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ANTIBACTERIAL AND *IN VITRO* ANTIOXIDANT ACTIVITIES OF SOME 4-AMINO-1,2,4-TRIAZOLE-5(4*H*)-THIONE DERIVATIVES

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ABSTRACT

This work represents the synthesis and characterization some new versatile intermediates namely 4-amino-1,2,4-triazole-5(4*H*)-thione derivatives in a one-step fusion method. The method works to be superior compared to the existing method by Reed and Handel which consists of four steps. The antibacterial activity of compounds is tested against *Escherichia coli, Bacillus subtilis, Pseudomonas aeruginosa, Staphilococcus aureus*. The compounds exhibited species specific activity. Among the tested compounds, **1a** and **1b** exhibited good activity at moderately low concentration (31.25µg/mL) against tested strains of bacteria. DPPH (1,1-diphenyl-2-picryl-hydrazyl), FRAP (ferric reducing antioxidant power) and Lipid peroxidation assays are performed to determine the *in vitro* antioxidant activities. The compound **1e** exhibited good antioxidant activity among the tested compounds.

Keywords: 1,2,4-triazole; Antibacterial; *In vitro*; Antioxidant; DPPH assay; FRAP assay; Lipid Peroxidation assay.

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1. INTRODUCTION

Triazole is a five membered ring system with three nitrogen hetero-atoms, which defines an

interesting class of compounds. It exists in two tautomeric forms, the 1,2,3-triazole and the 1,2,4-triazole . 1*H* and 4*H*- 1,2,4-Triazoles are considered to be pharmacologically important nucleus. The literature shows that 1,2,4-triazole possesses a wide range of biological activities. The considerable biological importance of triazoles has stimulated a lot of interest in its derivatives. 1,2,4-Triazoles have a wide range of therapeutic properties like antibacterial, antifungal, antimycobacterial and antiviral[1].

It has been reported that structural properties of triazoles, like moderate dipole character, hydrogen bonding capability, rigidity and stability under *in vivo* conditions are the main reasons for their superior pharmacological activities [2].There is an increasing demand for the preparation of new broad spectrum antibacterial compounds due to the developing resistance towards usual antibiotics. Therefore, we have synthesized some 4-amino - 1,2,4-triazole-5 (4*H*) derivatives possessing antimicrobial and antioxidant activities [3].

Hence in the present work we account, synthesis of some new 4-amino-1,2,4-triazole-5(4*H*)-thione derivatives, their structural determination by spectral analysis, *in vitro* antioxidant and antimicrobial analysis.

2. RESULTS AND DISCUSSION

The compounds **1a-1e** were synthesized as per the scheme (I) and purified by recrystallization.The purity was determined by TLC. Formation of the compounds was confirmed through analytical studies.This method is found to be a superior green method compared to the classical method described by Reid and Handel [4]. The classical method involves the conversion of the starting material acid to ester which in turn converted into acid hydrazide. Both steps involve five hours of refluxing respectively. The acid hydrazide should then be converted into corresponding potassium salt dithiocarbazinate in dry ether medium. Cyclization of this intermediate leads to 5-substituted 4-amino-3-mercapto 1,2,4-triazoles.

But the method reported here involves single step fusion reaction with good yield and purity.

2.1. Antibacterial assay

Antibacterial screening of the compounds was performed using four different bacterial strains both gram positive and gram negative. All the compounds exhibited activity against tested strains of bacteria. The compound **1a** exhibited better activity against *Escherichia coli* and *Staphilococcus aureus* strains at MIC 31.25 and 62.5µg/mL respectively. This might be due the -Cl group at para position of aromatic nucleus attached to 1,2,4-triazole. The compound 1b exhibited better activity against *Bacillus Subtilis* and *Pseudomonas aeruginosa* at MIC 31.25 and 62.5µg/mL respectively. The reason for this was assumed to be the presence of -Cl group at ortho position of aromatic nucleus.This observation is further supported by the literature studies [5-7].The compound **1c** exhibited better activity against *Pseudomonas aeruginosa* at MIC 62.5 µg/mL. The cause for this observation might be the $-OCH_3$ group at meta position of aromatic nucleus adjacent to 1,2,4-triazole. The compounds **1d** and **1e** exhibited poor activity against all the strains. The reason for this might be the $-CH_3$ and –OH groups at ortho and para positions respectively on the aromatic nucleus attached to 1,2,4-triazole moiety. The MICs for various strains of bacteria were determined and are depicted in Table 1.

