4. MATERIALS AND METHODS

4.1 Market survey to justify the topic of research:

Survey of Ashwagandha and Kalmegh products- A brief survey of stores dealing with Ashwagandha and Kalmegh products were carried out to gain information about the possibilities of microbial decontamination of the above products. The method followed is - Questionnaire method. A set of 22 questions were framed and individually the chemist and druggist were contacted, explained in detail and information collected. Data is analysed and graphical interpretation is done for relevant questions. (Annexure I attached).

4.2 Plant material Collection and authentication:

➢ Collection of samples: Roots of Ashwagandha and whole plant of Kalmegh has been collected from Natural Remedies and Pentacare Ayur Pharma, Bangalore.

➢ Both samples are subjected to aqueous extraction. Different methods have been tried out – like soxhlet, reflux and maceration methods – Soxhlet method of extraction has been found to give better yield. Hence this method is preferred for extraction.

➢ Ashwagandha and Kalmegh collected have been authenticated from University of Agricultural sciences, Bangalore. (Annexure II - Copy attached).

4.3 Gamma Irradiation treatment at doses of 5kGy and 10kGy:

Root powder of Ashwagandha and whole plant powder of Kalmegh were divided into 3 portions of 100gms each and packed individually in sterile polyethylene bags. Gamma irradiation was carried out at a commercial scale Cobalt 60 irradiation service facility, loaded with Cobalt-60 source with known strength, owned and operated by
Materials and Methods

Microtrol Sterilisation services Pvt. Ltd. at Bangalore. The applied dose levels were 5 and 10kGy and this absorbed dose was monitored with ceric/cerous dosimeters. (Annexure III attached).

For some studies, directly the non-irradiated and gamma irradiated powders were used and for some the non-irradiated and gamma irradiated aqueous extracts of both the plants were used.

Storage: All samples were stored at room temperature for further analysis at 0, 6 and 12th month after irradiation.

4.4 Preparation of Extracts:

Extracts of non irradiated and gamma irradiated samples were prepared during 0, 6 and 12th month, by soxhlet method using water as solvent. Initially, all samples were defatted with petroleum ether and then subjected to extraction.

Procedure for Defatting: 100g of air-dried powdered materials of all the samples were defatted by refluxing with 250ml petroleum ether (60°-80°) for 4 hrs. The residue was dried and then subjected to extraction by Soxhlet method using water as the solvent.

Procedure:

Extraction by Soxhlet method: 100g of defatted powdered materials of Ashwagandha and Kalmegh irradiated and non-irradiated samples were subjected to soxhlet extraction with 250ml water for 12 hrs. The extracts were concentrated by distilling the solvent at low temperature. They were further kept in polythene covers at room temperature for further analysis.
Non irradiated and gamma irradiated Ashwagandha and Kalmegh samples and aqueous extracts were stored in polythene covers at room temperature in a cool and dry place for analysis at 0, 6 and 12 months of storage. Further samples are identified as below (Table 4). So this sample identity will be followed for rest of the work.

**Table 4 - Sample identity**

<table>
<thead>
<tr>
<th>Sl. No</th>
<th>Samples</th>
<th>Sample Identity</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>Ashwagandha non-irradiated sample</td>
<td>A1</td>
</tr>
<tr>
<td>2</td>
<td>Ashwagandha gamma irradiated sample – dose 5kGy</td>
<td>A2</td>
</tr>
<tr>
<td>3</td>
<td>Ashwagandha gamma irradiated sample – dose 10kGy</td>
<td>A3</td>
</tr>
<tr>
<td>4</td>
<td>Kalmegh non-irradiated sample</td>
<td>K1</td>
</tr>
<tr>
<td>5</td>
<td>Kalmegh gamma irradiated sample – dose 5kGy</td>
<td>K2</td>
</tr>
<tr>
<td>6</td>
<td>Kalmegh gamma irradiated sample – dose 10kGy</td>
<td>K3</td>
</tr>
</tbody>
</table>

4.5 Comparative analysis of non-irradiated and gamma irradiated samples of Ashwagandha and Kalmegh at 0, 6 and 12 months of storage.

4.5.1 Pharmacognostical evaluation – Morphological analysis:

Morphological comparison of non-irradiated and gamma irradiated powder samples of Ashwagandha and Kalmegh was carried out. All samples were subjected to macroscopical identification based on colour, odour, taste and form of the drug and the morphological features of the powdered drug were compared with standard descriptions given in Indian Herbal Pharmacopoeia.\(^3,6\)

4.5.2 Powder Microscopical analysis during 0, 6 and 12 months of storage:

Further, powder microscopical analysis of non-irradiated and gamma irradiated powder samples of Ashwagandha and Kalmegh was carried out and all the samples were in accordance with the Indian Herbal Pharmacopoeia.\(^3,6\)
Materials and Methods

All samples were subjected to powder microscopy by the following method:

1. Small quantities of powder of each sample were taken in separate watch glasses. To this a few drops of chloral hydrate was added and heated for 2-3 minutes. Small amount of clarified drug was mounted on a slide with a drop of glycerin, covered with cover slip and observed under microscope for presence of calcium oxalate crystals.

2. To visualize lignified tissues, 1:1 ratio of phloroglucinol : concentrated Hydrochloric acid was added to the clarified mixture. After 2-3 minutes, small amount of drug was mounted on a slide with a drop of glycerin, covered with cover slip and observed under microscope.

3. To visualize starch grains, dilute Iodine solution was added to the clarified mixture. After 2-3 minutes, small amount of drug was mounted on a slide with a drop of glycerin, covered with cover slip and observed under microscope.

