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SYNTHESIS AND EVALUATION OF ANTIOXIDATIVE PROPERTIES OF SUBSTITUTED 10H-PHENOTHIAZINES WITH THEIR RIBOFURANOSIDES

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abstract: A series of novel substituted 10H-Phenothiazines were synthesized via Smiles rearrangement using specific precursors. Synthesized Phenothiazines in form of heterocyclic base were then treated with appropriate sugar to yield ribofuranosides. The compounds were evaluated for their antiradical and antioxidative properties through *in vitro* and *in vivo* studies in Swiss albino mice. The structural assignment of compounds was made on the basis of spectroscopic data and elemental analysis.

key words: phenothiazines; ribofuranosides; smiles rearrangement; Swiss albino mice; antioxidant activities.

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Introduction

In last few decades a considerable amount of attention has been focussed on synthesis of nitrogen and sulfur containing phenothiazines. The investigation of substituted 10H-Phenothiazines has steadily flourished because they exhibit a large scope of applications. These moieties are widely employed as antibacterial, antiviral, anti-inflammatory, anticancer, sedatives, tranquilizers agents etc. Slight change in substitution pattern in phenothiazine nucleus causes distinguishable difference in their biological activities [1-10]. Phenothiazines serve as heterocyclic base for the formation of ribofuranosides on treatment with sugar. These prepared ribofuranosides too possess similar chemotherapeutic activities [6-7]. The compounds were evaluated for their antiradical & antioxidative properties through *in vitro* and *in vivo* studies in Swiss albino mice [18].

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Results

Two series of substituted 10H-Phenothiazines were prepared by treating 2-aminobenzenethiols (I) with o-halonitrobenzene (II). In first series, substituted 10H-Phenothiazines (V_{a-b}) can be accomplished via Smiles rearrangement [10-12] (scheme-1) by condensation of formyl derivatives of diphenylsulphides (IV_{a-b}). The latter were obtained by the formylation of the diphenylsulphides (III_{a-b}), which in turn were obtained by condensation of 2-aminobenzenethiols (I) with o-halonitrobenzene (II) (R_6=Halo) in ethanolic sodium acetate solution. In second series, 10H-Phenothiazines (V_{c-d}) were prepared by condensation of (I) with (II), II containing a nitro group (R_6=Nitro) at both ortho positions to the halo atom, in ethanolic sodium hydroxide via Smiles rearrangement in situ. All synthesized phenothiazines (V_{a-d}) were treated with β-D-ribofuranosyl-1-acetate-1,3,5-tribenzoate (VI) in toluene and stirred in vacuuo on an oil bath, at 155-160°C for 10 hrs to form ribofuranosides (VII_{a-d}). The structure of all the synthesized compounds was characterized by correct spectroscopic data and elemental analysis (Table II).

The compounds were evaluated for antiradical activity and antioxidative properties on Swiss albino mice. Synthesized compounds showed mixed radical scavenging activity.

Results showed that there was significant decrease in lipid peroxidation (LPO) level and elevation in reduced glutathione (GSH) in Swiss albino mice.

Discussion

The structure assignment of these compounds was made on basis of spectral data and elemental analysis. In spectral data the characteristic IR bands and ^{1}H NMR data of compounds V_{a-d} and VII_{a-d} are presented in Table-I. Compounds V_{a-d} exhibit a single sharp peak in the region 3350-3110 cm^{-1} due to N–H stretching band which was found absent in compounds VII_{a-d} indicating site of ribosylation. Similarly, in compounds (V_{a-d}) N–H proton appeared as a singlet between δ 9.15-8.30 ppm which was found absent in ribofuranosides (VII_{a-d}). In mass spectra molecular ion peak are in accordance with their molecular weight (Table-II).

