Materials and methods

This chapter describes the materials used and the methods employed in the present study such as in-vitro multiplication of plants, oil extraction and its GC-MS analysis, antioxidant assays, total phenolic content determination, lipoxygenase (LOX) inhibition assay, DNA damage preventive analysis, histological and molecular analysis. Some other specific methods are, however, described in the respective chapters.

2.1. Experimental site and environmental conditions for maintenance of in-vitro and field grown plants

The plants were grown in the Botanical garden of Department of Botany, Banaras Hindu University (BHU), Varanasi. The city is geographically situated at 25° 18’ N latitude, 80° 01’ E longitude and at an altitude of 128.93 m in the North-Gangetic Plain. It is situated 126 m above the mean sea level. Varanasi is located in the eastern part of Uttar Pradesh. It enjoys sub-tropical climate and is very often subjected to extreme weather conditions like heat of summers and cold of winters with an average rainfall of 1080.4 mm. Generally about 85% of the total rainfall is received during summer monsoon season i.e. from June to September. An occasional mild shower occurs during winter season as well. The daily maximum and minimum temperature during the year usually increases from February to June and then decrease from July to December, thereafter dropping rapidly with the minimum value in January.

2.2. Plants used

Eleven medicinal plants were selected for the study, namely, Aloe vera, Amaranthus hypochondriacus, Andrographis paniculata, Artemisia annua, Azadirachta indica, Celastrus paniculatus, Curcuma longa, Nigella sativa, Psoralea corylifolia, Tinospora cordifolia, and Triticum aestivum. Some of these were multiplied in field and the others were micropropagated under in-vitro conditions for facilitating continuous supply throughout the study. All the plants were identified and authenticated by the taxonomists of the Ayurvedic department of BHU, Varanasi and the voucher specimens were deposited.
2.3. *In-vitro multiplication*

2.3.1. Chemicals and reagents used

- Sucrose (Himedia, India)
- Agar powder (Himedia, India)
- Tween-20 (Himedia, India)
- Sodium hypochlorite [NaClO] (E-Merck, India)
- Mercuric chloride [HgCl₂] (E-Merck, India)
- Absolute alcohol (Himedia, India)
- NaOH [Sodium hydoxide] (Himedia, India)
- HCl [Hydrochloric acid] (Himedia, India)
- Stock solutions chemicals to prepare MS medium [Murashige and Skoog, 1962] (E-Merck; Himedia; Sigma-Aldrich)
- BAP [6-Benzylaminopurine] (Sigma-Aldrich)
- NAA [1-Naphthaleneacetic acid] (Sigma-Aldrich)
- IAA [Indole-3-Acetic Acid] (Sigma-Aldrich)
- Extran (E-Merck, India)

2.3.2. Glasswares

All the glasswares used for conducting different experiments in the entire study were purchased from Borosil® company, India. The glasswares used included conical flasks, beakers, petri-dishes, pasteur pipettes, test-tubes, measuring cylinders etc. For cleaning, the glasswares were dipped overnight in extran (liquid detergent) solution followed by their washing in running tap water. They were later dried in hot air oven (Precision Instruments, India).

2.3.3. Plant materials used

The nodal segments or shoot apical meristems required for *in-vitro* propagation of the plants under tissue culture conditions were collected from the Botanical garden, BHU, Varanasi. The plant materials were collected in polyethylene bags (25cm x 15cm) containing about 100 ml of tap water, for keeping them fresh, and were brought to the lab. These materials were used as explants for *in-vitro* propagation.

2.3.4. Culture media and nutrients

All the *in-vitro* raised plants were grown in semi-solid MS medium (Murashige and Skoog, 1962), supplemented with various concentrations of plant growth regulators like BAP, NAA, and IAA. These cytokinins and auxins were used for shooting and rooting, respectively.
chemical composition of the MS medium used in the study along with the amount of each chemical required in full-strength medium are given in the Table 2.1.

Table 2.1. Composition of MS (Murashige and Skoog, 1962) medium.

