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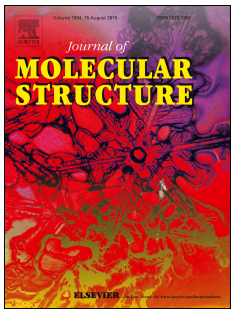

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DNA-binding studies and biological activities of new nitrosubstituted acyl thioureas

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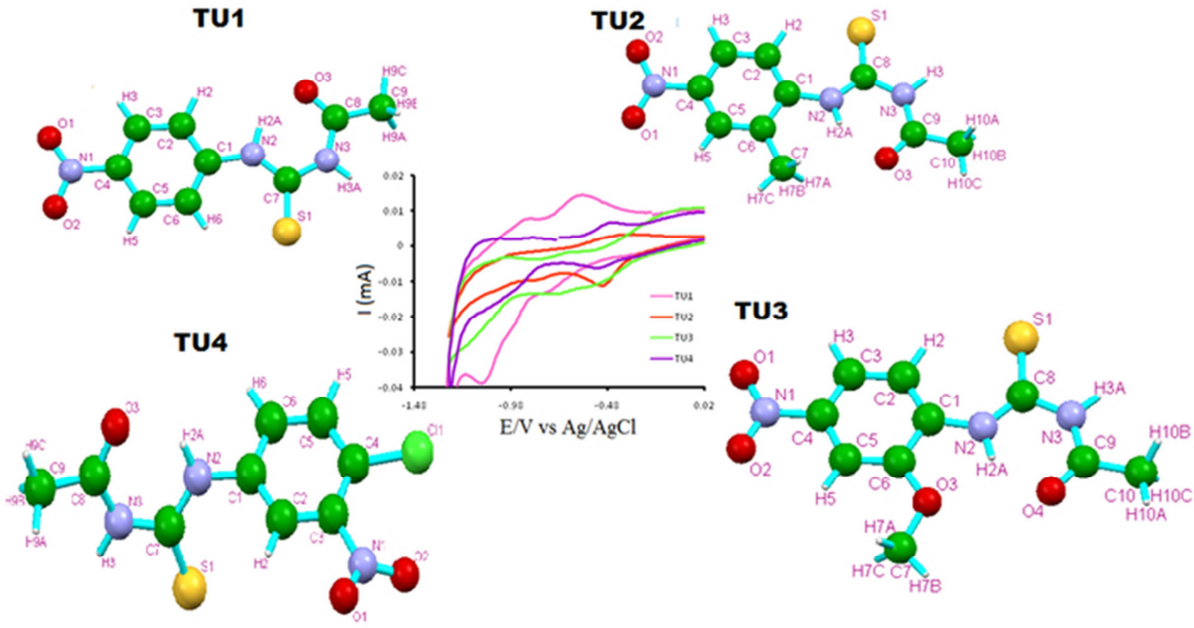


DNA-binding studies and biological activities of new nitrosubstituted acyl thioureas

Abstract:

Four new nitro substituted acylthioureas i.e. 1-acetyl-3-(4-nitrophenyl)thiourea (TU1), 1-acetyl-3-(2-methyl-4-nitrophenyl)thiourea (TU2), 1-acetyl-3-(2-methoxy-4-nitrophenyl)thiourea (TU3) and 1-acetyl-3-(4-chloro-3-nitrophenyl)thiourea (TU4) have been synthesized and characterized (by C^{13} and H^{1} nuclear magnetic resonance, Fourier transform infrared spectroscopy and single crystal X-ray diffraction). As a preliminary investigation of the anti-cancer potencies of the said compounds, DNA interaction studies have been carried out using cyclic voltammetry and UV-vis spectroscopy along with verification from computational studies. The drug-DNA binding constants are found to be in the order, K_{TU3} 9.04 x 10^6 M^{-1} > K_{TU4} 8.57 x 10^6 M^{-1} > K_{TU2} 6.05 x 10^6 M^{-1} > K_{TU1} 1.16 x 10^6 M^{-1}. Furthermore, the antioxidant, cytotoxic, antibacterial and antifungal activities have been carried out against DPPH (1,1-diphenyl-2-dipicrylhydrazyl), Brine shrimp eggs, gram positive (*M. luteus*, *S. aureus*) and gram negative (*B. bronchiseptica*, *S. typhimurium*, *E. aerogens*) and fungal cultures (*F. fumigatus*, *F. Mucor*, *F. niger*, *F. flavus*) respectively.

Keywords: Thiourea, DNA binding studies, cyclic voltammetry, UV-vis spectroscopy, antimicrobial activities, cytotoxicity

1. Introduction:

Thioureas, having a considerably wide range of applications, are the cousin compounds of ureas in which oxygen has been replaced with sulfur. The properties of urea and thiourea differ significantly because of the difference in electronegativity between sulfur and oxygen [1]. Thioureas possess broad spectrum of biological activities including antiviral [2], antibacterial [3], antifungal [4], antitubercular [5], herbicidal [6], insecticidal [7], and pharmacological properties [8], act as corrosion inhibitors, antioxidants and are polymer components [9].

Thiourea derivatives can be easily synthesized in good yield [10], and substituted thioureas have recently gained much interest in the preparation of wide variety of biologically active compounds [11]. Acylthiourea derivatives are well known for their biological activities like bactericidal, fungicidal, herbicidal, insecticidal and regulation of plant growth [12]. Herein, we have reported, the synthesis of four new nitrosubstituted

acylthioureas with complete characterization (FTIR, multinuclear NMR, single crystal XRD) and biological activities (DNA-binding, antioxidant, cytotoxic and antimicrobial activities).

As we previously reported in our review article on the biological applications of selenoureas and their homologues [8], thioureas are an important class of organic compounds with effective anti-cancer applications. However, their UV-vis spectra and cyclic voltammograms are not very helpful in the investigation of their ability to bind with DNA. So, we have synthesized nitro substituted thioureas with an aim to quantify the DNA interaction of the synthesized compounds from the results of their UV-vis spectroscopic and cyclic voltammetric analyses.

2. Materials and methods:

2.1 General

Melting points were determined in a capillary tube using electrothermal melting point apparatus model MP-D *Mitamura Riken Kogyo (Tokyo, Japan).* Infrared spectra were taken on a Thermo Scientific NICOLET 6700 *Fourier* transform infrared spectroscope (FT-IR). H^1 and C^{13} nuclear magnetic resonance (NMR) were recorded on a Jeol JNM-LA 500 FT-NMR. $Si(CH_3)_4$ was used as internal reference. Suitable single crystal of each nitrosubstituted acyl thiourea was mounted on a glass fiber and the intensity data were collected on a *Brucker* kappa APEXII CCD diffractometer using graphite monochromator having M_o Kα radiation (λ = 0.71073 Å) at 296 K. The structures were solved by direct methods and refined by full-matrix least squares against F^2 of data using SHELXL97 (Sheldrick, 1997) software [13]. Basic crystal data and description of diffraction experiment are given in Table 1.

Table 1. Crystal diffraction data of TU1, TU2, TU3 and TU4

	TU1	**TU2**	**TU3**	**TU4**
Empirical formula	$C_9H_9N_3O_3S$	$C_{10}H_{11}N_3O_3S$	$C_{20}H_{22}N_6O_8S_2$	$C_9H_8ClN_3O_3S$
Formula weight	239.25	253.28	538.55	273.69
Temperature (K)	296(2)	296(2)	296(2)	296(2)

Wavelength (Å)	0.71073	0.710173	0.710173	0.710173
a [Å]	5.1519(4)	8.7665(11)	3.9876(12)	4.484(2)
b [Å]	9.0702(7)	7.7560(7)	8.973(3)	23.617(11)
c [Å]	11.9097(9)	17.384(2)	16.899(6)	10.749(5)
α [degree]	71.175(4)	90	81.083(12)	90
β [degree]	82.499(4)	100.680(3)	88.322(13)	97.151(14)
γ [degree]	86.865(5)	90	79.505(13)	90
Volume (°A3)	522.20(7)	1161.5(2)	587.3(3)	1129.5(9)
Crystal system	triclinic	monoclinic	triclinic	monoclinic
Space group	P -1	P 21/c	P -1	P 21/c
Index ranges	$-6 \le h \le 6$, $-11 \le k \le 11$, $-15 \le l \le 15$	$-11 \le h \le 11$, $-9 \le k \le 10$, $-22 \le l \le 21$	$-5 \le h \le 4$, $-11 \le k \le 11$, $-22 \le l \le 21$	$-5 \le h \le 5$, $-29 \le k \le 21$, $-13 \le l \le 13$
Absorption coefficient (μ)	0.305	0.279	0.287	0.522
F(000)	248	528	280	560
Goodness-of-fit on F2 (S)	1.044	1.035	0.922	0.968
R factor (%); R_1, R_2.	0.0652, 0.0425	0.0602, 0.0422	0.2252, 0.0650	0.1452, 0.0556

The computational study (DFT) was carried out using GaussView 5.0 software.

