CHAPTER  4

MATERIAL & METHODS......
4.1. Plant material

Nine plants species were collected from Jhajjar and Rohtak district of Haryana as shown in Figure 4.1. Plants were selected based on their use in traditional and Ayurvedic system of medicines. Nine plants were selected and identified from botany department of Maharshi Dayanand University, Rohtak (India). The identified plants were further authenticated with the help of flora of Haryana (Jain et al., 2000) and the voucher specimens were deposited in the herbarium of Centre for Biotechnology, M. D. University, Rohtak. The voucher numbers are given respectively as *Achyranthes aspera* (CBT 002), *Aegle marmelos* (CBT 003), *Argemona mexicana* (CBT 004), *Callistemon lanceolatus* (CBT 008), *Capparis aphylla* (CBT 009), *Catharanthus roseus* (CBT 012), *Commelina bengalensis* (CBT 013), *Justicia adhatoda* (CBT 017) and *Syzygium cumini* (CBT 022).

![Figure 4.1 - Map of Haryana showing sites of collection (Jhajjar & Rohtak).](image)

4.2. Sample preparation and extraction of crude extracts

Total nine plants were taken in the study out of which leaves of eight plants and stem of one plant i.e. *Capparis aphylla* were collected from Haryana (30.73°N76.78°E), India. The plant material was washed with tap water, chopped into small pieces and air dried.
under shade for two weeks and, then oven dried at 40 °C for 18-24h. The dried plant material
was grinded and powdered with a mortar and pestle. The powder was weighed (50 g for each
plant sample) and the Soxhlet’s method was used for extraction. The five solvents (250 ml for
each sample): petroleum ether, chloroform, acetone, methanol and water were used in
ascending order of polarity (Harborne, 1998; Rajesh and Sharma, 2002). The combined
suspensions were filtered twice, first under vacuum through a double layer of Whatman filter
paper and then by gravity through a single sheet of Whatman No. 1 filter paper. The solvents
were removed from the clear supernatant by means of vacuum distillation at 30-35°C using a
Buichi Rotary Evaporator. The remaining solid was referred to as the crude extract.

4.3. Pathogens

Pathogenic strains of *Aspergillus fumigatus* [ITCC 4517 (IARI, Delhi), *Aspergillus
flavus* [ITCC 5192 (IARI, Delhi), *Aspergillus niger* [ITCC 5405 (IARI, Delhi), were
employed in the current study. All the *Aspergillus* strains were cultured in the laboratory on
Sabouraud dextrose agar plates.

4.3.1. Culture of pathogens

The pathogenic strains of *Aspergillus* were cultured on Sabouraud Dextrose Agar
(SDA) plates. The plates were inoculated with stock cultures of *A. fumigatus* (ITCC 4517),
*A. flavus* (ITCC 5192) and *A. niger* (ITCC 5405) and incubated for 96 h in BOD incubator at
37 °C (Chhillar et al., 2008). These cultures were used as the source of spores required for
performing experiments. SDA (Hi-Media, Mumbai) was used for the fungal cultures. SDA
was mixed in distilled water, boiled gently until it gets dissolved and pH was adjusted to 6.0.
Dispensed the media into flask and cotton plugs were applied. The media was sterilized by
autoclaving (121°C for 15 minutes). Antibiotics were then added in cooled media and poured
(20 ml) in the sterilized petriplates.

4.4. Antimycotic activity

4.4.1. Microbroth-dilution assay

The spores of Aspergillus were harvested from 96 h cultures and treated with various
extracts of different plants in a 96-well culture plate. The plates were incubated at 37°C and
examined macroscopically after 48 h for the growth of *Aspergillus* mycelia (Annette et al.,
1995; Yadav et al., 2005).

4.4.2. Disc-diffusion assay

This test was performed in radiation-sterilized Petri plates of 10.0 cm diameter
Sterilized discs (5.0 mm of Whatman paper) impregnated with various extracts of different plants were placed on the surface of agar plates already inoculated with *Aspergillus* spores (1 x 10⁶). The plates were incubated at 37°C and examined at 48 h for the zone of inhibition, if any, around the discs (Indian Pharmacopoeia, 1996; Yadav et al., 2005).