Experimental

All the melting points were determined in open capillary tubes and are uncorrected. IR spectra were recorded in KBr on NICOLET-MEGNA FT-IR 550 spectrometer and the ^{1}H NMR spectra on JEOL AL-300 spectrometer (300 MHz) in $CDCl_3$/DMSO-d_6 using TMS, as an internal standard (chemical shifts are measured in δ ppm), and ^{13}C NMR spectra (Table-III) in $CDCl_3$ were measured. The purity of the compounds was checked by TLC using silica gel "G" as adsorbent, visualizating these by UV light or Iodine chamber.

Synthesis of 2-amino-2´-nitrodiphenylsulfides (III_{a-b})

2-aminobenzenethiol (I) (0.01 mole) was dissolved in ethanol (20 ml) containing (0.01 mole) of anhydrous sodium acetate in 50 ml R.B. flask. Then halonitrobenzne (II) (0.01 mole) in 10 ml ethanol was added to above R.B flask. The reaction mixture was refluxed for 4-5 hrs and concentrated in an ice bath overnight. The solid separated out was filtered, washed with 30% ethanol and recrystallized from methanol to get diphenylsulfides (III_{a-b}).

Synthesis of 2-formamido-2´-nitrodiphenylsulfides (IV_{a-b})

The diphenylsulphides (III_{a-b}) (0.01 mole) obtained were refluxed for 4 hrs. in 90% formic acid (20 ml). The content was then poured into a beaker containing crushed ice. A solid separated out was filtered, washed with water until the filtrate was neutralized. The formyl derivatives of diphenylsulphides (III_{a-b}) were crystallized from benzene.

Synthesis of Phenothiazine (V_{a-b})

Formyl derivative (IV_{a-b}) (0.01 mole) in acetone (15 ml) with an alcoholic solution of potassium hydroxide (0.2 g in 5 ml ethanol) was refluxed and heated for 30 minutes. Again a second lot of potassium hydroxide (0.2 g in 5 ml ethanol) was added to the reaction mixture and further refluxed for 4 hrs. The content was poured into beaker containing crushed ice and filtered. The residue obtained was repeatedly washed with cold water and finally with 30% ethanol and then crystallized from benzene to get Phenothiazine (V_{a-b}).

Table I The ^{1}H NMR and IR spectral data of synthesized compounds.

Compd No.	^{1}H NMR (δ ppm from TMS)		IR (KBr : ν_{max} cm^{-1})				
	>NH	Ar-H multiplet	>NH	O ‖ N→O	C–Cl	C–Br	C–O–C
V_a	8.30	6.89-7.34	3350	-	820	-	-
V_b	8.89	6.45-8.03	3250	-	780	660	-
V_c	9.15	6.90-8.14	3110	1530, 1360	760	-	-
V_d	9.10	6.53-7.52	3190	1500, 1350	810	-	-
VII_a	-	6.72-7.86	-	-	820	-	1095
VII_b	-	6.91-7.98	-	-	760	630	1150
VII_c	-	6.21-7.69	-	1545, 1390	800	-	1165
VII_d	-	6.83-7.89	-	1520, 1390	760	-	1070

Synthesis of Nitrophenothiazines (V_{c-d})

A mixture of reactive halonitrobenzene (II) 0.01 mole (R_6=NO_2), substituted 2-aminobenzenethiol (I) 0.01 mole, sodium hydroxide (0.01 mole) and absolute ethanol (25 ml) was refluxed for two hours. The reaction mixture was concentrated on water bath, cooled and filtered. The precipitate was washed well with hot water and ethanol and crystallized from acetone to get nitrophenothiazine insitu.

Synthesis of substituted N-(2´,3´,5´-tri-o-benzoyl-β-D-ribofuranosyl) phenothiazine (VII_{a-d})

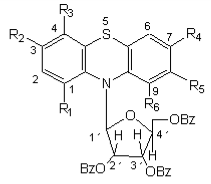
To a concentrated solution of (V_{a-d}) (0.002 mole) in toluene, β-D-ribofuranose-1-acetate-2,3,5-tribenzoate (VI) (0.002 mole) was added and stirred, in vacuum, on an oil bath, at 155-160°C, for 15 minutes. The vacuum was broken and the reaction was protected from moisture, by using a guard tube. Stirring was further continued for 10 hr. with application of vacuum for 15 minutes after every hour. The melt was dissolved in methanol, boiled for 10 minutes and cooled to room temperature. The precipitate was filtered and the filtrate was

evaporated to dryness. The viscous residue, thus obtained was dissolved in ether, filtered, concentrated and kept in a refrigerator overnight to get crystalline ribofuranoside (Scheme-I).