2.2 Synthesis:

All chemicals, organic solvents and reagents were purchased from Sigma-Aldrich, Fluka and E. Merck. Organic solvents acetone (99.9 % pure), and *n*-hexane (95 % pure) were distilled, purified & dried according to reported methods. Potassium thiocyanate (98 % pure), acetyl chloride (99 % pure), 4-nitroaniline (99 % pure), 4-chloro-3-nitroaniline (99 % pure), 2-methyl-4-nitroaniline (99 % pure) and 2-methoxy-4-nitroaniline (99 % pure) were used without further purification.

Acetyl chloride was added to the solution containing potassium thiocyanate in dry acetone and stirred for about 3 hours then respective nitroaniline was added into the

reaction mixture and further refluxed for about 5 hours to obtain the desired thioureas [14].

2.3 DNA-binding study by cyclic voltammetry:

Commercial *Salmon* DNA was solubilized in doubly distilled water to prepare a stock solution of 6×10^{-4} M from which working concentrations of DNA were prepared. Concentration of the stock solution was measured by UV absorbance (with *Shimadzu* 1800 spectrphotometer) at 260 nm using an epsilon value of 6600 $M^{-1}cm^{-1}$. This DNA was protein free because A260/A280 > *1.8*. Solution preparation for DNA binding studies was carried out according to our previously reported method [15, 16]. Cyclic voltammetry was performed on Biologic SP-300 cyclic voltammeter running with EC-Lab Express V 5.40 software (made in *France*). Before every reading, the working electrode was polished with alumina powder and rinsed with distilled water. Analytical grade KCl was used as supporting electrolyte and nitrogen gas (99.9 %) was purged through the mixture to avoid interference from oxygen. A setup having three electrodes system, i.e. working (platinum disc electrode with a geometric area of 0.071 cm^2s^{-1}), reference Ag/AgCl and auxiliary electrode (platinum electrode with geometric area much greater than working electrode) was used for cyclic voltammetric studies. The decrease in the peak current provided information about DNA binding constant whereas the shifts in the peak potentials were useful for the determination of the mode of interaction of the drug with DNA. Drug-DNA binding constant was determined with the help of following equation:

$$\log (1/ [DNA]) = \log K + \log (I/ I_o - I) \quad (1)$$

where K is the binding constant and I_o and I are the peak currents of free drug and DNA-bound drug respectively [14].

2.4 DNA-binding study by UV-vis spectroscopy:

For the DNA binding constants with the help of UV-vis spectroscopy at first the spectra of the analytes before the addition of DNA were recorded by taking solvent in the reference cell and solution of the analyte in the sample cell. Then the spectra were

recorded by adding different concentration of DNA, in solutions having constant concentration of the compound. The whole experiment was carried out by keeping the volume and concentration of the compound constant while varying the amount of DNA [16]. The equilibrium constants (binding constant) is calculated by fitting data in the *Benesi-Hildebrand* equation (2).

$$A_o/ A\text{-}A_o = (\varepsilon_G/ \varepsilon_{H\text{-}G} - \varepsilon_G) + (\varepsilon_G/ \varepsilon_{H\text{-}G} - \varepsilon_G)(1/ K[DNA]) \quad (2)$$

Where A_o and A are the absorbance of the free compound and of the compound–DNA complex, **ε**G and **ε**H-G are the molar extinction coefficients of free compound and of the compound–DNA complex respectively.

2.5 Antioxidant assay:

Oxidant reducing abilities of all the four nitrosubstituted thiureas were determined with the help of 1,1-diphenyl-2-picrylhydrazyl radical in DMSO to produce 1,1-diphenyl-2-picrylhydrazine. The decrease in the absorption of 1,1-diphenyl-2-picrylhydrazyl (DPPH) was monitored to calculate the percentage scavenging according to the following formula [17]:

$$\text{Scavenging activity (\%)} = A_o - A/A_o \times 100 \quad (3)$$

Where A_o is the absorbance of free DPPH and A is the absorption of DPPH–drug mixture with increasing concentration of drug. To a solution of DPPH (3.9 mg of DPPH in 100 mL DMSO) were added the increasing concentrations (1 mM) of nitrosubstituted thioureas. The decrease in absorption was monitored after 5 min at a wavelength of 517 nm of DPPH with the help of spectrophotometer. All the readings were taken in triplicate and average of all the readings was used.

2.6 Cytotoxic assay:

Cytotoxic effect of synthesized thioureas was determined using brine shrimp assay following the method as described by McLaughlin and Rogers [18]. 50 mg of compound was dissolved in 1 mL of DMSO to the prepared stock solution of each sample. Further 1000 µg/mL, 100 µg/mL and 10 µg/mL dilutions were prepared from the stock solution. Terbinafine was used as the standard drug. 1 mg of standard was dissolved in 1 mL of

DMSO to prepare stock solution. Three concentrations i.e. 10 µg/mL, 1 µg/mL and 0.1 µg/mL were used. Sea water produced by dissolving 34 g of sea salt in 1000 mL of water. Solution was stirred constantly, sea water was aerated in open mouth beaker for sufficient time. Hatching of shrimp eggs was carried out in compartmentalized rectangular dish (22 × 23 cm), which was filled with sea water. One large and other small compartment with separating wall containing holes were present in the dish. Eggs were poured in large compartment. Lamp light was showered on smaller compartment, after covering larger compartment with aluminum foil. Newly hatched nauplii moved towards small compartment due to light. These were picked with pasture pipette and dispensed in small beaker having sea water. Initially all vials were labeled, followed by addition of 2 mL sea water in all vials. After that 0.1 mL sample solution from their respective stock solutions were added in the respective vials. Ten nauplii were counted using pasture pipette and 3X magnify glass and transferred to vials. Simultaneously, volume was raised up to 5 mL with sea water, so that it does not affect the shrimps and desired concentration of sample is attained. These vials were incubated for 24 hours at room temperature. After that shrimps were taken out from vials using pasture pipette and counted under magnifying glass. Finally lethal dose (LD_{50}) of the thioureas was determined using finny (1971) software.

2.7 Antibacterial assay:

Antibacterial activity was determined by using disc diffusion method as described by Islam *et al.* (2013) [19], against two gram positive (*M. luteus* ATCC 10240, *S. aureus* ATCC 6538) and three gram negative strains (*B. bronchiseptica* ATCC 4617, *S. typhimurium* ATCC 14028, *E. aerogens* ATCC 13048). Nutrient broth was used for the maintenance of bacterial cultures for 24 hours at 4 ˚C. Next day cold cultures of these strains in nutrient broth (*Merck, Germany*) were used for assay. 4 mg of each nitrosubstituted thioureas was dissolved in 1 mL of DMSO, for the production of stock solution of each complex. Final concentration of 30 µg/disc of each complex was used in the assay. Cefixime-USP and roxythromycin were used as standard drugs. Stock of each standard drug was prepared by dissolving 4 mg 1 mL of DMSO. DMSO was used as negative control.

Bacterial inoculum was prepared in nutrient broth, by dissolving 2 g of broth in 100 mL of water and pH was maintained at 7. Nutrient agar medium was used for the growth of bacterial strains that was produced by dissolving 2 g in 100 mL of water. Filter paper discs of 6 mm in size ware prepared from Whatman no. 1 filter paper. Media, filter paper discs along with other apparatus required in this assay were autoclaved for sterilization. After autoclaving, whole experiment was carried out in microbiological safety cabinet. Solidified plates of nutrient agar were labeled and respective bacterial strain was streaked. Then 7.5 µL of each complex solution was absorbed on disc. These discs were placed on respective places in petri plate. These petri plates were incubated for 24 hours at 28 ˚C. Zones of inhibitions were measured after 24 hours. The following formula was used to calculate the percent bacterial inhibition.