### 4.4.3. Spore-germination-inhibition assay

The various test samples in 90 ±l µl of culture medium were prepared in 96-well flat bottom micro-culture plates (Nunc Nunclon) by double-dilution method. The wells were prepared in triplicates for each extract. The wells of the culture plates were inoculated with 10 ±l µl of spore suspension containing 100 ± 5 spores. The plates were incubated at 37 0C for 16 h and then examined for spore germination under an inverted microscope. The number of germinated and non-germinated spores was counted and the percent spore germination inhibition (PSGI) was calculated using following formula:

\[
PSGI = 100 - \frac{\text{No. of spores germinated in drug treated well} \times 100}{\text{No. of spores germinated in control well}}
\]

The activity of the preparations was represented as the MIC90 which inhibit the germination of spores in the range of 90–100%. The lowest concentration of the testing compound, which resulted in >90% inhibition of germination of spores in the wells was considered as MIC90 (Surender and Janaiah, 1987; Chhillar et al., 2006).

### 4.5. Qualitative phytochemical analysis

All the extracts obtained from nine plants were subjected to various tests for the identification of various bioactive constituents (Harborne, 1980) (Table 4.1).

**Table 4.1-** Preliminary phytochemical estimation methodology for medicinal plants.

<table>
<thead>
<tr>
<th>Sr. No</th>
<th>Phytochemical/Test</th>
<th>Methodology</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Alkaloids (a)</td>
<td>The alcoholic or aqueous extract (3 gm.) of the plant was dissolved in 5ml of distilled water then 2M hydrochloric acid was added until an acid reaction occurs, Dragendorff’s reagent (1ml) was added and an orange red precipitate was produced immediately.</td>
<td>(Kokate, 2005)</td>
</tr>
<tr>
<td>(a)</td>
<td>(Dragendorff’s Test)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(b)</td>
<td>(Wagner’s Test)</td>
<td>The alcoholic extract of the drug (2 gm) was acidified with 1.5% v/v of hydrochloric acid and a few drops of Wagner’s reagent were added. A yellow or brown precipitate was formed.</td>
<td></td>
</tr>
<tr>
<td>(c)</td>
<td>(Hager’s Test)</td>
<td>To alcoholic extract of the drug (2 gm) was taken in a test tube, a few drops of Hager’s reagent added. Formation of yellow precipitate confirmed the presence of alkaloids</td>
<td></td>
</tr>
</tbody>
</table>
2 | Flavanoids  
(Shimoda test) | To dry extract (15 mg), ethanol (1ml.) was added and dropped small piece of Magnesium ribbon. The drop wise addition of conc. HCl leads to the development of colour ranging from orange to red was confirmatory for flavanoids. | (Kokate, 2005) |
3 | Phenols  
(Ferric Chloride test) | The extract (10 mg) was added in 1ml of 1% ferric Chloride solution, a purple or red colour indicated the presence of phenols. | (Harborn, 1980) |
4 | Saponins  
(Haemolytic test) | The extract (0.5 gm) was boiled with water for 2 minutes in a test tube. After cooling the mixture was vigorously shaken and lefted for 3 minutes. The amount of honeycomb frothing may decide the presence (Frothing) and absence (No frothing) of saponins. | (Harborn, 1980) |
5 | Sterols/ Terpenes  
(a) Hoss’s Reaction  
(b) Lieberman Burchard Reaction  
(c) Moleschott’s reaction | In this test, the extract was taken in chloroform (2ml) and concentrated sulphuric acid was poured from side of the test tube. The colour of the ring at the junction of the two layers was noted. A violet green colour indicated the presence of cholesterol, sitosterol. A red colour ring showed the presence of sterol / terpenes. To 1ml of extract, 2 ml of acetic anhydride solution, 2ml of concentrated sulphuric acid was added. The change in the colour from red to blue was a test for sterols/terpenes. To 1 gm of the extract was mixed with 5ml of distilled water, 2ml of conc. Sulphuric acid was poured from the side of the tube and the colour was noted. Red colour changed to violet showed their presence. | (Harborn, 1980) |
6 | Tannins  
(a) Ferric chloride test  
(b) Lead acetate test | 1 to 2 ml of extract, a few drops of 5% aqueous ferric chloride solution was added. A bluish black colour was produced which disappears on addition of few ml of dilute sulphuric acid followed by the formation of a yellowish- brown precipitate. In 5ml of an aqueous extract, few drops of 1% solution of lead acetate were added. A yellow red precipitate was formed. | (Kokate, 2005) |

