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2.1. Chemicals

L-α-Lecithin (phosphatidylcholine) was obtained from CSIR Biochemical center, Delhi. 2-Thiobarbituric acid (TBA) , ferritin, lactoferrin, transferrin, reduced glutathione (GSH), L -ascorbate, bathophenanthroline disulphonic acid (BPS), etylenediamine tetraacetic acid (EDTA), ethylene glycol bis (aminoethyl ether) - N,N,N',N' - tetraacetic acid) (EGTA), ferrozine, sodium formate, catalase (EC 1.11.1.6), superoxide dismutase (SOD) (EC 1.15.1.1), horseradish peroxidase (EC 1.11.1.7), vitamin E (α - tocopherol) and trichloracetic acid (TCA) were purchased from Sigma Chemical Company, USA. Malondialdehyde (MDA) was from Fluka Chemical Company. All other chemicals and reagents were of analytical grade and were from E. Merck or BDH, Bombay.

2.2. Preparation of Liposomes

Liposomes were prepared essentially by the method of Gregoriadis and Ryman (1972). One ml (10% hexane solution) containing 100 mg of lecithin was dissolved in 5 ml of chloroform in a round bottom flask. Shaking was done well on the REMI-cyclo mixer. This solution of lecithin in chloroform was evaporated to dryness in the rotary vacuum evaporator (Buchi type) (Scientific Instrument). A thin film of lipid was obtained and was gently resuspended under the stream of nitrogen in 50 ml of 10 mM sodium phosphate buffer (pH 7.4). The milky suspension was then sonicated in an ultrasonic disintegrator (MSE) with an exponential probe at 8 microns peak to peak in an ice bath, with intermittent 10 periods of 60 seconds with cooling periods of 30

seconds. Maximum care was taken to avoid the contact of oxygen while preparing the liposomes. The liposomes thus prepared were used immediately in all experiments.

2.3. Estimation of Lipid Peroxidation

Lipid peroxidation was estimated by the method of Konings and Drijver (1979) and expressed in terms of malondialdehyde (MDA) formed per mg of lipid (Kale and Sitasawad, 1990a). To determine the concentration of MDA in the suspension, 1 ml liposomes with or without drug / chemical was transferred into the centrifuge tubes followed by addition of 1 ml of suspension medium (0.15 M KCl + 10 mM Tris HCl) to which 0.5 ml of 30% trichlor acetic acid TCA was added. Thereafter 0.5 ml of 52 mM thiobarbituric acid (TBA) was added. The tubes were covered with aluminium foil and placed in a waterbath shaker for 30 minutes at 80° C. The tubes were cooled in an ice waterbath for 10 minutes and centrifuged at room temperature for 10 minutes at 3,000 rpm in REMI-T8 table top centrifuge. The absorbance of the clear supernatant was measured against a reference blank of distilled water at 531.5 nm (Figure IV) in spectrophotometer (UV 260, Shimadzu).