4.5.3 Physicochemical analysis during 0, 6 and 12 months of storage:

- Gamma irradiated and non-irradiated samples of both plants were analyzed for Ash value, Extractive value and Moisture content according to IP162.

**a. Ash value:** This determines the amount of inorganic substance present in the plant sample. Total ash and acid-insoluble ash values were determined for air-dried samples.

Procedure:

**i. Total ash value:**

About 2 gm of powdered drug was weighed accurately into a tared silica crucible and incinerated at 450°C in a muffle furnace until free from carbon. The crucible was cooled and weighed. Percentage of total ash was calculated with reference to air-dried substance.
ii. Acid-insoluble ash:

Total ash obtained was boiled with 25ml of 2N HCl for few minutes and filtered through an ashless filter paper. The filter paper was transferred into a tared silica crucible and incinerated at 450\(^\circ\)C in muffle furnace until free from carbon. The crucible was cooled and weighed. Percentage of acid insoluble ash was calculated with reference to air-dried substance.

b. Extractive value: This determines the amount of active constituents present in the sample. Water soluble and alcohol soluble extractive values were determined for both gamma irradiated and non-irradiated samples of both the plants.

i. Water soluble extractive value: 5gms of powdered drug was treated with 100ml of water in a stoppered flask with frequent shaking during first 6 hours and allowed to stand for 18 hours. It was filtered after 24 hours. 25ml of the filtrate was evaporated in a tared dish and weighed. The same procedure was repeated for all the samples and percentage of water-soluble extractive value was calculated for all the samples.

ii. Alcohol soluble extractive value: 5gms of powdered drug was treated with 100ml of alcohol in a stoppered flask with frequent shaking during first 6 hours and allowed to stand for 18 hours. It was filtered after 24 hours. 25ml of the filtrate was evaporated in a tared dish and weighed. The same procedure was repeated for all the samples and percentage of alcohol soluble extractive value was calculated.

c. Moisture Content: Approximately 2gms of non-irradiated and gamma irradiated (5kGy and 10kGy) Ashwagandha and Kalmegh powders were weighed separately in weighing bottles and placed in hot air oven at 105\(^\circ\)C for 30 minutes. The weighing bottles were carefully removed, cooled in a desiccator, weighed and % of moisture content calculated and tabulated.
4.5.4 Phytochemical evaluation of Ashwagandha and Kalmegh at 0, 6 and 12 months of storage:

Qualitative evaluation of extracts.

Extracts obtained were subjected to various chemical tests to detect the chemical constituents present in them.

Procedure:

DETECTION OF ALKALOIDS

Extracts were dissolved individually in dilute Hydrochloric acid and filtered. The filtrates were used to test for the presence of alkaloids.

a) Mayer’s Test

Filtrates were treated with Mayer’s reagent (Potassium Mercuric iodide).

Formation of a yellow cream precipitate indicates the presence of alkaloids.

b) Wagner’s test

Filtrates were treated with Wagner’s reagent (Iodine in potassium iodide).

Formation of brown/reddish brown precipitate indicates the presence of alkaloids.

c) Dragendroff’s test

Filtrates were treated with Dragendroff’s reagent (solution of potassium bismuth iodide). Formation of red precipitate indicates the presence of alkaloids.

d) Hager’s test

Filtrates were treated with Hager’s reagent (saturated picric acid solution).

Formation of yellow coloured precipitate indicates the presence of alkaloids.

DETECTION OF CARBOHYDRATES

Extracts were dissolved individually in 5 ml distilled water and filtered. The filtrates were used to test for the presence of carbohydrates.

a) Molisch’s Test
Filtrates were treated with 2 drops of alcoholic α-naphthol solution in a test tube and 2 ml of Conc. Sulphuric acid was added carefully along the sides of the test tube. Formation of violet ring at the junction indicates the presence of Carbohydrates.

b) **Benedict’s test**

Filtrates were treated with Benedict’s reagent and heated on water bath. Formation of orange red precipitate indicates the presence of reducing sugars.

c) **Fehling’s test**

Filtrates were hydrolysed with dil. HCl, neutralized with alkali and heated with Fehlings A & B solutions. Formation of red precipitate indicates the presence of reducing sugars.

**DETECTION OF GLYCOSIDES**

Extracts were hydrolysed with dil. HCl, and then subjected to test for glycosides.

a) **Modified Borntrager’s Test**

Extracts were treated with Ferric Chloride solution and immersed in boiling water for about 5 minutes. The mixture was cooled and shaken with an equal volume of benzene. The benzene layer was separated and treated with ammonia solution. Formation of rose-pink colour in the ammoniacal layer indicates the presence of anthranol glycosides.

b) **Legal’s test**

Extracts were treated with sodium nitroprusside in pyridine and methanolic alkali. Formation of pink to blood red colour indicates the presence of cardiac glycosides.

**DETECTION OF SAPONINS**

a) **Froth Test**

Extracts were diluted with distilled water upto 20ml and this was shaken in a graduated cylinder for 15 minutes.
Materials and Methods

Formation of 1 cm layer of foam indicates the presence of saponins.

b) Foam test

Small amount of extract was shaken with little quantity of water. If foam produced persists for ten minutes it indicates the presence of saponins.

DETECTION OF PHYTOSTEROLS

a) Salkowski’s Test

Extracts were treated with chloroform and filtered. The filtrates were treated with few drops of Conc. Sulphuric acid, shaken and allowed to stand.