### 4.6. Chromatographic Methods

Chromatography is the method of choice in handling the problem of isolation of a compound of interest from a complex natural mixture. Therefore, the chromatographic methods used during the present work are described briefly.

#### 4.6.1. Equilibration of Chromatographic Chamber

About 1.0 cm of height of solvent system was taken in a clean dry glass chamber. The chamber was covered with air tight lid and allowed to soak with vapours of solvent. The inner side of the back wall of chamber was lined with a piece of filter paper. The lower edge of the filter paper was dipped into the solvent present at the bottom of the chamber to ensure the even distribution of the solvent vapours throughout the volume of developing tank.

#### 4.6.2. Thin layer chromatography (TLC)

Fifteen micro litres of neat plant extract at 100 mg/ml was applied 2 cm from the base of aluminium-backed silica plates (Merck 60F254, Germany) cut to size (10x5 cm). The plates were dried for 15 minutes at room temperature and separately developed in the various
solvent combinations. The plates were prepared in duplicates for each solvent combination (‘A’ and ‘B’) and developed in glass tanks closed with aluminium foil. Plate ‘A’ was used as a reference chromatogram to visualize the separated spots under visible light and UV irradiation at 365 nm and sprayed with vanillin (Eloff, 2001). The plate was carefully heated at 105 ºC for optimal colour development. The Rf values (Retention factor) of the spots on the plate were computed and recorded.

In this method retention of solute, whether by partition or adsorption are described by their migration relative to that of the eluting agent. This linear flow in one direction, Rf, is defined as distance travelled by mobile phase.

\[ R_f = \frac{\text{distance travelled by solute}}{\text{distance travelled by solvent}} \]

4.6.3. Development of Chromatogram

The plates loaded with sample fraction were kept in the chromatographic chamber containing the mixture of appropriate solvents in ratio of standardised amount. The chamber was closed with an air tight lid to saturate it properly. The solvent was allowed to rise up to a height of about 9.0 cm at room temperature. After developing the chromatogram, the plates were removed from the tank, the solvent front was marked and they were allowed to dry in the air. The various components in the fraction were detected with UV light and by spraying the group specific reagents.

4.6.4. Column Chromatography

Column Chromatography (CC) consists of a column of particulate material such as silica or alumina that has a solvent passed through it at atmospheric, medium or low pressure. The separation can be liquid/solid (adsorption) or liquid/liquid (partition). The columns are usually glass or plastic with sinter frits to hold the packing. Most systems rely on gravity to push the solvent through, but medium pressure pumps are commonly used in flash CC. The sample was dissolved in solvent and applied to the front of the column (wet packing), or alternatively adsorbed on a coarse silica gel (dry packing). The solvent elutes the sample through the column, allowing the components to separate.

The silica gel Column Chromatography was performed to obtain active component of our interest. The silica gel was suspended in petroleum ether and packed in a glass of 5x 35 cm size. The column was equilibrated with appropriate solvent (Petroleum ether) for 16 h before loading the sample. Slurry of sample fraction was prepared in appropriate solvent/s (Petroleum ether) and loaded on pre-equilibrated silica gel column carefully. The polarity was increased by addition of ethyl acetate (EtAC) at an interval of 1% until final component separate out. The components of sample fraction were eluted with 200 ml of suitable
solvent/s (Petroleum ether) at a flow rate of 1.0 ml/min followed by different ratio of Suitable solvent/s: Ethyl. Numbers of sub-fractions collected in test tubes were allowed to concentrate under a stream of cold air. Sub-fractions containing similar constituents were combined (monitored by TLC fingerprinting). The sub-fractions showing similar profile of Rf values were pooled and dried in vacuo-rotavapour.

