3. Materials and Methods

All the experiments were carried out by following standard published protocols without modification, unless otherwise mentioned. All chemicals and reagents used in the experiments were of analytical/HPLC grade and obtained from Sigma-Aldrich (St. Louis, MO, USA) and Merck (Mumbai, India). Solvents used for extraction of plant materials were of HPLC grade and procured from Hi-Media (Mumbai, India). Ultra pure water used for antioxidant assays and HPLC analysis was prepared in-house using a Milli-Q system. Cell culture media (DMEM and RPMI – 1640), antibiotics (cell culture tested) and lymphocyte separation medium (Histopaque 1077) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Trypsin-versene, L-glutamine and fetal bovine serum (FBS) were procured from PAA Biotech, Germany. Bacterial cultures were procured from MTCC, IMTECH, Chandigarh and Microbiology laboratory of Global Hospital, Hyderabad. Cancer cell lines were purchased from NCCS, Pune and maintained in respective media as recommended.

3.1. Plant materials and preparation of extracts

*Raphanus sativus* L. was purchased fresh from local supermarket in Hyderabad, India and processed on the same day itself. It was separated into root, stem, and leaves, washed thoroughly with distilled water and freeze dried. The dried samples were ground to powder and stored air tight at – 20°C until further analysis. The powdered plant material was extracted three times with solvents of varying polarity such as methanol, acetone, ethyl acetate, chloroform and hexane at room temperature for 24 h with mass to volume ratio of 1:40 (g/ml) and evaporated to dryness under vacuum on a rotary evaporator at 40°C. Water extract of *R. sativus* was prepared as above by soaking dried powder in distilled water and stirred using a magnetic stirrer at a low speed for 24 h. The extract was filtered using Whatman No. 1 paper and subsequently lyophilized in a lyophilizer at 5 μm Hg pressure at – 50°C. Dried residue of all extracts was subsequently redissolved in methanol for HPLC analysis and antioxidant activity. All extracts were filtered through 0.45 μm membrane filters and stored at –80°C until use. For antibacterial activity, dried residue of extracts was redissolved in respective solvents and sterilized by filtration using 0.22 μm membrane filters and stored at –80°C until use. For chemoprotective and chemopreventive activity of *R. sativus*, all extracts were
evaporated to dryness, dissolved in DMSO and stored at -80°C until use. All extracts were diluted appropriately with culture media and sterilized by filtration using 0.22 μm membrane filters prior to treatment of lymphocytes and cancer cell lines. The final concentration of DMSO used in culture medium was less than 0.2%.

3.2. Phytochemical analysis of *R. sativus*

3.2.1. Total polyphenolic content

Total polyphenolic content of root, stem and leaves of *R. sativus* extracts was determined using Folin–Ciocalteau reagent as described by Kim *et al* (2003) with slight modification. Different *R. sativus* extracts were made up to 2.0 ml with distilled water followed by 1.0 ml of 10% Folin–Ciocalteau reagent. After vortexing briefly, 1.0 ml of 10% sodium carbonate was added and mixture was allowed to stand at room temperature for 1 h. After incubation period, absorbance was measured at 760 nm using an UV-Vis spectrophotometer (Shimadzu, Japan). The total concentration of polyphenolic compounds in *R. sativus* extracts was measured as catechin equivalent and expressed as mg/g of dry extract. Experiments were performed in triplicate.

3.2.2. Total isothiocyanate content

The total isothiocyanate content of root, stem and leaves of *R. sativus* was estimated according to the method described by Zhang *et al* (1992a). Briefly, 5.0 μl of extract was added rapidly to tube containing 2.0 ml of methanol, 1.8 ml of 50 mM of sodium borate buffer (pH 8.5) and 0.2 ml of 8 mM of 1,2-benzenedithiol. The mixture was heated at 65°C for 1 h, cooled to 25°C and absorbance was measured at 365 nm. The content of isothiocyanates was calculated from a linear standard equation derived from benzyl isothiocyanate (BITC) and expressed as mg/g of dry extract. Experiments were performed in triplicate.

3.2.3. HPLC – DAD analysis

Polyphenolics in different parts of *R. sativus* were analyzed by Shimadzu LC10 HPLC system equipped with a photo diode array (PDA) detector. Separation of phenolics was carried out using a Luna C<sub>18</sub> column (250 mm x 4.6 mm i.d.; particle size, 5 μm) with a C<sub>18</sub> guard column. The mobile phase consisted of 6% acetic acid in 2 mM
sodium acetate (solvent A) and acetonitrile (solvent B). A solvent gradient was maintained by varying proportion of solvent A to solvent B in the following manner: a linear gradient of 0 - 15% of B for 45 min, 15 – 30% B for 15 min, 30 – 50% B for 5 min and 50 – 100% B for 5 min. The set time of recording chromatogram and spectra was 70 min, while total running time was 80 min including 10 min post run at initial conditions for equilibration of column. The column temperature was set at 30°C. The flow rate was 1.0 ml/min and injection volume was 20 µl. The PDA detector was set at 280, 320 and 360 nm for simultaneous monitoring of hydroxybenzoic acid (HBA)/flavanol (FLA) derivatives, hydroxycinnamic acid (HCA) derivatives and flavonol (FL) derivatives respectively.

