3.16 To evaluate the chemical stability of the shampoo by comparing TLC fingerprint profile of freshly prepared shampoo formulation with TLC fingerprint profile of shampoo formulation after six months and by TLC densitometry.

Experimental
4 EXPERIMENTAL

4.1 MATERIALS AND METHODS

4.1.1 Collection and authentication of plant material
The plants were selected on the basis of their ethno-medicinal importance and literature survey. The authenticated plant material for study, namely, leaves of *Eucalyptus globulus* Labill., *Ocimum kilimandscharicum* Baker and *Melaleuca leucadendron* (L.)L. were collected from Medicinal Plants Garden of University Institute of Pharmaceutical Sciences, Panjab University, Chandigarh, while seeds of *Pongamia glabra* Vent and bark of *Cinnamomum zeylanicum* Blume were procured from Yucca Enterprises, Mumbai. The specimens were also deposited in the Herbarium-cum-Museum of University Institute of Pharmaceutical Sciences. All drug samples were authenticated at the Department of Raw Materials Herbarium and Museum (RHMD), by Dr. H.B Singh, Chief scientist and Head, RHMD, NISCAIR, New Delhi vide ref. no. NISCAIR/RHMD/Consult/2011-12/1807/107.

4.1.2 Extraction of volatile oils
The drugs collected were subjected to hydrodistillation for extraction of volatile oils in Clevenger’s apparatus individually and by cold expression process for extraction of fixed oil.

4.1.3 Determination of organoleptic and physical properties of extracted oils
The organoleptic properties like appearance/ colour, odour, taste, solubility and specific gravity of extracted oils were determined. Organoleptic properties served as a means of assessing the purity and quality of the oil as well as for identification.
4.1.4 Screening of oils for antifungal activity against *Malassezia furfur* Collection of culture

Pure culture of *Malassezia furfur* (MTCC: 1374) was obtained from Institute of Microbial Technology (IMTECH), Chandigarh. The culture was maintained in Emmons modified medium (Hi-Media). Composition of Emmons modified medium-

- Sabourauds Glucose Broth: 30.0g
- Agar: 20.0g
- Special Pepton: 10.0g
- Dextrose: 20.0g
- Distilled water: 1.0 L

4.1.5 Method of antifungal activity

Antifungal effects of all the five test oils were evaluated by Disc diffusion method. Standard size Whatman No. 1 filter paper discs with 6.0 mm in diameter and sterilized were used to determine the antifungal activity.

4.1.6 Determination of minimum inhibitory concentration (MIC)

The MIC of the oils was determined by the micro dilution broth method. The MIC of these oils was compared with that of ketoconazole and tea tree oil taken as standards.

4.1.7 Thin Layer Chromatography fingerprint profile of selected oils

The oils selected on the basis of antifungal study were subjected to thin layer chromatography. Sample was prepared by diluting the test oils in chloroform in 1:10 concentration. Precoated TLC plates were used. The sample was applied using micro capillary. TLC Chamber was saturated with the solvent system (mobile phase) for 10-15 minutes. The solvent system consisted of toluene and ethyl acetate in 97:3 respectively. The TLC was developed using ascending chromatographic technique. The coloured zones were located without derivatization under the long wavelength UV (366 nm) and short wavelength (254 nm). A spray reagent consisting of 1% vanillin in concentrated sulphuric acid was used as a derivatizing agent to visualize the spots in white light.

4.1.8 Gas Chromatography – Mass Spectroscopy profile of cinnamon oil and kapur tulsi oil
The cinnamon oil and kapur tulsi oil were analyzed by GC-MS technique for identification of their chemical constituents. Gas chromatograph-Mass Spectrometer (Model- Polaris Q, Thermoelectron Corporation, Germany) equipped with DB-5 column (30 m x 0.25 mm x 0.25 mm) was used for analysis.

4.1.9 Evaluation of shampoo formulation by official methods as prescribed by Bureau of Indian Standards

The shampoo formulation was evaluated by official methods such as:

a) Determination of non-volatile alcohol soluble matter in shampoo

b) Determination of pH of shampoo

c) Determination of foam height

4.1.10 Evaluation of shampoo formulation by official and unofficial methods

The shampoo formulation was evaluated by the following methods:

a) Detergency evaluation

b) Wetting test

c) Measure of surface tension

d) Viscosity profile

e) Conditioning effects on isolated human hair by Scanning Electron Microscopy

4.1.11 Safety evaluation tests of the shampoo formulation

Safety evaluation tests of the shampoo formulation included the following methods:

a) Draize eye irritation test on rabbits

b) Draize skin irritation test on rabbits

4.1.12 Stability evaluation of the shampoo formulations

The shampoo formulation was evaluated for the stability during its shelf life. The shampoo formulation was evaluated for:

a) Physical stability

b) Chemical stability
4.1.13 Evaluation of shampoo formulation for antifungal activity
The shampoo formulation was evaluated for antifungal activity against Malassezia furfur by disc diffusion method and compared with the antifungal activity of marketed shampoos.

4.1.14 Determination of Minimum Inhibitory Concentration (MIC) of shampoo formulation
The MIC of shampoo formulation was determined by the micro dilution broth method.

4.2.1 EXPERIMENTAL SECTION
4.2.2 Extraction of volatile oils
The drugs collected were subjected to hydrodistillation for extraction of volatile oils in Clevenger’s apparatus individually. For extraction of cinnamon oil, the bark of Cinnamomum zeylanicum was powdered and 150 g of powdered bark was subjected to hydrodistillation for 5-6 hours in Clevenger’s apparatus. The oil obtained was dried over anhydrous sodium sulphate and yield of the oil was estimated. The oil was preserved in well-closed glass vials at 4 ºC for further studies.