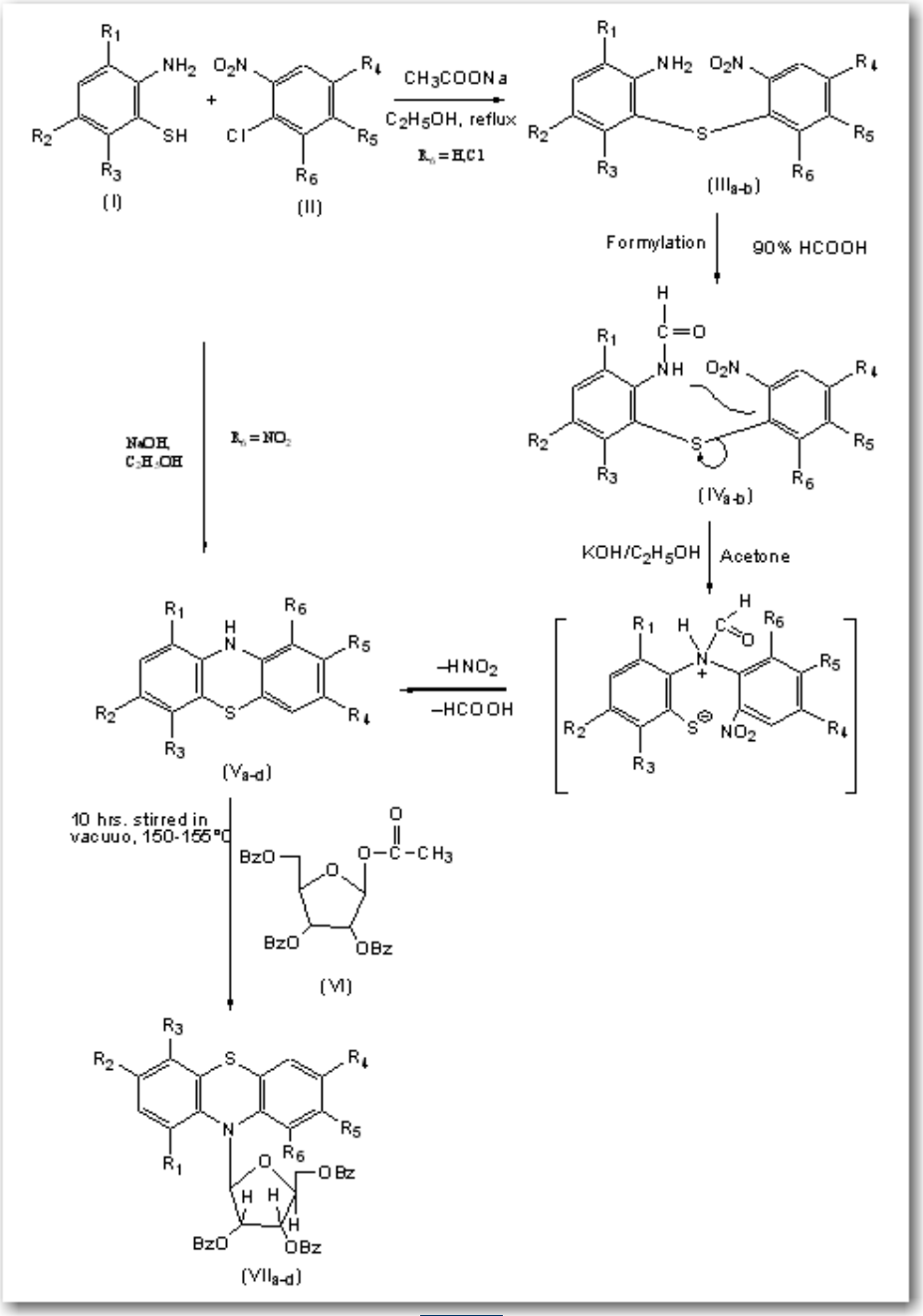
Table II Characterization data of phenothiazines and ribofuranosides.