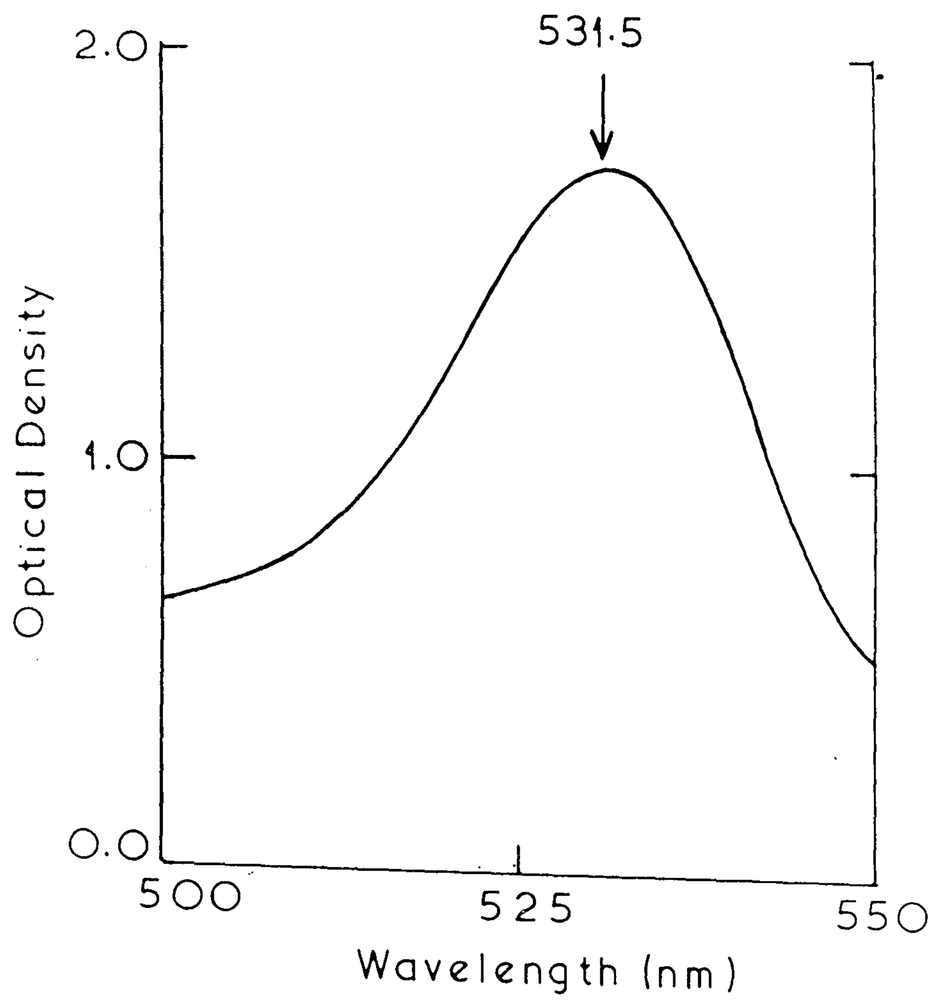


Figure IV : Spectra of MDA showing maximum absorption at 531.5 nm.

2.4. Calculation of MDA formed

The amount of MDA formed in a sample is estimated according to equation,

$$\text{nanomoles MDA} = \frac{V \times OD}{0.152}$$

Where, V = final volume of the test solution,

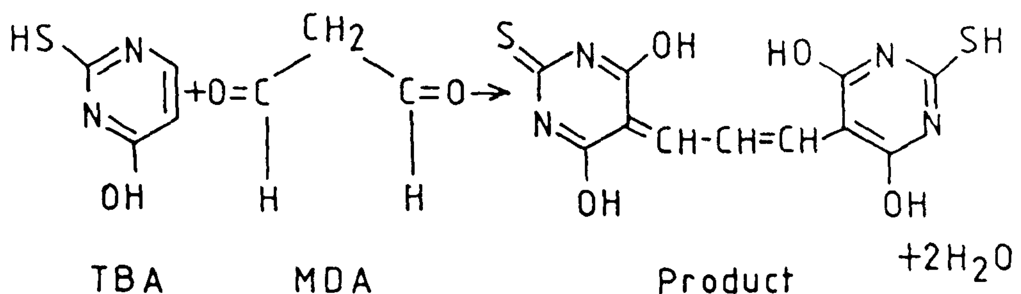
OD = Optical density at 531.5 nm, and

e = 1.52×10^5 mM^{-1} cm^{-1} (extinction coefficient)

$$\text{nanomoles MDA / mg lipid} = \frac{3 \times OD}{0.152 \times \text{Amount of lipid in the cuvette}}$$

2.5. Lipid peroxide - TBA Reaction

TBA reacts with lipid peroxides and the product of the reaction was found to have the structure shown below (Pryor, 1976)



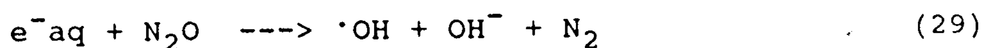
Malondialdehyde gives the same product upon reaction with TBA.

The formation of MDA from lipid peroxides is shown in Figure II. (Here, RH indicates lipid molecule).

2.6. Generation of Restricted Free Radical Sources

It is of interest to have a more restricted free radical source and which could be generated with the help of N_2, N_2O, O_2 + sodium formate.

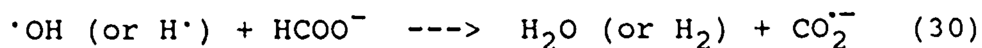
N_2O converts the solveted electrons into $^{\cdot}OH$ radicals according to reaction 29



$$e^{-}_{aq} + N_2O \text{ ---> } {}^{\cdot}OH + OH^{-} + N_2 \qquad (29)$$

The solution saturated with N_2O by purging gives, on irradiation, 90% $^{\cdot}OH$ and 10% $H^{\cdot}$ radicals.