For extraction of eucalyptus oil, kapur tulsi oil and cajeput oil, fresh leaves of plants of Eucalyptus globulus, Ocimum kilimandscharicum and Melaleuca leucadendron were taken and cut into small pieces. About 150 g of leaves of each plant were subjected to hydrodistillation separately for 5-6 hours to get their respective oil. The oils obtained were dried over anhydrous sodium sulphate and percentage yield of each oil was estimated. The oils were preserved in glass vials at 4 ºC for further studies.

The karanj oil from seeds of Pongamia glabra was obtained by cold expression process. About 150 g of seeds were taken to extract the fixed oil. The % age yield of the oil was estimated. All the oils were preserved in separate vials and preserved suitably for further studies. The oils were freshly extracted time to time as and when required. The % age yield of oils was calculated by using formula:
Percentage (% age) yield of oil = \frac{\text{volume of oil in ml}}{\text{wt of plant material taken}} \times 100

4.2.2 Determination of organoleptic and physical properties of extracted oils

The organoleptic properties like appearance/colour, odour, taste, solubility and specific gravity of extracted oils were determined. Organoleptic properties served as a means of assessing the purity and quality of the oil as well as for identification.

Method

The oils were placed separately in transparent bottle over a white background and the colour and clarity were observed; the characteristic odour of each oil was determined by sniffing; and to determine its characteristic feel to the touch, the oil was rubbed between thumb and forefingers.

The solubility of oils was determined in different solvents such as water, ethyl alcohol, methanol, chloroform and petroleum ether.

Specific gravity of the essential oils varied between 0.696-1.188 at the specific temperature. The specific gravity of the oils was expressed as the ratio of the weight of the volume of oil to that of an equal volume of pure water when both were determined at 25º C (Knevel and DiGangi, 1977).

4.2.3 Screening of oils for antifungal activity against Malassezia furfur using Disc diffusion method

4.2.3.2 Method of antifungal activity

Antifungal effects of all the five test oils were evaluated by Disc diffusion method (Rios et al., 1988). Standard size Whatman No.1 filter paper discs, 6.0 mm in diameter, sterilized in dry heat at 140ºC in an oven for 1 hour, were used to determine antifungal activity. Sabourauds dextrose agar medium was prepared with 1% corn oil. After sterilization, it was poured into sterilized petri plates and allowed to solidify. Using a sterile cotton swab, culture was swabbed on the surface of medium plates. Sterilized filter paper discs were soaked in neat, undiluted (100%) concentration of oils. The discs saturated with oil were aseptically placed over plates containing medium with the help of sterilized forceps. Along with the five test oils, discs saturated with tea tree oil and ketoconazole, both taken
as standards were also aseptically placed over plates containing medium for comparison of antifungal activity of test oils. Three replicates were maintained for each sample. The plates were incubated at 32°C and the zone of inhibition was observed after 4 days. Control was maintained with filter paper discs dipped in distilled water.

4.2.3.3 Determination of Minimum inhibitory concentration (MIC)

The MIC of the oils showing antifungal activity was determined by the micro dilution broth method (National Committee of Clinical Laboratory Standard, 1993). A stock solution of 25.6 mg/ml of the plant oil was prepared in Mueller Hinton broth (Difco). Further, serial double dilutions were made in a range from 25.6 mg/ml to 0.05 mg/mL. A 100 μl aliquot of each dilution and Mueller Hinton broth without oil (positive and negative controls) were put in the wells of a microtiter plate. The inoculated microtiter plates were then incubated at 37°C for 72 h. After examining turbidity visually, 40 μl of 0.02 mg/ml 2, 3, 5 triphenyl tetrazolium chloride (TTC) was added to each microplate well and incubated at 37 °C and re-examined after 30 mins. The MIC was calculated as the lowest concentration of the oil that prevented growth of the culture. All samples were examined in duplicate in three separate experiments. The MIC of these oils was compared with the MIC of tea tree oil and ketoconazole taken as standard, against *M. furfur*.

4.2.3.4 Evaluation of the antifungal activity of oils in different combinations

Some studies have reported that combinations of essential oils have greater antimicrobial activity than their individual components and studies also suggested that the components of essential oils are responsible for providing synergistic or potentiating effects probably.

Therefore, oils showing antifungal activity against *M. furfur* in present study were evaluated for antifungal activity in different combinations to check their antagonist or synergistic effects. The combinations evaluated under this part of study were

(a) Cinnamon oil + kapur tulsi oil
(b) Cinnamon oil + cajeput oil
(c) Cinnamon oil + kapur tulsi oil + cajeput oil
(d) Kapur tulsi oil + cajeput oil

To form the combinations of oils, 100% concentration of oils was used and they were mixed in equal ratio. The antifungal activity of combinations of oils was also evaluated by
Disc diffusion method, as discussed in section 4.2.3.2 and the combinations were also evaluated for their Minimum Inhibitory Concentration by micro broth dilution method, as discussed in section 4.2.3.3. On the basis of this study, most active combination was selected as the active ingredient for the formulation of an antidandruff shampoo.

4.2.4 Thin Layer Chromatography fingerprint profile of selected oils

Thin Layer Chromatography is one of the widely used techniques for rapid identification of drugs and their formulations. TLC provides visual semi-quantitative information on the active constituents of drugs. It provides a chromatographic drug fingerprint, and is therefore suitable for monitoring the identity and purity of drugs.

The oils selected on the basis of antifungal study were subjected to the thin layer chromatography. The oils subjected to TLC were cinnamon oil and kapur tulsi oil.