Compd. No.	*R_1*	*R_2*	*R_3*	*R_4*	*R_5*	*R_6*	*Mol. Formula*	*Yield %*	*M.P. (°C)*	*Elemental analysis Found (calcd.)*		
										C	*H*	*N*
V_a	Cl	H	CF_3	H	H	H	$C_{13}H_7NClF_3S$	34	280	51.80	2.33	4.68
										(51.74)	(2.32)	(4.64)
V_b	F	Br	H	H	Cl	Cl	$C_{12}H_5NBrCl_2FS$	42	160	39.62	1.38	3.89
										(39.56)	(1.37)	(3.85)
V_c	Cl	H	CF_3	NO_2	H	NO_2	$C_{13}H_5N_3O_4ClF_3S$	40	295	39.92	1.29	10.77
										(39.85)	(1.28)	(10.73)
V_d	Cl	H	CF_3	COOH	H	NO_2	$C_{14}H_6N_2O_4ClF_3S$	52	240	43.10	1.57	7.21
										(43.02)	(1.54)	(7.17)
VII_a	Cl	H	CF_3	H	H	H	$C_{39}H_{27}NO_7ClF_3S$	22	120	62.86	3.64	1.91
										(62.78)	(3.62)	(1.88)
VII_b	F	Br	H	H	Cl	Cl	$C_{38}H_{25}NO_7BrCl_2FS$	31	100	56.42	3.12	1.76
										(56.35)	(3.09)	(1.73)
VII_c	Cl	H	CF_3	NO_2	H	NO_2	$C_{39}H_{25}N_3O_{11}ClF_3S$	35	95	56.11	3.04	5.06
										(56.02)	(2.99)	(5.03)
VII_d	Cl	H	CF_3	COOH	H	NO_2	$C_{40}H_{26}N_2O_{11}ClF_3S$	46	110	57.53	3.40	3.36
										(57.45)	(3.1)	(3.35)

Table III ^{13}C NMR spectral data of phenothiazines and ribofuranosides.



S. No.	R_1	R_2	R_3	R_4	R_5	R_6	^{13}C NMR spectral data
V_a	Cl	H	CF_3	H	H	H	δ 148.7 (C–1), δ 118.4, 129.1, 116.2 (C–2, C–3, C–4); δ 114.8 (C–6); δ 139.9, 111.4, 142.3 (C–7, C–8, C–9);
V_b	F	Br	H	H	Cl	Cl	δ 142.4 (C–1); δ 113.4 (C–2); δ 141.1, 113.9 (C–3, C–4); δ 119.2, 113.6, 138.3 (C–6, C–7, C–8); δ 137.4 (C–9)
V_c	Cl	H	CF_3	NO_2	H	NO_2	δ 130.1 (C–1), δ 117.4, δ 115.6 (C–2, C–3); δ 130.6 (C–4); δ 115.4, 142.9, 111.4 (C–6, C–7, C–8); δ 148.1 (C–9)
V_d	Cl	H	CF_3	COOH	H	NO_2	δ 132.2 (C–1), δ 119, δ 113.9 (C–2, C–3); δ 128.4 (C–4); δ 112.1, 138.4, 113.2 (C–6, C–7, C–8); δ 146.4 (C–9)
VII_a	Cl	H	CF_3	H	H	H	δ 148 (C–1), δ 117.9, 130.1,. 116.4, (C–2, C–3, C-4); δ 114.1 (C–6); δ 141.1, 112.3, 145.2 (C–7, C–8, C–9); δ 96.1 (C–1′); δ 81.51, 80.91 (C–2′. C–3′), δ 95.3 (C–4′)
VII_b	F	Br	H	H	Cl	Cl	δ 143.4 (C–1), δ 114.3 (C–2); δ 143.2, 115.1 (C–3, C–4); δ 121.1, 116.1, 140.2 (C–6, C–7, C–8); δ 139.1 (C–9); δ 96.8 (C–1′), δ 83.41, 80.42(C–2′, C–3′), δ 94.2 (C–4′)
VII_c	Cl	H	CF_3	NO_2	H	NO_2	δ 131.5 (C–1), δ 119.6, 118.1 (C–2, C–3); δ 132.4 (C–4); δ 117.1, 143.7, 113.6 (C–6, C–7, C–8); δ 149.2 (C–9); δ 93.9 (C–1′); δ 82.96, 83.10(C–2′, C–3′) δ 96.4 (C–4′)
VII_d	Cl	H	CF_3	COOH	H	NO_2	δ 132.4 (C–1), δ 118.6, 117.2 (C–2, C–3); δ 126.9 (C–4); δ 117.2, 139.2, 115.6 (C–6, C–7, C–8); δ 147.1 (C–9); δ 97.4 (C–1′); δ 82.98, 81.26(C–2′, C–3′), δ 98.4 (C–4′).


