Materials and Methods
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Chemicals used:

1) Medium
   a) HiKaryo XL™ RPMI – 1640 HIMEDIA Lab. Pvt. Ltd., Mumbai
   b) PB – MAX™ Karyotyping Medium GIBCO, Life Technologies, USA

2) Heparin (Beparine) Biological Evans Ltd., Hyderabad

3) HiKaryo XL™ Colchicine HIMEDIA Lab. Pvt. Ltd., Mumbai

4) Bleomycin Biochem Pharmaceutical Industries Ltd., Mumbai

5) Giemsa stain HIMEDIA Chemicals, Mumbai

6) Cytochalasin – B EMD Milipore Corp., USA

7) Radiation 6 MV X – ray Photons, Siemens, Oncor Expression Medical Linear Accelerator

Plant material:

The bark, stem and leaves of *A. scholaris* were collected after proper identification and authentication of the plant. The average age of the plants from where samples were collected was more than 50 years. All sampled plants were from Bardoli taluka, Gujarat, India.
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Extraction methods:

The bark, stem and leaves of *A. scholaris* were collected separately and dried for 2-3 days at room temperature. After drying, they were ground crumbly. The finely ground powder was placed in a porous bag (thimble) made of strong filter paper. The thimble was placed in the chamber of Soxhlet apparatus. Two separate solvents (methanol and double distilled water) were used for extraction. The extraction assembly was set up as described [143]. Hot continuous extraction was carried out. After 5-6 refluxing cycles, a drop of solvent from the siphon tube was collected and observed for residue mark after evaporation. When no residue was found from the evaporated drop so collected, the refluxing cycle was terminated. The solvent containing extract siphoned out into the distillation still was collected and allowed to cool. The liquid content was evaporated and condensed. This was stored in sterile labelled container at 4°C to 8°C until further use. Extracts were re-dissolved in sterile pyrogen free water and sterilized using syringe filters prior to use.

Lymphocyte culture:

The method of Hungerford [144] was followed. In a sterile culture vial, 5 ml of PB – MAX™ Karyotyping Medium was taken. To this was added, 50 µl of Heparin and 0.6 ml of human peripheral whole blood. The incubation temperature was kept at 37°C for duration of 72 hours. At the 69th hour, 100 µl of colchicine was added. After 2 hours of colchicine treatment, the cultures were terminated. A treatment of pre-warmed hypotonic Potassium Chloride (0.075M) solution at 37°C for 25 min was given followed by fixative (3:1 Methanol: Acetic acid) washes so that clear white pellet was obtained. This was suspended in half ml of fixative before final preparation of slides. On chilled sterile slides, 4-5 drops of cell suspension were dropped from a convenient height. The slides were air dried, immediately blind coded and routinely stained in 2% Giemsa staining solution. Optimum staining time varied between slides and the stain batches, however, was generally of the order of about 5-7 minutes. For counting chromosomal aberrations (CAs) one hundred well spread metaphase plates, each containing not less than 44 chromosomes were considered. Both chromosome as well as chromatid types of aberrations were scored.
Micronucleus assay:

The cytokinesis blocked micronucleus (CBMN) assay was carried out according to the method of Fenech and Morley [145] with some modifications [146]. To inhibit cytokinesis, 6 µg/ml Cytochalasin-B was added to each culture (5 ml HiKaryo XL™ RPMI – 1640 medium + 50 µl heparin + 0.6 ml blood) after 44 hours of initiation of culture. The cultures were harvested at 72\textsuperscript{nd} hour. The lymphocytes were subjected to a mild hypotonic (0.56% KCl) treatment for 9-12 minutes and then centrifuged. The cells were fixed in Carnoy's fixative (3:1 Methanol:Acetic acid) for 10 minutes, centrifuged and re-suspended in a small volume of fixative. Air dried preparations were carried out on pre-cleaned coded slides to avoid observer bias. The slides containing cells were stained with May-Grünwald and counter stained with Giemsa. The slides were examined at 1000 X magnification using a light microscope. The basic principles and methods including detailed scoring criteria for all the genotoxicity and cytotoxicity end-points of the CBMN assay as described earlier [147] were followed.