% inhibition = A/B x 100 (4)

Where A is the linear growth (mm) in test sample and B is the linear growth (mm) in control.

2.8 Antifungal assay:

Antifungal assay was performed by following disc diffusion method as described by Islam *e t al.* (2013) [19]. Fungal cultures (*F. fumigates* FCBP 66, *Mucor*species FCBP 0300, *F. niger* FCBP 0198, *F. flavus* FCBP 0064) were maintained on SDA at 4 ˚C. 4 mg of each synthesized thiourea was dissolved in 1 mL of DMSO to prepare stock solution of each sample. Terbinafine was used as a standard drug (positive control) and DMSO was used as negative control. Stock solution of standard was prepared by dissolving 4 mg of standard drug in 1 mL of DMSO and 10 µg/disc concentration of standard drug was used. Fungal cultures were grown on SDA medium at 6.5 pH. Sabouraud dextrose agar (SDA) media was prepared by dissolving 6.5 g in 100 mL of distilled water and the pH was adjusted to 6.5. Whatman no.1 filter paper was used for the preparation of circular discs of 6 mm size. All apparatus required in the assay were autoclaved for sterilization. Experiment was carried out in microbiological safety cabinet. SDA media was poured in petri plates and left for solidification. Filter paper discs were used for the absorption of sample solution and 7.5 µL of compound solution absorbed on disc. After absorption of complex solution on disc, were placed in their respective places on solidified SDA plates.

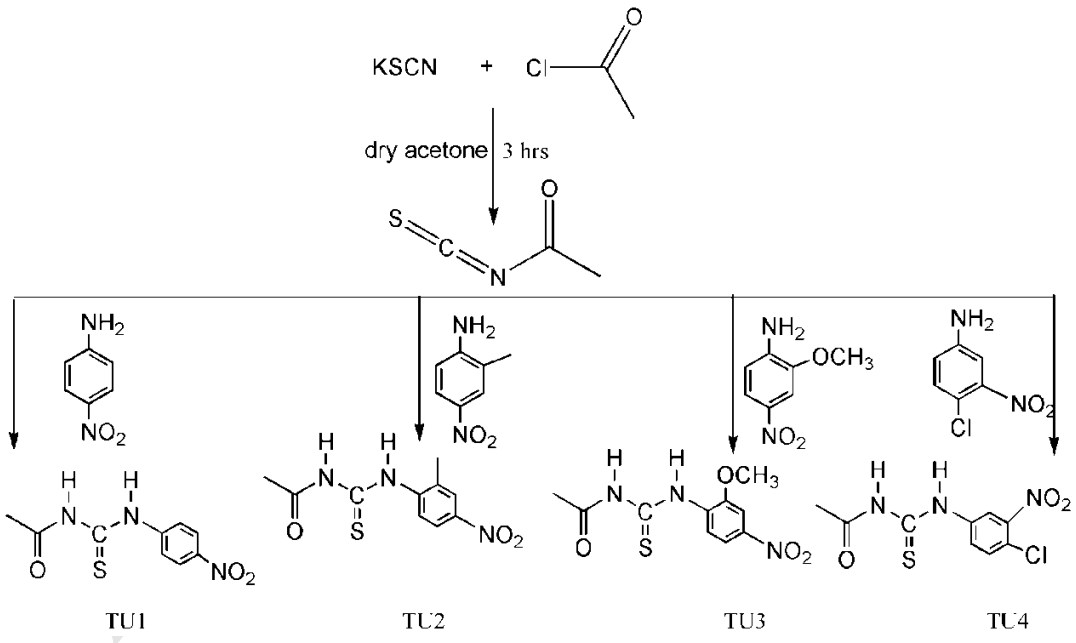
Contamination was prevented by wrapping petri plates with parafilm. After that plates were incubated for 24 hours at 28 °C. On next day inhibition zones were measured by using vernier caliper. Percent fungal inhibition was calculated by using equation 4.

3. Experimental:

3.1 1-acetyl-3-(4-nitrophenyl)thiourea (TU1)

TU1 was synthesized by the reaction of 0.2 g of potassium thiocyanate (0.00206 mol) with 0.15 mL (0.00206 mol) of acetylchloride, in dry acetone at continuous stirring for 3 hours. After the formation of white precipitates of potassium chloride (KCl), 0.427g (0.00206 mol) of 4-nitroaniline was added into the reaction mixture. The reaction was kept on stirring under reflux for further 5 hours and monitored with the help of thin layer chromatography (Scheme.1).

Scheme 1.



The reaction mixture was then poured in cold water and again stirred for at least two hours to get rid of the byproduct and impurities. The obtained solid product was filtered, washed with n-hexane and recrystallized in acetone.

Yield 92 %; **mp** 194 ˚C; **H^1 NMR (acetone) δ (ppm)** 13.092 (s, 1H), 10.587 (s, 1H), 8.328-8.122 (4H), 2.306 (s, 3H); **C^{13} NMR (acetone) δ (ppm)** 179.12, 172.83, 144.83, 144.02, 124.20, 123.26, 123.11, 23.33; **FTIR υ (cm^{-1})** 3188.3 (N-H, stretching), 2999.5 (Aromatic C-H, stretching), 1689.2 (C=O, stretching), 1578 (N-O, bending), 1507.5 (C-C, stretching), 1411.6 (C-H, bending), 1332.99 (N-O, bending), 1250.8 (C=S, stretching).

3.2 1-acetyl-3-(2-methyl-4-nitrophenyl)thiourea (TU2)

TU2 was synthesized following the same procedure as used for the synthesis of TU1, except that 0.4712 g (0.00206 mol) of 2-methyl-4-nitroaniline were added after stirring, for 2 hours instead of 4-nitroaniline.

Yield 92 %; **mp** 215 ˚C; **H^1 NMR (acetone) δ (ppm)** 12.70 (s, 1H), 10.607 (s, 1H), 8.469-8.109 (3H), 2.878 (s, 3H), 2.452 (s, 3H); **C^{13} NMR (acetone) δ (ppm)** 179.70, 172.75, 142.99-121.26, 23.25, 17.30; **FTIR υ (cm^{-1})** 3233.6 (N-H, stretching), 3001.0 (Aromatic C-H, stretching), 1693.1 (C=O, stretching), 1574 (N-O, bending), 1511.9 (C-C, stretching), 1332 (N-O, bending), 1257.8 (C=S, stretching).

3.3 1-acetyl-3-(2-methoxy-4-nitrophenyl)thiourea (TU3)

TU3 was also synthesized following the same procedure as used for the synthesis of TU1, except that 0.5208 g (0.00206 mol) of 2-methoxy-4-nitroaniline were added after stirring, for 2 hours instead of 4-nitroaniline.

Yield 90 %; **mp** 227 ˚C; **H^1 NMR (acetone) δ (ppm)** 13.188 (s, 1H), 10.491 (s, 1H), 8.172-8.132 (3H), 4.128 (s, 3H), 2.303 (s, 3H); **C^{13} NMR (acetone) δ (ppm)** 178.18, 172.25, 140.36-121.77, 56.63, 23.22; **FTIR υ (cm^{-1})** 3201.9 (N-H, stretching), 3000.6 (Aromatic C-H, stretching), 1702.1 (C=O, stretching), 1573 (N-O, bending), 1516.4 (C-C, stretching), 1421 (C-H, bending), 1349.9 (N-O, bending), 1243 (C=S, stretching), 1156 (C-O, stretching).

3.4 1-acetyl-3-(4-chloro-3-nitrophenyl)thiourea (TU4)

Again, TU4 was synthesized following the same procedure as used for the synthesis of TU1, except that 0.535 g (0.00206mol) of 4-chloro-3-nitroaniline were added after stirring, for 2 hours instead of 4-nitroaniline.

Yield 90 %; **mp** 149 ˚C; **H^1 NMR (acetone) δ (ppm)** 12.870 (s, 1H), 10.598 (s, 1H), 8.725-7.747 (3H), 2.299 (s, 3H); **C^{13} NMR (acetone) δ (ppm)** 131.67, 128.87, 120.53, 23.28; **FTIR υ (cm^{-1})** 3185.3 (N-H, stretching), 3019.8 (Aromatic C-H, stretching), 1686.0 (C=O, stretching), 1524.7 (N-O, bending), 1419.5 (C-H, bending), 1370.8 (N-O, bending), 1241 (C=S, stretching).