Appearance of golden yellow colour indicates the presence of triterpenes.

b) Libermann Burchard’s test

Extracts were treated with chloroform and filtered. The filtrates were treated with few drops of acetic anhydride, boiled and cooled. Conc. Sulphuric acid was added carefully along the sides of the test tube.

Formation of brown ring at the junction indicates the presence of phytosterols.

c) Tshugajeu test

Extracts were treated with chloroform and filtered. Excess of acetyl chloride and a pinch of Zinc Chloride was added, kept aside for some time till the reaction was complete and then warmed on water bath.

Appearance of eosin red colour indicates the presence of triterpenes.

DETECTION OF FIXED OILS & FATS

a) Stain Test

Small quantities of extracts were pressed between two filter papers.

An oily stain on filter paper indicates the presence of fixed oil.

DETECTION OF RESINS

a) Acetone-water Test
Extracts were treated with acetone. Small amount of water was added and shaken.

Appearance of turbidity indicates the presence of resins.

**DETECTION OF PHENOLS.**

a) **Ferric Chloride Test**

Extracts were treated with few drops of ferric chloride solution.

Formation of bluish black colour indicates the presence of phenols.

**DETECTION OF TANNINS**

a) **Gelatin Test**

To the extract, 1% gelatin solution containing sodium chloride was added.

Formation of white precipitate indicates the presence of tannins.

**DETECTION OF FLAVONOIDS**

a) **Alkaline Reagent Test**

Extracts were treated with few drops of sodium hydroxide solution.

Formation of intense yellow colour, which becomes colourless on addition of dilute acid, indicates the presence of flavonoids.

b) **Lead acetate Test**

Extracts were treated with few drops of lead acetate solution.

Formation of yellow colour precipitate indicates the presence of flavonoids.

c) **Shinoda Test**

To the alcoholic solution of extracts, a few fragments of magnesium ribbon and Conc. HCl was added.

Appearance of magenta colour after few minutes indicates presence of flavonoids.

d) **Zinc hydrochloric acid reduction Test**

To the alcoholic solution of extracts, a pinch of Zinc dust and Conc. HCl was added.

Appearance of magenta colour after few minutes indicates presence of flavonoids.
DETECTION OF PROTEINS AND AMINOACIDS

a) Xanthoproteic Test
The extracts were treated with few drops of concentrated Nitric acid solution.
Formation of yellow colour indicates the presence of proteins.

b) Ninhydrin test
To the extract, 0.25% ninhydrin reagent was added and boiled for few minutes.
Formation of blue colour indicates the presence of amino acid.

c) Biuret Test
The extracts were treated with 1 ml of 10% sodium hydroxide solution and heated. To this a drop of 0.7% copper sulphate solution was added.
Formation of purplish violet colour indicates the presence of proteins.

DETECTION OF DITERPENES

a) Copper acetate Test
Extracts were dissolved in water and treated with few drops of copper acetate solution. Formation of emerald green colour indicates the presence of diterpenes.

4.5.5 Quantitative evaluation of Ashwagandha aqueous extracts during 0, 6 and 12 months of storage:
All extracts were analysed for total alkaloids, withanolides and glycowithanolide content.

Procedure:
a. Estimation of total alkaloid content
3 gms of extracts was taken in a conical flask and treated with 100ml of mixture of 4 volumes of solvent ether and 1 volume of alcohol. To this 5 ml of dilute ammonia solution was added and frequently shaken for one hour.
Materials and Methods

The solution was filtered and taken in a separator. The residue was washed with 100ml of ether-alcohol mixture in 5 lots of 20ml each.

To this total ether alcohol solution, 30ml of 1 N Sulphuric acid was added and shaken thoroughly. It was allowed to separate. Lower layer was run off. Extraction was continued with 25ml and then with successive quantities each of 20ml of a mixture of 3 volumes of 0.5N Sulphuric acid and 1 volume of alcohol, until complete extraction of alkaloids was affected.

The mixed acid solution was washed with 10ml of chloroform. The chloroform layer is rejected and the acidic layer is made alkaline by adding excess ammonia.

This was then extracted with 25ml of chloroform, until complete extraction of alkaloids was affected.

Chloroform extract was washed with 10ml water, filtered through cotton plug and evaporated on water bath at 80°C.

The same procedure was followed for all other extracts. Total alkaloid content was calculated and results are tabulated.

b. Estimation of total withanolide content by gravimetry

5 gms of extract was taken in a separating funnel. To this 25ml of methanol and 25ml of water was added. It was defatted by extraction with 4 x 50ml hexane. This fraction was discarded. The remaining aqueous methanolic solution was extracted with 5 x 25ml ether. The ether extracts were combined, washed twice with water and evaporated to dryness to give withanolides.

c. Estimation of Glycowithanolides

The remaining aqueous solution following Withanolide estimation was extracted with chloroform:methanol:2:1 (3 times). The chloroform-methanol extracts were combined, evaporated to dryness to give glycowithanolide.
Same procedure was followed for all other samples. Glycowithanolide content was calculated.

4.5.6 Quantitative evaluation of Kalmegh aqueous extracts for total bitters as Andrographolide during 0, 6 and 12 months of storage:

All Kalmegh plant extracts were analyzed for total bitter as Andrographolide.

Procedure:

a. Estimation of total bitters as Andrographolide in Kalmegh extracts

3 gms of the extract was refluxed with 50ml of alcohol on water bath for half an hour. It was filtered and the above procedure was repeated twice. Alcohol was then evaporated and residue was repeatedly taken up with 25, 20, 15 and 15ml of hot water. This aqueous solution was shaken repeatedly with 25, 20 & 15ml of petroleum ether 60\(^0\)-80\(^0\)C in a separator and discarded. The above aqueous layer was extracted repeatedly with 25, 20 & 15ml of ethyl acetate. It was evaporated to dryness and weighed.