CH3COONa
C2H5OH, reflux
(I)
(II)
(IIIa-b)
Formylation
90% HCOOH
(IVa-b)
KOH/C2H5OH
Acetone
NaOH
C2H5OH
–HNO2
–HCOOH
(Va-d)
10 hrs. stirred in
vacuuo, 150-155°C
(VI)
(VIIa-d)

Scheme I.

Antiradical Activity

All the synthesized compounds $V_{(a-d)}$ and their ribofuranosides $VII_{(a-d)}$ were screened for their antiradical activity by 1,1-diphenyl-2-picryl hydrazyl (DPPH) radial scavenging assay and 2,2-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) ($ABTS^{\bullet+}$) radical cation decolorization assay.

The present study demonstrated that the synthesized compounds showed mixed radical scavenging activity in DPPH (Table IV) and $ABTS^{\bullet+}$ assays (Table V).

a) Compounds (V_c, VII_a) showed strong radical scavenging activity in DPPH assay that have DPPH% inhibition $\geq$ 50.

b) Compounds (V_a, VII_c, VII_d) showed moderate radical scavenging activity in DPPH assay that have DPPH% inhibition $\geq$ 30.

c) Compounds (V_b, V_d, VII_d) showed mild radical scavenging activity in DPPH assay that have DPPH% inhibition < 30.

d) Compounds (V_a, V_c, V_d, VII_a; VII_c, VII_d) were found to be more active in $ABTS^{+}$ assay which showed much decline in graph.

The study reveals that ribofuranosides (VII_a and VII_d) showed better antiradical effect than their respective bases in DPPH assay. Ribofuranosides (VII_a, VII_c, VII_d) showed better antiradical effect than their respective base in $ABTS^{\bullet+}$ assay.

DPPH Radical Scavenging Assay

Radical scavenging activity of compound $V_{(a-d)}$ and $VII_{(a-d)}$ against stable 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical was determined spectrophotometrically as described by Cuendet *et al.*,[13]. A stock solution 1 mg/ml of the compound was prepared in methanol. 50 µl of compounds were added to 5 ml of a 0.004% methanol solution of DPPH. After 30 min. incubation in dark at room temperature, the absorbance was read against a blank at 517 nm.