The generation of $O_2^{\cdot-}$ radicals was accomplished by irradiation in the presence of sodium formate and oxygen according to reactions 30 and 31



$${}^{\cdot}OH \text{ (or } H^{\cdot}) + HCOO^{-} \text{ ---> } H_2O \text{ (or } H_2) + CO_2^{\cdot-} \qquad (30)$$

$$CO_2^{\cdot-} + O_2 \text{ ---> } O_2^{\cdot-} + CO_2 \qquad (31)$$

N_2 saturated solution gives the radiation chemical yields (G values) around 2.7, 0.55 and 2.65 of $^{\cdot}OH$, $H^{\cdot}$ and e^{-}_{aq} respectively.

2.7. Measurement of release of iron from iron saturated proteins

The release of iron from the iron saturated protein in presence or absence of chemicals / enzymes was determined by the method described by Reif et al.(1988) using the iron chelator bathophenanthroline disulphonic acid (BPS) as a chromophore. Two ml solution in phosphate buffer (pH 7.3) containing iron saturated protein and BPS (1 mM) was irradiated to required dose and / or dose rate. The appearance of the Fe^{2+} $(BPS)_3$ complex was

monitored at 530 nm and iron release was determined by using e = 22.14 $mM^{-1}cm^{-1}$. All measurements were made on a spectrophotometer (UV 260, Shimadzu).

2.8. Extraction of Papaya / Bittergourd juice

Papaya / Bittergourd pieces without seeds were crushed in the juicer. The juice was collected by filteration followed by centrifugation at 4,000 rpm for 30 minutes in the REMI-T8 Table top centrifuge. Juice was collected, kept chilled and used within 10 hours. This preparation was considered 100% concentration.

2.9. Preparation of Mint / Brassica extract

The fresh mint leaves / brassica seeds were crushed into the pestle and mortar. The extract was filtered using Buchner funnel. The extract was centrifuged at 4,000 rpm for 30 minutes in a REMI-T8 Table top centrifuge. These extracts of mint leaves / brassica seeds were kept chilled and used within 10 hours. This concentration of mint leaves / brassica seeds were taken as 100%.

2.10. Irradiation

One ml of the reaction mixture was irradiated at required dose / dose rate in gamma chamber (204 TBq ^{60}Co) obtained from Isotope Division, Bhabha Atomic Research Center, (BARC) Bombay. All irradiations were done at room temperature. The dose rate was determined using Fricke's Fe^{2+}/Fe^{3+} dosimetry as described by Schested (1970). After irradiation, reaction mixtures were used immediately to determine the release of iron from the proteins and lipid peroxidation in liposomes.

2.11. Determination of Dose Rate

The solution consisted of 0.001M ferrous ammonium sulphate ($Fe(NH_4)_2(SO_4)_2$), 0.8 N H_2SO_4 and 0.001M NaCl was prepared as follows :

The sulphuric acid (4.4 ml) was added to the water (200 ml) first and allowed to cool at room temperature. Ferrous ammonium sulphate (80 mg and NaCl 12 mg) were then added and the solution shaken vigorously with a glass rod to dissolve the salts completely as quickly as possible.

The dosimeter solution (5 ml) was irradiated for small time periods (1, 2, 3, 4 and 5 minutes). The duration of radiation period was noted accurately. The O.D. of the ferric ions formed, was measured spectrophotometrically at 305 nm. At this wave length the molar extinction coefficient of the ferrous ion is particularly zero and that of the ferric ion, 2197 M^{-1} cm^{-1} at 25° C. Another ferric ion peak is located at 244 nm where the molar extinction coefficient is about twice as high, namely 4565 M^{-1} cm^{-1}. However, at this wave length the absorption of the ferrous ions cannot be neglected (about 20 M^{-1} cm^{-1}).

The optical density of the sample after irradiation is compared with that of the blank (unirradiated solution). A graph of $\triangle$ OD (OD irradiated - OD unirradiated) Vs time is plotted (Figure V) and the slope ($\triangle$ OD / min) measured.