4. Results and Discussion:

The title compounds TU1-TU4 were prepared using previously reported method, [14] as depicted in Scheme 1.

The synthesized nitrosubstituted acyl thioureas have been characterized on the basis of their spectroscopic data. H^1 and C^{13} NMR spectra were recorded in deuterated acetone. Three different types of protons gave their distinct signals in H^1 NMR, in case of TU1 and TU4 i.e. singlets for each of the two –NH protons, aromatic protons and methyl protons. The methyl and methoxy protons, of TU2 and TU3 respectively, gave an additional singlet, in their H^1 NMR spectra. In all of the synthesized thioureas, the -NH proton situated in between phenyl ring and C=S, is maximum desheilded owing to the intramolecular hydrogen bonding and thus gave a singlet at around 13 ppm and the -NH proton located in between the C=O and C=S is deshielded to a lesser extent thus providing a singlet in the region of 10-11 ppm [8, 15-17]. The aromatic ring protons gave their signals between 8 and 9 ppm. In C^{13} NMR, carbon bonded with sulfur provided a weak signal at about 180 ppm and carbonyl carbon appeared in the range of 172-175 ppm. Signals for aromatic carbons are visible in the region between 120-140 ppm [8, 15-17].

In FTIR, -NH of thioureas gave a broad band above 3100 cm^{-1} owing to the intramolecular hydrogen bonding between the oxygen of the carbonyl and -NH. Just around 3000 cm^{-1} Ar-H stretch was evident and carbonyl group appeared as an intense band in the region of 1700-1600 cm^{-1}. C=S was available between 1050 cm^{-1} and 1250 cm^{-1} [8, 15-17], for all the compounds (Figure 1.).

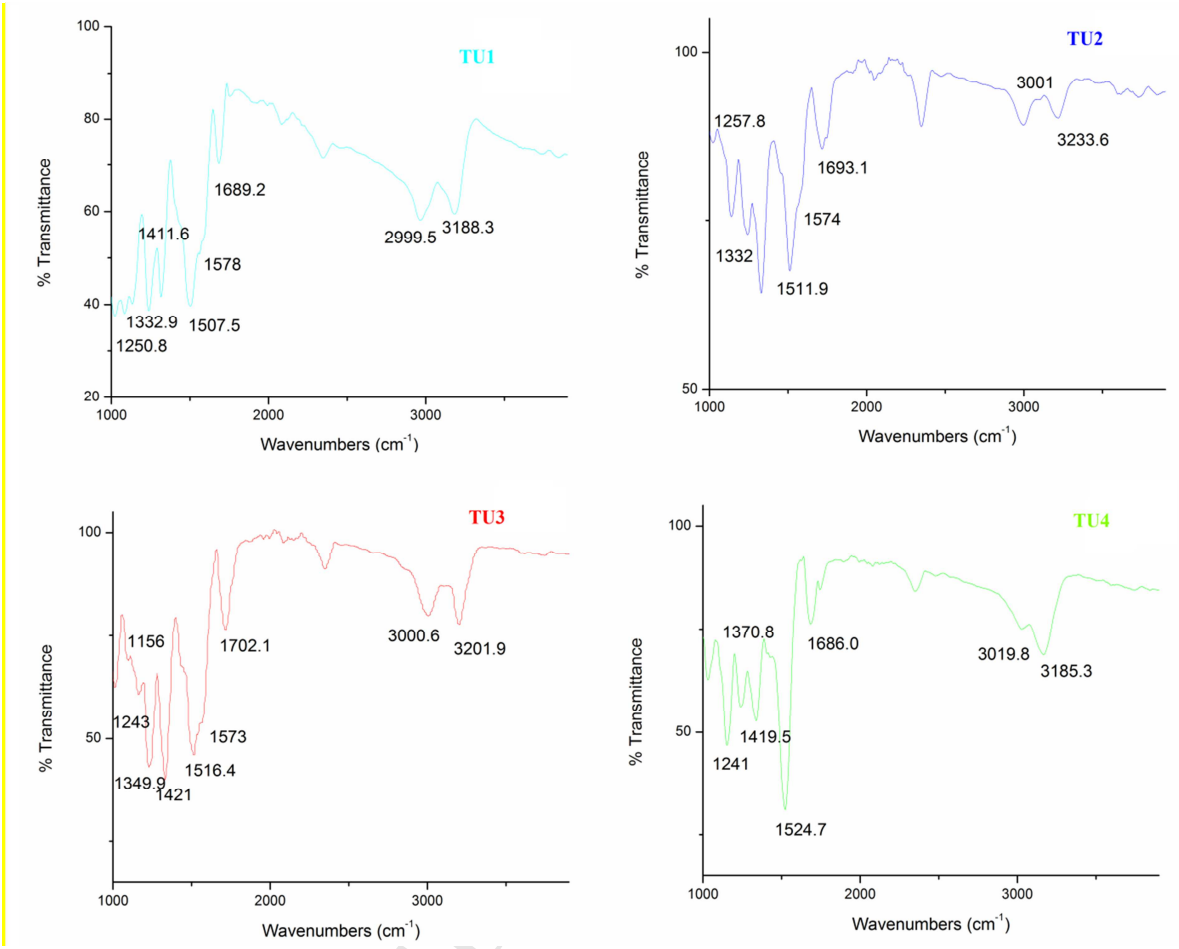


Figure 1. FTIR spectra of the synthesized thioureas.

4.1 Crystallography:

The crystal structures of TU1, TU2, TU3 and TU4, along with their nonbonding interactions, have been depicted in Figure 2-5 respectively and the selected bond lengths and bond angles for the crystal structures of TU1, TU2, TU3 and TU4 have been provided in the Tables 2-5.

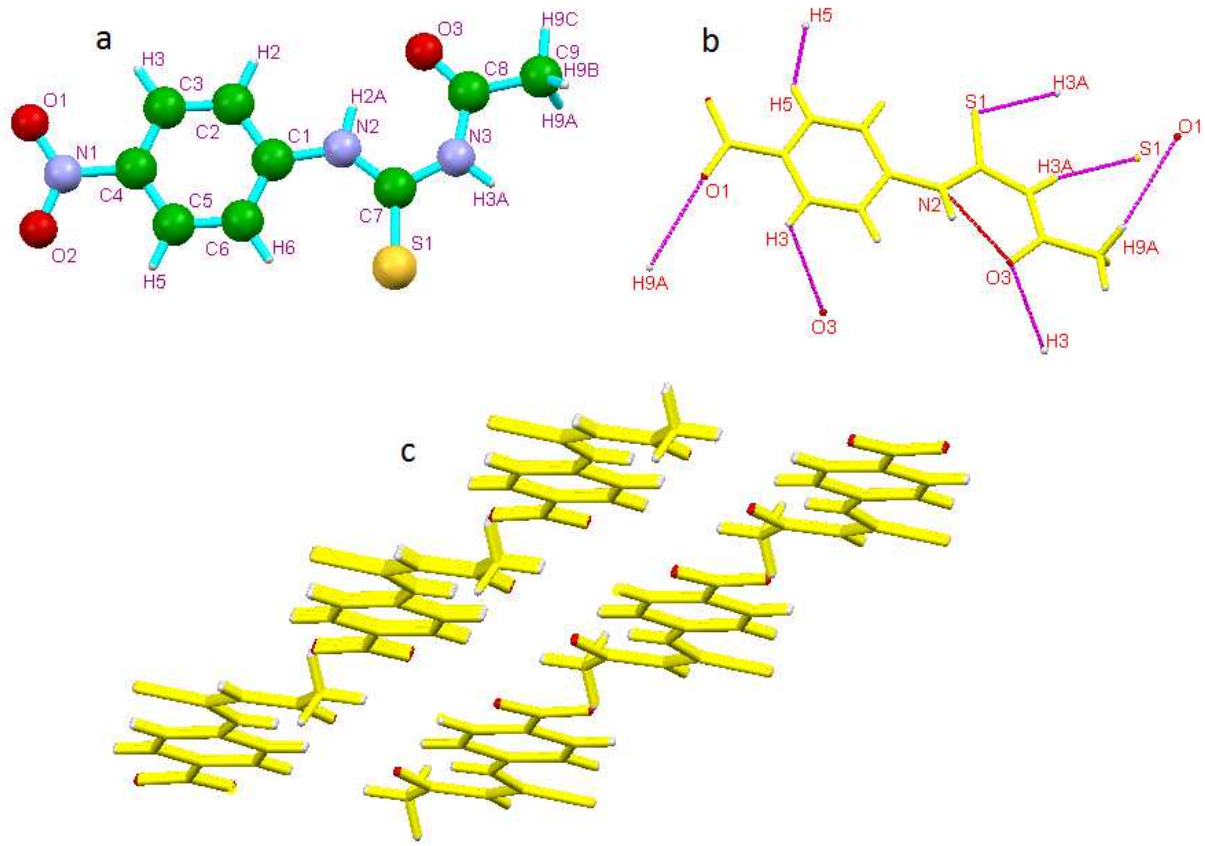


Figure 2. (a) Molecular diagram of TU1 with ball and stick arrangement, non-hydrogen atoms represented by 30 % probability boundary spheres and hydrogen atoms are spheres of arbitrary size, (b) expanded (red) and short contacts (magenta) of TU1 at vdW radii of 0.1 Å, with labeled contact atoms and (c) π-stacked layered supramolecular structure of TU1.