Procedure

a) Sample preparation

Sample was prepared by diluting the cinnamon oil in chloroform in 1:10 concentration and same procedure was followed for the preparation of sample of kapur tulsi oil for TLC.

b) Activation of precoated plate

Freshly opened box of precoated plates usually do not require activation. However plates exposed to high humidity or kept on hand for long time may have to be activated by placing in oven at 110-120°C for 30 minutes prior to sample spotting.

c) Application of sample

Sample application is the most critical step for obtaining good resolution. The sample was completely transferred to the layer without damaging the layer. The sample was applied using microcapillary. A 1-10 µl volume of the sample was applied as band to keep the size of the starting zone between 8-10 mm.

d) Chamber saturation
The TLC chamber was saturated with the solvent system (mobile phase) for 10-15 min to get reproducible results. The solvent system consisted of toluene and ethyl acetate in 97: 3 respectively.

e) **Development of chromatogram**

The TLC was developed using ascending chromatographic technique. After development the plate was removed from the chamber and mobile phase was allowed to evaporate completely.

f) **Detection and visualization**

After the plates get dried completely, the coloured zones were located without derivatization under the long wavelength UV (366 nm) and short wave length (254 nm). A spray reagent consisting of 1% vanillin in concentrated sulphuric acid followed by heating in an oven at 105ºC was used as a derivatizing agent to visualize the spots in white light.

g) **Evaluation**

The Rf value of the spots developed on chromatogram were calculated as under

\[
Rf = \frac{\text{Distance travelled by spot}}{\text{Distance travelled by mobile phase}}
\]

(Sethi, 1999)

4.2.5 **Gas Chromatography - Mass Spectroscopy profile of Cinnamon oil and Kapur tulsi oil**

Gas Chromatography has been the method of choice for analysis of essential oils for many years (Tabacchi and Garnero, 1987). The constituents of essential oils are identified using a combination of different GC techniques, including GC in combination with mass spectrometry. GC-MS is the most powerful technique used to identify the components present in the oils (Adams, 1995).

The selected oils namely cinnamon oil and kapur tulsi oil on the basis of antifungal activity against *Malassezia furfur* were analysed by GC-MS technique for identification of their chemical constituents. Gas chromatograph- Mass Spectrometer (Model- Polaris Q, Thermoelectron Corporation, Germany) equipped with DB-5 column (30 m x 0.25 mm x 0.25 mm) was used for analysis. The oven temperature was programmed as isothermal at
40^\circ C for 1 min, then raised to 250^\circ C at 6^\circ C/min and held at this temperature for 4 min. Helium was used as carrier gas at the rate of 1.0 ml/min. Effluent of GC column was introduced directly into the source of the MS via a transfer line. Ionization voltage was 70ev and ion source temp was 230^\circ C. Scan range was 41-450 amu.

The constituents were identified by comparison of their retention indices with literature values and their mass spectral data with those from the Wiley mass spectral library.

4.2.6 Formulation of an Antidandruff Shampoo

4.2.6.1 Selection of appropriate shampoo base for formulation

Various shampoo bases were formulated and compared in terms of physical stability and foam height/stability. The different shampoo bases that were under trial are as follows:

a) Coconut oil base (B_1)
b) Liquid cream shampoo (B_2)
c) Clear liquid shampoo (B_3)

4.2.6.2 Formulation of Coconut oil shampoo base (B_1)

**Formula for 100 ml base**

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coconut oil</td>
<td>15.0 ml</td>
</tr>
<tr>
<td>Palm oil</td>
<td>5.0 ml</td>
</tr>
<tr>
<td>Potassium hydroxide 90%</td>
<td>3.0 ml</td>
</tr>
<tr>
<td>Sodium hydroxide 90%</td>
<td>1.0 ml</td>
</tr>
<tr>
<td>Sodium lauryl sulphate</td>
<td>69.0 ml</td>
</tr>
<tr>
<td>Ethyl alcohol</td>
<td>7.0 ml</td>
</tr>
</tbody>
</table>

**Method**

Heat coconut oil and palm oil separately. Add both the oils to potassium hydroxide and sodium hydroxide with slow agitation. Pour the mixture to sodium lauryl sulphate and mix the contents with constant stirring. Cool to add ethyl alcohol.

4.2.6.3 Formulation of Clear liquid shampoo base (B_2)

**Formula for 100 ml shampoo formulation –**

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Purified water</td>
<td>46ml</td>
</tr>
<tr>
<td>Sodium lauryl ether sulphate</td>
<td>46ml</td>
</tr>
</tbody>
</table>
Cocodiethanolamine 1ml
Cocobitaine 6ml

**Method**
Add SLES to purified water with constant stirring. Stir for 10 mins. Then add cocodiethanolamine into it with stirring. When the contents are mixed properly add cocobitaine into it.

### 4.2.6.4 Formulation of Cream shampoo base (B₃)

**Formula for 100 ml shampoo formulation**

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Purified water</td>
<td>33.0 ml</td>
</tr>
<tr>
<td>Sodium hydroxide</td>
<td>0.4 ml</td>
</tr>
<tr>
<td>EDTA</td>
<td>0.1 ml</td>
</tr>
<tr>
<td>SLS (30%)</td>
<td>50 ml</td>
</tr>
<tr>
<td>Coconut fatty acids</td>
<td>3.0 ml</td>
</tr>
<tr>
<td>Stearamide DEA</td>
<td>5.0 ml</td>
</tr>
<tr>
<td>Lauramide DEA</td>
<td>5.0 ml</td>
</tr>
<tr>
<td>Glycol stearate</td>
<td>4.0 ml</td>
</tr>
</tbody>
</table>

**Method**
Sodium hydroxide, EDTA, coconut fatty acids is added to 20ml water, mixed and heated at 55-60ºC to form the soap. This soap solution is then diluted with additional water. Then add stearamide DEA, lauramide DEA and glycol stearate. The contents are heated for 5 min for mixing completely.