<table>
<thead>
<tr>
<th>Stock solution</th>
<th>Constituents</th>
<th>Molecular formula</th>
<th>Amount in stock (mg/l)</th>
<th>Amount in medium (mg/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inorganic</td>
<td>Stock solution I (Macro-salts)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ammonium nitrate</td>
<td>NH₄NO₃</td>
<td>33,000</td>
<td>1,650</td>
</tr>
<tr>
<td></td>
<td>Potassium nitrate</td>
<td>KNO₃</td>
<td>38,000</td>
<td>1,900</td>
</tr>
<tr>
<td></td>
<td>Calcium chloride</td>
<td>CaCl₂.2H₂O</td>
<td>8,800</td>
<td>440</td>
</tr>
<tr>
<td></td>
<td>Magnesium sulphate</td>
<td>MgSO₄.7H₂O</td>
<td>7,400</td>
<td>370</td>
</tr>
<tr>
<td></td>
<td>Potassium dihydrogen ortho-phosphate</td>
<td>KH₂PO₄</td>
<td>3,400</td>
<td>170</td>
</tr>
<tr>
<td>Stock solution II (Micro-salts)</td>
<td>Potassium iodide</td>
<td>KI</td>
<td>166</td>
<td>0.83</td>
</tr>
<tr>
<td></td>
<td>Boric acid</td>
<td>H₃BO₃</td>
<td>1,240</td>
<td>6.20</td>
</tr>
<tr>
<td></td>
<td>Manganese sulphate</td>
<td>MnSO₄.7H₂O</td>
<td>4,460</td>
<td>22.30</td>
</tr>
<tr>
<td></td>
<td>Zinc sulphate</td>
<td>ZnSO₄.7H₂O</td>
<td>1,720</td>
<td>8.60</td>
</tr>
<tr>
<td></td>
<td>Sodium molybdate</td>
<td>Na₂MoO₄.2H₂O</td>
<td>50</td>
<td>0.25</td>
</tr>
<tr>
<td></td>
<td>Cobaltous chloride</td>
<td>CoCl₂.6H₂O</td>
<td>5</td>
<td>0.025</td>
</tr>
<tr>
<td></td>
<td>Cupric sulphate</td>
<td>CuSO₄.5H₂O</td>
<td>5</td>
<td>0.025</td>
</tr>
<tr>
<td>Stock solution III (Iron-EDTA)</td>
<td>Ferrous sulphate</td>
<td>FeSO₄.7H₂O</td>
<td>5,560</td>
<td>27.80</td>
</tr>
<tr>
<td></td>
<td>Sodium EDTA</td>
<td>Na₂EDTA.2H₂O</td>
<td>7,460</td>
<td>37.30</td>
</tr>
<tr>
<td>Organic</td>
<td>Stock solution IV (Organics)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Inositol</td>
<td>C₆H₁₂O₆</td>
<td>20,000</td>
<td>100.0</td>
</tr>
<tr>
<td></td>
<td>Nicotinic acid</td>
<td>C₆H₅NO₂</td>
<td>100</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td>Pyridoxine</td>
<td>C₅H₁₁NO₃.HCl</td>
<td>100</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td>Thiamine</td>
<td>C₁₂H₁₇ClN₄Os.HCl</td>
<td>20</td>
<td>0.1</td>
</tr>
<tr>
<td></td>
<td>Glycine</td>
<td>C₅H₅NO₂</td>
<td>400</td>
<td>2.0</td>
</tr>
</tbody>
</table>

2.3.5. Media preparation

2.3.5.1. Preparation of nutrient stock solutions

Four different stock solutions of nutrients, as shown in Table 2.1, were prepared by weighing and dissolving them one by one in double distilled water. The salts for preparing the stocks were weighed with the help of single pan electronic balance (Sartorious, India). Generally, 500 ml of stock I and 100 ml each of stock II, stock III and stock IV of MS medium were prepared at a time. After preparation, the stock solutions were poured in clean bottles, labelled and, stored at 4°C. Each stock was generally used within 2-3 weeks for media preparation. Stocks were checked for contamination at every use and only clean and uncontaminated solutions were used.
2.3.5.2. Preparation of growth regulators

Stock solution of about 2-3 ml volume and 1 mg ml\(^{-1}\) concentration for each growth regulator was prepared freshly as and when required. For preparing 10 ml stock solution, 10 mg of the growth regulator was weighed on single pan electronic balance and dissolved in 1 ml of its solvent (NAA and IAA in absolute alcohol, BAP in 1N NaOH). The final volume of the solution was made to 10 ml by adding 9 ml of double distilled water. Dilutions of the stocks of growth regulators were made if their lower concentrations were required in the medium.