Table 2. Selected bond lengths and bond angles for TU1

Bond lengths (Å)			**Bond angles (˚)**			
O3	C8	1.219(2)	O2	N1	O1	123.7(2)
N3	C8	1.376(3)	O1	N1	C4	118.5(2)
N3	H3A	0.860(2)	C1	N2	H2A	113.8(2)
N3	C7	1.389(3)	H2A	N2	C7	113.8(2)
N2	C7	1.337(2)	C7	N3	S1	118.9(1)
N2	H2A	0.860(2)	C7	N3	H3A	115.3(2)
N2	C1	1.402(3)	H3A	N3	C8	115.3(2)
N1	C4	1.461(3)	N3	C8	O3	122.1(2)
N1	O1	1.217(2)	C9	C8	O3	123.2(2)
S1	C7	1.656(2)				

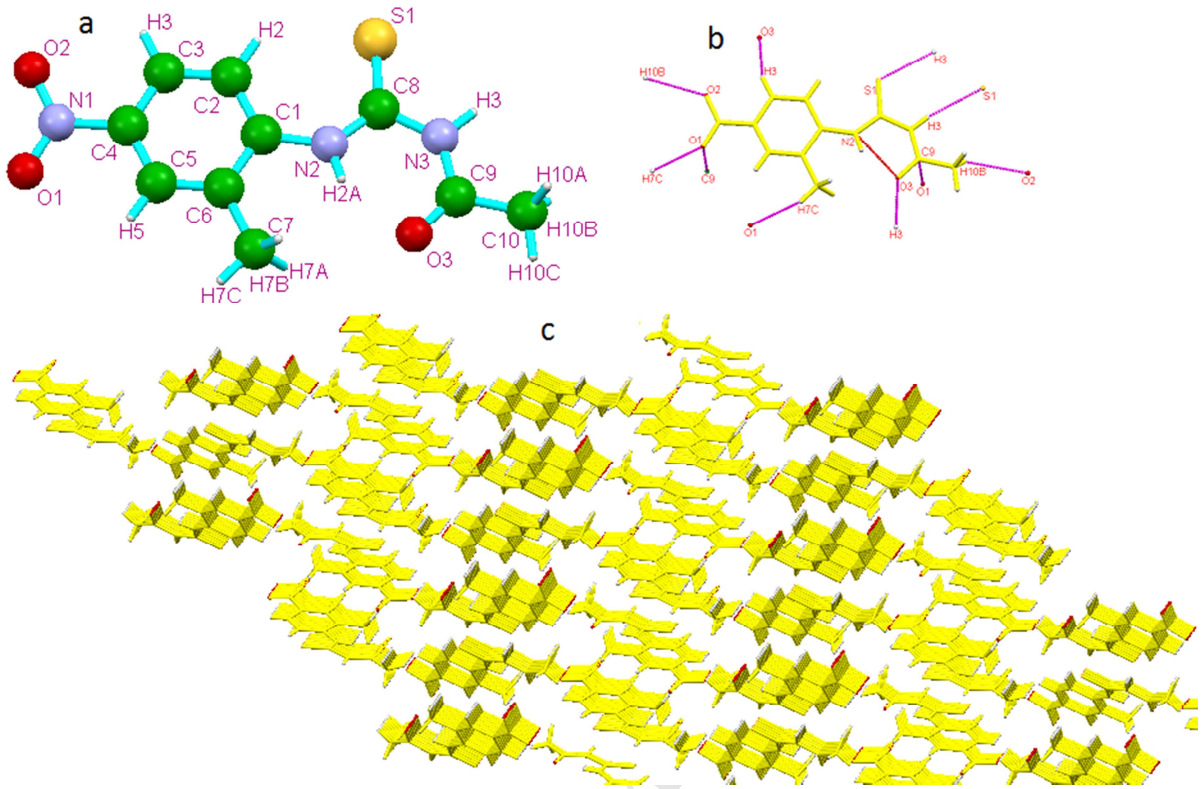


Figure 3. (a) Molecular diagram of TU2 with ball and stick arrangement, non-hydrogen atoms represented by 30 % probability boundary spheres and hydrogen atoms are spheres of arbitrary size, (b) expanded (red) and short contacts (magenta) of TU2 at vdW radii of 0.1 Å, with labeled contact atoms and (c) π-stacked layered supramolecular structure of TU2.

Table 3. Selected bond lengths and bond angles for TU2

Bond lengths (Å)			**Bond angles (˚)**			
O3	C9	1.217(2)	O2	N1	O1	122.7(2)
C9	N3	1.375(2)	C3	C4	CN1	118.5(2)
N3	H3	0.860(1)	N1	C4	C5	113.8(2)
N3	C8	1.391(2)	C5	C6	C7	113.8(2)
N3	H3	0.860(1)	C6	C1	N2	118.9(1)
N2	H2A	0.860(2)	C1	N2	H2A	115.3(2)
N2	C1	1.407(2)	H2A	N2	C8	115.3(2)
N1	C4	1.461(3)	N3	C8	S1	122.1(2)
N1	O1	1.214(2)	S1	C8	N2	123.2(2)

S1	C8	1.655(2)	C8	N3	H3	115.4(2)
N2	C8	1.341(2)	H2	C3	N3	115.3(2)
C6	C7	1.505(3)	O3	C9	N3	121.8(2)