The same procedure was followed for all other extracts. Total Andrographolide content was calculated and results are tabulated.

4.5.7 IR fingerprint analysis of Ashwagandha and Kalmegh at 0, 6 and 12 months of storage:

Non-irradiated and gamma irradiated samples of Ashwagandha and Kalmegh were subjected to IR fingerprint analysis. IR spectra were recorded on JASCO FT-IR 460-plus spectrometer, which showed different vibration levels of molecules by using KBr diffused reflectance technique. The peaks obtained in IR spectrum gave an idea about the functional groups in the extract. IR region ranges between 4000-400cm\(^{-1}\).
4.5.8 HPTLC Analysis

Withaferin A and Andrographolide are chosen as respective standards for analysis of phytochemicals by HPTLC method in Ashwagandha and Kalmegh respectively. HPTLC method was developed for evaluation of Withaferin A in Ashwagandha and Andrographolide in Kalmegh. The method was further validated and applied to evaluate non-irradiated and gamma irradiated aqueous extracts of Ashwagandha and Kalmegh.

4.5.8.1 Development of HPTLC method for estimation of Withaferin A.

**Instruments:** CAMAG Linomat 5 sample applicator, CAMAG TLC Scanner 3, CAMAG Reprostar 3, CAMAG TLC plate heater, Twin trough chamber, Dip tank, winCATS software –version 1.3.3.

**Stationary Phase:** Merck TLC plates silica gel 60 F 254 (10x10 cm).

**Mobile phase** constituted of Chloroform: Methanol in the ratio of 9:1.

**Preparation of standard solution:** 1mg of Withaferin A was dissolved in 10ml methanol to give a concentration of 100ng / μl.

**Preparation of sample solution:** 100mg of aqueous extracts of all Ashwagandha samples were dissolved in 10ml of methanol and filtered through Whatmann No.1 filter paper.

**Procedure:**

**Standard and Sample application:** 5μl-9μl of the standard solution and 10μl of all sample solutions were applied as bands on the plate.

**Development and scanning:** The spotted plates were individually developed upto 90mm in a previously saturated Twin Trough chamber. Chamber saturation was done
for 10 minutes. The developed plate was scanned at 254nm. Spectrum analysis was done for determination of $\lambda$ max of Withaferin A in the range of 190 – 380nm using D2 lamp. The nm at which the peak obtained maximum height and area was considered as $\lambda$ max. Chromatogram was scanned at $\lambda$ max to compare the peak area of sample and standard.

### 4.5.8.2 Validation\textsuperscript{163,164} of HPTLC method for estimation of Withaferin A

The developed method was validated in terms of pre-validation, limit of detection, limit of quantification, linearity, precision and accuracy.

**i. Pre-Validation:** The stability of the standard solution was tested in the solution and on the plate.

a) **Sample solution stability:** Chromatography of the sample solutions standing for different period of time (24hrs, 2hrs, 1hr, 0.5hr, <0.1hr) before development was carried out. The sample solution was prepared and stored at room temperature. The solution was spotted after different time intervals. The chromatogram was developed and scanned at 223nm. The peak areas obtained were compared to study the variation of the standard solution.

b) **Stability of sample on plate:** Sample solution was applied to different plates and development was done after different time intervals (24hrs, 2hrs, 1hr, 0.5hr, <0.1hr). Respective chromatogram was developed and scanned at 223nm. The peak areas obtained were compared to study the variation of the standard solution on the plate.

**ii. Limit of Detection:**

Limit of Detection is the minimum concentration of the sample required to give a peak with an area of 100-300. This was established by trial and error method. The selected concentration was spotted in duplicate. The chromatogram was developed,
scanned at 223nm and evaluated to confirm the LOD. The concentration showing minimum peak area is considered as limit of detection.

**Procedure:**

1mg of Withaferin A standard was dissolved in 100ml of methanol. This gave a concentration of 10ng/µl. From this standard stock solution, 1µl - 5µl were spotted in duplicate. The chromatogram was developed as described in method development. Scanning was done at 223nm. The concentration, which gave minimum peak area was reported as LOD.

**iii. Limit of Quantification:**

Limit of Quantification is the concentration within which the compounds can be quantified in the sample. Generally the limit of quantification is three times more than limit of detection.

**Procedure:**

1mg of Withaferin A standard was dissolved in 10ml of methanol. This gave a concentration of 100ng/µl. From this standard stock solution, 1µl - 5µl were spotted in duplicate. The chromatogram was developed as described in method development. Scanning was done at 223nm. The concentration, which gave peak area, that was three times greater than LOD was reported as LOQ.

**iv. Linearity:**

Linearity of an analytical method is its ability to elicit test results that are directly or by a well-defined mathematical transformation proportional to the concentration of analytes in sample within a given range. Detection of linearity involves determination of concentration required to prepare a linear graph. Linearity is reported in terms of range, regression value, standard deviation and Y value.
**Materials and Methods**

**Procedure:**

1mg of Withaferin A standard was dissolved in 10ml of methanol. This gave a concentration of 100ng/µl. From this standard stock solution, 5µl - 9µl were spotted. The chromatogram was developed as described in method development and scanned at 223 nm. Linear graph was plotted with concentration against Area under Curve.

**v. Precision**

The precision of an analytical method is the degree of agreement among individual test results when the method is applied repeatedly to multiple samplings of a homogeneous sample. Precision is a measure of repeatability. Intra day and Inter day variations were studied. The plates were prewashed with methanol upto 9.5cm, dried and then used for spotting the sample.