Treatment protocols:

Fourteen separate culture vials were set up as shown in (Table I). The first culture vial was kept untreated so as to act as a control. The second culture vial was treated with 15 µg/ml of BLM. Culture vials labelled three to eight received a dose of A. scholaris bark, stem and leaf extracts (aqueous and methanolic) separately, at the concentration of 50 µg/ml each. The selection of this particular dose was on the basis of initial observations carried out in the laboratory. The vials labelled nine to fourteen received a combined dose of extracts and BLM at the concentration of 50 µg/ml and 15 µg/ml respectively. All treatments were given after 24 hours of initiation of culture. The whole experimental set-up was repeated with blood samples collected from twelve healthy volunteers in the age group of 20 to 30 years, and sharing similar socio-economic condition.

G\textsubscript{2} Assay:

The experiments were carried out with blood samples collected from 12 healthy volunteers. A total of 12 culture vials from each individual were set up for lymphocyte cultures (as described above). These culture tubes were divided into two groups of six each (Table II). The first group was labelled as ‘G\textsubscript{0}’ while the
<table>
<thead>
<tr>
<th>Culture vials</th>
<th>Treatment</th>
<th>Dose</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Control</td>
<td>Nil</td>
</tr>
<tr>
<td>2</td>
<td>Bleomycin</td>
<td>15 µg/ml</td>
</tr>
<tr>
<td>3</td>
<td>Aqueous bark extract (ABE)</td>
<td>50 µg/ml</td>
</tr>
<tr>
<td>4</td>
<td>Methanolic bark extract (MBE)</td>
<td>50 µg/ml</td>
</tr>
<tr>
<td>5</td>
<td>Aqueous stem extract (ASE)</td>
<td>50 µg/ml</td>
</tr>
<tr>
<td>6</td>
<td>Methanolic stem extract (MSE)</td>
<td>50 µg/ml</td>
</tr>
<tr>
<td>7</td>
<td>Aqueous leaf extract (ALE)</td>
<td>50 µg/ml</td>
</tr>
<tr>
<td>8</td>
<td>Methanolic leaf extract (MLE)</td>
<td>50 µg/ml</td>
</tr>
<tr>
<td>9</td>
<td>ABE + Bleomycin</td>
<td>50 µg/ml + 15 µg/ml</td>
</tr>
<tr>
<td>10</td>
<td>MBE + Bleomycin</td>
<td>50 µg/ml + 15 µg/ml</td>
</tr>
<tr>
<td>11</td>
<td>ASE + Bleomycin</td>
<td>50 µg/ml + 15 µg/ml</td>
</tr>
<tr>
<td>12</td>
<td>MSE + Bleomycin</td>
<td>50 µg/ml + 15 µg/ml</td>
</tr>
<tr>
<td>13</td>
<td>ALE + Bleomycin</td>
<td>50 µg/ml + 15 µg/ml</td>
</tr>
<tr>
<td>14</td>
<td>MLE + Bleomycin</td>
<td>50 µg/ml + 15 µg/ml</td>
</tr>
</tbody>
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Table II  
Treatment protocol for G\textsubscript{2} assay

<table>
<thead>
<tr>
<th>Group</th>
<th>Vials</th>
<th>Treatment at 0 hr</th>
<th>Treatment at 70 hr</th>
</tr>
</thead>
<tbody>
<tr>
<td>☉</td>
<td>1</td>
<td>Control</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>Bleomycin (15 µg/ml)</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>Aqueous bark extract (50 µg/ml)</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>Methanolic bark extract (50 µg/ml)</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>Aqueous bark extract (50 µg/ml) + Bleomycin (15 µg/ml)</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>Methanolic bark extract (50 µg/ml) + Bleomycin (15 µg/ml)</td>
<td>-</td>
</tr>
<tr>
<td>☉</td>
<td>7</td>
<td>Control</td>
<td>Bleomycin (5 µg/ml)</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>Bleomycin (15 µg/ml)</td>
<td>Bleomycin (5 µg/ml)</td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>Aqueous bark extract (50 µg/ml)</td>
<td>Bleomycin (5 µg/ml)</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>Methanolic bark extract (50 µg/ml)</td>
<td>Bleomycin (5 µg/ml)</td>
</tr>
<tr>
<td></td>
<td>11</td>
<td>Aqueous bark extract (50 µg/ml) + Bleomycin (15 µg/ml)</td>
<td>Bleomycin (5 µg/ml)</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>Methanolic bark extract (50 µg/ml) + Bleomycin (15 µg/ml)</td>
<td>Bleomycin (5 µg/ml)</td>
</tr>
</tbody>
</table>
second as ‘G2’. In both groups, initial tubes were kept untreated so as to act as a control, the second received treatment of BLM (15µg/ml). The third and fourth tubes received treatments of 50 µg/ml aqueous and 50 µg/ml methanolic bark extracts respectively, while the fifth and sixth received treatments of 50 µg/ml aqueous bark extracts + 15 µg/ml BLM and 50 µg/ml methanolic bark extract + 15 µg/ml BLM respectively. All treatments were given at the initiation of cultures. All six tubes belonging to ‘G2’ group were treated with a mild dose of 5 µg/ml BLM at the 70th hour of incubation. At the 71st hour, the cells were treated with 100 µl of 0.001% colchicine. The cultures were terminated at the 72nd hour of incubation. Routine air dried preparations were made. Each slide was blind coded, conveniently stained in 2% Giemsa and scored under 100X oil immersion lens. To avoid observer’s bias all slides were scored twice by different observers. The average of total chromatid aberrations recorded by each observer has been tabulated.