2.3.5.3. Preparation of culture media

Depending on the required strength of the medium, the appropriate volumes of each of the four stock solutions I, II, III and IV were added in a measuring cylinder and final volume was maintained with double distilled water. To make a homogenous solution, the medium was poured in a beaker and was continuously stirred with a magnetic stirrer for about 15-20 min. Meanwhile, growths regulators as per the requirement were added to the solution. Subsequently, the pH of the medium was adjusted to 5.8 ± 0.02 with the help of 0.1N NaOH or 0.1N HCl using single electrode pH meter (CyberScan pH 510, Eutech Instruments). When pH of the medium was stabilized, required amount of 3% (w/v) sucrose was dissolved in it. For preparing semi-solid medium, 0.8% (w/v) agar powder was added to the medium. To melt the agar, medium was heated in a microwave oven (Kenstar, India) with occasional stirring till the media showed a uniform consistency. Thereafter, the media was poured in culture tubes (25 x 150 mm). About 10-12 ml of medium was dispensed into each culture tube. The culture tubes were marked and plugged with non-absorbent cotton wrapped in muslin cloth. They were kept in autoclavable plastic baskets and covered with aluminium foil before autoclaving so as to avoid wetting of the cotton plugs.

2.3.6. Sterilization of the media and equipments

The prepared media was sterilized in an autoclave (Modern Industrial Corporation, Bombay) at 121°C (250°F) and 15 psi pressure for 15 min. Double distilled water used for sterilization of explants was also autoclaved in 500 ml flasks. Other equipments and requirements like forceps, scalpels, pasteur pipettes, empty petri-dishes, empty test tubes plugged with cotton plugs, and petri-dish with filter papers were wrapped in aluminium foil and autoclaved in the same manner. After autoclaving, the culture tubes were placed in the test tube stands and kept
for cooling in the culture room in a slightly tilted position to prepare slants of the medium in order to provide larger surface area.

2.3.7. Surface sterilization of the explants

The plant materials like apical shoots or nodal segments (1.5-2 cm long) collected from the garden were kept for washing under running tap water for about half an hour. Thereafter, 2-3 drops of Tween-20 and 5-6 drops of sodium hypochlorite were added to 100 ml water containing explants followed by 15 min of shaking. The explants were again kept for washing under running tap water for about half an hour to remove the detergent from the surface of the explants. Subsequently, the explants were further sterilized in laminar air flow hood (Klenzaids Bioclean Devices Pvt. Ltd., India) by treating them with 70% ethanol for 30 sec followed by thrice washing with double distilled water. Thereafter, treatment with 0.02% (w/v) mercuric chloride solution (prepared in sterile double distilled water) for about 3-4 min with gentle shaking was done for disinfection of the explants followed by rinsing of the explants 3 to 4 times with double distilled water.

2.3.8. Inoculation of the explants

After surface sterilization of the explants, they were cut into suitable lengths (1.0-1.5 cm) by trimming the portions damaged due to surface sterilization process. All the explants were cultured in 25 x 150 mm culture tubes containing 10-12 ml of MS medium supplemented with different growth regulators. The in-vitro grown plantlets were sub-cultured after an interval of about 4 weeks by transferring them to the fresh medium.

2.3.9. Culture environment

All the cultures were grown in an air conditioned culture room maintained at 25 ± 2°C temperature and at 60% relative humidity. They were illuminated for 16/8h light/dark regime with 40 watt white fluorescent tubes fitted at a distance 30-35 cm in each culture rack. The cultures received light intensity from 2,000 to 3,000 Lux (PAR 50-70 µEm²s⁻¹) for 16 h per day (controlled by a timer).

