)-ylidene) hydrazinecarboxamide

UV spectra (nm)- 355.5, 314, 246; IR Spectra (Cm^{-1}) - 1739 (C=O), 3379 (N-H), 1099 (C=S), 1622(C=N), 3047, 2870($CH_{2\text{-}}$ methylene), 1427(N-N), 1340,1303(C-N),1533, 1496(NH_2); Raman Spectra (Cm^{-1}) - 1729 (C=O), 3218(N-H), 1101(C=S), 1651(C=N), 3105, 2869($CH_{2\text{-}}$ methylene), 1440(N-N), 1367(C-N),1534(NH_2); ^{1}H-NMR spectra (δ value) - NH $_{(Hydrazide)}$ –5.040, NH $_{(RING)}$- 8.762, 11.10, CH_2 methylene- 2.144, NH_2- 7.239; ^{13}C-NMR spectra (ppm) -168.54, 183.53, 162.72, 30.65. Yield- 69%; M.P. - 170℃; Colour – Turbid white.

4.1.5. 2-(6-oxo-2-thioxotetrahydropyrimidin-4(1H)-ylidene) hydrazinecarbothioamide

UV spectra (nm)- 385.5, 256; IR Spectra (Cm^{-1}) - 1834 (C=O), 3720(N-H), 1165(C=S), 1639(C=N), 2954($CH_{2\text{-}}$ methylene), 1294(C-

N), 1537(NH_2); Raman Spectra (Cm^{-1}) - 3255N-H, 1049(C=S), 1604(C=N), 3180($CH_{2-\ methylene}$), 1301(C-N), 1517(NH_2); ^{1}H-NMR spectra (δ value)- NH $_{(Hydrazide)}$ –7.908, NH $_{(RING)}$- 10.218, 11.923, CH_2 methylene- 4.64, NH_2 – 9.48; ^{13}C-NMR spectra (ppm) – 183.3, 175.39, 166.45, 154.81,82.49. Yield - 68%; M.P. - 174℃; Colour - Dark yellow.

4.1.6. N'-(6-oxo-2-thioxotetrahydropyrimidin-4(1H)-ylidene) Benzohydrazide

UV spectra (nm)- 370.48, 313.16, 289.48; IR Spectra (Cm^{-1}) - 1799,1690(C=O), 3428(N-H), 1014(C=S), 1600(C=N), 3155(Ar-H), 2880, 2669($CH_{2-\ methylene}$), 1528(Ar-C), 1106(N-N), 1396(C-N); Raman Spectra (Cm^{-1}) - 1762,1723(C=O), 3470, 3298 (N-H), 1002(C=S), 1673(C=N), 3005(Ar-H), 2878 ($CH_{2-\ methylene}$), 1543(Ar-C), 1194(N-N), 1367(C-N); ^{1}HNMR spectra δ value- NH $_{(Hydrazide)}$ –5.017, NH $_{(RING)}$ – 7.964, 11.818, CH_2 methylene- 3.533, Ar-H- 7.54-7.67; ^{13}C-NMR spectra (ppm) –168.85, 175.31, 167.80, 166.40, 162.79, 82.58, 128.76-130.69. Yield- 84%; M.P. - 168℃; Colour - reddish white

4.2. Microbial study

Procedure:

About 10 mg of newly synthesized 2-thioxodihydropyrimidine-2-dione derivatives were dissolved in 1 ml of DMSO solvent. Using 100µml of solution, the discs have been impregnated and placed on the solidified Mueller Hinton Agar medium for antibacterial assay and Potato Dextrose agar medium for antifungal assay to find out the antimicrobial activity of the compounds on each organism. The antimicrobial sensitivity assay was performed by using (Bauer et al. 1966) disc diffusion technique. The antimicrobial activities of the derivatives have been studied against ten microorganisms five bacterial and five fungal organisms and the results have been discussed below.

4.2.1. Antibacterial activity

The synthesized 2- thioxodihydropyrimidine – 2 - dione derivatives are subjected to antibacterial activity revealed in Fig.3. The antibacterial activities of all the synthesized compounds have been studied against three gram positive pathogenic strains Bacillus substilis, streptococcus pyogene and Staphylococcus aureus and two gram negative strains Escherichia coli and Pseudomonas aeruginosa. Table 1 shows the zone of inhibition of the derivatives and the resultant clustered column chart is shown in Fig. 4.
The compound OTTHPYBH shows excellent activity against all fungal organisms. PHTTO and DNPHTTO shows good activity against all fungal species when compared with OTHHPYHC and OTTHPYHCT which show moderate activity for all, except for staphylococcus aureus which shows poor activity.