**Procedure:**

**Intra day variation:** 1mg of Withaferin A standard was dissolved in 10ml of methanol. This gave a concentration of 100ng/µl. From this standard stock solution, 5µl of standard Withaferin A was spotted 6 times in the morning, developed and scanned at 223nm. The same stock solution was spotted on a fresh plate on the same day in the afternoon. The plate was developed and scanned at 223nm.

**Inter day variation:** The same method was followed but plates were spotted on two consecutive days to study Inter day variations. The chromatogram was developed and scanned at 223nm.

**vi. Accuracy**

The accuracy of an analytical method is the closeness of test results obtained by that method to the true value. Accuracy is reported in terms of % recovery.

The sample, standard and spiked sample were spotted. The chromatogram was developed and scanned at 223nm to study the % recovery.
Procedure:

5µl of sample solution, 5µl, 6µl, 7µl of standard Withaferin A and 5 µl of Ashwagandha sample spiked with 5µl, 6µl, 7µl of standard Withaferin A were spotted on the plate. The chromatogram was developed. Scanning was done at 223 nm. Areas of sample, standard and spiked samples were recorded and % recovery was calculated using the following formula.

\[
\text{% Recovery} = \frac{A + B}{C} \times 100
\]

Where,

- Area of sample = A
- Area of standard = B
- Area of sample + Area of standard (spiked) = C

4.5.8.3 Application of the validated method to quantify Withaferin A in non-irradiated and gamma irradiated aqueous extracts of Ashwagandha at 0, 6 and 12 months of storage:

The validated HPTLC method was applied to quantify Withaferin A in non-irradiated and gamma irradiated aqueous extracts of Ashwagandha.

Procedure:

**Preparation of standard solution:** 1mg of Withaferin A was dissolved in 10ml methanol to give a concentration of 100ng / µl.

**Preparation of sample solution:** 100mg of aqueous extracts of non-irradiated and gamma irradiated (5kGy and 10kGy) samples of Ashwagandha were dissolved in 10ml of methanol and filtered through Whatmann No.1 filter paper.
**Materials and Methods**

**Procedure:**

The chromatographic estimation was performed using the following conditions: stationary phase was precoated silica gel 60 F 254 aluminium sheets (10 x 10cm) and the mobile phase used was Chloroform:Methanol::9:1 v/v. The chamber saturation time employed was 10 minutes and the developing distance was 9cm. Scanning wavelength of 223nm with a slit dimension of 5.0 x 0.45mm, band length of 6mm and scanning speed of 10 mm/s were employed.

**Standard and Sample application:** 8μl of the standard solution and 10μl of all sample solutions were applied as bands on the plate.

**Development and scanning:** The spotted plates were individually developed upto 90mm in a previously saturated Twin Trough chamber. Chamber saturation was done for 10 minutes. The developed plate was scanned at λ max of 223 nm and peak areas of standard and samples were compared. The amount of Withaferin A present in each extract was calculated by comparing the peak area of standard and respective samples.

The following formula was used to quantify the active constituent,

\[
\text{% Withaferin A} = \frac{\text{Area of sample} \times \text{conc. of standard}}{\text{Area of standard} \times \text{conc. of sample}} \times 100
\]

**Derivatization:** The plates spotted with sample and standard were derivatized with 200ml of Anisaldehyde-Sulphuric acid reagent. 200ml of derivatising reagent was charged into dip tank. The chromatogram was dipped in this, immediately removed and dried.

**Documentation:** The images of the derivatized plates were recorded using CAMAG Reprostar 3. Plate was placed in CAMAG Reprostar, suitable light was selected and release button was clicked. The electronic image of the chromatogram was documented in the system. HPTLC pattern of Ashwagandha aqueous non-irradiated...
and gamma irradiated (5kGy and 10kGy) extracts along with standard Withaferin A is documented. Rf values and Area Under Curve of both standard and sample were recorded and % actives calculated.

4.5.8.4 Development of HPTLC method for estimation of Andrographolide.

Similar procedure as in section 4.5.8.1 was followed for estimation of Andrographolide and the data is summarized in Table 5. Spectrum showing λ max for Andrographolide is recorded.

4.5.8.5 Validation of HPTLC method for estimation of Andrographolide

The developed method was validated in terms of pre-validation, limit of detection, limit of quantification, linearity, precision and accuracy. Validation of the developed HPTLC method was according to 4.5.8.2.

i. Pre-Validation: Similar method as in section 4.5.8.2 - i was followed. Standard andrographolide solution was spotted. The chromatogram was developed and scanned at 231nm. The peak areas obtained were compared to study the variation of the standard solution.

ii. Limit of Detection:

Procedure:

1mg of Andrographolide standard was dissolved in 100ml of methanol. This gave a concentration of 10ng/µl. From this standard stock solution, 1µl-5µl was spotted in duplicate. The chromatogram was developed as described in method development. Scanning was done at 231nm. The concentration that gave minimum peak area was reported as LOD.
iii. Limit of Quantification:

**Procedure:**

1mg of Andrographolide standard was dissolved in 10ml of methanol. This gave a concentration of 100ng/µl. From this standard stock solution, 1µl - 5µl was spotted in duplicate. The chromatogram was developed as described in method development. Scanning was done at 231 nm. The concentration that gave peak area that was three times greater than LOD was reported as LOQ.

iv. Linearity:

**Procedure:**

1mg of Andrographolide standard was dissolved in 10ml of methanol. This gave a concentration of 100ng/µl. From this standard stock solution, 1µl - 5µl was spotted. The chromatogram was developed as described in method development and scanned at 231 nm. Linear graph was plotted with concentration against Area under Curve.

v. Precision

Plates were prewashed with methanol upto 9.5cm, dried and then sample is spotted. Similar method as in section 4.5.8.2 - v was followed.