According to the definition of the G value :

$$G = \frac{\triangle \text{ molecules / ml x 100}}{\text{D (ev)}}$$

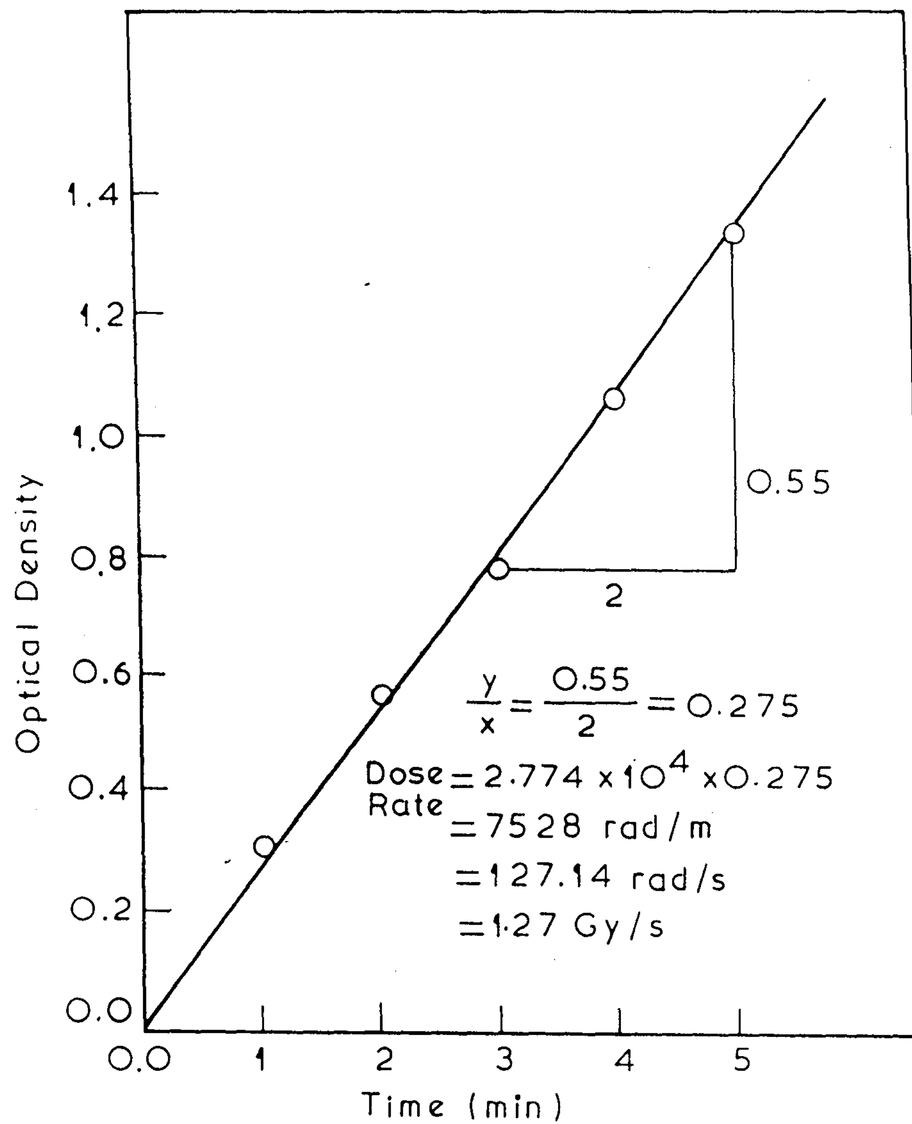


Fig. V

Formation of Fe^{3+} on irradiation of Fricke's Dosimetry solution.

Where D (ev) is the dose in ev / ml. Therefore,

$$D(ev) = \frac{\Delta \text{ molecules / ml} \times 100}{G}$$

In case of spectrophotometry, we have

$$\text{molecules/ ml} = \frac{\Delta OD \times N \times 10^{-3}}{d\ e\ l}$$

The conversion of D (ev) to Drad is

$$D\ (ev) = 6.245 \times 10^{13}\ \text{Drad}$$

and the dose equation can now be written as

$$\text{Drad} = \frac{\Delta OD \times N \times 10^{-3} \times 100}{G\ d\ e\ l \times 6.245 \times 10^{13}}$$

Where,

ΔOD = Difference in OD between the irradiated sample and the blank

N = Avogardro's number (= 6.02×10^{23} molecules / mole)

G = No. of ferrous ions oxidized per 100 ev of absorbed energy (GFe^{+3} = 15.6)

d = Specific density of the dosimeter solution (= 1.024 g / cm^3 for 0.8N NH_2SO_4)

e = Molar extinction coefficient (= 2197 M^{-1} cm^{-1} at 25^oC)

l = Optical path length of the spectrophotometric cell (= 1.0 cm)

After substituting these values,

$$\text{Dose Rate} = 2.75 \times 10^4 \times \Delta OD \text{ in rad}$$

Where,

ΔOD is a slope i.e. ΔOD / min

Rad being an old unit of absorbed radiation, it was converted into Gray (Gy) by following relation,

100 Rad = 1 Gy.