Table 1. Antibacterial activity of synthesised compounds

Micro Organisms	Compds.	Concentration in µg/mL						MIC (µg/mL)
		500	250	125	62.5	31.25	15.62	
E.coli	1a	-	-	-	-	-	+	31.25
ATCC 25922	1b	-	-	-	-	+	+	62.5
	1c	-	-	-	+	+	+	125
	1d	-	-	-	+	+	+	125
	1e	-	-	-	+	+	+	125
Bacillus	1a	-	-	-	-	+	+	62.5
Subtilis	1b	-	-	-	-	-	+	31.25
ATCC 6633	1c	-	-	+	+	+	+	250
	1d	-	-	-	+	+	+	125
	1e	-	-	+	+	+	+	250
Pseudomonas	1a	-	-	+	-	+	+	125
aeruginosa	1b	-	-	-	-	-	+	62.5
(re cultured)	1c	-	-	-	-	-	-	62.5
	1d	-	+	+	+	+	+	250
	1e	-	+	+	+	+	+	250
Staphilococcus	1a	-	-	-	-	+	+	62.5

Aureus	1b	-	-	-	+	+	+	125
ATCC 25923	1c	-	-	-	+	+	+	125
	1d	-	-	+	+	+	+	250
	1e	-	-	+	+	+	+	250

2.2. DPPH radical scavenging activity

The DPPH assay of **1e** gave maximum activity among all the synthesized 1,2,4-triazoles (78.16%). This was very much close to that of the standard GSH (71.76%). This enhanced activity of the compound **1e** might be attributed due to the presence of -OH group at para position of aromatic nucleus substituted to 1,2,4-triazole. The result is given in the **Fig. 1**.

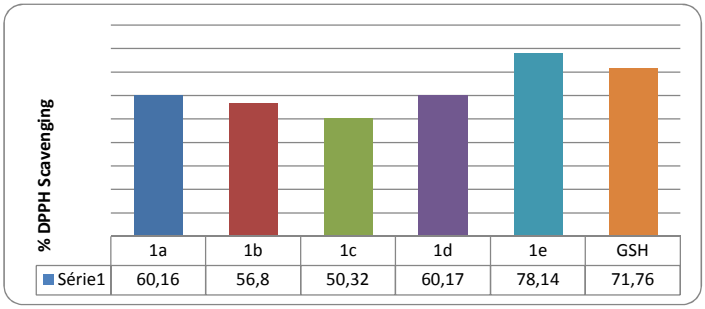


Fig.1. DPPH radical scavenging assay of **1a-1e**

2.3. FRAP assay

FRAP assay measures the reducing power of the antioxidant molecule i.e. FRAP assay also evaluates the capacity of compound to transfer a hydrogen atom (protection against lipid peroxidation and glutathione oxidation) as well as the capacity of compounds to transfer a single electron. Substances which have reduction potential react with potassium ferricyanide (Fe^{3+}) to form potassium ferrocyanide (Fe^{2+}) which then reacts with ferric chloride ($FeCl_3$) to form ferric ferrous complex that has an absorption maximum at 700 nm.

In FRAP assay, increased absorbance of the compounds indicates increased reducing power. Compounds with higher absorption (100µg/mL) showed a higher reducing power (**Fig.2**). These results clearly reveal that compounds have antioxidant activity. Among the synthesized

compounds, **1d** and **1e** showed maximum reducing activity which might be due to the presence of electron releasing methyl and hydroxyl group on aromatic nucleus attached to 1,2,4-triazole moiety.