2.3.10. Acclimatization of the plantlets to external environment

In-vitro generated plantlets were taken out of the culture tubes and washed thoroughly under running tap water for about 30 min so as to remove any medium attached to them. Thereafter,
the plantlets were potted in plastic pots (8 cm height, 7 cm diameter) containing a mixture of sterile sand and soil in 3:1 ratio. The plantlets were irrigated with tap water and were covered with polyethylene bags to maintain high humidity. The potted plantlets were kept in laboratory conditions at 25 ± 2°C in artificial light from white fluorescent tubes to acclimatize them to the external environment. The polyethylene bags from the plantlets were removed after 4-5 weeks for a shorter period initially (2-4 h per day) and gradually the time period was increased.

2.3.11. Field transplantation

After acclimatization of the plantlets in the laboratory conditions, the plantlets (about eight months old) were moved outdoors for a portion of the day for their hardening and to gradually introduce them to the direct sunlight, dry air, and cold nights. They were transferred to small earthen pots containing a mixture of soil and compost for some days followed by their transplantation in the Botanical garden of Department of Botany, BHU, Varanasi.

2.4. Oil extraction and GC-MS analysis

2.4.1. Plant material

Seeds of the plant from which the oil had to be extracted were collected from ayurvedic garden and were identified and authenticated by the taxonomists at the Department of Ayurveda, BHU, Varanasi. The fresh seeds were separated from their fruit capsules and were semi-crushed. These semi-crushed seeds were used for oil extraction.

2.4.2. Essential oil extraction

The essential oil from the semi-crushed seeds was extracted using hydro-distillation. The semi-crushed seeds (200 g) were packed in a Clevenger apparatus (Perfit, India) with 400 ml of water and were submitted to hydro-distillation. The extraction was carried out for 8 h. The isolated oil was collected from the graduated receiver. For the removal of water traces from the oil, the extract was treated with anhydrous sodium sulphate (Na₂SO₄). The oil samples were stored in sealed vials in a refrigerator at 4°C until used for analysis.

2.4.3. Gas chromatography-mass spectrometry (GC-MS) analysis

GC-MS analysis of the essential oil derived from the seeds was performed on Shimadzu GCMS-QP-2010 plus system. RTx-5 Sil MS column (30 m x 0.25 mm ID, 0.25 µm film
thickness) was used for the analysis. The operating conditions of the column were as follows: oven temperature program from 80°C to 210°C at 4°C/min with hold time of 2 min and from 210°C to 300°C at 15°C/min with hold time of 5 min, and the final temperature was kept for 20 min. The injector temperature was maintained at 270°C, the volume of injected sample was 0.2 μl; pressure 85.4 kPa, total flow 76.8 ml/min, column flow 1.21 ml/min, linear velocity 40.5 cm/sec, purge flow 3.0 ml/min, split ratio: 60.0; ion source temperature 230°C; scan mass range of m/z 40-600; and interface line temperature 280°C. The identification of compounds was performed by comparing their mass spectra with data from NIST05 (National Institute of Standards and Technology, US), WILEY 8, and FFNSC1.3 (Flavour and Fragrance Natural and Synthetic Compounds) libraries.

2.5. Antioxidant and lipoxygenase inhibition assays

2.5.1. Chemicals and reagents required

- DPPH [2, 2-diphenyl-1-picrylhydrazyl] (Sigma-Aldrich)
- ABTS [2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)] (Sigma-Aldrich)
- TPTZ [2,4,6-tripyridyl-s-triazine] (Sigma-Aldrich)
- Sodium acetate [NaOAc] (HiMedia, India)
- Ferric chloride [FeCl₃·6H₂O] (HiMedia, India)
- Trolox solution [6-hydroxy-2,5,7,8-tetramethychroman-2-carboxylic acid] (Sigma-Aldrich)
- Potassium persulfate (HiMedia, India)
- Folin-Ciocalteu reagent (Sigma-Aldrich)
- Gallic acid (Sigma-Aldrich)
- NDGA [Nordihydroguaiaretic acid] (Cayman Chemical)
- Quercetin (Cayman Chemical)
- Linoleic acid (MP Biomedicals, India)
- Ferrous sulphate [FeSO₄] (HiMedia, India)
- Sodium carbonate [Na₂CO₃] (HiMedia, India)
- Methanol (HiMedia, India)
- Chloroform (HiMedia, India)

2.5.2. Plant extracts preparation

Plant material from each plant was weighed to 10 g and coarsely ground using pestle and mortar. The ground powder of each plant material was extracted in 100 ml of the desired solvent to prepare the respective solvent extract. The prepared extract was subjected to centrifugation at 4000 x g (Remi Centrifuge, India) for 30 min at 25°C. The supernatant was
evaporated to dryness at 40°C for 2-4 days to form a thick concentrated extract. The extracts were preserved at 4°C in brown bottles until use.