The identification of each compound was based on comparing retention time and spectral matching against those of standards and also by co-elution. A match in both UV spectrum and retention time gives positive identification of a compound. A match only in UV spectrum but not in retention time gives tentative identification of a compound, i.e., identified as a derivative of that particular polyphenolic group. The standards used for the identification of polyphenolics in *R. sativus* were catechin, protocatechuic acid, vanillic acid, syringic acid, *o*-coumaric acid, sinapic acid, ferulic acid, myricetin and quercetin. Polyphenolics in *R. sativus* were quantified using linear regression equations derived from authentic standards. Accordingly, HBAs, HCAs, FLAs and FLs were measured as protocatechuic acid, ferulic acid, catechin and quercetin equivalent respectively and expressed as mg/g of dry weight.

### 3.2.4. Gas Chromatography – Mass spectrometry analysis

The bioactive compounds in ethyl acetate and hexane extract of *R. sativus* were analyzed by GC–MS, using an Agilent 6890N gas chromatograph coupled to an Agilent 5975N mass spectrometer. A HP-5MS capillary column (30 m x 0.25 mm I.D, 0.25 µm film thickness) was used for gas chromatographic separation. The column temperature was set at 60°C for 3 min, then programmed to 280°C at a rate of 10°C/min and held at 280°C for 10 min, for ethyl acetate extract. For analysis of hexane extract, GC oven temperature was programmed from 60°C to 280°C at a rate of 10°C/min and held at 280°C for 8 min. The injection volume was 2 µl with a split ratio of 1:5; injector temperature was held constant at 220°C. Helium was used as carrier gas at a flow rate of
1.1 ml/min. The mass spectrometer was operated in electron impact (EI) mode with ionization energy of 70eV and source temperature was held at 280°C. MS spectra were obtained in the mass range of m/z 43 – 350. Identification of analytes was based on comparison with mass spectra of the Wiley mass spectral database and with available literature data.

3.3. Antioxidant activity of R. sativus

3.3.1. Ferric reducing activity (FRAP assay)

The FRAP assay was done according to the method described by Benzie and Strain (1999) with some modification. This method is based on reduction of TPTZ-Fe³⁺ complex to TPTZ-Fe²⁺ form in the presence of antioxidants. The stock solutions included acetate buffer (300 mM, pH 3.6), 2, 4, 6-tripyridyl s-triazine (TPTZ) solution (10 mM in 40 mM HCl) and ferric chloride (FeCl₃·6H₂O) solution (20 mM). The fresh working FRAP solution was prepared by mixing 25 ml acetate buffer, 2.5 ml TPTZ solution and 2.5 ml FeCl₃ solution. Extracts were made up to 2.0 ml with distilled water and 1.0 ml of FRAP solution was added. An intense blue color developed was measured at 593 nm, after an incubation period of 20 min. The absorbance was related to absorbance changes of a ferrous sulphate solution (0 – 100 μM) tested in parallel. All results were based on three separate experiments and antioxidant capacity was expressed as μM FeSO₄/ g of dry extract. Quercetin and Butylated Hydroxy Toluene (BHT) were used as positive control.

3.3.2. Reducing power

The reducing power of extracts was evaluated according to the method described by Yen and Chen (1995) with slight change. Briefly, different amounts of R. sativus extracts (0.05 – 1.0 mg/ml) were incubated with 2.5 ml of phosphate buffer (0.2 M, pH 6.6) and 2.5 ml of 1% potassium ferricyanide [K₃Fe(CN)₆] at 50°C for 20 min. The reaction was terminated by adding 2.5 ml of 10% TCA solution and the mixture was centrifuged at 3000 rpm for 10 min. The supernatant (1.0 ml) was mixed with 2.5 ml of distilled water and 1.0 ml of 0.1% ferric chloride (FeCl₃) solution and absorbance was measured at 700 nm after incubation at room temperature for 10 min. Quercetin and BHT (10 – 250 μg/ml) were used as positive control. Experiments were performed in triplicate.
3.3.3. Metal chelating activity

The chelating capacity of *R. sativus* extracts on Fe\(^{2+}\) ions was determined according to the method of Dinis *et al* (1994), wherein Fe\(^{2+}\) chelating potential of extracts was monitored by measuring ferrous iron – ferrozine complex at 562 nm. Briefly, extracts (0.05 – 1.0 mg/ml), quercetin, BHT and EDTA (10 – 250 µg/ml) were made up to 4.7 ml with distilled water and then mixture was allowed to react with 0.1 ml of Ferrous chloride (2.0 mM) and 0.2 ml of ferrozine (5.0 mM) for 20 min. Absorbance of mixture was measured at 562 nm against a blank, which contained distilled water, instead of extracts/EDTA/standard antioxidants.