4.2.2. Antifungal activity

The antifungal activities of all the synthesized 2-thioxodihydropyrimidine-2-dione derivatives have been studied against five fungal species namely, Aspergillus flavus, Aspergillus Niger, Fusarium oxysporum, penicillin chryogenum and Trigoderma viride by using disc diffusion method. Fig. 5 shows zone of inhibition of the derivatives and the resultant clustered column chart is shown in Fig. 6. From Table 2, most of the synthesized compounds in the present investigation have shown moderate to good activity against all the five fungal species probably.
The synthesized compound OTHHPYHC shows moderate activity against all five fungal species whereas PHTTO, DNPHTTO shows good to moderate activity against Penicillim chryogenum. OTTHPYHCT show very low activity against Aspergillus flavus, Aspergillus niger. OTTHPYBH shows poor activity against fungal species expect for Trigoderma veride which shows a mild activity.

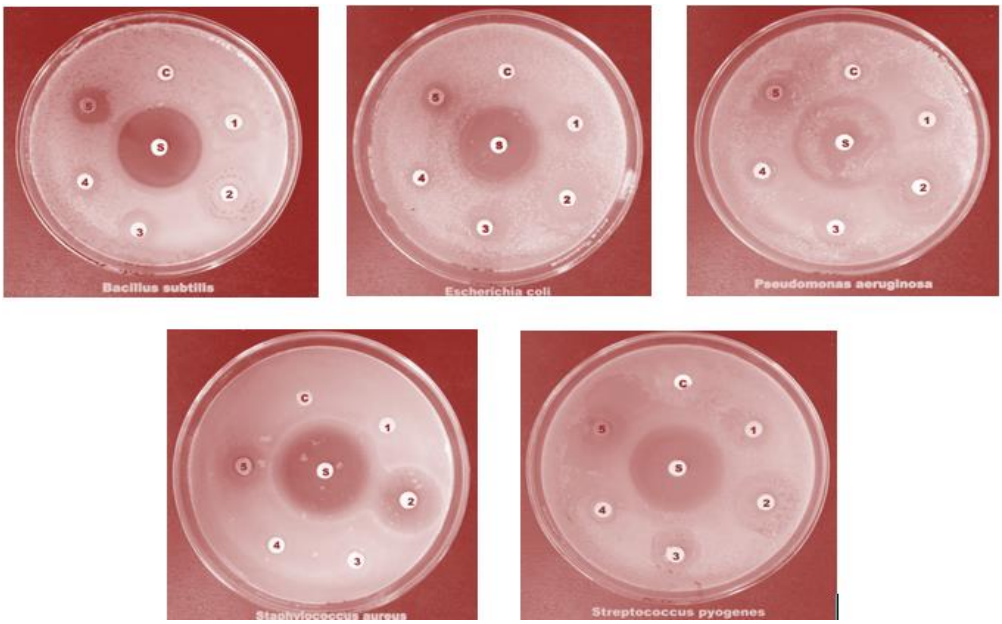


Fig. 3: Antibacterial activity of 2-thioxodihydropyrimidine-2-dione derivatives.