**Procedure:**

Intra day variation: 1mg of Andrographolide standard was dissolved in 10ml of methanol. This gave a concentration of 100ng/µl. From this standard stock solution, 2µl of standard Andrographolide was spotted twice on same day and developed.

Inter day variation: The same method was followed. The plates were spotted and developed on two consecutive days. Co-efficient of variation was compared.

vi. Accuracy:

**Preparation of standard solution:** 1mg of Andrographolide was dissolved in 10ml methanol to give a concentration of 100ng /µl.
**Preparation of sample solution:** 100mg of aqueous extract of Kalmegh was dissolved in 10ml of methanol and filtered through Whatmann No.1 filter paper.

**Procedure:**

5μl of sample solution, 1μl, 2μl, 3μl of standard Andrographolide and 5 μl of sample spiked with 1μl, 2μl, 3μl of standard Andrographolide were spotted on the plate. The chromatogram was developed. Scanning was done at 231nm. Areas of sample, standard and spiked samples were recorded and % recovery was calculated.

**4.5.8.6 Application of the validated method to quantify Andrographolide in non-irradiated and gamma irradiated aqueous extracts of Kalmegh at 0, 6 and 12 months of storage.**

Similar method of analysis was carried out for determination of Andrographolide in K1, K2 and K3 samples of Kalmegh. \( \lambda \) max of Andrographolide was found to be at 231nm. Sample and standard solutions were prepared, spotted as 6mm bands on precoated plates and developed in Chloroform: Methanol :: 7:1v/v. The plates were further dried and scanned and documented after derivatising with 10% sulphuric acid. HPTLC pattern of Kalmegh aqueous non-irradiated and gamma irradiated (5kGy and 10kGy) extracts along with standard Andrographolide was captured. Rf values and AUC for standard and samples were noted and % actives calculated.
### Table 5. Compiled data for HPTLC analysis of Ashwagandha and Kalmegh

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<th>Parameters</th>
<th>Ashwagandha Standard – Withaferin A</th>
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<tr>
<td>Application instrument</td>
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<tr>
<td>Mobile Phase</td>
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<td>Chloroform:methanol (7:1)</td>
</tr>
<tr>
<td>Development Chamber</td>
<td>Twin Trough Glass Chamber</td>
<td>Twin Trough Glass Chamber</td>
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<tr>
<td>Development Distance</td>
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<td>9 cm</td>
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<tr>
<td>Tank saturation time</td>
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<td>10 minutes</td>
</tr>
<tr>
<td>Development time</td>
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<td>15 minutes</td>
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<tr>
<td>λ max</td>
<td>223nm</td>
<td>231nm</td>
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<td>CAMAG TLC SCANNER 3</td>
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<td>Documenting instrument</td>
<td>CAMAG REPROSTAR 3</td>
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<tr>
<td>Derivatising agent</td>
<td>Anisaldehyde sulphuric acid</td>
<td>10% sulphuric acid</td>
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### 4.5.9 Toxicological analysis of Ashwagandha and Kalmegh at 0, 6 and 12 months of storage:

Experimental protocols of animal experiments were duly approved by Institutional Animal ethics Committee, IAEC of KLE College of Pharmacy, Bangalore-560010 (Annexure IV).
Swiss albino mice/Wistar Albino rats were maintained in our animal house (12:12 dark:light cycle), with adequate ventilation, hygienic conditions on normal pelleted diet (M/s Lipton India, Bangalore) and water *ad libitum*. A group of animals were housed in polypropylene cage of 42 X 27 X 13cm paddy husk bed covered with stainless steel wire mesh 28 X 20.5cm with provision for water and feed. Experimental animals were purchased from IISc, Bangalore. The animals were subjected to Toxicological study as per OECD / OCED guideline 425 (modified, adopted 23rd march 2006)\(^\text{165}\).

**ACUTE (ORAL) TOXICITY STUDY**

Acute oral toxicity study for non-irradiated and gamma irradiated samples of Ashwagandha and Kalmegh were carried out using OECD / OCED guideline 425 (modified, adopted 23rd march 2006). The test procedure minimizes the number of animals required to estimate the oral acute toxicity of a substance. The test also allows the observation of signs of toxicity and can also be used to identify chemicals that are likely to have low toxicity.

**Principle of the test.**

The test is sequential test that uses a maximum of five animals. A test dose of 2000 or exceptionally 5000mg/kg may be used in situation where experiment has information indicating that the test material is likely to be non - toxic.

**Procedure:** As suggested, after acclimatization of animals for 4-5 days, study was carried out as follows:

- Healthy, young adult Swiss albino mice/Wistar Albino rats, female nulliparous and non pregnant were used for this study. Food, but not water was with held for 3-4 hours and further 1-2 hours post administration of sample under study.
Dosed one animal at the test dose by oral route.

Since, this first test animal survived, four other animals were dosed (orally) on subsequent days, so that a total of five animals were tested. The same test was repeated after 6 and 12 months of storage.

Observation.

Animals were observed individually at least every 5 minutes once during first 30 minutes after dosing, periodically at 2hrs during the first 24 hours (with special attention during first four hours) and daily thereafter, for a total of 14 days.