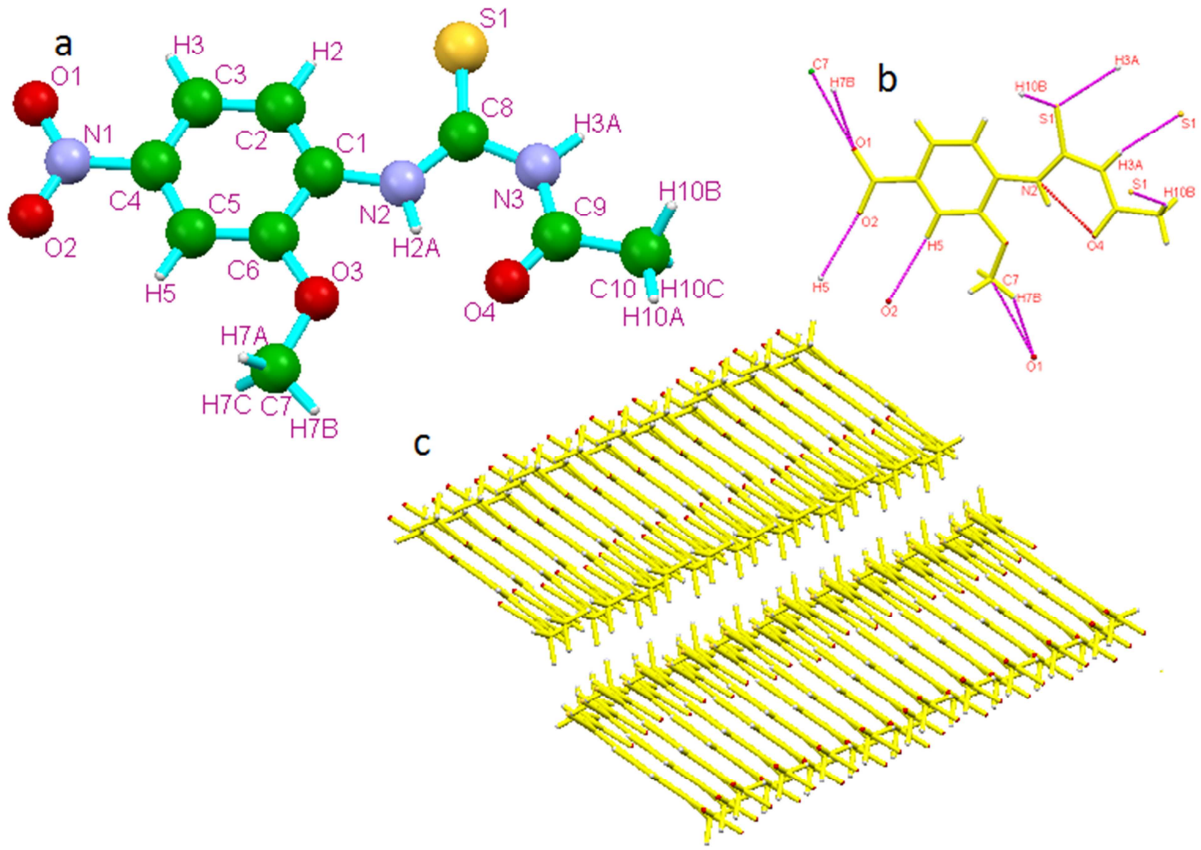


Figure 4. (a) Molecular diagram of TU3 with ball and stick arrangement, non-hydrogen atoms represented by 30 % probability boundary spheres and hydrogen atoms are spheres of arbitrary size, (b) expanded (red) and short contacts (magenta) of TU3 at vdW radii of 0.1 Å, with labeled contact atoms and (c) π-stacked layered supramolecular structure of TU3.

Table 4. Selected bond lengths and bond angles for TU3

Bond lengths (Å)			**Bond angles (˚)**			
N1	O1	1.208(6)	O1	N1	C4	118.5(4)
N1	C4	1.460(6)	N1	C4	C3	118.6(4)
C4	C5	1.367(6)	N1	C4	C5	118.2(4)
C4	C3	1.368(7)	C5	C6	O3	124.2(4)
C5	C6	1.375(6)	C6	O3	C7	118.6(4)
C6	O3	1.355(5)	O3	C1	C6	115.0(4)
C7	O3	1.423(6)	C1	N2	H2A	114.2(4)
C6	C1	1.394(7)	H2A	N2	C8	114.2(4)
C1	N2	1.400(6)	S1	C8	N3	117.3(3)
N2	H2A	0.860(4)	N2	C8	S1	127.9(4)
N2	C8	1.332(6)	C8	N3	H3A	115.4(4)
N3	C8	1.380(5)	C8	N3	C9	115.4(4)

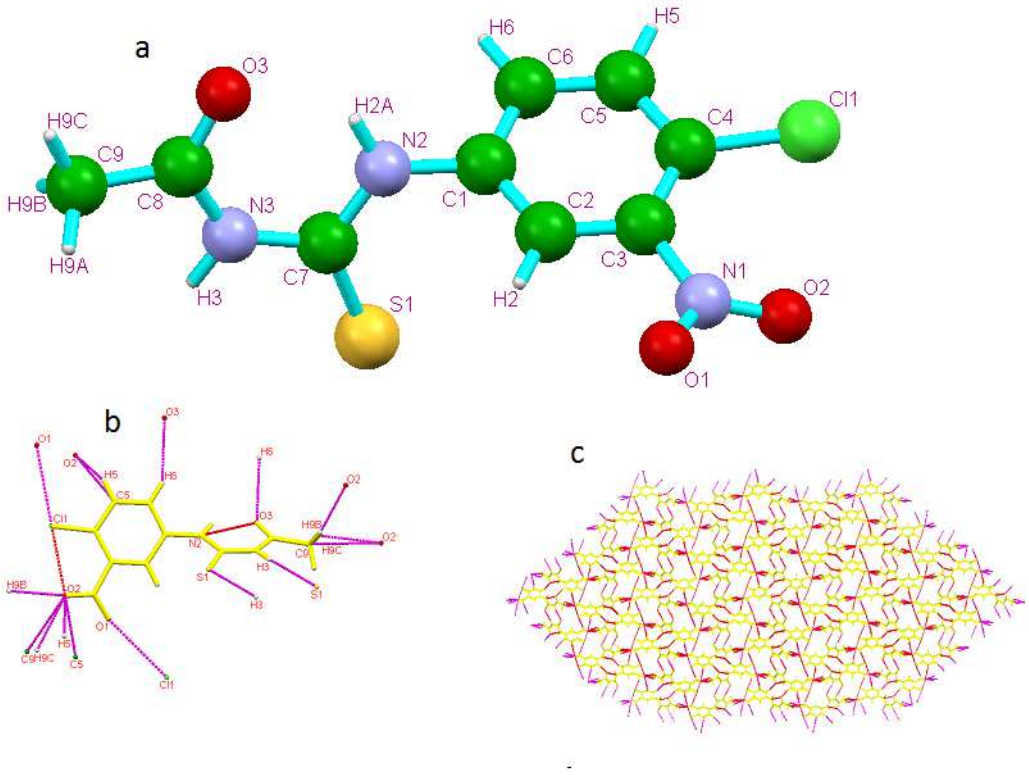


Figure 5. (a) Molecular diagram of TU4 with ball and stick arrangement, non-hydrogen atoms represented by 30 % probability boundary spheres and hydrogen atoms are spheres

of arbitrary size, (b) expanded (red) and short contacts (magenta) of TU4 at vdW radii of 0.1 Å, with labeled contact atoms and (c) π-stacked layered supramolecular structure of TU4.

Table 5. Selected bond lengths and bond angles for TU4

Bond lengths (Å)			**Bond angles (˚)**			
N1	O1	1.207(6)	C9	C8	O3	122.7(4)
N1	C3	1.463(6)	C9	C8	N3	115.2(4)
C3	C2	1.370(6)	C8	N3	H3	115.0(4)
C3	C4	1.379(6)	H3	N3	C7	115.0(4)
C4	Cl1	1.721(5)	N3	C7	S1	120.4(3)
C4	C5	1.371(7)	S1	C7	N2	124.4(3)
C1	C6	1.382(6)	C7	N2	H2A	180.0(4)
C1	N2	1.422(6)	C1	N2	H2A	118.0(4)
N2	H2A	0.860(3)	N2	C1	C2	120.9(4)
N2	C7	1.338(5)	N2	C1	C6	119.2(4)
C7	N3	1.374(6)	C2	C3	N1	115.9(4)
H3	N3	0.861(3)	N1	C3	C4	123.0(4)

It can be seen that both inter and intramolecular hydrogen bonding are present in all of these four nitrosubstituted acyl thioureas rendering them remarkable supramolecular chemistry. TU1 forms a π-stacked layered supramolecular structure with methyl of the acetyl moiety of one layer situated at so called trans position to the methyl of the acetyl functionality of the adjacent layer. π-stacking of phenyl rings is also evident in the zig-zag patterned layers of TU2. TU3 forms a bamboo fence like supramolecular structure with methoxy substituent of each layer pointed outwardly towards the next layer. TU4 forms a beautiful motif like supramolecular structure.

4.2 DNA binding studies:

4.2(a) By UV-vis spectroscopy and cyclic voltammetry:

The DNA-binding constants calculated by cyclic voltammetry using Equation 2, are found to be in close agreement with the ones calculated by UV-vis spectroscopy. The

voltammograms of all the synthesized thioureas give oxidation, reduction peak in a region between -1V-1V. Compounds show quasi reversible behavior which is evident by:

- Change of potential of the oxidation and reduction peaks at different scan rates.
- Ratio of oxidation and reduction peak currents not exactly equal to one.
- Difference of potential between the oxidation and reduction peaks not close to 60 mV.

The representative cyclic voltammograms for TU3 have been depicted in Figure 7(a).