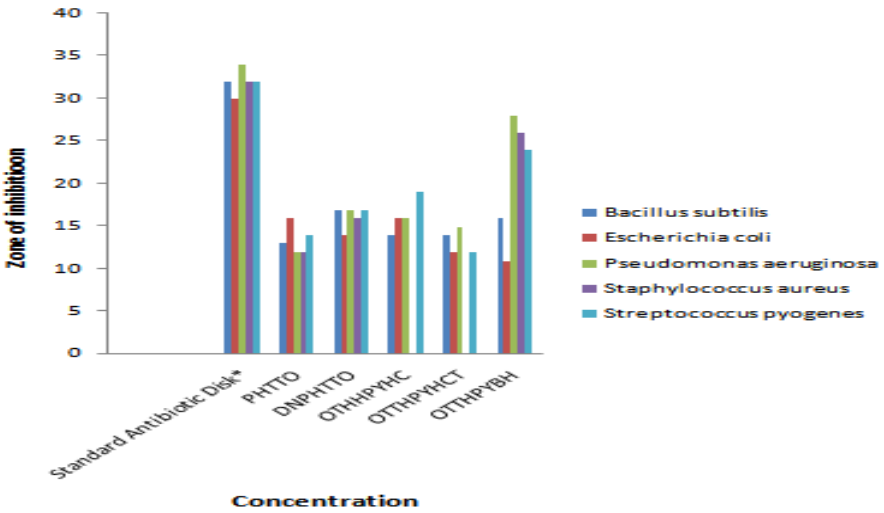


Fig. 4: Cluster Column Chart for Antibacterial Activity of 2-Thioxodihydropyrimidine-2-Dione Derivatives.

STANDARD: *CIPROFLOXACIN CONTROL: DMSO

Table 1: Antibacterial Activity [Disc Diffusion Method] of Hydrazone Derivatives of 2- Thioxodihydropyrimidine-2-Dione

COMPOUND NAME		Bacillus subtilis	Escherichia coli	Pseudomonas aeruginosa	Staphylococcus aureus	Streptococcus pyogenes
Zone of inhibition (mm)	Ciprofloxacin*	32	30	34	32	32
	PHTTO	13	16	12	12	14
	DNPHTTO	17	14	17	16	17
	OTHHPYHC	14	16	16	-	19
	OTTHPYHCT	14	12	15	-	12
	OTTHPYBH	16	11	28	26	24

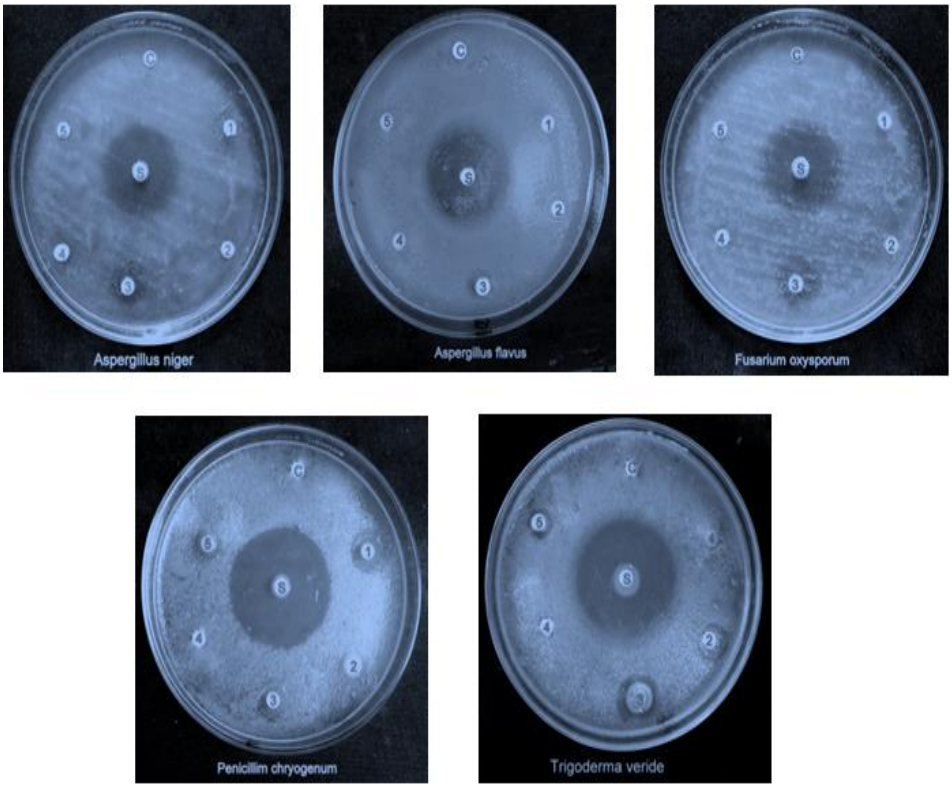


Fig. 5: Antifungal Activity of 2-Thioxodihydropyrimidine-2-Dione Derivatives.