The ability of extracts to chelate ferrous ion was calculated using the following equation:

\[
\text{Chelating effect (\%)} = \left( \frac{\text{Ab}_{\text{control 562}} - \text{Ab}_{\text{sample 562}}}{\text{Ab}_{\text{control 562}}} \right) \times 100.
\]

Experiments were done in triplicate.

3.3.4. Inhibition of peroxides in linoleic acid system

The capacity of *R. sativus* extracts to inhibit formation of peroxides in linoleic acid system was determined according to the thiocyanate method (Osawa and Namiki, 1981). This method is based on the capacity of peroxides to catalyze oxidation of Fe\(^{2+}\) to Fe\(^{3+}\). The Fe\(^{3+}\) produced is linked to thiocyanate anion, yielding a red complex, which is measured spectrophotometrically at 500 nm. The linoleic acid emulsion was prepared by mixing 0.284 g of linoleic acid and 0.284 g of Tween 20 as an emulsifier and made up to 50 ml with phosphate buffer (0.04 M, pH 7.0). The reaction was set up by mixing *R. sativus* extracts, quercetin and BHT (250 µg/ml) in 2.5 ml phosphate buffer (0.04 M, pH 7.0) with 2.5 ml of linoleic acid emulsion. It was then incubated in the dark at 37°C. At regular intervals during incubation, degree of oxidation was measured by sequentially adding 4.7 ml of ethanol (75%), 0.1 ml of ammonium thiocyanate (30%) and 0.1 ml of ferrous chloride (0.02 M in 3.5% HCl) to 0.1 ml of sample solution. After incubation at room temperature for 3 min, peroxide level was determined by measuring absorbance at 500 nm. A control was performed with linoleic acid emulsion without extracts or standards. The increase in absorbance is directly proportional to the concentration of peroxides present in the samples.
3.3.5. DPPH radical-scavenging activity

DPPH radical-scavenging activity of *R. sativus* extracts was determined as previously described (Burits and Bucar, 2000). The capacity of extracts to scavenge lipid-soluble 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical, which results in bleaching of purple color exhibited by stable DPPH radical, is monitored at an absorbance of 517 nm. Briefly, 1.0 ml of extracts (0.05 – 1.0 mg/ml) and quercetin/BHT (10 – 250 µg/ml) in ethanol were added to 4 ml of 0.004% methanolic solution of DPPH. After incubation for 30 min at room temperature in the dark, absorbance was read against a blank at 517 nm. Tests were carried out in triplicate.

The ability of extracts and quercetin/BHT to scavenge DPPH radical was calculated using the following equation:

\[
\text{Radical scavenging activity (\%)} = \left[ \frac{A_0 - A_1}{A_0} \right] \times 100
\]

Where \( A_0 \) was absorbance of negative control (containing all reagents except test compounds) at 517 nm and \( A_1 \) was absorbance of the extracts or quercetin/BHT at 517 nm. DPPH scavenging activity of extracts and standard was expressed as IC\(_{50}\), which was interpolated from a graph constructed, using percent inhibition (Y-axis) against concentration (X-axis) of extracts and standards.

3.3.6. Superoxide radical-scavenging activity

The ability of *R. sativus* extracts, quercetin and BHT to quench generation of superoxide radicals was determined according to the method of Nishikimi *et al* (1972) with a slight change. Superoxide radicals were generated in PMS-NADH system by oxidation of NADH and analyzed by NBT reduction. 0.1 ml of extracts (0.05 – 1.0 mg/ml) and quercetin/BHT (10 – 250 µg/ml) were mixed with 2.9 ml of phosphate buffer (40 mM, pH 7.4), 1.0 ml of nitroblue tetrazolium (NBT) solution (150 µM in 40 mM phosphate buffer, pH 7.4) and 1.0 ml of NADH (468 µM in 40 mM phosphate buffer, pH 7.4). The reaction was initiated by addition of 1.0 ml of phenazine methosulphate (60 µM in 40 mM phosphate buffer, pH 7.4) to reaction mixture. After incubation at 25°C for 5 min, absorbance was measured at 560 nm. Negative control was subjected to the same procedures as extracts, except that only solvent was added for negative control. All measurements were made in triplicate.

The ability of extracts and quercetin/BHT to scavenge superoxide radical was
calculated using the following equation:

Superoxide radical scavenging activity (%) = \[A_0 - A_1/A_0\] x 100.

Where \(A_0\) was absorbance of negative control at 560 nm and \(A_1\) was absorbance of the extracts or quercetin/BHT at 560 nm. \(IC_{50}\) value, which represents concentration of extracts and standards that caused 50% inhibition, was determined by a linear regression analysis.