A continuous passage of the solvent aids the setting of the packing particles. The active constituents are determined using spectrophotometric methods.

4.7. Spectroscopic Techniques

Spectroscopy is the study of the interaction of electromagnetic radiation (EMR) with matter. NMR spectroscopy is the study of interaction of radio frequency (RF) of the EMR with unpaired nuclear spins in an external magnetic field to extract structural information about a given sample.

An analytical Varian-NMR-vnmrs 600 instrument operating at proton frequency of 600 MHz was used for ¹H and ¹³C. The isolated pure compound was weighed (10-30 mg) and dissolved in deuterated CDCl₃ since the compound was soluble in CHCl₃. Each sample were dissolved in 0.7 ml CHCl₃ and transferred into NMR tubes (5 mm). The chemical shifts of various kinds of protons plotted in ppm scale using tetramethylsilane (TMS) as internal standard were obtained. The coupling constants (J value) were expressed in Hz. The notations used in the text were s, d, t, m and br for singlet, doublet, triplet, multiplet and unresolved bond signals, respectively.

NMR spectroscopy is routinely used by chemists to study chemical structure of simple molecules using simple one dimensional technique (1D-NMR). Two-dimensional techniques (2D-NMR) are used to determine the structure of more complicated molecules. The organic chemist is principally concerned with the study of carbon compounds. As a consequence, 1D and 2D NMR involving protons (¹H) and carbons (¹³C) were carried out.

4.7.1. One Dimensional NMR

4.7.1.1. 1D-Proton NMR (¹H-NMR)

Proton NMR is a plot of signals arising from absorption of radio frequency (RF) during an NMR experiment by the different protons in a compound under study as a function of frequency (chemical shift). The area under the plots provides information about the number of protons present in the molecule, the position of the signals (the chemical shift) reveals information regarding the chemical and electronic environment of the protons, and the splitting pattern provides information about the number of neighboring (vicinal or geminal) protons.
4.7.1.2. 1D-Carbon NMR (13C-NMR)

Similar to proton NMR, carbon NMR is a plot of signals arising from the different carbons as a function of chemical shift. The signals in $^{13}$C-NMR experiments normally appear as singlets because of the decoupling of the attached protons. Different techniques of recording of the 1D carbon NMR has been developed so that it is possible to differentiate between the various types of carbons such as the primary, secondary, tertiary and quaternary from the 1D $^{13}$C-NMR plot. The range of the chemical shift values differs between the 1H (normally 0-10) and $^{13}$C NMR (normally 0-230) that arises from the two nuclei having different numbers of electrons around their corresponding nuclei as well as different electronic configurations.

4.7.2. Two dimensional NMR (2D-NMR)

Two-dimensional NMR is useful as compared to one-dimensional NMR because the two dimensional spectra provide more information than one dimensional spectra about a molecule and gives a detail information regarding the structure of a molecule, particularly in case of molecules that are too complicated to work with using one dimensional NMR. Currently, the common 2D-NMR experiments that appear in papers concerned with structural elucidation of natural products include the homonuclear 1H, 1H-COSY (CORrelated SpectroscopY).

1H, 1H-COSY is one of the most useful experiments. It is a plot that shows coupling among neighboring protons involving $2J$, $3J$ as well as $4J$. It provides information on the connectivity of the different groups within the molecule. On both axes are shown the $^1$H-NMR of the compound. By drawing a straight line from any of the dark spots to each axis, one can see which protons couple with one another, and which are therefore attached to neighbouring carbons.