### 4.2.7 Stability evaluation of formulated shampoo bases

To check the physical stability, all the three bases were divided into parts. One part of each base was kept at 45ºC and other part at 5ºC for a period of one month. After one month the bases were evaluated for the organoleptic properties, like any change in appearance, colour and odour and for phase separation. Bases were also evaluated for the foam volume and stability, as it is one of the most important criterions in shampoo’s performance.
On basis of results of above tests, final base was selected for the formulation of antidandruff shampoo.

4.2.8 Formulation of antidandruff shampoo

On the basis of antifungal studies and stability evaluation of different bases (B₁, B₂, B₃), antidandruff shampoo was formulated using formula as under-

For 100ml formulation-

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cinnamon oil</td>
<td>0.5 ml</td>
</tr>
<tr>
<td>Kapur tulsi oil</td>
<td>0.5 ml</td>
</tr>
<tr>
<td>Sodium lauryl ether sulphate</td>
<td>46 ml</td>
</tr>
<tr>
<td>Cocodiethanolamine</td>
<td>6 ml</td>
</tr>
<tr>
<td>Cocobetaine</td>
<td>1 ml</td>
</tr>
<tr>
<td>Purified water</td>
<td>46 ml</td>
</tr>
</tbody>
</table>

Method

Add cinnamon oil and kapur tulsi oil to purified water with constant stirring. To this, add SLES with constant stirring. Stir for 10 minutes and then add cocodiethanolamine into it with stirring. When properly mixed, add cocobetaine and mix thoroughly. pH should vary between 5 - 6.

4.2.9 Evaluation of antidandruff shampoo formulation

4.2.9.1 Evaluation of formulated shampoo for the ideal properties as prescribed by Bureau of Indian Standards in IS 7884:2004

Collection of hair wig

Artificial hair wig (non remi virgin hair) was procured from one of the beauty saloon in Ambala cantt, which was required to evaluate ideal properties of the shampoo formulation. Selection of hair type was based on the fact that normal non-remi hair is original human hair and is the least expensive. These are suitable to check the performance of hair care products including shampoos, as they are free from any chemical or acid treatments, which is generally given to make tangle free hair. Generally isolated human hairs are subjected to acid bath for removing the cuticle and for reducing the friction among hair. But to test the
performance of shampoo virgin hair (which are free from any chemical treatment) are mostly preferred.

According to Bureau of Indian Standards the ideal properties a shampoo must possessed are mentioned below. The shampoo formulation was evaluated for the ideal properties.

a) **Ease of application**: The shampoo formulation was observed visually and keeping it on the hand to evaluate that the shampoo should be viscous enough to stay in the hands before application to the hair and scalp and during application, the shampoo must spread easily and disperse quickly over the head and hair.

b) **Rinsing**: The hair were washed with water and it was observed that the shampoo should rinse out easily and should not leave a residual tackiness or stickiness and should not form a dulling film on the hair.

c) **Easy wet combing**: After washing the hair they were dried with clean and dry cotton towel and subjected to combing with regular nylon comb. Ideally the hair should comb through easily without tangling.

d) **Manageability**: The hairs were combed after they get dried to observe that the hair should be left in a manageable condition when combed dry. There should not be fly away or frizziness.

e) **Luster**: The hairs were observed for having luster, as ideal shampoo should impart luster to the hair.

f) **Body**: The hair should have body when dried. That is hair should not limp or over conditioned. The hairs were observed for having body after getting dried.

g) **Fragrance**: The shampoo was observed that it should not contain any objectionable odor and should leave fresh refreshing scent on the hair. This could be the major factor in consumer acceptance of the product.

h) **Economical**: While making formulation the stress was given to this specification to make the product as simple as possible. Shampoo formulation was developed using only those raw materials that were necessary to accomplish the desired goal.
4.2.9.2 Evaluation of shampoo formulation by official methods as prescribed by Bureau of Indian Standards in IS 7884:2004 (for surfactant based shampoo)

a) Determination of non-volatile alcohol soluble matter in shampoo

Procedure: About 10g shampoo formulation was weighed and transferred into 150 ml beaker. It was evaporated on a steam bath to almost complete dryness. The residue was digested with 50 ml of 96 % ethyl alcohol by heating on a steam bath for about 2 min. Filtered the hot alcoholic solution through a sintered glass filter fitted to buchner flask. Washed the beaker and the residue five times with 30 ml portions of ethyl alcohol.

Transferred the filtrate in the buchner flask to a weighed, wide mouth flat-bottomed flask. Evaporated nearly to dryness on a water bath and dried off the remaining alcohol by rotating the flask on water bath. Heated the flask in a hot air oven at a temperature of 105ºC, until constant mass. Calculated mass percent of residue obtained.

\[
\text{Mass, percent of residue (Y)} = \frac{\text{Mass of residue obtained} \times 100}{\text{Mass, in gram, of the material taken for test}}
\]

Dissolved the residue in 50 ml of distilled water and added two drops of methyl red indicator solution in it. Titrated the solution with silver nitrite solution using the same quantity of all reagents except the sample.

Calculated the chloride content in shampoo in terms of molecular mass of sodium chloride (X) in percent by the formula:

\[
\text{Sodium Chloride in percent (X)} = \frac{V \times 0.5844}{M}
\]

Where

\(V=\) volume, in ml, of standard silver nitrate solution required for the material minus volume in ml, of standard silver nitrate solution required for the blank

\(M=\) mass, in gm, of the material taken for test.