The assay was carried out in triplicate and the percentage of inhibition was calculated using the following formula.

$$\% \text{ Inhibition} = \frac{AB - AA}{AB} \cdot 100$$

Where: AB = Absorption of blank AA = Absorption of test

ABTS Radical Cation Decolorization Assay

The 2,2-azinobis(3-ethybenzothiazoline-6-sulphonic acid) radical cation (ABTS) decolorization test was also used to assess the antioxidant activity of compounds V_{a-d} and VII_{a-d}. The $ABTS^{\bullet+}$ assay was carried out using the improved assay of Re *et al.*,[14]. In brief, $ABTS^{\bullet+}$ was generated by oxidation of ABTS with potassium persulphate. For this purpose ABTS was dissolved in deinoized water at a concentration of 7mM, and potassium persulphate added to a concentration of 2.45mM. The reaction mixture was left at room

temperature overnight (12-16 h) in the dark before use; the ABTS solution then was diluted with ethanol to an absorbance of 0.700 ± 0.020 at 734 nm. After addition of 1 ml of the diluted ABTS solution to 10 µl of compound and mixing, absorbance readings were taken at 30°C at intervals of exactly 1-6 min. later. All determinations were carried out in triplate.

Table IV Antiradical activity of synthesized compounds.

Compd. No.	DPPH % inhibition of 1 mg/ml of the compound
V_a	47.44 ± 1.4
V_b	25.68 ± 1.1
V_c	52.89 ± 1.6
V_d	18.71 ± 1.2
VII_a	53.21± 1.4
VII_b	20.41 ± .09
VII_c	42.41± 0.6
VII_d	30.99 ± 1.5

Inhibition (%) of DPPH radical scavenging activity of various compounds at particular concentration. Stock solution of crude compound was prepared as 1mg/ml in methanol. Fifty microlitres of samples of particular concentration were added to 5 ml of 0.004% methanol solution of $DPPH^{\cdot}$. After 30 min. incubation in dark at room temperature, the absorbance was read against a blank at 517 nm.

Table V Antiradical activity of synthesized compounds.

Compd. No.	ABTS activity at different time intervals minutes				
	0 min.	1 min.	2 min.	4 min.	6 min.
V_a	0.722	0.109	0.106	0.102	0.101
V_b	0.739	0.691	0.690	0.690	0.690
V_c	0.732	0.285	0.283	0.282	0.280
V_d	0.727	0.145	0.143	0.142	0.140
VII_a	0.738	0.068	0.065	0.054	0.050
VII_b	0.729	0.617	0.613	0.612	0.612
VII_c	0.722	0.150	0.150	0.150	0.150
VII_d	0.730	0.059	0.048	0.045	0.039

(ABTS activity at different time intervals) of Phenothiazines(V)

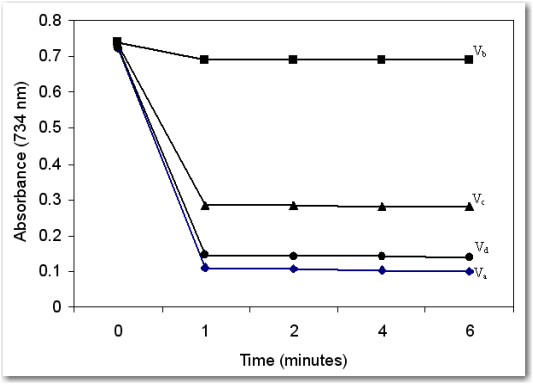


Fig. 1 *The effect of time on the suppression of absorbance of ABTSby phenothiazines(V). After addition of 1ml of diluted ABTS solution (A 734 nm = 0.700±0.020) to 10 µl of the compound the absorbance reading was taken at 30°C exactly 1 min., after initial mixing and up to 6 min. All determinations were carried out in triplicates.*

(ABTS activity at different time intervals) of Ribofuranosides(VII)

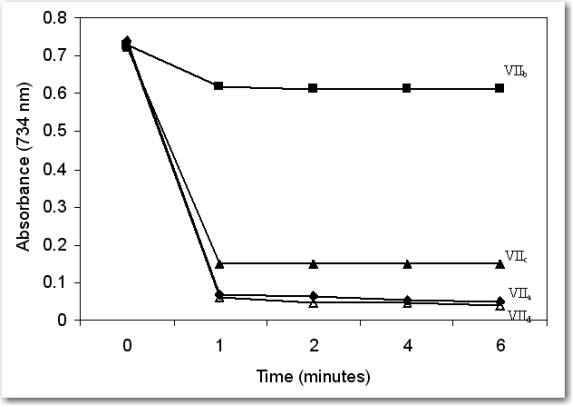


Fig. II *The effect of time on the suppression of absorbance of ABTS by ribofuranosides(VII). After addition of 1ml of diluted ABTS solution (A 734 nm = 0.700±0.020) to 10 µl of the compound the absorbance reading was taken at 30°C exactly 1 min., after initial mixing and up to 6 min. All determinations were carried out in triplicates.*