2.5.3. DPPH radical scavenging assay

DPPH scavenging assay was used to evaluate the free-radical scavenging activity of the various solvent extracts (aqueous, methanolic, and chloroform) of the selected plants. 10 μl of each solvent extract was added to 100 μl of 0.2 mM methanolic solution of DPPH. After vigorous shaking of the reaction mixture, it was incubated at 25°C for 5 min (Mensor et al., 2001). The absorbance of the mixture was measured at 520 nm. Percent inhibition of DPPH free radical was calculated as:

\[
\text{% Inhibition} = \left( \frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}} \right) \times 100
\]

where, \(A_{\text{control}} = \text{absorbance of control; } A_{\text{sample}} = \text{absorbance of sample}\)

2.5.4. Ferric reducing antioxidant potential

FRAP assay was determined using freshly prepared FRAP reagent. The reagent was prepared by mixing 300 mM/l NaOAc (sodium acetate, pH 3.6) buffer with 10 mM/l TPTZ (2,4,6-tripyridyl-s-triazine) solution in 40 mM HCl and 20 mM/l FeCl₃.6H₂O (ferric chloride solution) in the ratio of 10:1:1 (Benzie and Strain, 1996). Freshly prepared reagent was warmed to 37°C in a water bath before use. For performing the assay, 100 μl of each plant extract was treated with 3 ml freshly prepared FRAP reagent. The reaction mixture was incubated for 4 min at 25°C, thereafter absorbance was measured at 593 nm. FRAP values of the samples were calculated using the standard curve of FeSO₄ solution. Results were expressed as μmol Fe(II)/g dry weight of plant extract.

2.5.5. Trolox equivalent antioxidant capacity

TEAC assay was used to determine the free radical scavenging capacity of the prepared solvent extracts using ABTS radical cation (Re et al., 1999). ABTS radical cation was prepared by reacting 7 mM ABTS stock solution with 2.45 mM potassium persulfate in a volume ratio of 1:1. The mixture was incubated in the dark for 16 h at room temperature before use. 100 μl of the sample was mixed with 3.8 ml ABTS working solution. The reaction mixture was incubated for 5 min at room temperature and the absorbance was measured at
734 nm. Trolox solution was used as an antioxidant standard. The results were expressed as μmol Trolox/g dry weight of plant extract.

2.5.6. Total phenolic content determination

The total phenolic content was determined using Folin–Ciocalteu method (Mallick and Singh, 1980). 200 μl of each test sample was mixed with 500 μl of Folin-Ciocalteu reagent. After incubating the mixture for 3 min, 2 ml of 20% Na₂CO₃ solution was added was added to each sample. Thereafter the reaction mixture was vortexed, followed by boiling for 1 min in a water bath and then cooling. The absorbance was measured at 650 nm. Gallic acid served as the reference standard, and the results were expressed as mg GAE/g (mg Gallic acid equivalent/g) dry weight of plant extract.

2.5.7. Lipoxygenase (LOX) inhibition assay

Anti-inflammatory activity of the plant extracts were estimated by using LOX inhibition assay (Malterud and Rydland, 2000). In the assay, linoleic acid served as the substrate and soybean lipoxygenase as enzyme. The enzyme solution was pre-incubated for 5 min at 25°C, with solvent extracts of all the plants. In these test samples, the reaction was initiated by the addition of 0.2 μM linoleic acid solution and 0.2 M borate buffer (pH 9.0). The reaction mixture was incubated at 25°C for 5 min. An ethanolic solution of linoleic acid was used to stop the reaction and the absorbance was measured at 234 nm. NDGA served as a positive control in the assay. Lipoxygenase percentage inhibition was calculated by the formula:

\[
\text{% Inhibition} = \left( \frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}} \right) \times 100
\]

where, \( A_{\text{control}} \) = absorbance of control; \( A_{\text{sample}} \) = absorbance of sample