4.8. Gas Chromatography/Mass Spectrometry (GC/MS)

In GC/MS a mixture of compounds to be analyzed is initially injected into the GC where the mixture is vaporized in a heated chamber (injector). The gas mixture travels through a GC column carried by a carrier gas, where the compounds become separated as they interact with the stationary phase of the column. The separated compounds then immediately enter the mass spectrometer that generates the mass spectrum of the individual compounds. Advantages of GC analysis include an improved separation of closely related isomers and simple coupling to MS detectors for identification through the fragmentation pattern.
The GC-MS investigation of plant extracts were carried out using Shimadzu QP-2010 plus with thermal desorption system TD-20 gas chromatography equipped with an Turbomolecular pump (58L/Sec for He), Rotary pump 30L/min (60Hz) and Column (Inert Cap Pure-WAX) flow up to 4ml/min which was operated in EI mode (1 pg octafluoronaphthalene m/z 272 S/N >200). Helium was the carrier gas at a flow rate of 1ml/min. The injector was operated at 250°C and the column temperature was programmed as follows; 35°C for 5min to 4°C/min, then gradually increased to 250°C for 10min. Identification of constituent of the extract was achieved on the basis of their retention indices determined with a reference to a homologous series of phytoconstituents and by comparison of their mass spectral fragmentation patterns (NIST database/chemstation data system) with data previously reported in literature (Sathyaprabha et al., 2010).

4.8.1. Mass Spectrometry (MS)

MS is an analytical technique that involves generating charged particles (ions) from molecules of the analyte. The generated ions are analyzed to provide information about the molecular weight of the compound and its chemical structure. There are many types of mass spectrometers and different sample introduction techniques which allow a wide range of samples to be analyzed. The widely utilized practice of coupling Gas Chromatography (GC) with Mass Spectrometry (MS) was routinely employed for the analysis of the compositions of various essential oils. The electron impact and fast atom bombardment spectra (JOEL-JMS-DX303) were recorded and m/z values of peaks were used to calculate the molecular mass and fragmentation pattern of compound.

4.9. Other Spectroscopic Methods

These include the infrared (IR) spectroscopy which offers information relating to the functional groups, and the ultraviolet (UV) spectroscopy which reveals information relating to the presence of sites of unsaturations in the structure. These two methods are becoming less important in structure elucidation of natural products due to the superiority of information obtained from the NMR experiments with much less sample amounts.

4.9.1. Fourier Transforms Infrared Spectroscopy

Fourier transform infrared (FTIR) spectroscopy is form of IR spectroscopy and it is measurement technique for collecting infrared spectra. Instead of recording the intensity of energy absorbed when the frequency of the infra-red light is non constant (monochromatic), the infra red light is guided through an interferometer. After passing through the sample under investigation, the measured signal is the interferogram. Performing a mathematical
Fourier transform on this signal results in a spectrum identical to that from conventional (dispersive) infrared spectroscopy.

Spectral data were collected on a FTIR spectrometer (Model FTS 7000; Varian Inc., Palo Alto, CA, USA) coupled to an infrared microscope (model 600 UMA; Varian) using a 15 xVarian objective and fitted with a liquid N₂ cooled MCT 64x 64 element array Stingray (Varian) focal plane array detector. The Varian system was controlled by IBM compatible PC running WIN IR PRO 3.0 software (Varian). The absorbance spectra were acquired in reflectance mode at a spectral resolution of 8 cm⁻¹ with 64 scans co-added. Apodization was performed using a triangular function. The system enabled 4096 spectra to be acquired from a sample area of approx. 350 μm² in approx. 2 min.

4.9.2. Ultraviolet-Visible Spectroscopy

UV-visible spectroscopy related to the spectroscopy of photons in the UV-visible region. UV-visible spectroscopy uses light in the visible ranges or its adjacent ranges i.e. near ultraviolet (UV) and near infrared (NIR) ranges. The colour of the chemicals involved is directly affects the absorption in the visible ranges. Molecules undergo electronic transitions in the ranges of the electromagnetic spectrum.