4.5.10 Pharmacological activity of Ashwagandha – Anti-stress activity by Forced Swim Endurance test:

Naive, healthy, Swiss albino mice weighing between 18-30gm were employed for study. The chosen experimental animals were maintained in our animal house (12:12 dark:light cycle), with adequate ventilation, hygienic conditions maintained on normal pelleted diet (M/s Lipton India, Bangalore) and water ad libitum. A group of six animals were housed in polypropylene cage of 26 X 19 X 13cm on paddy husk bed and covered with stainless steel wire mesh 28 X 20.5 cm with provision for water and feed. Experimental animals were purchased from IISc, Bangalore. All experiments were performed in our Psychopharmacology laboratory illuminated with 11W CFL lamp, free from acoustic stress and after acclimatization of animals for 4-5 days in this laboratory. All experiments were performed during 10:00 am to 4:00 pm to reduce influence of circadian rhythm on the observation.

FORCED SWIM ENDURANCE TEST:

Forced swim endurance test (FST), was employed with modification to suit out our laboratory conditions. For all experiments, group refers to group of six animals.
Materials and Methods

(Briefly, mice were individually placed in acrylic cylinder 24×12cm filled 10cm high with water at 25±2°C.

Four groups of naive, healthy, Swiss albino mice of either sex, weighing between 18-30gm were grouped and treated with saline 0.2ml/animal, A1 (Ashwagandha non-irradiated aqueous extract), A2 (Ashwagandha gamma irradiated at dose of 5kGy) and A3 (Ashwagandha gamma irradiated at dose of 10kGy) orally through gavage tube at a dose of 100mg/kg body wt. After an hour of drug administration, animals were forced to swim for 5 minutes in the cylinder and the duration of swimming, immobility and climbing were observed and recorded. Swimming was defined as animal making active swimming motions, immobility as animal making only the those movements necessary to keep its head above the water and climbing as animal making vigorous movements with its forepaws in and out of the water, usually directed against the walls. The same was carried out at 5th and 24th hour of drug administration. Mean duration of swimming, immobility and climbing of various groups of animals are tabulated.

STATISTICAL ANALYSIS

Data was analyzed using Graphpad Prism 5.0 version. The results were expressed as the mean±SEM. The significance of the mean difference between the control group and each treatment group was determined by Newmann-Keul’s test. The level of $P<0.001$ and $P<0.05$ was used as the criterion of statistical significance.

4.5.11 Pharmacological activity of Kalmegh – Anti-inflammatory activity by Carrageenan induced rat paw edema

Chemicals and drugs:

Carrageenan (SD fine chem., Mumbai)

Diclofenac Sodium Standard (Commercial source)
Aqueous extracts of Kalmegh [non-irradiated (K1) and gamma irradiated at doses of 5kGy (K2) and 10kGy (K3) respectively].

Study design: To compare the anti-inflammatory activity, 30 Wistar Albino rats weighing between 150-200g were chosen and kept on overnight fasting. They were further randomly divided into 5 groups of 6 animals each.

Group I – Normal Control

Group II – Treated with standard Diclofenac sodium 15mg/kg p.o.

Group III – Treated with non-irradiated aqueous extract of Kalmegh – 200mg/kg p.o.– K1

Group IV - Treated with gamma irradiated (5kGy) aqueous extract of Kalmegh – 200 mg/kg p.o. – K2

Group V -Treated with gamma irradiated (10kGy) aqueous extract of Kalmegh – 200 mg/kg p.o. – K3

All animals were injected with 0.1ml of freshly prepared carrageenan suspension, into sub plantar region of left hind paw to induce inflammation.

Procedure: Acute inflammation was performed according to the method of Winter et al\textsuperscript{166}. All animals received vehicle/test sample/std. diclofenac sodium orally and after one hour, all animals were injected with 0.1ml of 1% freshly prepared carrageenan suspension into the sub plantar region of the left hind paw. The paw was marked with ink at the level of the lateral malleolus and immersed in mercury upto the marked place. The paw volume was measured using plethysmograph before injection, immediately after injection and again repeatedly at 1\textsuperscript{st}, 2\textsuperscript{nd}, 3\textsuperscript{rd}, 4\textsuperscript{th}, 5\textsuperscript{th} and 24\textsuperscript{th} hour after injecting carrageenan. The change in paw volume compared to 0 hour was calculated on each consequent observation hour for each group and noted down. The percentage inhibition of paw volume was calculated for each group from the formula,
Percentage inhibition = \( \frac{V_c - V_t}{V_t} \times 100 \)

Where, \( V_c \) – Increase in paw volume of Control group, and

\( V_t \) – Increase in paw volume in test groups

**STATISTICAL ANALYSIS**

Data were analyzed using GraphPad InStat version 3. The results were expressed as the mean ± SD. The significance of the mean difference between the control group and each treatment group was determined by Tukey’s multiple comparison test. The level of \( P<0.001 \) and \( P<0.05 \) was used as the criterion of statistical significance. The results are tabulated and graphically interpreted.

**4.5.12 Microbial Analysis** of non-irradiated and gamma irradiated samples of Ashwagandha and Kalmegh during 0, 6 and 12 months of storage:

A1, A2, A3 and K1, K2, K3 samples, stored at room temperatures were subjected to microbiological analysis at 0, 6 and 12 months after irradiation.

**Chemicals and Reagents:** Nutrient broth, Nutrient agar, Sabourd dextrose agar, MacConkey agar, Eosin Methylene Blue (EMB) agar, Lactose broth, Selenite-F broth, Brilliant green agar, Bismuth sulphite agar, Cetrimide agar, Mannitol salt agar, Kovac’s reagent and Vogel-Johnson agar were purchased from Hi-media, Mumbai.