3.3.7. Hydrogen peroxide scavenging activity

The method of Sinha (1972) originally designed for the estimation of antioxidant enzyme, catalase, was adopted to evaluate hydrogen peroxide scavenging effect of \(R. sativus\) extracts. Extracts (0.05 – 1.0 mg/ml) and quercetin/BHT (10 – 250 µg/ml) were incubated with 0.6 ml of \(H_2O_2\) (40 mM in a phosphate buffer, 0.1 M pH 7.4) in the dark for 10 min. A negative control was set up in parallel with entire reagent except extract or standard. After incubation, remaining \(H_2O_2\) was allowed to react with 1.0 ml of dichromate in acetic acid (5% potassium dichromate and glacial acetic acid in the ratio of 1:3) in a boiling water bath for 10 min. Absorbance of green color developed was determined at 620 nm. All experiments were performed in triplicate.

The percentage scavenging of \(H_2O_2\) by \(R. sativus\) extracts and standards were calculated using the following equation:

\(H_2O_2\) scavenging activity (%) = \[A_0 - A_1/A_0\] x 100.

Where \(A_0\) was absorbance of negative control and \(A_1\) was absorbance of the extracts or standards. \(H_2O_2\) scavenging activity of extracts and standards was expressed as \(IC_{50}\), which was interpolated from a graph constructed using percent inhibition (Y-axis) against concentration (X-axis) of extracts and standards.

3.3.8. Nitric oxide radical scavenging activity

The method of Rai et al (2006) based on spontaneous generation of nitric oxide (NO•) from sodium nitroprusside (SNP)-buffered solution was used to assess NO• scavenging ability of \(R. sativus\) extracts. Briefly, 0.5 ml of SNP (10 mM) in phosphate buffered-saline was mixed with 0.5 ml of \(R. sativus\) extracts (0.05 – 1.0 mg/ml) and incubated in the dark at room temperature for 2.5 h. A control was set up as above, but sample was replaced with same amount of water. After incubation, 1.0 ml of sulfanilic
acid reagent (0.33 % sulfanilic acid in 20 % glacial acetic acid) was added to 0.5 ml of reaction mixture. After 5 min, reaction mixture was incubated further with 1.0 ml 0.1 % naphthylethlenediamine dihydrochloride (NEDD) for 30 min at 25 °C. Absorbance of chromophore formed was read at 540 nm. Results were expressed as a percent of scavenged nitric oxide with respect to negative control. Quercetin and BHT (10 – 250 µg/ml) were used as positive control. All analyses were done in triplicate.

The percentage scavenging of NO• by R. sativus extracts and standards were calculated using the following equation:

\[
\text{NO• scavenging activity (\%)} = \left[ \frac{A_0 - A_1}{A_0} \right] \times 100.
\]

Where \(A_0\) was absorbance of negative control and \(A_1\) was absorbance of extracts or standards. NO• scavenging activity of extracts and standard was expressed as IC\(_{50}\), which was interpolated from a graph constructed using percent inhibition (Y-axis) against concentration (X-axis) of the extracts and standards.

3.4. Antibacterial activity of R. sativus

3.4.1. Test organisms and culture condition
A collection of ten organisms including four Gram-positive and six Gram-negative organisms were used for this study. *Bacillus subtilis* (MTCC 2391), *Escherichia coli* (MTCC 1563) and *Pseudomonas aeruginosa* (MTCC 6642) were obtained from Microbial Type Culture Collection, IMTECH, Chandigarh, India. Clinical isolates such as *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Enterococcus faecalis*, *Salmonella typhimurium*, *Klebsiella pneumoniae*, *Enterobacter aerogenes* and *Enterobacter cloacae* were obtained from Microbiology laboratory of Global Hospital, Hyderabad. All strains were tested for purity by standard microbiological methods. The bacterial stock cultures were maintained on Mueller Hinton Agar (MHA) slants and stored at 4°C.

3.4.2. Determination of antibacterial activity
An agar-well diffusion method was employed for evaluation of antibacterial activity (Perez *et al*, 1990). The bacterial strains were reactivated from stock cultures by transferring into Mueller Hinton Broth (MHB) and incubating at 37°C for 18 h. A final inoculum containing \(10^6\) colony forming units (\(1 \times 10^6\) CFU/ml) was added aseptically to MHA medium and poured into sterile Petri dishes. Different test extracts at a
concentration of 1 mg/ml were added to wells (8 mm in diameter) punched on agar surface. Plates were incubated overnight at 37°C and diameter of inhibition zone (DIZ) around each well was measured in mm. Experiments were performed in triplicates. Antibiotics such as penicillin, ampicillin, streptomycin, ciprofloxacin and ofloxacin at a concentration of 100µg/well were used as positive reference to determine sensitivity of microorganisms tested. Negative controls were prepared using solvents such as methanol, acetone, ethyl acetate, chloroform and hexane.

3.4.3. Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of *R. sativus*

MIC and MBC of extracts and antibiotics were determined by tube broth dilution assay (Muroi and Kubo, 1996). A serial 2-fold dilution, ranging from 4.06 - 0.008 mg/ml of extracts and antibiotics (positive control) were prepared and 500 µl of each dilution was incubated with 2.5 ml of MHB containing 50 µl of inocula (1 x 10^6 CFU/ml) at 37°C for 24 h. The MIC was determined as lowest concentration that demonstrated no visible growth by macroscopic evaluation. After determination of MIC, tubes showing no turbidity were diluted 100-fold with drug-free MHB and incubated at 37°C for 48 h. The lowest concentration of tube that showed no visible growth in drug-free cultivation was considered as the MBC. Both MIC and MBC assays were done in triplicates.