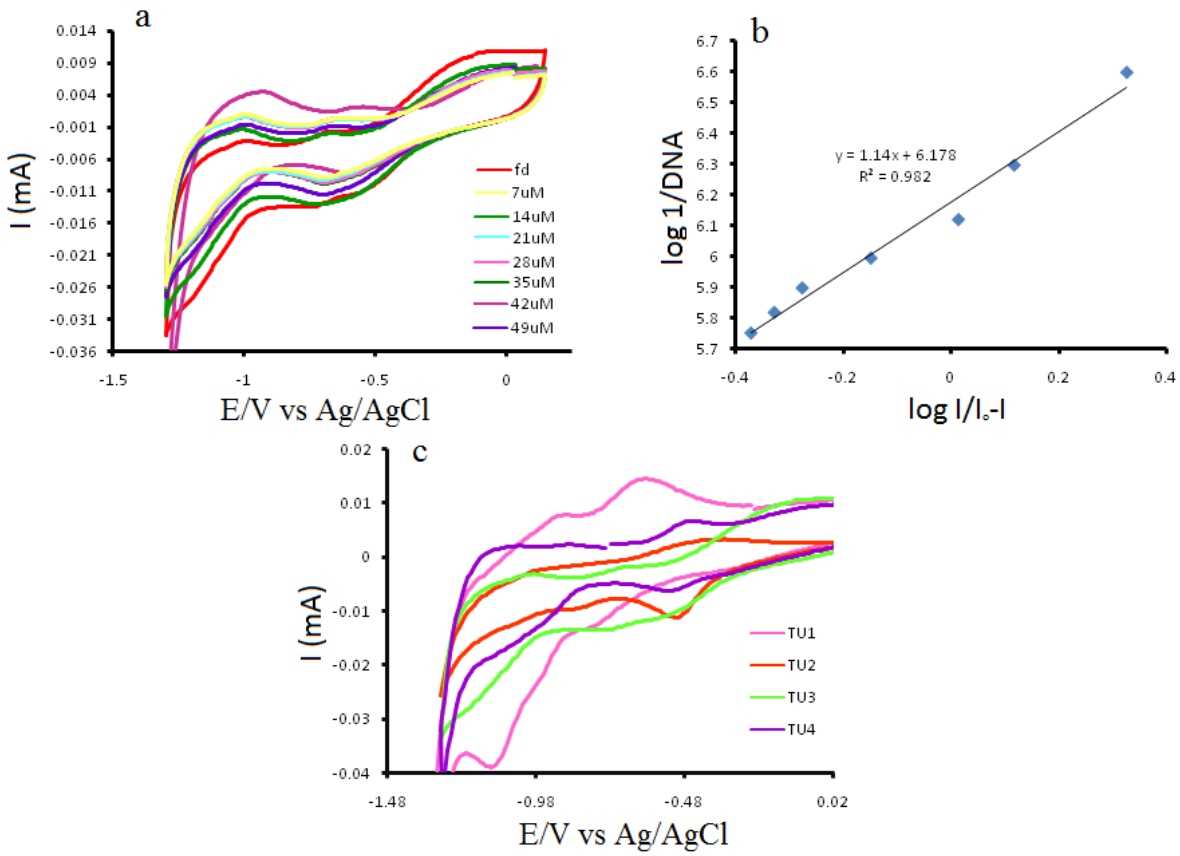


Figure 7. (a) Representative cyclic voltammograms for sample TU3 solution free of DNA and TU3 solution with different concentrations of DNA (b) Graphical representation of the plot of log 1/DNA vs. log I/I_o-I for the calculation of DNA binding constant K_{TU3}

for TU3 and (c) cyclic voltammograms of TU_1 (1-Acetyl-3-(4-nitrophenyl)thiourea) in pink, TU_2 (1-Acetyl-3-(2-methyl-4-nitrophenyl)thiourea) in red, TU_3 (1-Acetyl-3-(2-methoxy-4-nitrophenyl)thiourea) in bright green and TU_4 (1-Acetyl-3-(4-chloro-3-nitrophenyl)thiourea) in plum.

The peak at –0.79713 V for nitro was scanned with different additions of the DNA. By the addition of the DNA (7-49 µM) there is a shift in the peak potential to -0.8072 V, -0.9719 V, -0.9881 V, -0.9981 V, -0.9982 V, -0.9983 V and -1.007 V with a decrease in the peak current. This decrease in the peak current confirms the formation of TU3-DNA adduct which diffuses comparatively slowly towards the electrodes and decrease the current. Negative shift in the potential is in favor of electrostatic mode of interaction. All the compounds show similar type of peaks (Figure 7-b) and same mode of interaction with the DNA. Representative UV-vis spectroscopic data of DNA binding for TU4 confirms the cyclic voltammetric results by showing hypochromism and blue shift of the TU4-DNA adducts relative to free TU4 [8, 15-17], (Figure 6).

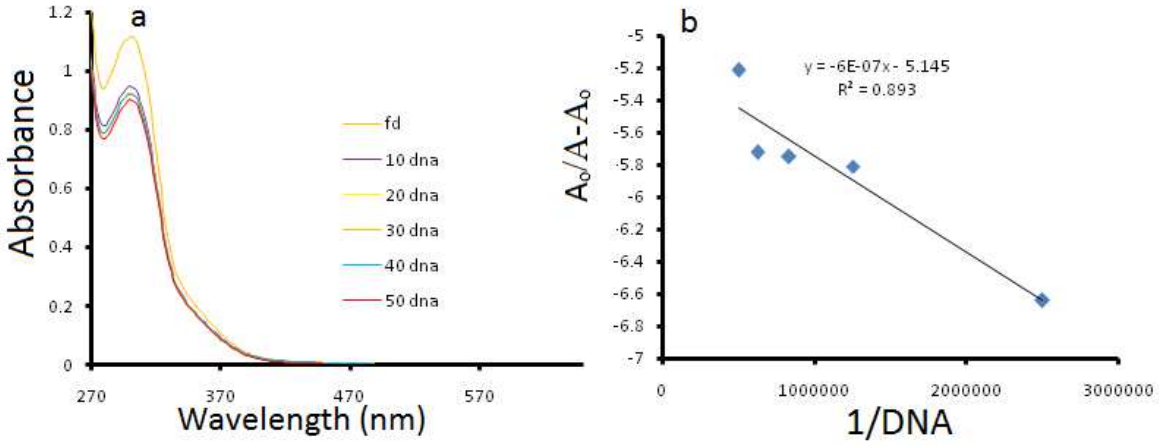


Figure 6. (a) Representative plot of absorbance vs. wavelength for TU4 sample without DNA and with addition of 10 µM, 20 µM, 30 µM, 40 µM and 50 µM DNA solution (b) Graphical representation of $A_o/A\text{-}A_o$ vs. 1/DNA to calculate binding constant K_{TU4}.

DNA binding parameters for all the compounds have been presented in Table 6.

Table 6. Calculations of DNA binding constant by UV-vis spectroscopy and cyclic voltammetry for TU1, TU2, TU3 and TU4

Samples	UV-vis data			Cyclic voltammetry data		
	K(M^{-1})	**In K**	**ΔG(kJM^{-1})**	**K(M^{-1})**	**In K**	**ΔG(kJM^{-1})**
TU1	1.16 x 10^6	13.964	-34.6	5.20 x 10^6	15.464	-38.7
TU2	6.05 x 10^6	15.616	-38.7	1.24 x 10^6	14.031	-34.8
TU3	9.04 x 10^6	16.017	-39.7	1.51 x 10^6	14.228	-35.2
TU4	8.57 x 10^6	15.964	-39.5	2.40 x 10^6	14.691	-36.4

4.3 Computational study:

The oxidation and reduction potential of TU3 is observed to be least among the four thioureas and is in accordance with the computed HOMO and LUMO values (Table 7), which can be attributed to the presence of methoxy group at the ortho position of phenyl ring in TU3.

Table 7. Observed oxidation and reduction potentials in cyclic voltammetry and computed HOMO, LUMO values for TU1, TU2, TU3 and TU4.

Samples	Oxidation Potential (V)	HOMO (eV)	Reduction potential (V)	LUMO (eV)
TU1	-0.917332	-0.23455	-1.1259	-0.09796
TU2	-0.847337	-0.22384	-0.854572	-0.09627
TU3	-0.797130	-0.19976	-0.794254	-0.07549
TU4	-1.00521	-0.23548	-1.00825	-0.12099

It can also be seen in Figure 8. that the orbitals are polarized to a much greater extent in TU3, compared to those in the rest of the thioureas, owing to the presence of a more polar methoxy group on the phenyl ring of TU3.