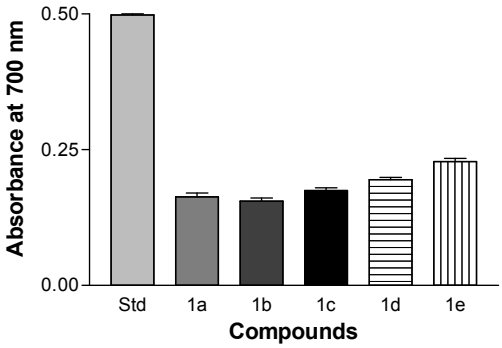


Fig.2. *In vitro* FRAP assay

2.4. Lipid Peroxidation Assay

Lipid peroxidation involves the formation and propagation of lipid radicals with numerous deleterious effects, including destruction of membrane lipids, metabolic disorders and inflammation, and production of malondialdehyde (MDA) is a hallmark of this process [8]. Inhibition of lipid peroxidation was assessed by amount of MDA produced. Lipids in egg yolk undergo rapid nonenzymatic peroxidation in the presence of ferrous sulphate. These compounds showed good inhibition of lipid peroxidation. Therefore this suggests that the compounds exhibited significantly to the inhibition of lipid peroxidation. Among the compounds tested, compound **1e** exhibited very good activity (**Fig.3**).This could be attributed due the presence of -OH group on aromatic nucleus substituted to 1,2,4-triazole moiety. These results are also supported by similar studies documented in available literature [9,10].

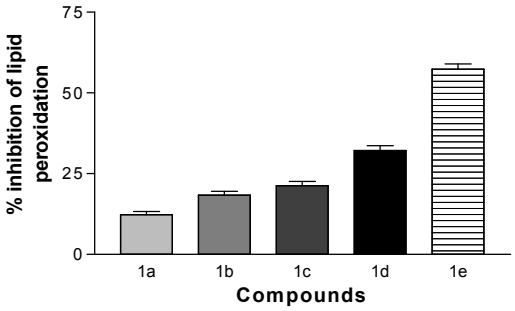


Fig.3. *In vitro* Lipid Peroxidation Assay

3. EXPERIMENTAL

3.1. Materials and Methods:

All reagents were purchased from Sigma-Aldrich and used without further purification. Open capillary method was used for determination of melting points; correction factor was not applied to the values reported. Purity of the compound was checked on silica gel precoated TLC plates and visualised using UV chamber. Elemental analysis was carried out by using VARIO EL-III (Elementar Analysensysteme GmBH). The structure of synthesized compounds was characterized by IR, ^{1}H NMR, ^{13}C NMR and LCMS analysis. IR spectra were recorded on Shimadzu 8400S FTIR spectrometer using KBr pellets. ^{1}H NMR spectrum was recorded in DMSO-d_6 at 400MHz and ^{13}C NMR spectrum was recorded in DMSO-d_6 at 100MHz. SHIMADZU UV-2550 double beam spectrophotometer with 1cm matched quartz cell was used to measure the absorbance.

The 1,2,4-triazoles (**1a-1e**) were synthesized (Scheme II) from commercially available substituted Phenylaceticacids **1**. Thiocarbohydrazide **2** was synthesized by Taguchi method [6]. The synthesis of thiocarbohydrazide (Scheme I) required for the reaction was obtained by condensing hydrazine hydrate and carbon disulphide in the ratio 3:1. It takes 6 hours of heating the reaction mixture at 343K. Substituted Phenylacetic acid and thiocarbohydrazide were fused under solvent free condition on an oil bath at 413K for one hour to get the title

products. Synthesized compounds were recrystallized from methanol, dried and melting point was determined. Purity of the compounds is determined by HPLC. The structure of all new compounds was established by IR, ^{1}H-NMR, ^{13}C-NMR, LCMS and elemental analysis. The IR spectra of all the synthesized compounds showed absorption bands due to -NH stretching band in the region 3310-3242 cm^{-1}, -NH_2 stretch at 3194-3161 cm^{-1}, -C=N- stretch at 1307-1259 cm^{-1} and the C=S stretching band at 772-663 cm^{-1}. These data confirmed formation of triazoles. In ^{1}H NMR and ^{13}C NMR spectra, the signals of the protons and carbons of the synthesized compounds were analyzed on the basis of their chemical shifts (δ).The peak integrating for single proton around the region δ 13.5 ppm was assigned to NH-SH tautomeric proton. The aromatic proton signals appeared in the region δ 6.77-7.46 ppm , A single peak appeared at δ 5.5 ppm was assigned for two protons of -NH_2 group attached to triazole ring. These ^{1}H NMR data confirmed the formation of synthesised compounds. In ^{13}C NMR spectra of the newly synthesized compounds, the phenyl ring carbons were resonated in the range of δ 120-137 ppm. The -C=N- peak was observed at δ 150-159 ppm and the C=S peak at δ 163-168 ppm. The methylene carbon attached to triazole ring resonated around δ 27-39 ppm. LCMS spectra also showed the respective molecular ionic peaks for the all the triazole derivatives.