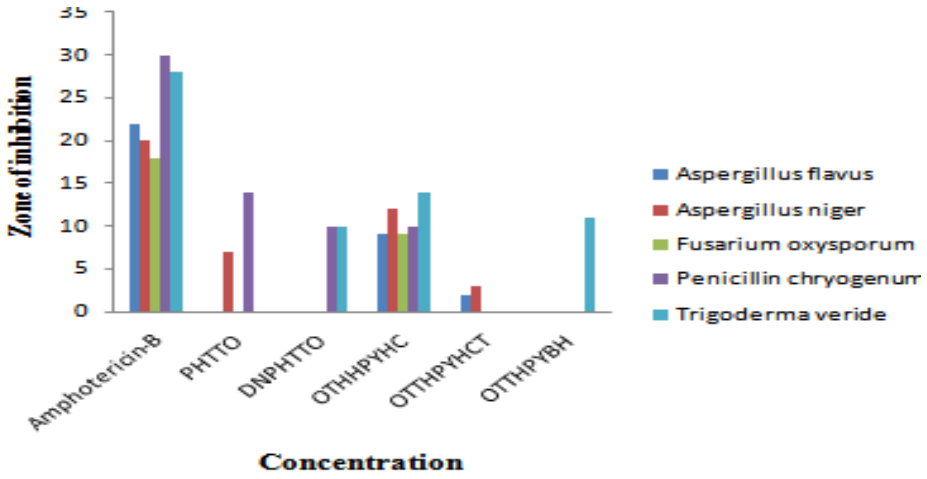


Fig. 6: Cluster Column Chart for Antifungal Activity of 2-Thioxodihydropyrimidine-2-Dione Derivatives.

Standard: *Amphotericin-B Control: DMSO.

Table 2: Antifungal Activity [Disc Diffusion Method] of Hydrazone Derivatives of 2- Thioxodihydropyrimidine-2-Dione

	COMPOUND NAME	Aspergillus flavus	Aspergillus niger	Fusarium oxysporum	Penicillim chryogenum	Trigoderma veride
Zone of inhibition (mm)	Amphotericin-B	22	20	18	30	28
	PHTTO	-	07	-	14	-
	DNPHTTO	-	-	-	10	10
	OTHHPYHC	09	12	09	10	14
	OTTHPYHCT	02	03	-	-	-
	OTTHPYBH	-	-	-	-	11

4.3. Toxicity study

MTT assay for cell viability:
The MTT assay is based on the application of MTT dye, which works with the action of mitochondrial enzyme. It is mainly performed to measure the functionality of human and animal cells. The MTT assay (Mossman 1983) is the best known method for determining mitochondrial dehydrogenase activity in the living cells. The mitochondrial enzymes are responsible for reduction of MTT a yellow tetrazolium dye MTT- 3-4, 5-dimethylthiazol-2-yl-2, 5-diphenyltetrazolium bromide to a purple formazan product by NADH. MTT formazan is insoluble in water, and it forms purple needle-shaped crystals in the cells. Only viable cells will convert MTT and give a purple colour, whereas dead cells will not be able to convert MTT. Therefore prior to measuring the absorbance, an organic solvent is required to solubilize the crystals. Additionally, the cytotoxicity of MTT formazan makes it difficult to remove cell culture media from the plate wells due to floating cells with MTT formazan needles, giving significant well-to-well error (Alley & Lieber 1984).

PROCEDURE:
Cells were maintained in DMEM medium, supplemented with 10% Fetal Bovine Serum, at 37ºC in humidified atmosphere with 5% CO_2. Colo 320 cells were plated in 96 well flat bottom tissue culture plates at a density of approximate l 1.2 X 10^4 cells / well and allowed to attach overnight at 37ºC. The medium was then discarded and cells were incubated with different concentrations of the extract for 24 hours. After the incubation, medium was discarded and 100µl fresh medium was added with 10µl of MTT 5mg/ml. After 4 hours, the medium was discarded and 100µl of DMSO was added to dissolve the formazan crystals. Then, the absorbance was read at 570 nm in a microtitre plate reader. The assay was performed for all 10 fractions obtained from column chromatography. Cell survival was calculated by the following formula and the values are tabulated in Table 3 and respective cluster chart is shown in Fig.7. The IC_{50} values obtained are greater than 100 hence all the five synthesized compounds show moderate activity.