2.6. DNA damage preventive analysis

DNA damage preventive capacities of the test plant extracts were performed by using pBluescript II SK(−) DNA (Imlay et al., 1988). The plasmid DNA was isolated using a QIAprep Spin Mini prep Kit according to the manufacturer’s instructions. pBluescript II SK(−) (250 ng) was incubated with 0.5 mM FeSO₄, 25 mM H₂O₂ and 50 mM phosphate buffer (pH 7.4) in the presence or absence of plant extracts (0.2 μg/ml). The total reaction mixture of 12 μl was incubated for 1 h at 37°C. The test samples were then analyzed on 1% agarose gels at 70 V at room temperature to analyse the extent of DNA damage by Fenton’s reaction and
DNA protective capacities of the plant extracts. Quercetin (1 mM) served as a positive control. Gels were scanned using Gel documentation system and the densitometric analysis of the bands was performed using Quantity One analysis software (Bio-Rad).

2.7. Histological analysis

2.7.1. Chemicals and reagents required

- Formalin (E-Merck, India)
- Histo-Grade xylene (E-Merck, India)
- Alcohol (E-Merck, India)
- Paraffin wax (E-Merck, India)
- Hematoxylin and eosin (H&E) stain (Himedia, India)
- DPX mountant (Himedia, India)
- Egg albumin (Himedia, India)

2.7.2. Fixation and processing of the tissue samples

All the control and treated skin tissue samples whose histological analysis had to be conducted, were excised from the mice (approx. 1cm² size) and were fixed in 10% formalin for 48 h at room temperature. The fixed samples were dehydrated using absolute alcohol. After dehydration, the samples were cleared from alcohol by using Histo-Grade xylene. Thereafter, the samples were infiltrated with paraffin wax (m.p. 58-60°C) and embedded into it to form blocks on its cooling overnight. The blocks were kept in deep freezer for 2-3 h before sectioning.

2.7.3. Microtomy

Serial sections of the samples were cut at 6 µm thickness using a rotary microtome (Weswox Optik, India). The ribbons of the sections were adhered on to the surface of the glass slides (Blue Star Co., India) with the help of egg albumin. The glass slides with the tissue sections were marked accordingly and kept on slide warmer overnight for easy fixation of the sections to the slide, due to melting of the wax.

2.7.4. Staining of the sectioned samples

The sectioned samples were de-paraffinized in xylene till the complete clearing of the wax. Thereafter, the sections were gradually hydrated to an aqueous state by placing the slide through a series of alcohols, starting at 100% to 95%, 80%, and 70%, followed by rinsing with water. The hydrated sections were then stained with hematoxylin (5-7 min) and eosin (1-
2 min) (H&E) stain. The stained sections were again dehydrated using the above mentioned alcohol series in the reverse order (70%–100%), followed by xylene treatment. Mounting was done using DPX mountant and the sections were covered with coverslips. All the histological slides were observed under Leitz Laborlux microscope and photographed using Leica DFC 290 digital camera system (Leica Microsystems, Wetzlar, Germany) connected to Intel® Pentium® D computer (Model dx2280 MT, HP Compaq, California, USA) installed with ‘Motic-software’.

2.8. In-silico studies

2.8.1. Bioinformatics tools and databases used

- PDB [Protein structure database] (http://www.rcsb.org/)
- PMDB [Protein Model Database] (http://mi.caspur.it/PMDB/)
- Ramachandran plot of RAMPAGE and PDBSum servers
- ProtParam (http://web.expasy.org/protparam/)
- WHAT IF (http://swift.cmbi.ru.nl/whatif/)
- PDBSum (http://www.ebi.ac.uk/pdbsum)
- ResProx [Resolution-by-proxy] (http://www.resprox.ca/)
- Q-SiteFinder (http://bmbpcu36.leeds.ac.uk/qsitefinder/)
- MetaPocket 2.0 (http://projects.biotec.tu-dresden.de/metapocket/)
- Docking server (http://www.dockingserver.com/web)
- Patchdock server (http://bioinfo3d.cs.tau.ac.il/PatchDock/)
- STRING 9.0 (http://string-db.org/)
- Discovery studio 3.0
- DS MODELER
- CHARMM forcefield [Chemistry at HARvard Macromolecular Mechanics]
- LOOPER
- Verify3D
- Profile 3D
- ERRAT
- ProSA servers [Protein Structure Analysis]