The spectral characterization of a representative analogue 4-amino-3-(4-chlorobenzyl)-1*H*-1,2,4-triazole-5(4*H*)-thione (**1a**) is presented as follows. The IR spectrum of **1a** showed absorption bands at 3242 cm^{-1} and 3161 cm^{-1}were due to -NH and -NH_2 groups respectively. A sharp absorption band at 1294 $cm^{-1}$was assigned for the -C=N- group of the molecule and an absorption band due C=S stretch was seen at 663 cm^{-1}. A band representing C-Cl stretch was found at 765 cm^{-1}. The ^{1}H NMR spectrum showed a singlet at δ 13.55 ppm integrating for one NH-SH tautomeric proton present in the triazole ring. A singlet seen at δ 5.55 ppm was assigned to two protons of -NH_2 group attached to the triazole ring. Another singlet at 4.04 ppm represented two protons of methylene group connecting triazole and phenyl rings. Multiplet was seen at 7.3-7.39 ppm represented four protons of aromatic ring. In the ^{13}C NMR spectrum, the peak at δ 38.90 ppm was assigned for CH_2 carbon. Two peaks at δ 151ppm and δ 166.10 ppm represent C=N and C=S carbon

respectively. The chemical shifts of the aromatic ring carbons were appeared at δ128.36-134.42ppm. It was assigned for different aromatic carbons as C_1-134.42ppm, C_2 & C_6-130.75ppm, C_3& C_5- 128.36ppm, C_4-134.42ppm. LCMS spectrum showed a molecular ion peak at m/z 241.33 (M+1) corresponding to the molecular formula $C_9H_9ClN_4S$ (Mol. mass 240.7). Elemental analysis supported the further confirmation of the structure of the synthesised compounds. Similarly, structure of all the compounds was determined and the data presented in the experimental section.

3.2. General Procedure for the synthesis of Thiocarbohydrazide (2)

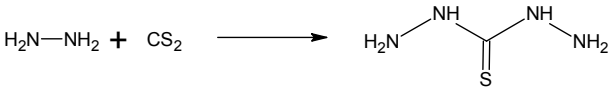
Thiocarbohydrazide was prepared by Taguchi method [11]. In a round bottom flask 85% hydrazine hydrate (0.3mol, 13.9 ml) was placed, which was stirred at 283K. Carbon disulfide (0.1mol, 6ml) was added drop wise over about one hour at temperature below 288K. Later the mixture was agitated for 30 min at room temperature. Then it was heated at 343K for 6 hrs. The reaction mixture was cooled to room temperature and precipitate was filtered, washed with ice water and dried.

3.2.1. General Procedure for the Green Synthesis of (1a-1e)

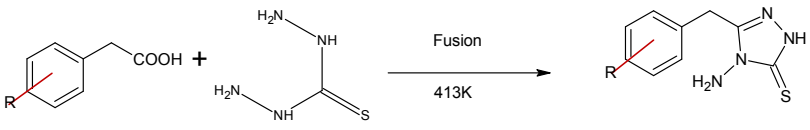
The well triturated equimolar mixture of thiocarbohydrazide **2** and corresponding substituted phenyl acetic acid **1**(**Scheme II**) was fused in a round bottom flask for one hour on oil bath at 413K. The reaction mixture was cooled to room temperature and washed with 5% sodium bicarbonate solution to remove unreacted acid and again washed with water. The compound was dried and recrystallized from methanol [12].