In Vivo Studies in Swiss Albino Mice

The compounds were further treated for evaluation of antioxidative properties in Swiss albino mice. Results showed that there was significant decrease in lipid peroxidation (LPO) level and elevation in reduced glutathione (GSH) in Swiss albino mice.

Material and methods

1. Animals. Swiss albino mice were obtained from, Jawaharlal Nehru University, New Delhi, India. Random-bred, males Swiss albino mice (8 weeks old), weighing 24 ± 2 gm were used for experiments. These animals were maintained in the animal house at temperature of 24° ± 3°C. They were housed in polypropylene cages and fed standard mice feed from Hindustan Lever Ltd.,India. Tap water was provided to the animals.

2. Chemicals. Compound synthesized, Sodium chloride, Tris potassium chloride, Trichloro acetic acid, 5-dithiobis-2-nitrobenzoic acid (DTNB), acetic acid, Thiobarbituric acid, n-butanol, Pyridine.

3. Experimental design. All the synthesized compounds V_{a-d} and their sulfones VII_{a-d} were given at dose of 1mg/kg body weight intra-peritonealy to Swiss albino mice 2 hrs before sacrifice. For each compound five animals were used. The LPO level and GSH content were estimated in liver of mice.

Biochemical Studies

- Lipid peroxidation assay. The LPO level in liver was measured in terms of thiobarbitituric and reactive substance (TBARS) by the method of Ohkhawa, et al. (1979)[16]. Absorbance in the assay was read at 532 nm (Table VI)

- Sulfhydral group assay (GSH). The level of acid-soluble sulfhydral groups was estimated in liver as total non-protein sulfhydral groups using the method described by Moron et al., (1979) [17], Reduced glutathione (GSH; obtained from Sisco Research Laboratories, Bombay, India) was used as a standard to calculate the micromoles of SH/g of tissue. Absorbance in the assay was read at 412 nm using a systronic spectrophotometer (Systronics Type 108; Naroda, Ahmedabad, India) (Table VII).

Statistical Analysis

Results of the biochemical studies were evaluated using student's 't' test.

Table VI Antioxidative properties of compounds in the Liver in Swiss Albino Mice.

Treatment compound No.	LPO (n mole/mg tissue) 8.24±0.65
V_a	6.71 ± 0.6
V_b	6.5 ± 0.5
V_c	6.2 ± 0.21, p< 0.005
V_d	6.51 ± 0.5
VII_a	6.7 ± 0.18, p< 0.05
VII_b	6.78 ± 0.18
VII_c	6.81 ± 0.16
VII_d	6.51 ± 0.17, p< 0.05

Table VII Antioxidative properties of compounds in the liver in Swiss Albino Mice.

Treatment compound No.	GSH (n mole/100 gm tissue) 4.06±0.14
V_a	4.15 ± 0.25
V_b	4.2 ± 0.21
V_c	5.01 ± 0.12,p<0.005
V_d	4.19 ± 0.21
VII_a	4.90 ± 0.11,p<0.005
VII_b	4.7 ± 0.13,p<0.05
VII_c	4.8 ± 0.15,p<0.05
VII_d	5.01 ± 0.12,p< 0.05

The above value shows there was significant increase in GSH content of liver in animals treated with compounds V_c, VII_a, VII_d, also in these animals the value of LPO was significantly decreased, showing potent antioxidant activities in Swiss albino mice. However, other compounds show increase in GSH content and decrease in LPO level but not statistically significant.

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