To calculate percent non-volatile alcohol soluble matter, subtract the mass percent of sodium chloride determined as (X) from the mass percent of the residue (Y) obtained.
Percent non-volatile alcohol soluble matter = Y - X

b) Determination of pH of shampoo
The pH of shampoos has been shown to be important for improving and enhancing the qualities of hair, minimizing irritation to the eyes and stabilizing the ecological balance of the scalp.

Procedure: The pH of the 10% solution of shampoo formulation in distilled water was determined at 25°C using pH meter.

c) Determination of foam height (foam volume and stability)
Although foam generation has little to do with the cleansing ability of shampoos, yet it is of paramount importance to the consumer and is therefore an important criterion in evaluating shampoos.

Procedure: The artificial sebum was prepared for the test. The sebum composition was chosen to include a variety of functional groups similar to that in actual sebum (Gloor, 1978). The actual formula for preparing artificial sebum in this study was olive oil 20%, coconut oil 15%, oleic acid 15%, paraffin wax 15% and cholesterol 20%.

Foam volume was tested by using the blender method. The test was carried out in distilled water, hard water and using standard soil (artificial sebum). A quantity of 0.25 ml of sebum in hexane was used for 4 g of the shampoo. 40 ml of 10 % shampoo solution in distilled water was blended for 5 sec in a kitchen hand blender. The height of the foam generated was measured immediately and after 3 min. The test was repeated in hard water and in the presence of artificial sebum.

4.2.9.3 Evaluation of shampoo formulation by other official and unofficial method

a) Detergency evaluation

Procedure: For this test tresses of non-remi hair were used. The hair tresses were prewashed with 5% SLS solution, dried and cut into 10 inch, 3g swatches. The hair swatch (3g) was suspended in 20ml of a 10% sebum solution in hexane for 15 min with intermittent shaking. The swatch was removed, the solvent evaporated at room temperature and the hair swatch weighed to determine the sebum load. Fifteen
swatches were treated similarly and the soil levels were found to range from 9.96 to 11.05%. Each swatch was then split into two equal samples of 1.5g each: one for the shampoo treatment and the other to act as an internal control to overcome the tress-to-tress variation in soil levels. The control swatch was left untreated. The test swatch was washed with 0.1ml of a 10% shampoo solution using the finger method. It was then dried using hair dryer and further dried in an oven at 60°C for 4 hrs to ensure uniform moisture content. The sebum remaining in the test swatch after shampooing and that in the unwashed control swatch, was then extracted using 20 ml of hexane in stopper flask for 30 min on a rotary shaker. The hexane solution was then evaporated to dryness and the sebum extracted from the test and control swatches was weighed. Detergency was evaluated as percentage of sebum removed after shampooing.

\[
\text{Detergency} = 100 \cdot \left(1 - \frac{T}{C}\right) \times 100,\text{ where } T = \text{weight of sebum in test swatch} \text{ and } C = \text{weight of sebum in control swatch.}
\]

b) Wetting test
Procedure: The rate of wetting or the wetting ability of surface-active agents is commonly used to determine their comparative efficacies. The canvas disc wetting test method was used in this study to determine the wetting ability of the shampoo formulation.

Different types of canvas were tried for the test and the one that gave an effective balance between time saving and testing efficiency was chosen for the test. The canvas was cut into 1 inch diameter discs having an average weight of 0.44 g. The disc was immersed just below the surface of a 1% shampoo solution and the stopwatch started. The time required for the disc to begin to sink was noted as the wetting time.

c) Measurement of surface tension
Surface tension of the shampoo formulation was measured by Drop weight method using stalagmometer. Measurement was carried out with a 10% shampoo dilution in distilled water at room temperature (Bahl et al., 2006). Formula applied here was:
\[
\frac{R_1}{R_2} = \frac{M_1}{M_2}
\]

Where \(R_1\) is a surface tension of unknown sample, \(R_2\) is a surface tension of reference (water), \(M_1\) is a mass of sample drops and \(M_2\) is a mass of water drops.

d) **Viscosity profile**

Product rheology plays an important role in defining and controlling many attributes such as shelf life stability and product aesthetics such as clarity, ease of flow on removal from packaging and spreading on application to hair and product consistency in the package.

The flow characteristics of non-newtonian materials are usually not measured with a single data point, because their viscosity is dependent on the shear rate. The best approach is to take multipoint measurements.

**Method**

The viscosity profile of the shampoo formulation was measured using the Brookfield Synchroelectric viscometer model RVT at 25°C. The viscosity profile was recorded at different r.p.m, at 0.5, 1, 2.5, 5, 10, 20, 50 and 100 r.p.m.

**e) Conditioning effects on isolated human hair by Scanning Electron Microscopy**

The conditioning effect is one of the most important attributes of a modern shampoo. The term conditioning implies that the shampoo must leave the hair smooth, lustrous and easily manageable. The scanning electron microscope (SEM) is a valuable tool to demonstrate the effects of different shampoos. It is known that cuticle cells overlap in a telescopic fashion over the central cortex and it is on these cuticle cells that the effects of conditioning are seen. This implies that the lesser the uplift of the scales, the better the conditioning effects of shampoo on hair.

In this study the conditioning effect of the shampoo formulation was studied using SEM. And also the conditioning effects of the shampoo formulation were compared with the conditioning effects of marketed shampoos. Two marketed
shampoos of popular brands were selected for this study and coded as MS\textsubscript{1} and MS\textsubscript{6}.

**Method**

**Hair samples:** Hair sample were collected from 25 year old volunteer, whose hair were maintained at an average length of 30 cm. The hair had never been bleached, permed, dyed or subjected to any other treatments except for the regular use of a shampoo and conditioner. The hair sample was collected by retrieving those that fell out naturally during combing from the same head. The hair were divided into three groups (1) Control (2) Test shampoo treated (10%) (3) Marketed shampoo treated with formulation MS\textsubscript{1} and MS\textsubscript{6}.