X-ray crystallographic structure of **1c** and **1e** were already reported by the authors and given in **Fig.4** and **Fig.5** respectively [12,13].

Scheme I:



Scheme II:



1 **2** **TR**

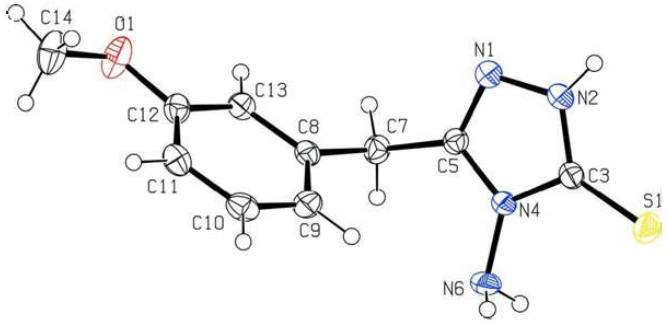


Fig.4. Crystal structure of

4-Amino-3-(3- methoxybenzyl)-1*H*-1,2,4-triazole-5(4*H*)-thione (**1c**)

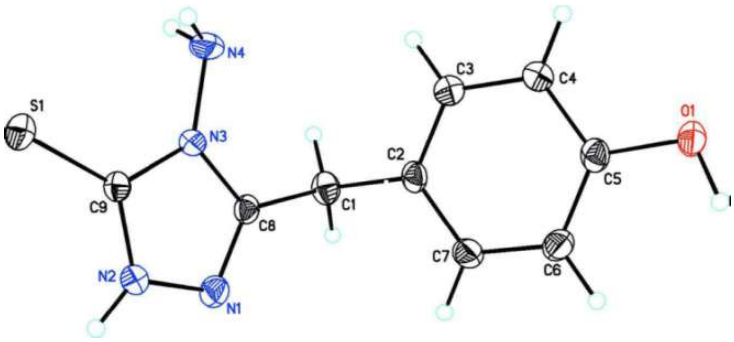


Fig.5. Crystal structure of
4-Amino-3-(4-hydroxybenzyl)-1*H*-1,2,4-triazole-5(4*H*)-thione (**1e**)

3.2.2. Spectral details

4-Amino-3-(4-chlorobenzyl)-1*H*-1,2,4-triazole-5(4*H*)-thione (1a)

The compound **1a** was obtained as a white solid prepared following the general procedure yield 82%; m.p.449K; IR(KBr)cm^{-1} :3242, 3161(NH, NH_2), 2934 (Ar. C-H str.), 1558(NH bend), 1489(C=C ring str.), 1294 (CN str.), 765 (C-Clstr.), 663 (C=S); ^{1}H NMR (400 MHz, DMSO-d_6): δppm,13.55(1H,s, of NH-SH), 7.3-7.39 (4H, m, of Ar-H), 5.55(2H, s, of NH_2), 4.04 (2H, s, of CH_2); ^{13} C NMR (100 MHz, DMSO- d_6): δ ppm, 38.90 (CH_2), 128.12, 128.36, 130.75, 131.48, 134.42, 134.88 (Aromatic carbons), 151 (C=N), 166.10 (C=S); Anal. cal. for $C_9H_9ClN_4S$: C, 44.91; H, 3.77; N, 23.28. Found: C, 44.87; H, 3.69; N, 23.22.LCMS (ESI-MS) *m/z* 241.33(M+1).

4-Amino-3-(2-chlorobenzyl)-1*H*-1,2,4-triazole-5(4*H*)-thione (1b)

The compound **1b** was obtained as a white solid prepared following the general procedure, yield 80%; m.p.465K; IR(KBr)cm^{-1}:3280, 3171(NH, NH_2), 2930 (Ar. C-H str.), 1597(NH bend), 1494(C=C ring str.), 1307 (CN str.), 752 (C-Cl str.), 700(C=S);^{1}H NMR (400 MHz, DMSO-d_6): δppm,13.50(1H,s, of NH-SH), 7.26-7.46 (4H, m, of Ar-H), 5.58(2H, s, of NH_2), 4.12 (2H, s, of CH_2); ^{13} C NMR (100 MHz, DMSO- d_6): δ ppm, 38.80 (CH_2), 126.23, 127.41,

129.57, 130.12, 133.22, 134.33 (Aromatic carbons), 150.11 (C=N), 163.21 (C=S); Anal. cal. for $C_9H_9ClN_4S$: C, 44.91; H, 3.77; N, 23.28. Found: C, 44.77; H, 3.65; N, 23.18.LCMS (ESI-MS) *m/z* 241.39(M+1).