**Radiation Treatments:**

From each of the twelve different volunteers, peripheral blood sample was drawn and distributed in equal quantities in four separate sodium heparinised vacutainer tubes. The first vacutainer was sham exposed while the second, third and fourth were irradiated to 2, 4 and 6 Gy X-rays respectively. Two sets of three culture vials each were set up from every vacutainer. The first set was processed for lymphocyte culture, while the second for CBMN assay. In each set, the first culture vial served as control while remaining two received treatments of methanolic (50 µg/ml) and aqueous (50 µg/ml) extracts from bark of *A. scholaris* respectively. Exposure of cells to methanolic and aqueous bark extracts was done under three different conditions to check three different possible activities of the plant:

(i) **Radio-protective activity:** Cultures were set up from blood samples and treated with extracts at 0 hour of incubation. These cultures, after 24 hours of incubation, were irradiated and then re-cultured for another 48 hours. This was to rule out whether the presence of components in extract may not allow the damage to take place.

(ii) **Radio-modulatory activity:** Cultures were set up from sham and irradiated blood samples and treated with methanolic and aqueous extracts at 0 hour of
initiation of cultures. This was done to rule out the possibility of possible radiomodulatory properties of the extracts.

(iii) **Radio-mitigatory activity:** Cultures were set up from sham and irradiated blood samples, cultured and then treated with extracts after 24 hours of initiation of culture. This was done to rule out the possibility of possible DNA damage repair capability of extracts.

**Phytochemical Screening:**

(A) Qualitative screening: Qualitative screening of bioactive compounds present within bark, stem and leaf of *Alstonia scholaris* was carried out following standard protocols of Harbone [148], Trease and Evans [149].

1. **Alkaloids**
   - **Wagner’s test:** The plant extract was added in a test tube. Few drops of Wagner’s reagent were added along sides of the test tube. A reddish brown precipitate was formed which confirmed the presence of alkaloids.
   - **Dragendorff’s test:** The plant extract was mixed with few drops of acetic acid followed by Dragendorff’s reagent. Orange red precipitates were formed indicating the presence of alkaloids.

2. **Flavonoids:** About 5ml of diluted ammonia solution was added to aqueous filtrate of the plant extract followed by the addition of concentrated sulphuric acid. A yellow coloration was observed in the extract which indicated the presence of flavonoids.

3. **Amino acid (Ninhydrin test):** Drops of Ninhydrin reagent were added to the plant extract, purple colour appeared which confirmed the presence of amino acids.

4. **Carbohydrates (Fehling’s test):** The plant extract was boiled in water bath at 60°C in a test tube. 5ml of Fehling’s solution was added in the test tube. A red precipitate formation indicated the presence of carbohydrate.

5. **Phenolic compound (Ferric chloride’s test):** The plant extract was mixed with 2 ml of distilled water. Few drops of 5%ferric chloride were added along the walls of the test tube. A dark green colour showed the presence of phenolic compound.
6. **Terpenoids (Salkowski test):** To the plant extract, 2 ml of chloroform was added followed by few drops of concentrated sulphuric acid. A reddish brown precipitate indicated the presence of terpenoids.

7. **Cardiac glycosides (Keller-Kiliiani test):** The plant extract was dissolved in 5ml of glacial acetic acid. Few drops of 5%ferric chloride were added followed by few drops of concentrated sulphuric acid. A greenish blue colour indicated the presence of glycosides.

8. **Oils and Fats (Spot’s test):** A drop of extract was pressed between two filter papers. Oil stain on the paper indicates the presence of fixed oil and fats.

9. **Steroids and Sterols (Lieberman’s test):** To 1 ml of plant extracts 10 ml of chloroform and conc. sulphuric acid was added along the sides of the test tubes. The lower sulphuric acid fraction turned yellow with green fluorescence and the upper layer turned red which indicated the presence of steroids and sterols.