Hair samples in the control group were left untreated to see the original condition of the hair. Hair strands in the other two groups were subjected to the wash cycle. The hair strands were immersed in 20 ml of the shampoo solution and agitated mildly on a rotary shaker for 10 min. The solution was siphoned off and the hair strands were subjected to rinsing for a period of 1 min with 20 ml of distilled water and repeated once. The rinse water was siphoned off and the cycle repeated 20 times. Finally the hair was dried by gently pressing between pieces of filter paper.

**Scanning Electron Microscopy (SEM)**

Surface morphology of the hair was examined by Scanning Electron Microscopy (Leo 430, Leo Electron Microscopy Ltd., England). The hair samples were mounted directly on the SEM sample stub, using double side stitching tape and coated with gold film under reduced pressure. The photomicrographs were taken with the hair oriented with the axis of the hair shaft at an angle of 45\degree to the incident electron beam, but with the tip end of the hair facing the electron beam. The micrographs were taken at three different magnifications- 500x, 1500x and 3000x. The results were most satisfactory and informative at magnification 1500x. Any gross changes in the hair structure could be seen at this magnification. The 3000x magnification gave a close up view of the individual scales and its fine surface details. For our purpose, we found 1500x to be the most satisfactory and informative magnification. The photomicrographs were taken for hair from control, test shampoo treated and marketed shampoo treated groups.
4.2.10 Comparison of the shampoo formulation with some leading brands of shampoos available in the market

The shampoo formulation was compared with the leading brands of antidandruff shampoos available in the market. This part of study was based to compare the effectiveness of the shampoo formulation with already established brands in the market. Six marketed shampoos were selected for the study. The codes were given to antidandruff shampoo formulated under this study and to all five brands of marketed shampoos as TS, MS\(_1\), MS\(_2\), MS\(_3\), MS\(_4\), and MS\(_5\) and MS\(_6\) respectively. The marketed shampoos were evaluated for amount of non-volatile alcohol soluble matter, pH, foam volume and foam stability, detergency, surface tension, percentage of solid contents, viscosity, wetting ability and conditioning effect on hair. The results obtained for these tests were compared.

4.2.11 Safety evaluation tests of the shampoo formulation

As mentioned in IS 4011:1997 which specified “Safety evaluation of cosmetics.” It included-

(a) Draize eye irritation test on rabbits

(b) Draize skin irritation test on rabbits

4.2.11.1 Draize Eye Irritation Test

The objective of Eye irritation test in rabbit was to evaluate the eye irritancy potential of antidandruff shampoo formulation on the basis of its ability to cause injury to the cornea, iris and conjunctivae when applied to the eye.

Test animals

Newzealand white (1.5-2 Kg) male rabbits were used for the study. They were procured from the in-house bred animals after approval by IAEC under project BIO- IAEC 394-12/01. The animals were housed singly in a stainless steel wire mesh cages at an ambient temperature of 22 ± 3°C and standard 30-70 % relative humidity with 12 hours fluorescent light and 12 hours dark cycle. The animals were acclimatized for minimum of seven days to laboratory diet (Nutrilab rabbit feed). Clean and potable water was provided *ad libitum* throughout the acclimatization and experimental period.
Procedure
A series of 12 animals was used for testing. The animals were divided into two groups G1 and G2 consisting of six animals in each group. Both eyes of each experimental animal were examined for 24 hours before treatment starts. Only those animals showing absence of signs of eye irritation / inflammatory changes or ocular defects were selected. Eye irritancy potential of a substance was evaluating on the basis of its ability to cause injury to the cornea, iris and conjunctivae when the substance applied to the eye.

On treatment day, 0.1 ml of 1% antidandruff shampoo formulation in distilled water was instilled into the left eye of each animal, after gently pulling the lower lid away from the eyeball. The right eye was remaining untreated and served as the reference control. The rabbits were fixed in rabbit-holder during application.

In the G1 group, the treated eyes were washed with 20 ml of luke warm water (37 ±1°C), 2 seconds after instillation of test shampoo formulation. In the G2 group, the treated eyes were washed with 20 ml of luke warm water, 4 seconds after instillation of the test shampoo formulation.

Observations
a) Body Weight
Body Weight of animals under study were recorded on the day of application (day 1) and on 7th day.

b) Clinical Signs and Mortality
Rabbits were observed once daily for clinical signs and mortality throughout the experimental period. The eyes of each animal were examined at 24, 48 and 72 hours, 4th day and 7th day after application. A preparation that elicits corneal and iris lesions, which were not cleared by the seventh day, would be considered as a severe eye irritant.

(c) Scoring
The cornea was scored on the basis of density of opacity and total area involved. The iris was scored on the intensity or degree of inflammation exhibited; and the
conjunctiva was scored on the extent of chemosis, redness and discharge. The Draize scale for scoring ocular lesions is given in Table 4.