**Procedure:**

1. **Determination of total microbial count:**
   1. 1 gm of test substance was suspended in buffer solution pH 7.2 to produce 10 ml, in a sterile container.
   2. The samples were incubated at 37°C for 60 minutes.
   3. Sterile nutrient agar and Sabourd’s dextrose agar media plates were prepared as per standard procedures.
4. 0.1ml of the clear supernatant from the container was placed on the surface of the previously prepared sterile nutrient agar and Sabouroud’s dextrose agar media plates for determination of bacteria and fungi respectively.

5. Sample on the surface of the medium was spreaded using sterile autoclaved spreader uniformly maintaining aseptic conditions in laminar air flow cabinet. Alternatively, 1ml of sample solution was poured into a sterile petri plate, above which 15ml of respective media’s were poured.

6. The plates were inverted and incubated for 24 hours to 48 hours at 37°C ±1°C for total bacterial count and at 28°C ±1°C for total fungal count, respectively.

7. Plates were examined for microbial growth, the number of colonies were counted and expressed in terms of colony forming units per gram or per ml of the substance (CFU/gm/ml).

II. Identification test for Escherichia Coli:

1 gm of test substance was placed in a sterile screw-capped container with 10 ml of nutrient broth, shaken and incubated at 37°C for 18 to 24 hours.

a) Primary test: 0.1 ml of the enrichment culture was spread on MacConkey agar plate using sterile autoclaved spreader uniformly maintaining aseptic conditions, in laminar airflow cabinet. Alternatively, 1ml of sample solution was poured into a sterile petri plate, above which 15ml of respective media’s were poured.

Plates were incubated at 37°C ±1°C. Extensive growth of bacterial colonies and few colonies surrounded with yellow zones in MacConkey agar plates confirms the presence of E.coli. Plates were examined for microbial growth, the number of colonies were counted and expressed in terms of colony forming units per gram or per ml of the substance (CFU/gm/ml).
b) **Secondary tests**: 0.1 ml of the enrichment culture was spreaded on EMB agar plates using sterile autoclaved spreader uniformly maintaining aseptic conditions in laminar air flow cabinet. Simultaneously 0.1 ml of enrichment culture was incorporated in tubes containing 5 ml of peptone water and also 1ml samples were put in a sterile petri plate on to which 15ml media was poured. Plates and tubes were incubated at 37°C±1°C, examined for the formation of metallic sheen in the agar plates and formation of indole (To test for indole add 0.5 ml of Kovac’s reagent, shake well and allow to stand for one minute; if a red colour is produced in the reagent layer - indole is present). The formation of metallic sheen and indole in the secondary test indicates the presence of *Escherichia coli*. Plates were examined for microbial growth, the number of colonies were counted and expressed in terms of colony forming units per gram or per ml of the substance (CFU/gm/ml).

III. **Identification test for *Salmonella***:

1 gm of test substance was placed in a sterile screw-capped container with 10 ml of nutrient broth, shaken and incubated at 37°C for 1 hr.

a) **Primary test**: 1.0 ml of the enrichment culture was added to tubes containing 10 ml of Selenite F broth and incubated at 37°C for 24 hours. Tubes were observed for the microbial growth and for the colour of the broth. Microbial growth with no colour in tubes shows presence of *Salmonella*.

b) **Secondary test**: Cultures from the preliminary test tubes were inoculated onto the brilliant green agar and bismuth sulphite agar plates. Plates were incubated at 37°C for 24hr. Small, transparent and colourless, or opaque, pinkish or white (frequently surrounded by a pink or red zone) colonies on brilliant green agar and black or green colonies on bismuth sulphite agar confirms the presence of *Salmonella typhi*. Plates
Materials and Methods

were examined for microbial growth, the number of colonies were counted and expressed in terms of colony forming units per gram or per ml of the substance (CFU/gm/ml).

IV. Identification test for *Pseudomonas*:

a) Preliminary test: 1 gm of the sample was placed in a sterile screw capped jar containing 10 ml of Cetrimide broth and incubated at 37\(^0\) C for 24 hours. It was subcultured on a plate containing a layer of Cetrimide agar and incubated at 37\(^0\)C for 24-48 hours, examined for the growth by Gram’s stain. If there are microbial colonies, it indicates the absence of *Pseudomonas*.

b) Secondary test: Since the preliminary test for *Pseudomonas* was negative, the confirmatory secondary tests were not performed.

V. Identification test for *Staphylococcus aureus*:

1 gm of sample was placed in a sterile screw capped jar containing 10 ml of nutrient broth and incubated at 37\(^\circ\) C for 24 hours. It was further subcultured on a plate containing a layer of mannitol salt agar medium and Vogel-Johnson agar medium and incubated at 37\(^0\) C for 24 hours. Yellow colonies in mannitol salt agar medium and black colonies surrounded by yellow zones in Vogel-Johnson agar medium indicate the presence of *Staphylococcus aureus*.

Plates were examined for microbial growth, the number of colonies were counted and expressed in terms of colony forming units per gram or per ml of the substance (CFU/gm/ml).
4.6 Post Market survey on practical issues of gamma irradiation:

Survey on Practical issues of gamma irradiation of Ashwagandha and Kalmegh products-

A brief survey among practitioners, health care providers, distributors and consumers dealing with Ashwagandha and Kalmegh products were studied to gain information about the practical issues of gamma irradiation. Data was collected and the results are interpreted in the form of graph. The method followed was by questionnaire method (Annexure V). A set of 17 questions were framed and individually the individuals were contacted, explained in detail and information collected. Data has been collected and graphical interpretation has been done for relevant questions.