10. **Saponins (Foam’s test):** The plant extract was mixed with 5ml of distilled water and shaken vigorously for 10 minutes. Foam formation confirmed the presence of saponins.

11. **Tannins (Ferric chloride test):** The plant extract was mixed with 5%ferric chloride solution. The formation of blue, blue-black or brownish green coloration indicated the presence of tannin.

12. **Gums and Mucilage:** The plant methanolic extract (200µl) was mixed with 5 ml distilled water and 2-3 drops of ruthenium red solution. A pink color formation indicated the presence of gums and mucilage.

13. **Vitamins:** The plant extract was treated with dinitrophenyl hydrazine and concentrated sulphuric acid. The formation of yellow precipitate suggested the presence of vitamins.

(B) **Quantitative analysis:**

1. **Estimation of total alkaloids [150]:** About 5g of plant sample was placed in a beaker and extracted with 10% ethanol. The mixture was covered and allowed to stand for 4 hours. It was then filtered and the extract was concentrated on a water bath until it reached a quarter of its original volume. Concentrated ammonium hydroxide was added drop by drop to the extract
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until its precipitation was completed. The whole solution was allowed to settle and the precipitates were collected and washed with diluted ammonium hydroxide and then filtered. The residue (alkaloids) was dried and weighed.

2. Estimation of total Flavonoid [151]: The total quantity of flavonoids was estimated by Aluminium Chloride method. The standard aliquots of Quercetin (10-100 µg/ml) were prepared. To 0.5 ml of standard solution, 1.5 ml of 95 % ethanol, 0.1 ml of 10 % aqueous Aluminium Chloride, 0.1 ml of 1M Potassium Acetate and 2.8 ml of distilled water were added, and then incubated for 30 min at room temperature. The absorbance of reaction mixture at 415 nm with UV spectrophotometer was recorded. In the blank solution, 10 % Aluminium Chloride was substituted with the same amount of distilled water. While for test samples, 0.5 ml of plant extracts were used in place of standard solution. Flavonoid content was calculated from the calibration curve.

3. Estimation of total Phenolic compound [152]: The total amount of phenol was estimated using Folin-Ciocalteu reagent. The standard Gallic acid (10-100 µg/ml) aliquots were prepared in test tubes. Aqueous and methanolic extracts in the concentration range of 1 mg/ml were prepared. 1ml of each sample was mixed with 0.25 ml of Folin-Ciocalteu's reagent and 1.25 ml of 20 % Sodium Carbonate solution. The mixture was allowed to react for 40 minutes at room temperature. After the reaction period, the contents were mixed and the blue colour developed was measured at 725 nm in comparison with the standard. The amount of total phenols was calculated from calibration curve as gallic acid equivalent by the following formula:

\[ T = \frac{C \cdot V}{M} \]

where, \( T \) is total content of Phenolic compounds (mg per gm of plant extract), \( C \) is the concentration of gallic acid established from the calibration curve (mg/ml) \( V \) is the volume of extract (ml) and the \( M \) is gram weight of plant extract.

4. Estimation of total steroids and sterols: Total sterol content was measured using spectrophotometer by Liberman-Burchard method [153, 154].
Chloroform was added to 1ml of plant extract to make-up the volume to 5ml in test tube. In this was added 2 ml of Liberman-Burchard reagent. The tubes were covered with black paper and kept under dark for 15 minutes. The green coloured complex formed was spectrophotometrically measured at 640 nm. Diosgenin was used as a standard for constructing a calibration curve.

5. **Estimation of total tannins**: The total amount of tannin was estimated by Folin-Denis method [155]. Standard tannic acid (5mg/ml) was prepared in distilled water. Accurately weighed 0.5 gm of the powdered material was transferred to 250 ml conical flask. To this was added 75ml of distilled water, gently boiled for 30 min and centrifuged at 2000 rpm for 20 min. The supernatant was collected in 100 ml volumetric flask to make up the volume. 0.5ml of the sample extract was added to the test tube containing 0.5 ml of Folin-Denis reagent, 1 ml of Sodium Carbonate solution and diluted to 100 ml with water and shaken well. The absorbance was read at 700 nm after 30 min.