The above mentioned bioinformatics tools and databases were used in the in-silico study for comparative homology modeling of some proteins and their molecular docking analyses with certain phytocompounds. Protein sequence database of NCBI was used to obtain the protein sequences of the proteins and their homologous template structures for predicting
their 3D structures were obtained from PDB resources using advanced BLAST. DS MODELER of Discovery studio was employed for comparative homology modelling of the proteins. ProtParam server was used for computation of various physical and chemical parameters for the selected proteins. LOOPER and CHARMM forcefield programmes were used for loop refinement and structural simulation. For checking the quality of constructed models, Verify3D, Profile 3D, ERRAT, ResProx, and ProSA servers were employed. Stereochemical properties of the modeled protein structures were evaluated using Ramachandran plot of RAMPAGE and PDBSum databases. Standard bond lengths and bond angles of the model were determined using WHAT IF. Protein–protein functional association network was predicted using STRING database. Quantitative analyses of the models were performed through VADAR. Successfully modeled and the most reliable structures of proteins were deposited in PMDB database. For active site prediction of the proteins, Q-SiteFinder, MetaPocket 2.0, and PDBSum servers were used.Docking analyses were done using Docking server and Patchdock server.

2.9. In-vivo studies and molecular analysis

2.9.1. Chemicals and reagents required

- TRIzol reagent (Invitrogen)
- PBS [Phosphate buffered saline] (Sigma-Aldrich)
- Chloroform [CHCl₃] (HiMedia, India)
- Ethanol (HiMedia, India)
- Isopropanol (Sigma-Aldrich)
- DNase (Sigma-Aldrich)
- Taq DNA polymerase (Genei, India)
- Deoxynucleotide triphosphates (dNTPs) and Primers (Genei, India)
- DEPC [Diethyl pyrocarbonate] (Sigma-Aldrich)
- Tris Base (HiMedia, India)
- EDTA [Ethylenediaminetetraacetic acid] (HiMedia, India)
- Glacial acetic acid (Sigma-Aldrich)
- Glycerol (Sigma-Aldrich)
- Bromophenol Blue (HiMedia, India)
- Xylene cyanol (HiMedia, India)
- Agarose (HiMedia, India)
- Ethidium bromide [EtBr] (Sigma-Aldrich)
2.9.2. Preparation of reagents

**DEPC water**

- 0.1 ml of DEPC was added per 100 ml of milliQ water
- Left overnight
- Autoclaved

**10X RBC lysis buffer**

- 8.26 g of NH₄Cl
- 1.19 g of NaHCO₃
- 200 µl of EDTA [0.5 M, pH8]
- Added to 100 ml DEPC water
- Adjusted pH to 7.3
- Filter sterilized and stored at 4°C

**50X TAE buffer (Tris Acetate EDTA, 1 l)**

- 100 ml EDTA (50 mM, pH 8.0)
- 242 g Tris Base
- 57.1 ml Glacial acetic acid
- 842.9 ml DEPC water

**0.8 % Agarose gel (50 ml)**

- 1 ml TAE (50X)
- 0.4 g Agarose
- 49 ml DEPC water

**6X Gel loading dye (4 ml)**

- 10 mg Bromophenol Blue
- 10 mg Xylene cyanol
- 1.2 ml Glycerol
- 2.8 ml DEPC water

2.9.3. Experimental model

Laboratory male mice (*Mus musculus*) of the Parkes (P) strain of about 8 to 10 weeks of age and weighing around 20 g each were used for the study. The animals were housed in polypropylene cages at 22 ± 10°C with 50 ± 10% relative humidity and were subjected to a 12h light/12h dark cycle in the animal house facility of Department of Zoology, BHU, Varanasi. Mice were acclimatized for 1 week before use while providing them with food and
water *ad libitum*. Experiments were conducted according to the University guidelines of the ethical committee for research animal experimentation.