4-Amino-3-(3- methoxybenzyl)-1*H*-1,2,4-triazole-5(4*H*)-thione (1c)

The compound **1c** was obtained as a white solid prepared following the general procedure, yield 83%; m.p.417K; IR(KBr)cm^{-1}:3310, 3194(NH, NH2), 2939 (Ar. C-H str.), 1583(NH bend), 1481(C=C ring str.), 1259 (CN str.), 772(C=S);^{1}H NMR (400 MHz, DMSO-d_6): δppm,10.06(1H,s, of NH-SH), 6.77-7.23(4H, m, of Ar-H), 5.54 (2H, s, of NH_2), 3.90 (2H, s, of CH_2), 3.72 (3H, s, of OCH_3); ^{13} C NMR (100 MHz, DMSO- d_6): δ ppm, 30.11(CH_2), 54.96 (OCH_3), 120.98, 121.23, 129.16, 129.48, 136.95, 137.13 (Aromatic carbons), 159.29 (C=N), 168.76 (C=S); Anal. cal. for $C_{10}H_{12}N_4OS$: C,50.83; H, 5.12; N, 23.71. Found: C, 50.77; H, 5.07; N, 23.68.LCMS (ESI-MS) *m/z* 237.4(M+1).

4-Amino-3-(2-methylbenzyl)-1*H*-1,2,4-triazole-5(4*H*)-thione (1d)

The compound **1d** was obtained as a white solid prepared following the general procedure, yield 85%; m.p.453K; IR(KBr)cm^{-1}:3277, 3167(NH, NH2), 2926 (Ar. C-H str.), 1582(NH bend), 1450(C=C ring str.), 1298 (CN str.), 739(C=S);^{1}H NMR (400 MHz, DMSO-d_6): δppm,13.48(1H,s, of NH-SH), 7.09-7.21(4H, m, of Ar-H), 5.56(2H, s, of NH_2), 3.99 (2H, s, of CH_2), 2.28 (3H, s, of CH_3);^{13} C NMR (100 MHz, DMSO- d_6): δ ppm, 19.29 (CH_3), 27.95 (CH_2), 125.59,126.57,129.36, 133.69, 136.58 (Aromatic carbons), 150.93 (C=N), 168.97 (C=S); Anal. cal. For $C_{10}H_{12}N_4S$: C, 54.52; H, 5.49; N, 25.43. Found: C, 54.45; H, 5.45; N, 25.39.LCMS (ESI-MS) *m/z* 221.56(M+1).

4-Amino-3-(4-hydroxybenzyl)-1*H*-1,2,4-triazole-5(4*H*)-thione (1e)

The compound **1e** was obtained as a white solid prepared following the general procedure, yield 82%; m.p.475K; IR(KBr)cm^{-1}:3330 (OH broad str.)3213, 3024 (NH, NH2), 2754 (Ar. C-H str.), 1589 (NH bend), 1489 (C=C ring str.), 1290 (CN str.), 700(C=S);^{1}H NMR (400 MHz, DMSO-d_6): δppm,13.46(1H,s, of NH-SH), 9.26 (1H, s, of OH), 6.66-7.07 (4H, m, of

Ar-H), 5.50(2H, s, of NH_2), 3.88 (2H, s, of CH_2); NMR (100 MHz, DMSO- d_6): δ ppm, 39.22 (CH_2), 125.26, 126.96, 129.25, 130.73, 133.88, 133.88 (Aromatic carbons), 150.32 (C=N), 164.55(C=S); Anal. cal. for $C_9H_{10}N_4OS$: C,48.63; H, 4.53; N, 25.21. Found: C, 48.58; H, 4.48; N, 25.15;