3.4.4. Effect of pH on inhibitory zone against pathogenic bacteria

The ethyl acetate extracts of root, stem and leaves at a concentration of 100mg/10ml were adjusted with sterile 0.1 N HCl and NaOH to have pH ranging from 3.0 to 9.0. Then pH adjusted extracts were filtered with 0.22 µm membrane and used within 60 min. Sterile distilled water adjusted to different pH as above was used as an acid/alkali control solution to ascertain whether observed change in bacterial growth was because of acidic/alkaline pH or because of extracts. pH-unadjusted extracts were used as controls. Antibacterial activity of pH adjusted and control samples were evaluated by agar-well diffusion method. Experiments were performed in triplicates.
3.4.5. Effect of temperature on stability of ethyl acetate extracts

The ethyl acetate extracts of root, stem and leaves at a concentrations of 100mg/10ml were incubated in a water bath for 30 min at 25, 50, 75 and 100°C respectively. The incubated extracts were then cooled and stored at –80°C until use. Untreated extracts were used as controls. Antibacterial activity of treated and control samples were determined by agar-well diffusion method Experiments were done in triplicates.

3.5. Cytoprotective activity of R. sativus

3.5.1. Isolation of lymphocytes

5.0 ml of peripheral blood was collected by venepuncture from healthy male non-smoking donors (not exceeding age of 30 years), who had not been exposed to any known mutagens at least for six months prior to the experiment. All procedures involving human subjects were conducted as per the guidelines laid down in the Declaration of Helsinki. All subjects were informed about purpose of the experiment and verbal consent was obtained from all subjects. Lymphocytes were isolated by centrifugation in a density gradient of Histopaque 1077. Blood was carefully layered over Histopaque 1077 and centrifuged at 400 x g for 30 min at room temperature. After centrifugation, upper layer was discarded and opaque interface containing mononuclear cells at the top of Histopaque 1077 was transferred into a clean centrifuge tube. To mononuclear cells was added 10ml of RPMI 1640 media and cells were again centrifuged at 250 x g for 10 min. The resultant pellet was washed twice with RPMI 1640 media and centrifuged as before and finally resuspended in 0.5 ml of RPMI 1640 media. Trypan blue exclusion assay was used to assess viability of isolated lymphocytes. Briefly, 0.1ml of cell suspension was mixed with 0.9ml of trypan blue solution (0.2% in PBS), and blue stained cells were counted as non-viable cells using a haemocytometer. The percentage viability was calculated based on the percentage of unstained cells. The viability of lymphocytes was found to be about 92%. The final concentration of lymphocytes was adjusted to about 2 x 10^5 cells/ml. Lymphocyte isolation was carried out in the dark to minimize DNA damage.
3.5.2. Effect of *R. sativus* on viability of lymphocytes

The lymphocytes were incubated with root, stem and leaves of *R. sativus* extracts (5 - 100µg/ml) diluted appropriately with culture media at 37°C for 3 h. Lymphocytes grown in media containing an equivalent concentration of DMSO served as control. After incubation, lymphocytes were harvested by centrifugation at 800 x g for 5 min at 4°C and viability was assessed by MTT assay (Mosmann, 1983). Amount of formazan was determined by measuring absorbance at 540 nm, using an ELISA plate reader. The data were presented as percent post-treatment recovery (% live cells), whereas absorbance from non-treated control cells was defined as 100% live cells.

3.5.3. Protective effect of *R. sativus* on H$_2$O$_2$-induced cytotoxicity in lymphocytes

The lymphocytes were exposed to a range of H$_2$O$_2$ concentration (25 - 500µM) dissolved in ice-cold PBS at 37°C for 10 min in the dark to choose a desired dose of concentration with significant cytotoxicity. After incubation, lymphocytes were harvested and viability was assessed by MTT assay as described previously (Mosmann, 1983). To determine protection against H$_2$O$_2$-induced cell death, lymphocytes were supplemented with *R. sativus* extracts (5 - 100 µg/ml) for 3 h at 37°C before exposure to 200µM of H$_2$O$_2$ for 10 min at 37°C. Positive controls were treated only with H$_2$O$_2$ (without addition of *R. sativus* extract) and negative controls were treated with PBS without addition of H$_2$O$_2$. After incubation, cells were harvested and viability was assessed by MTT assay as described previously.