Table 4: Draize scale for scoring lesions

1. CONJUNCTIVA

A. Redness (refers to palpebral and bulbar conjunctivae excluding cornea and iris only)

<table>
<thead>
<tr>
<th>Vessels normal</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vessels definitely injected above normal</td>
<td>1</td>
</tr>
<tr>
<td>More diffuse deeper crimson red, individual vessels not easily discernible</td>
<td>2</td>
</tr>
<tr>
<td>Diffuse beefy red</td>
<td>3</td>
</tr>
</tbody>
</table>

B. Chemosis

<table>
<thead>
<tr>
<th>Any swelling above normal (includes nictitating membrane)</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Obvious swelling with partial eversion of the lids</td>
<td>2</td>
</tr>
<tr>
<td>Swelling with lids about half closed</td>
<td>3</td>
</tr>
<tr>
<td>Swelling with lids about half closed to completely closed</td>
<td>4</td>
</tr>
</tbody>
</table>

C. Discharge

<table>
<thead>
<tr>
<th>Any amount different from normal (does not include small amount observed in inner canthus of normal animals)</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Discharge with moistening of the lids and hair just adjacent to the lids</td>
<td>2</td>
</tr>
<tr>
<td>Discharge with moistening of the lids and hair and considerable area around the eye</td>
<td>3</td>
</tr>
</tbody>
</table>

Score (A + B + C) x 2 = Total possible maximum = 20

2. IRIS

D. Values

<table>
<thead>
<tr>
<th>Normal</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Folds above normal, congestion, swelling (any one or all of these or combination of any thereof), iris still reacting to light, (sluggish reaction is positive)</td>
<td>1</td>
</tr>
</tbody>
</table>
No reaction to light, haemorrhage, gross destruction (any one or all of these).............. 2

Score D x 5  
Total possible maximum = 10

3.  CORNEA  
Score

E.  Opacity – Degree of density (area taken for reading)

No opacity................................................................................................................................. 0
Scattered or diffuse area – details of iris clearly visible...................................................... 1
Easily discernible translucent areas, details of iris slightly obscured................................. 2
Opalescent areas, no details of iris visible, size of pupil barely discernible ...................... 3
Opaque, iris invisible.................................................................................................................. 4

F.  Area of Cornea Involved

One quarter (or less) but not zero............................................................................................ 1
Greater than one quarters - less than one - half............................................................... 2
Greater than one - half but less than three quarters...................................................... 3
Greater than three quarters up to whole area....................................................................... 4

Score  E x F x 5  
Total possible maximum = 80

Interpretation of results

The numeric values corresponding to each animal, tissue and observation time was recorded. The data relating to the conjunctivae was designated by the letters A (redness), B (chemosis) and C (discharge), those relating to the iris designated by the letter D and those relating to the cornea by the letters E (degree of opacity) and F (area of opacity).

The total score for the eye was the sum of all scores obtained for the cornea, iris and conjunctivae. The total possible score would be 110. The critical score was about 30. Score exceeding 30 tend to represent irreversible damage. Scores less than 30 tend to clear away, leaving no visible sign of damage. A preparation eliciting more than mild reaction that lasts for more than 2 days on the ocular mucosa would not be considered safe as an eye area cosmetic.

4.2.11.2 Draize skin irritation Test
The objective of this skin irritation test in rabbit was to assess the possible skin irritation effects likely to arise from exposure of the skin to the test shampoo formulation following single application. This study provided a rational basis of risk assessment in man.

**Test animals**

Newzealand white (1.5-2 Kg) male rabbits were used for the study. They were procured from the in-house bred animals after approval by IAEC under project BIO- IAEC 394-12/01. The animals were housed singly in a stainless steel wire mesh cages at an ambient temperature of 22 ± 3°C and standard 30-70 % relative humidity with 12 hours fluorescent light and 12 hours dark cycle. The animals were acclimatized for minimum of seven days to laboratory diet (Nutrilab rabbit feed). Clean and potable water was provided *ad libitum* throughout the acclimatization and experimental period.

**Procedure**

A set of 6 animals was used for testing. Two positions on the dorsal area of the trunk were designated for the patch on each animal. One patch will be used for the application of positive control and another patch for the application of test shampoo formulation.

**Preparation of animals**

Approximately 24 hours before the treatment initiation, hair was carefully removed from the dorsal area of the trunk of the all test animals by clipping without abrading the skin. Only animals with a healthy intact epidermis by gross observation were selected for the experimentation.

**Dose Formulation**

Test shampoo formulation was diluted to a test concentration of 10% using distilled water and 20 % of sodium lauryl sulphate (positive control) was prepared using distilled water for application of test shampoo formulation.

On test day, two areas of the back, spaced approximately 2-3 cm apart were designated for the position of the patches. 0.5 ml of the diluted test shampoo formulation (10%) was applied at posterior patch and 20% sodium lauryl sulphate (positive control) was applied at anterior patch simultaneously to each rabbit. Each patch was covered with approximately 10 cm² (2 x 5 cm) of 12-ply cotton gauze. The test patches were held in their respective
positions with non-irritating adhesive tape and wrapping the whole trunk with a cotton bandage. The test shampoo formulation was kept in contact with skin for 4 hours. At the end of contact period, the protective covering and patches were removed and applied area was wiped with distilled water and dried with tissue paper and local skin reactions were recorded.

**Observations:**

**Body Weight**

Body weight of animals under study was recorded, on the day of application and at termination.

**Clinical Signs and Mortality**

Animals were observed once daily for clinical signs and mortality till the termination of the experiment.

**Grading of skin reactions**

The test patches were removed after 4 hours of application and the skin sites were scored at post removal (0 hour) of the test patch and at 48 hour later using following scale given in Table 5.