6. **Estimation of total saponin [156]**: The plant sample was extracted with 100 ml of 20% aqueous ethanol. The sample was heated over a water bath for 4 hours with continuous stirring at about 50°C. The mixture was filtered and the residue re-extracted with another 200 ml of 20% ethanol. The combined extract was reduced to 40 ml over water bath at about 90°C. The concentrate was transferred into 250 ml separator funnel and 20 ml of diethyl ether (CH₃CH₂)₂O was added and shaken vigorously. The aqueous layer was recovered while the ether layer was discarded. The purification step was repeated. 60 ml of n-butanol was added and the combined n-butanol extract was washed with 10 ml of 5% aqueous sodium chloride twice. The remaining solution was heated on a water bath, after evaporation the sample was dried in the oven to a constant weight. The percentage of saponin content was calculated.

7. **Estimation of total Terpenoids [157]**: Total terpenoid content in the extracts were assessed by soaking 500 mg of plant powder separately in alcohol for 24 hrs and then filtered. The filtrate was extracted with
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petroleum ether; the ether extract was treated as total terpenoids. The residue obtained was dried and weighed.

8. Estimation of total Glycosides [150]: A 0.02 % solution of Digitoxin in Chloroform-Methanol (1:1v/v) mixture (1ml = 0.2 mg) was prepared and transferred into different volumes, viz. 1, 2, 3, 4 and 5 ml (equivalent to 0.2, 0.4, 0.6, 0.8 and 1 mg of Digitoxin respectively) each to dry Erlenmeyer flasks. The solvent in each flask was evaporated on a water bath and each residue was dissolved in 0.35 ml 90 % alcohol, final volume was adjusted to 10 ml with distilled water by means of graduated pipette. Freshly prepared Baljet's reagent (10 ml) was added to each flask and set aside for one hour at room temperature, then diluted with 20 ml of distilled water and the absorbance (A) of the developed orange-red colour was measured at 495 nm using a suitable spectrophotometer against a blank in which Digitoxin solution was replaced by 10 ml of distilled water. For each concentration, the mean of three determinations was taken for plotting the standard curve, depicting the concentration against absorbance (A). Percentage of total glycosides = A × 100/17 g % of total glycosides calculated as Digitoxin.

Antioxidant Potential:

a) Total antioxidant activity: The total antioxidant capacity of the extracts was evaluated by the phosphomolybdenum method according to the procedure described by [158]. A. scholari s bark extract (0.3ml) was combined with 3 ml of reagent solution (0.6 M Sulfuric acid, 28 mM Sodium Phosphate and 4 mM Ammonium Molybdate). The tubes containing the reaction solution were incubated at 95°C for 90 min. Then the absorbance of the solution was measured at 695 nm using a UV-VIS spectrophotometer (UVmini-1240) against blank after cooling down to room temperature. Methanol (0.3 ml) in the place of extract was used as the blank. The total antioxidant activity was expressed as the number of gram equivalent of ascorbic acid. The calibration curve was prepared by mixing ascorbic (50, 100, 150, 200, 250 and 300μg/ml) with methanol.

b) Superoxide anion scavenging activity [159]: The activity was tested using Nitroblue Tetrazolium (NBT) method. The reaction mixture consisted of 1ml
of NBT solution (156 μM) and sample solution at different concentrations (50, 75, 100, 125, 150, 175, 200 and 225 μg/ml). The reaction was started by adding 100 μl of Phenazine Methosulfate solution (60 μM PMS) in phosphate buffer (pH 7.4) to the reaction mixture followed by incubation at 25°C for 5 min and the absorbance was measured at 560 nm against blank. Gallic acid was used as the standard. The percentage of super oxide anion scavenging was calculated as:

% of superoxide anion scavenged =

\[
(1 - \text{absorbance of the sample/absorbance of the blank}) \times 100
\]

c) Free radical scavenging activity: Free radical scavenging activity was evaluated using ascorbic acid as standard antioxidants. The radical scavenging activity was measured using the stable radical DPPH according to the method described by [160] with some modifications. Various concentrations (2, 4, 8, 16, 32, 64 and 128 μg/ml) of the extracts were added to 4 ml of a 0.004 % methanol solution of DPPH. The mixture was shaken and left for 30 min at room temperature in the dark, and the absorbance was then measured with a spectrophotometer at 517 nm. All determinations were performed in triplicate. The antioxidant activity was calculated as the percent inhibition caused by the hydrogen donor activity of each sample according to the following:

Inhibition (%) = (1 – absorbance of the sample/absorbance of the blank) × 100

Statistical analysis:

The data were analysed using Student’s t-test to compare two different treatment groups, while ANOVA analysis was carried out to compare differences between and within treatment groups. P-values of 5 % and 0.1 % were considered as ‘significant’ and ‘highly significant’ respectively.