3.5.4. Genotoxicity of *R. sativus* and H$_2$O$_2$ towards lymphocytes

The lymphocytes were incubated with *R. sativus* extracts (5 - 50µg/ml) diluted appropriately with culture media at 37°C for 3 h in the dark to determine their genotoxic effect. DNA damage was induced *ex-vivo* in parallel by exposing lymphocytes to a range of H$_2$O$_2$ concentrations (25 - 500 µM) at 37°C for 10 min to select a desired dose with significant DNA damage. After incubation, cells were centrifuged, washed twice with PBS and were resuspended in low-melting agarose for the comet assay.
3.5.5. Genoprotective effect of *R. sativus* on H$_2$O$_2$-induced DNA damage in lymphocytes

The lymphocytes were supplemented with *R. sativus* extracts (5–50µg/ml) for 3 h at 37°C before exposure to 200 µM of H$_2$O$_2$ for 10 min at 37°C in the dark. Positive controls were treated only with H$_2$O$_2$ (without addition of *R. sativus* extract) and negative controls were treated with PBS without addition of H$_2$O$_2$. After incubation, lymphocytes were harvested and resuspended in low-melting agar for the comet assay.

3.5.6. Alkaline single cell gel electrophoresis (comet) assay

DNA damage was assessed using the comet assay by the method of Tice *et al* (1990). A 10-µl aliquot of both treated and untreated lymphocytes were suspended in 70 µl of 1.0 % low-melting agarose (premelted solution at 37°C) in PBS and spread onto microscope slides precoated with 1% normal-melting agarose. A coverslip was placed over molten agarose, and slides were incubated at 4°C in the dark for a minimum of 30 min. Once top layer of agarose that contained cells had solidified, coverslip was removed and coated with a third layer of 1.0 % low-melting agarose. After solidification of third layer, samples were then placed in cold lysis solution (10 mM Tris, 2.5 M NaCl, 100 mM EDTA, 1% sodium sarcosinate (pH 10) with fresh 1% Triton X-100 and 10% dimethyl sulfoxide) for 2 h at 4°C. After cell lysis, slides were aligned in a horizontal gel electrophoresis tank (Amersham) and DNA was allowed to unwind for 20 min in a freshly prepared alkaline electrophoresis buffer (300 mM NaOH, 1 mM EDTA; pH 13.0) at 4°C in the dark. DNA was electrophoresed at 25 V and 300 mA for 20 min at 4°C. After electrophoresis, slides were washed thrice with a neutralizing buffer (0.4 M Tris – HCl; pH 7.5), left to dry for 30 min and fixed in 75% ethanol for 10 min. After staining with propidium iodide (10mg/ml), comets from individual cells were examined using a fluorescence microscope. For each experiment, 50 comets were scored for their size and shape by computerized image analysis (TriTek CometScore$^\text{TM}$). The extent of DNA damage was expressed in two ways (i) mean percentage of tail DNA, reflecting proportion of DNA that has migrated from head and (ii) olive tail moment (OTM), which represents product of tail length and percentage DNA in the tail.
3.6. Chemopreventive efficacy of *R. sativus*

3.6.1. Cell lines and culture condition

HeLa (human cervical epithelial carcinoma cell line), A549 (human alveolar basal epithelial carcinoma cell line), MCF-7 (human mammary epithelial carcinoma cell line) and PC-3 (human prostate epithelial carcinoma cell line) were obtained from the National Centre for Cell Science, Pune, India. All cell lines except MCF-7 were grown in a DMEM culture media supplemented with 2 mM L-glutamine, 10% FBS, penicillin (50 IU/ml) and streptomycin (50 µg/ml) at a temperature of 37°C in a humidified incubator with a 5% CO₂ atmosphere and passaged twice weekly to maintain sub-confluent state. MCF-7 cells were grown in a RPMI 1640 culture media supplemented with 2 mM L-glutamine, 10% FBS, penicillin (50 IU/ml) and streptomycin (50 µg/ml) at a temperature of 37°C in a humidified incubator with a 5% CO₂ atmosphere and passaged thrice weekly to maintain sub-confluent state.

3.6.2. Cell viability assay

The viability of cells was assessed by MTT (3, 4, 5-dimethylthiazol-2-yl)-2-5-diphenyltetrazolium bromide) assay, which is based on the reduction of MTT by mitochondrial dehydrogenase of intact cells to a purple formazan product (Mosmann, 1983). Cells were plated into wells of 96-well plates at a concentration of 1 × 10⁴ cells/well in 100 µl volume, and incubated for 24 h at 37°C in a humidified atmosphere containing 5% CO₂ to allow adherence, prior to exposure to *R. sativus* extracts diluted appropriately with culture media. Briefly, cells were treated with predetermined concentrations (0 - 25 µg/ml) in triplicate and grown in a humidified atmosphere at 37°C and 5% CO₂ for 24, 48 and 72 h. After the treatment, media containing *R. sativus* extract were carefully removed by aspiration. 100 µl of 0.4 mg/ml MTT in PBS was added to each well and incubated in the dark for 4 h. After incubation period, formed formazan crystals were solubilized by addition of 100µl of DMSO to each well and kept in an incubator for 4 h. Amount of formazan was determined by measuring absorbance at 540 nm, using an ELISA plate reader. The data were presented as percent post-treatment recovery (% live cells), whereas absorbance from non-treated control cells was defined as 100% live cells. The percent recovery (% live cells) was plotted (Y-axis) against concentration (X-axis) of *R. sativus* extract, where IC₅₀ values could be
interpolated from the graph. Anticancer drug - etoposide (0 – 25 µg/ml) was used as positive reference compound to determine sensitivity of cancer cell lines. Cells grown in media containing an equivalent concentration of DMSO served as negative control.