2.9.4. Total RNA isolation

2.9.4.1. Isolation from skin samples

Total RNAs were isolated from skin and blood samples of different treatment groups using TRIzol reagent as per manufacturer’s protocol. Tissue samples (100 mg) were collected in 1 ml PBS solution before grinding in liquid nitrogen in pestle and mortar. Homogenization was done using 600 µl TRIzol reagent. After spinning the homogenate at 12,000 x g at 4°C, the supernatant was transferred in a new tube and left at room temperature (RT) for 5 min. To the supernatant, 200 µl chloroform was added, vortexed and left at RT for 5 min. Centrifugation was performed at 12,000 x g for 15 min at 4°C. The top aqueous phase (~600 µl) was transferred to 1.5 ml tube containing 500 µl of isopropanol, left at RT for 10 min, mixed by inverting and centrifuged at 12,000 x g at 4°C. The RNA pellets were washed twice with 1 ml of 75% ethanol and air dried. The pellet was resuspended in 50 µl DEPC treated water. Forty units of RNase free DNase was added to it to remove any contaminating DNA followed by heating at 70°C for 5 min for denaturing DNase. The recovered RNA was stored at –80°C. The quality of RNA was checked on 1% agarose gel and its concentration was determined by measuring the absorbance at 260 nm.

2.9.4.2. Isolation from blood samples

The samples were treated with 3 times volume of RBC lysis buffer twice, followed by vortexing for 15 sec, incubation for 15 min at RT, and centrifugation for 10 min at 2000 x g (4°C). Supernatant was discarded and the centrifugation step was repeated with the pellet using 1 ml RBC lysis buffer. The final WBC pellet was lysed and homogenized with 1 ml TRIzol reagent. The rest of the procedure was similar to that of the skin sample RNA isolation.

2.9.5. cDNA synthesis and semi-quantitative RT-PCR

cDNA synthesis of isolated mRNA from treatment groups was performed using Reverse Transcription System kit (Sigma, USA), using oligo dT primers, according to the instructions given in the manufacturer's protocol. Expression patterns of the genes were analyzed by RT-PCR performed with cDNA using gene-specific primers. PCR was performed in 25 µl final volume of reaction mixture containing 100 ng DNA, 2.5 µl 10X PCR buffer with 15 mM
MgCl₂, 100 μM dNTPs, 0.2 U Taq DNA polymerase and 10 pmol of each gene specific primer (Bangalore Genei, India). The amplification conditions used were as follows: 95°C for 10 min for denaturation, followed by 35 cycles of 94°C for 1 min. The annealing temperatures were taken according to the Tm (melting temperature) of the respective primers while extension was done at 72°C for 2 min. (Rojas et al., 2006). Biorad MJ Mini Thermal Cycler was used for performing PCR. All the reagents pertaining to RNA isolation and RT-PCR were prepared in DEPC treated water and the glasswares used were also treated with DEPC water. Sequences of the primer sets were designed using Primer 3 software. Experiments were repeated three times with triplicate samples from each treatment group. GAPDH was used as endogenous control gene for quantification.

2.9.6. Agarose gel electrophoresis

The appropriate amount of agarose was weighed according to its required concentration. Thereafter, it was dissolved in 1X TAE buffer and boiled. When the temperature of the agarose solution reached 50-55°C, ethidium bromide was added to a final concentration of 0.5 μg/ml, mixed well and poured in a gel tray with a well comb in place. The gel was left at room temperature for 20-30 min for polymerization. Appropriate amount of loading dye was added to the samples prior to loading and the gel was run in 1X TAE at 75 V for 1.5 h. Intensities of the appropriate amplified products on agarose gels were quantified using Gel Doc 2000 system and volume tool of the Quantity one software (BioRad USA). All the reactions were performed thrice so as to verify the reproducibility of the results.

2.10. Statistical analysis and data visualization

All the values are expressed as mean ± Standard error (SE). Microsoft Excel (2007) and Sigma Plot (v.11.0) were used for statistical and graphical evaluations. All the experiments were performed thrice using a minimum of three replicates. Data were analyzed by using SPSS (version 16.0) statistical software, and statistical analysis was carried out using one way ANOVA, wherever applicable. Duncan multiple range test was used to compare the significance of differences between test samples at P values of < 0.05. Heatmap preparation and clustering of the genes using RT-PCR data was created using the R package gplots. Clustering was based on complete linkage and pairwise Euclidean distances, using hclust (hierarchical cluster) function in R.