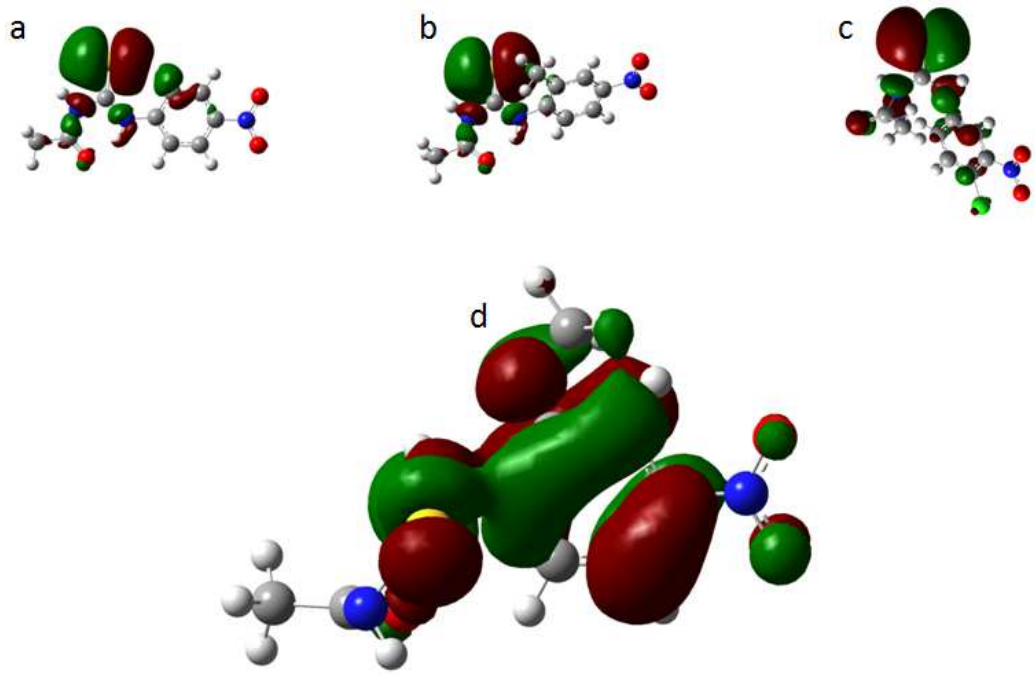


Figure 8. Orbital diagrams of (a) TU1, (b) TU2, (c) TU4 and (d) TU3.

4.4 DPPH scavaenging activity:

The percent scavenging of synthesized compounds against DPPH is shown in Figure 9.

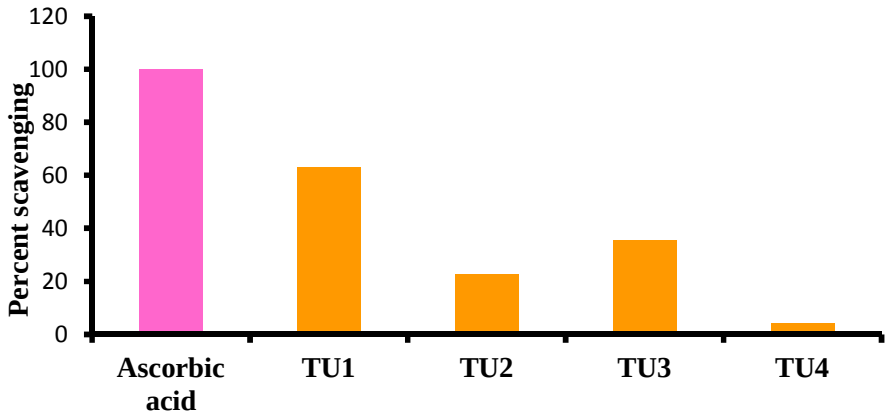


Figure 9. Percent scavenging of TU1, TU2, TU3 and TU4 against reference ascorbic acid.

The better antioxidant activity of TU1 and TU2 can be attributed to their planar structures which favor the free radical intermediate structure formation by delocalization of elcectron over the wider part of the molecule [20]. IC_{50} value for TU1 which showed more than 50 % scavenging is found to be 250 µM.

4.5 Cytotoixicity:

Owing to the same functionality, all thioureas show promising but almost same percent cytotoxicity Figure 10.

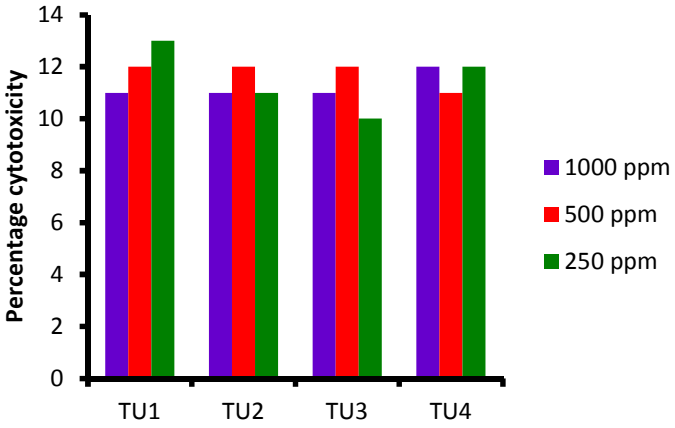


Figure 10. Percentage cytotoxicity graph for TU1, TU2, TU3 and TU4.

The LD_{50} value is found to be 55 ppm for TU1, TU2 and TU3 and 60 ppm for TU4.

4.6 Antibacterial activity and antifungal activity:

Except TU_3, all of the thioureas show moderate antibacterial activity 36-40 % (Table 8), against gram positive bacterial strains and lower or no antibacterial activity against gram negative bacterial strains, this could be because of the well known structural difference in the cell membranes of the gram positive and gram negative bacteria [21], but the actual mechanism of the drugs against respective bacterial membranes is not the subject of this research article.

Table 8. Bacterial inhibition chart of thioureas against gram positive and gram negative bacterial strains.

Samples (µg/disc)	*E.Aerogens* ±SE	*S.Typhimurium* ±SE	*M.Lutues* ±SE	*B.Bronchiseptaca* ±SE	*S.Auaurea* ±SE
TU1	6.5±0.075	0	6.5±0.075	0	6.5±0.075
TU2	7.5±0.075	0	6.5±0.075	6.5±0.075	6.5±0.075
TU3	0	6.5±0.075	7±0.075	0	0
TU4	7±0.078	6.5±0.075	6.5±0.075	6.6±0.075	6.5±0.075

Among all of the synthesized thioureas, TU4 shows better activity against both positive and negative bacterial strains. This can be attributed to chloro substituent available at the phenyl ring in TU4, as better antimicrobial activities associated with different compounds having chloro substitution has been reported in literature as well [22].

All of the synthesized thioureas have shown about 39 % fungal inhibition (Table 9), against all fungal cultures. This almost similar percent fungal inhibition can be correlated to the similar functional moiety i.e. thio and nitro substituted phenyl rings available in all the synthesized compounds which may inhibit fungal growth following similar mechanism [23].

Table 9. Fungal inhibition data for the four thioureas

Samples(µg/disc)	*F.Fumagatus* ±SE	*F. Mucor* ±SE	*F.Niger* ±SE	*F.Flaws* ±SE
TU1	7.5±0.722	7.5±0.711	7.5±0.712	7.5±0.712
TU2	7.5±0.717	7.5±0.717	7.5±0.717	7.5±0.717
TU3	7.5±0.719	7.5±0.707	7.5±0.719	7.5±0.719
TU4	7.5±0.715	7.5±0.715	7.5±0.715	7.5±0.715

5. Conclusions:

In case of all the synthesized thioureas, the observed decrease in peak potential with increase in DNA concentration in cyclic voltammetry and blue shift with hypochromism in UV-vis spectroscopy suggest the electrostatic binding mode of interaction of drug with DNA. The DNA binding constants calculated by both techniques

are in the order of $10^6\,M^{-1}$ for all of the four nitrosubstituted acyl thioureas, showing their good binding potential with DNA. The planar thiourea i.e. TU1 shows good antioxidant activity. Moderate biological activities are shown by TU1, TU2 and TU3. TU4 which is chloro substituted is found to have better biological activities, among all of the synthesized thioureas.

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Highlights:

- Synthesis and characterization of new nitrosubstituted acyl thioureas
- DNA-binding studies by UV-vis spectroscopy and cyclic voltammetery
- Biological assays of the synthesized thioureas