Viability % = Test OD/ Control OD X 100

Cytotoxicity % = 100 – Viability%

Table 3: Cytotoxicity Analysis of Hydrazone Derivatives of 2- Thioxodihydropyrimidine-2-Dione

Sample	Concentration	Control	Sample	%of viability	%of toxicity	IC50 value
	Control	1.121	1.121	100	0	
PHTTO	50µg	1.121	1.009	90.00	9.99	213.03
	100µg	1.121	0.912	81.35	18.64	
	150µg	1.121	0.853	76.09	23.90	
DNPHTTO	50µg	1.121	0.912	81.35	18.64	181.01
	100µg	1.121	0.861	76.80	23.19	
	150µg	1.121	0.731	65.20	34.79	
OTHHPYHC	50µg	1.121	0.951	84.83	15.16	173.8
	100µg	1.121	0.833	74.30	25.69	
	150µg	1.121	0.715	63.78	36.21	
OTTHPYHCT	50µg	1.121	0.9001	80.29	19.70	171.41
	100µg	1.121	0.831	74.13	25.86	
	150µg	1.121	0.711	63.42	36.57	
OTTHPYBH	50µg	1.121	1.004	89.56	10.43	204.3
	100µg	1.121	0.902	80.46	19.54	
	150µg	1.121	0.834	74.39	25.61	

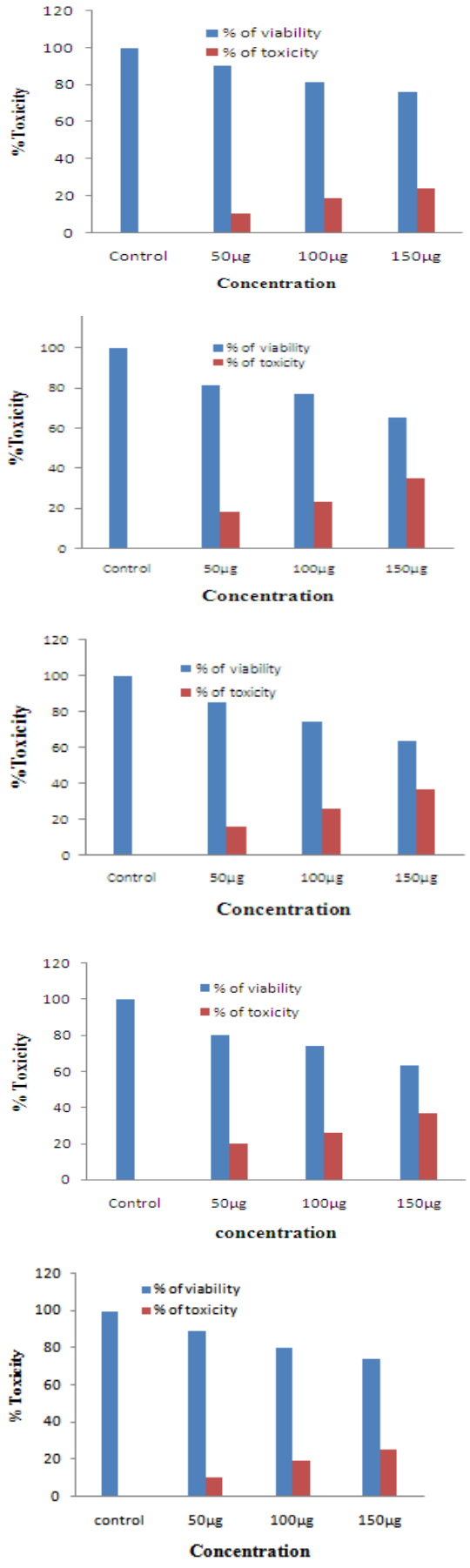


Fig. 7: Cluster Chart of Cytotoxicity Study of 2-Thioxodihydropyrimidine-2-Dione Derivatives.

5. Conclusion

All the synthesized compounds were characterized by UV absorption which favours π-π* transition for compounds attached with phenyl group and n-π*, n-σ* for other substituted compounds with lone pair of electrons. IR frequencies were calculated which acts as a supportive information of the presence of functional group. NMR both ^{1}H and ^{13}C are used to calculate the total no. of protons and carbon atom present in synthesized compound. Biological study of the compound reveals good to moderate bacterial activity for all the synthesized compound and moderate to low activity for antifungal. The requirement of new drugs to kill infectious species that affects human and animals cells. Since the biological study shows moderate activity we performed toxicity study in addition to determine the toxic nature of the synthesized compound in order to conform the usage of synthesized compounds in drug field. And probably from the IC_{50} value obtained all the compounds show moderate activity and hence in future we focus to have a deep study of the compounds in medicinal and other fields.

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