3.3. Antimicrobial studies

3.3.1. Antibacterial activity

The newly synthesized compounds were screened for their antibacterial activity against *Escherichia coli, Bacillus subtilis, Pseudomonas aeruginosa, Staphilococcusaureus* bacterial strains by disc diffusion method. The discs measuring 6.25mm in diameter were punched from Whatman No. 1 filter paper. Batches of 100 discs were dispensed to each screw capped bottles and sterilized by dry heat at 140 °C for an hour. The test compounds were prepared with different concentrations using 10% DMSO. One millilitre containing 100 times the amount of chemical required in each disc was added to each bottle which contains 100 discs. The discs of each concentration were placed in triplicate in nutrient agar medium seeded with fresh bacteria separately. The incubation was carried out at 37.8°C for 24 h. Solvent and growth controls were kept [13].

3.4. *In vitro*Antioxidant assays:

3.4.1. DPPH radical scavenging activity

DPPH is stable and commercially available organic nitrogen radical and has a UV-visabsorption maximum at 515 nm. Upon reduction, the deep violet colour of the solution fades. The progress of the reaction is monitored by a spectrophotometer at 515nm.

The ability of **1a -1e** to act as hydrogen donor was measured *in vitro* by stable free radical as 1,1-diphenyl-2-picryl-hydrazyl free radical ($DPPH^{\bullet}$) as described by Blois [14]. Antioxidant reacts with $DPPH^{\bullet}$, converts it to a colourless, nonradical1,1-diphenyl-2-picrylhydrazine ($DPPH_2$). The decolouration from violet indicates the scavenging potential of the antioxidant compound in terms of hydrogen donating ability. The reaction mixture contained 1 ml of 0.3 mM solution of $DPPH^{\bullet}$ in methanol and 1 ml of the test compounds or Glutathione (GHS) at 50µg/ml concentrations. After shaking the mixture, it was allowed to stand for 20 minat room temperature. SHIMADZU UV-2550

double beam spectrophotometer with 1cm matched quartz cell was used to measure the absorbance at 515 nm. The percentage of inhibition was calculated using the equation,

% Inhibition = (Control – Test) / Control x100

3.4.2. FRAP assay

Antioxidant activity was determined by FRAP assay as described by Oyaizu, 1986. According to this method 100 µg/mL of each sample and standard ascorbic acid in DMSO were prepared and mixed (2.5 mL) with phosphate buffer (2.5 mL, 0.2 mole, pH 6.6) and 1.0 % potassium ferricyanide (2.5 mL). The mixture was incubated at 50 ºC for 20 minutes. Aliquots of 10 % trichloro acetic acid (2.5 mL) were added to the mixture, centrifuged at 5000 rpm for 10 min. The upper layer of solution (2.5 mL) was mixed with distilled water (2.5 mL) and a freshly prepared ferric chloride solution (0.5 mL, 0.1 %) and allowed to stand for 30 minutes in dark to complete the reaction. The control solution was prepared as above, taking water in place of samples. The absorbance was measured at 700 nm [15].

3.4.3. Lipid Peroxidation Assay

A modified Thiobarbituric acid-reactive species (TBARS) assay was used to measure the lipid peroxide formed, using egg yolk homogenates as lipid-rich media. Adapted TBARS method was employed to measure the antioxidant capacity. Briefly, egg homogenate (100 µL of 1:25, v/v in phosphate-buffered saline (PBS) of PH 7.4 and 10 µL of 100 µg/mL concentration of compound samples were added to a test tube as well as added 25mmol/L freshly prepared $FeSO_4$ made up to 300 µL with PBS. Homogenate was incubated at 37 °C for 15 min and then the reaction was stopped by adding 50 µL 15% w/v Trichloroacetic acid (TCA) and centrifugation (3,500 rpm) for 15 min. An aliquot of 200 µL from supernatant was mixed with 100 µL thiobarbituricacid (TBA) and heated at 95 °C for 30 min. After cooling, absorbance of the samples was measured using a spectrophotometer at 532 nm [16].

% Inhibition of lipid peroxidation = $100 \times (A_0 - A_1)/A_0$, where A_0 and A_1 are the absorbance of control and the compounds or standards respectively.

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