### 3.6.3. Light microscopic study

Light phase contrast microscopy was used to observe morphological changes in cancer cell lines treated with *R. sativus* extracts. Briefly, cells were cultured in 6 wells plate and treated with different concentrations (0 – 25 µg/ml) of hexane extract of *R. sativus* root for 24 h at 37°C in a CO₂ incubator. After incubation period, photomicrographs were taken under a phase contrast microscope.

### 3.6.4. Fluorescence Imaging

Propidium iodide (PI) staining method was used to observe apoptotic morphological changes in cells treated with hexane extract of *R. sativus* root. Briefly, cells were cultured in 6 wells plate and treated with hexane extract of *R. sativus* root (0 – 25 µg/ml) and incubated for 24 h at 37°C in a CO₂ incubator. After incubation period, cells were washed with PBS, fixed in absolute alcohol for 30 min at 4°C, rehydrated with PBS and incubated with 100 µl of propidium iodide (25 µM) at 37°C for 5 min. Photomicrographs were taken under a fluorescent microscope.

### 3.6.5. DNA fragmentation assay by diphenylamine assay

DNA fragmentation assay allows determining quantity of DNA that is fragmented upon treatment of cancer cells with anticancer or cytotoxic agents. This assay is based on the principle that extensively fragmented double stranded DNA can be separated from chromosomal DNA upon centrifugal separation and determined as described by Burton, 1968.

Briefly, cells were cultured in 6-wells plate and treated with different concentrations (0 – 25 µg/ml) of hexane extract of *R. sativus* root for 24 h at 37°C in a CO₂ incubator. After incubation, both treated and untreated cells were lysed and homogenized in a lysis buffer (10mM Tris-HCl, 1mM EDTA, 0.5% Triton X-100, pH 8.0). Homogenates were centrifuged at 27,000 g for 20 min to separate high molecular weight chromatin from cleavage products. Pellet was resuspended in TE buffer (10 mM Tris-
HCl, 1 mM EDTA, pH 8.0). The resulting pellet and supernatant fraction were reacted with diphenylamine reagent (DPA 1.5g + 1 ml Conc.H₂SO₄ + 100 ml glacial acetic acid + 0.50 ml 2% acetaldehyde) and allowed to stand at room temperature for 16 - 20h, before spectrophotometric determination at 600 nm. The data are expressed as percentage of fragmented DNA which is measured using the following formula,

\[
\% \text{ fragmented DNA} = \frac{\text{DNA in supernatant}}{\text{DNA in pellet + supernatant}} \times 100
\]

3.6.6. RT-PCR for expression of genes related to apoptotic pathway

3.6.6.1. Extraction of RNA

Total RNA was extracted from cancer cells (5 x 10⁶) treated with hexane extract of R. sativus root (0 – 25 µg/ml) for 12 h at 37°C in a CO₂ incubator using TRIZOL reagent according to the manufacturer’s specifications. Briefly, 1.0 ml of TRIZOL reagent was added directly to T₂₅ flask containing monolayer culture of cancer cells and cells were lysed by repeated pipetting. The contents were then transferred into a microcentrifuge tube and kept at room temperature for 5 min for complete dissociation of nucleoprotein. To this was added 0.1 ml of bromochloropropane/ml of TRIZOL reagent used, shaken gently for 15 sec and allowed to stand for 5 – 15 min at room temperature, centrifuged the resulting mix at 12,000 x g for 15 min at 4°C. The aqueous phase was transferred to a fresh microcentrifuge tube and added 0.5 ml of isopropanol/ml of TRIZOL reagent used. The content was mixed, allowed to stand at room temperature for 5 – 15 min and centrifuged at 12,000 x g for 10 min at 4°C. Supernatant was decanted; RNA pellet was washed twice with 75% ethanol and centrifuged at 12,000 x g for 5 min at 4°C. The RNA pellet was air dried briefly and dissolved in DEPC treated water.

3.6.6.2. Determination of RNA concentration

RNA concentration was estimated using a dual beam UV spectrophotometer at wavelength 260 nm and for contaminating proteins at 280 nm using following formula,

“Absorbance 260 nm x Dilution factor x 0.040 = RNA µg/µl”
The ratio of absorbance obtained at 260 nm and 280 nm was used to check RNA quality. A ratio of 1.8 – 2.0 indicated the presence of high quality RNA with minimal contaminating proteins. The quality of RNA was checked by formaldehyde gel electrophoresis of RNA solution on an ethidium bromide stained 1.0% agarose gel in MOPS buffer. Good quality RNA was indicated by the presence of at least two bands, which correspond to the presence of 28s and 18s respectively and a third band indicates 5s that shows the presence of high quality RNA.