<table>
<thead>
<tr>
<th><strong>Table 5: Grading of skin reactions</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>1. Erythema and Eschar formation</strong></td>
</tr>
<tr>
<td>No erythema ..........................................................</td>
</tr>
<tr>
<td>Very slight erythema (barely perceptible). ................................</td>
</tr>
<tr>
<td>Well-defined erythema. ........................................................</td>
</tr>
<tr>
<td>Moderate to severe erythema..........................</td>
</tr>
<tr>
<td>Severe erythema (beef redness) to slight eschar formation (injuries in depth)....</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>2. Oedema formation</strong></th>
<th><strong>Score</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>No oedema ..........................................................</td>
<td>0</td>
</tr>
<tr>
<td>Very slight oedema (barely perceptible) ................................</td>
<td>1</td>
</tr>
<tr>
<td>Slight oedema (edges of area well defined by definite raising).................</td>
<td>2</td>
</tr>
</tbody>
</table>
Moderate oedema (raised approximately 1 millimetre) .............................................. 3
Severe oedema (raised more than 1 millimetre and extending beyond area of exposure) .......................................................................................................................... 4

**Interpretation of results:**

Calculation of Primary Irritation Index and Grading of Irritancy Potential using the Draize Scheme:

The scores for erythema and oedema at the 4-hour post removal of the test patch and 48-hour readings were totaled for the 6 test rabbits and this total was divided by 6 and multiplied by 2 (number of observation) i.e., 6/12 to give the primary irritation index of the test shampoo formulation. The test shampoo formulation would be classified according to the following scheme devised by Draize (1959).

<table>
<thead>
<tr>
<th>Primary Irritation Index</th>
<th>Classification of Irritancy</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Non-irritants</td>
</tr>
<tr>
<td>&gt; 0 to 2</td>
<td>Mild irritant</td>
</tr>
<tr>
<td>&gt; 2 to 5</td>
<td>Moderate irritant</td>
</tr>
<tr>
<td>&gt; 5</td>
<td>Severe irritant</td>
</tr>
</tbody>
</table>

4.2.12 **Stability studies of the shampoo formulation**

Stability and acceptability of organoleptic properties (odour, color) of shampoo formulation during the storage period indicate that it is chemically and physically stable. According to the specification given by Bureau of Indian Standards, the clear liquid shampoo when examined visually, should be free from any sedimentation. There should be no visible sign of phase separation and should be free from any agglomerated particles when formulated and the formulation should retain the properties during its shelf life also.

4.2.12.1 **Physical stability of the shampoo formulation**

**Method**

To test the physical stability of shampoo formulation, samples were placed at temperature 5°C, 45°C and at room temperature for duration of six months. After a period of one-month physical stability of samples of the shampoo formulation was inspected. The physical stability was inspected for three months for any change in the appearance of the
formulation, pH, foam height, detergency, and viscosity. These tests were repeated after a period of six months.

### 4.2.12.2 Chemical stability of the shampoo formulation

To test the chemical stability of the shampoo formulation, following methods were used:

#### 4.2.12.2.1 TLC finger print profile of shampoo formulation

To evaluate the chemical stability of the shampoo formulation, the TLC finger print profile of freshly prepared shampoo formulation was compared with the TLC finger print profile of the shampoo formulation after six months.

**Procedure**

The sample for TLC were prepared for both fresh shampoo formulation and also the formulation kept for stability studies for six months. The solution for both was prepared by dissolving 0.1 ml of the formulation in 10 ml methanol. The solvent system used as mobile phase was toluene and ethyl acetate in 9.7: 0.3. The chromatogram of fresh formulation was compared with the chromatogram of shampoo formulation after six months.

#### 4.2.12.2.2 TLC Densitometry

**Procedure**

The thin layer chromatogram of freshly prepared shampoo formulation and shampoo formulation after six months, developed on the precoated TLC plates was subjected to scanning through TLC densitometry under UV at 254 nm using CAMAG TLC scanner 3 and CATS software version 4.06.

### 4.2.13 Evaluation of shampoo formulation for antifungal activity and its comparison with the antifungal activity of marketed shampoo formulations

The test shampoo formulation TS and marketed shampoos MS\(_1\) containing standard antifungal agent ketoconazole and MS\(_2\) containing tea tree oil and rosemary oil as antifungal agent, were evaluated for antifungal activity against *Malassezia furfur* by Disc diffusion method. The results of antifungal activity of test formulation and marketed
formulations MS₁ containing ketoconazole and MS₂ containing herbal ingredients were compared to check the effectiveness of test shampoo formulation against dandruff.

**Procedure**
Antifungal effects of TS, MS₁ and MS₂ were evaluated by Disc diffusion method. The broth culture of *Malassezia furfur* was swabbed over the Sabourauds dextrose agar medium by using sterile cotton buds. *M. furfur* was developed as white to tan cream in colour and smooth pasty yeast like appearance over the medium. Sterile filter paper discs (6 mm diameter Whatman no. 32) impregnated with samples to be tested were placed in the center of separate plates containing medium. Three replicates were maintained for each sample. The plates were incubated at 32°C and the zone of inhibition was observed after 4 days. Control was maintained with filter paper discs dipped in sterile WFI.

**4.2.14 Determination of minimum inhibitory concentration (MIC) of shampoo formulation**
The MIC of the shampoos showing antifungal activity was determined by the micro dilution broth method (National Committee of Clinical Laboratory Standard, 1993). A stock solution of 25.6 mg/ml of the shampoo was prepared in Mueller Hinton broth (Difco). Further, serial double dilutions were made in a range from 25.6 mg/ml to 0.05 mg/ml. 100 µl aliquot of each dilution and Mueller Hinton broth with positive and negative controls were put in the wells of a microtiter plate. The inoculated microtiter plates were then incubated at 37°C for 72 h. After examining turbidity visually, 40 µl of 0.02 mg/ml 2, 3, 5 triphenyl tetrazolium chloride (TTC) was added to each microplate well and incubated at 37 °C and re-examined after 30 mins. The MIC was calculated as the lowest concentration of the shampoo that prevented growth of the culture. All samples were examined in duplicate in three separate experiments. The MIC of these shampoos was compared with the MIC of marketed antidandruff shampoo of herbal origin and marketed antidandruff shampoo containing ketoconazole, both taken as standards against *M. furfur*. 