One mg of the pure compound was dissolved properly in 2.0 ml of methanol and filtered through a membrane of 0.22 µ pore size. The sample was taken into a cuvette and scanned at wavelengths between 200 to 600 nm with an increment of 100 nm using Shimadzu spectrophotometer (UV-2450).

4.10. Study of physical/biochemical properties

The colour, melting point and solubility behaviour of the purified compound was determined as described by Furniss et al., (1989).

4.11. Toxicity studies

4.11.1. Acute toxicity

All the bio-extracts at the range 100mg to 1000mg/kg were administered orally to the groups of rats comprised six rats in each group. Mortality, toxic symptoms if any and general behaviour was observed for 14 days.

4.11.2. Haemolytic assay

Human erythrocytes, collected from apparently healthy individuals, were washed three times with PBS by centrifugation at 1500 rpm for 10 min. A 2% erythrocyte suspension was incubated at 37 °C for 1 h with different concentrations of extracts ranging from 500.00 to 3.9 µg/ml plant extracts. After incubation, cells were pelleted at 5000 rpm for 10 min. The supernatant was collected and the A450 was determined using a spectrophotometer (UV Vis
Spect Lambda Bio 20, Perkin Elmer). In negative control sets, only buffer was used for background lysis, whereas in positive controls, lysis buffer was used for completely lysing the erythrocytes. For each sample the percentage of maximum haemolytic activity was determined (Yadav et al., 2005).

4.11.3. Single cell gel electrophoresis assay (Comet assay)

1 ml of blood was taken from a healthy donor by venipuncture. Briefly, blood is diluted 1:1 with Phosphate buffered saline (PBS) and layered over 600 ul Histopaque and centrifuged at 800 X g for 20 min. The ‘buffy’ coat was aspirated into 3-5 ml of PBS and centrifuged at 250 X g for 10 min to pellet the lymphocytes. The pellet was resuspended in ~1 ml of PBS and counted using a haemocytometer. Nearly 2X 10^4 cells per 100 μL of medium are taken for each dose of the test material (Costa et al., 2008). One ml of each dose was made in medium and lymphocytes added to it. The eppendorff was inverted to mix the cells and test material. The eppendorff-tubes were properly wiped with alcohol and kept in the incubator at 37°C for 3 hours. After the treatment, the cells are centrifuged at 3000 rpm for 5 min to pellet the lymphocytes. The test substance was aspirated and discarded. The pellet was then resuspended in 100 ml of PBS and 10 ml removed for trypan blue viability test. 100 ml of 1% low melting point agarose (LMPA) was added and 80 ml of the suspension layered onto the base slides, which was precoated with 1% agarose, and then covered with a cover slip. The slides were put on ice packs until Agarose layer hardens (~5 to 10 min).

Gently slide off cover slip and add a third agarose layer (90 μL LMPA) to the slide. Cover it with a new cover slip and then left for 10 min on a chilled metal plate in order for solidification of the agarose layer to occur. Following this, the cover slip was removed, and the slides were submerged in alkali lysis solution (2.5 M NaCl, 10 mM Trizma, and 100 mM EDTA) at pH> 13 overnight at 4 °C (Klaude et al., 1996). Thereafter, the glass slide was incubated in fresh, cold electrophoresis buffer (0.3M NaOH and 1mM EDTA, pH 13) in a horizontal electrophoresis tank (Model POWER PAC 200, Bio-Rad Co., USA) for 30 min at room temperature to allow for DNA unwinding. Electrophoresis was performed for 10 min at 25 V and 300 mA in a chamber cooled in an ice bath. After electrophoresis, the glass slides were neutralized in 0.4M Tris-HCl (pH 7.5) buffer, washed twice in distilled water, and left overnight for drying at room temperature. The slides were stained following the silver staining method (Nadin et al., 2011). Stained slides were examined using an Olympus Microscope model-BX60 fitted with an Olympus-DP72 camera. The classification of comet category and their tail measurements were carried out according to Garcia et al., (2007).