3.6.6.3. Preparation of cDNA

The cDNA was synthesized from RNA template using RevertAid™ first strand cDNA synthesis kit according to the manufacturer’s instructions. The reaction was started with the incubation of reaction mixture containing total RNA at a concentration of 5 µg/µl and 1.0 µl of oligo (dT)₁₈ made up to 12.0 µl with DEPC treated water at 70°C for 5 min. The contents were chilled on ice and further reaction was carried out with addition of 10mM of dNTP mix, 20 U/µl of RNase inhibitor, cDNA reaction buffer and 200 U/µl of M-MuLV reverse transcriptase to a total volume of 20 µl. Reverse transcription was carried out at 42°C for 60 min, followed by termination of reaction at 70°C for 10 min and finally, chilled on ice. The cDNAs were stored at -80°C, until PCR amplification.

3.6.6.4. Reverse Transcription – PCR

PCR was performed with 1.0 µl of cDNA in a total volume of 20 µl using Gen – Master Mix. The reaction mixture contained 1x PCR buffer (65 mM TrisHCl, pH 8.8, 16 mM (NH₄)₂SO₄, 2.75 mM MgCl₂), 2.5 U DFS-Taq DNA polymerase, 0.4 mM of dNTPs and 10 pM of each set of gene-specific upstream and downstream primers (Table 3.1). PCR cycling conditions were followed for each gene as per temperature profile mentioned in Table 3.2 and β – actin was amplified separately as a control to avoid any competitive PCR. RT-PCR products were separated by electrophoresis on ethidium bromide stained 1.8% agarose gel and visualized in a Bio-Rad gel documentation system. The relative expression of apoptosis-related genes was then determined using Image Pro-Plus (Media Cybernetics, Inc., Silver Spring, MD) software and compared to control values.
3.7. Statistical analysis

Results calculated from triplicate data were expressed as means ± standard deviations. The data were compared by least significant difference test using Statistical Analysis System (SAS, ver.9.1). Graphing, curve fitting and IC$_{50}$ were performed using GraphPad Prism (ver.5.0a).

Table 3.1
Oligonucleotide primer sequences used for amplification of apoptosis related genes and internal control β–actin gene.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Size (bp)</th>
<th>Primer sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>BCI-2</td>
<td>367</td>
<td>5’ – AGATGTCCAGCCAGCTGCACCTGAC – 3’</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5’ – AGATAGGCACCCAGGGTGATGCAAGCT – 3’</td>
</tr>
<tr>
<td>BCI-XL</td>
<td>310</td>
<td>5’ – GTGGAAGAGAAGGACAGCATGAGG – 3’</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5’ – CCCGGAAGAGTTCATTCACTAC – 3’</td>
</tr>
<tr>
<td>Bax</td>
<td>246</td>
<td>5’ – TTTGCTTCAGGGTTTCATCC – 3’</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5’ – CAGTTGAAGTGGCCGTCAGA – 3’</td>
</tr>
<tr>
<td>p53</td>
<td>368</td>
<td>5’ – TGGCCCTTCCTCAGCATCTTTAT – 3’</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5’ – GTTGGGCAGTGCTCGCTTATG – 3’</td>
</tr>
<tr>
<td>Caspase 3</td>
<td>419</td>
<td>5’ – CGGTCTGTACAGATGTCGAT – 3’</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5’ – TAAACCAGGTGCTGGAGGTATG – 3’</td>
</tr>
<tr>
<td>β–actin</td>
<td>253</td>
<td>5’ – CTGTCTGCCGCGACCACCACCAT – 3’</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5’ – GCAACTAAGTCAAGTCCGC – 3’</td>
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Table 3.2
Temperature profile used for amplification of apoptosis related genes and β – actin control gene

<table>
<thead>
<tr>
<th>Step</th>
<th>BCl-XI/Bax/Cas3</th>
<th>BCl-2</th>
<th>p53</th>
<th>β – actin</th>
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<tbody>
<tr>
<td></td>
<td>1 cycle</td>
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<tr>
<td>Initial denaturation</td>
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<tr>
<td></td>
<td>94°C 3 min</td>
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<tr>
<td>Denaturation</td>
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<td>95°C 45 sec</td>
<td>94°C 45 sec</td>
<td>94°C 45 sec</td>
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<tr>
<td>Primer annealing</td>
<td>55°C 45 sec</td>
<td>64°C 45 sec</td>
<td>60°C 45 sec</td>
<td>52°C 45 sec</td>
</tr>
<tr>
<td>Synthesis</td>
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<td>72°C 90 sec</td>
<td>72°C 90 sec</td>
<td>72°C 90 sec</td>
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<tr>
<td></td>
<td>1 cycle</td>
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<tr>
<td>Final extension</td>
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<tr>
<td></td>
<td>72°C 7 min</td>
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