Chapter V

STANDARDIZATION
OF EXTRACT OF
GYMNEMA
SYLVSTRE
AN INTRODUCTION TO THE STANDARDIZATION OF HERBAL DRUGS

Medicinal plants constitute a source of raw materials for both traditional systems of medicine (e.g. Ayurvedic, Chinese, Unani, Homeopathy, and Siddha) and modern medicine. Nowadays, plant materials are employed throughout the industrialized and developing world as home remedies, over-the-counter drugs, and ingredients for the pharmaceutical industry. As such, they represent a substantial proportion of the global drug market. Most rural populations, especially in the developing world, depend on medicinal herbs as their main source of primary health care.

Plants have been used for medicine from time immemorial because they have fitted the immediate personal need and are easily accessible and inexpensive. With the passage of time, there has been more demand of herbal medicines due to a general disillusionment with conventional medicine. The desire for a “natural” lifestyle has resulted in an increasing utilization of alternative or complementary therapies with the natural products in general and herbal medicines in particular.

The demand is high but there is a shortage of supply of genuine herbal medicines. Herbal products represent a number of unique problems when quality aspects are considered. These are because of the nature of the herbal ingredients present therein, which are complex mixtures of different secondary metabolites that can vary considerably depending on environment and genetic factors.

In almost all the traditional systems of medicine, the quality control aspects have been considered from its inception itself by the Rishis and later by the Vaidyas and Hakims. However, in modern concept it requires necessary changes in their approach. Quality can be defined as the status of a drug that is determined by identity, purity, content, and other chemical, physical, or biological properties, or by the manufacturing processes. Quality control is a term that refers to processes involved in maintaining the quality and validity of a manufactured product.

For the quality control of traditional medicines, the traditional methods are procured, studied, documented and then the traditional information about identification and quality assessment is interpreted properly in terms of modern assessment.
Quality assurance is an integral part of traditional medicine, which ensures that it delivers the required quantity of quality medicament.

A simple chromatographic technique such as TLC may provide valuable additional information to establish the identity of the plant material. This is especially important for those species that contain different active constituents. TLC fingerprinting is of key importance for herbal drugs made up of essential oils, resins, and gums, which are complex mixtures of constituents that no longer have any organic structure. It is a powerful and relatively rapid solution to distinguish between chemical classes, where macroscopy and microscopy will fail.

The fingerprinting and marker compound analyses are nowadays getting momentum for the standardization of traditional medicinal formulations. Here, the concentration of the secondary metabolites, which are the major constituents of herbal drugs, is studied, which provides valued scientific standardization procedures. This technique not only helps in establishing the correct botanical identity but also helps in regulating the chemical sanctity of the herbs.

For standardization and quality assurance, three attributes viz. authenticity, purity and assay are desirable. Authenticity relates to proving that the material is true i.e., it corresponds to the right identity. Authentication itself involves many parameters including gross morphology, microscopy, chemical analysis etc.

Purity pertains to evaluating that there are no adulterants present in the plant material. Purity depends upon the absence of foreign matter, whether organic or inorganic, while quality refers essentially to the concentration of the active constituents or components, the product is used and it’s economic and commercial value is estimated. Based on the concentration and nature of the constituents though, crude drug may conform to all the official standards of purity and be of good quality (Mukherjee, 2002).

**OBJECTIVES**

1. To prepare and standardize the ethanolic *Gymnema sylvestre* extract.
2. To prepare the water soluble and water insoluble fractions of ethanolic *Gymnema sylvestre* extract.
Preparation of *Gymnema sylvestre* ethanolic extracts (water soluble and water insoluble fractions)

The air dried leaves of *Gymnema sylvestre* were purchased from the local market (Khari Baoli), Delhi, India and drug was authenticated by Dr. H. B. Singh, Head, Raw Materials, Herbarium and Museum, NISCAIR, Pusa, New Delhi, India. (Ref. NISCAIR/RHMD/Consult/2008-09/980/11).

The air dried powdered leaves of *Gymnema sylvestre* (1 kg) were extracted with (70%) ethanol in Soxhlet’s apparatus for 72 hours. The solvent was removed under reduced pressure. The extract was concentrated and yield was 10.8% (w/w) in terms of starting material.

The ethanolic extract of *Gymnema sylvestre* was further subfractionated into water-soluble (W-S) fraction and water-insoluble (W-INS) fractions by the method reported by Alam et al. (2005). The ethanolic extract was stirred in distilled water at room temperature and filtered to give water-soluble (W-S) fraction and water-insoluble (W-INS) fractions. The yield obtained of W-S and W-INS were 60% and 40% respectively in terms of total ethanolic extract.

**Parameters for standardization of *Gymnema sylvestre* ethanolic extract**

The following parameters should be taken into the considerations:

1. Extractive values
   - Hot Extraction
2. Ash value
   - Total ash
3. Determination of pH
4. Foaming Index
5. Preliminary Phytochemical Screening
6. Microbial Contamination
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1. EXTRACTIVE VALUES

The amount of an extract that a drug yields in a particular solvent is often an approximate measure of the amount of certain constituents that the drug contains. The drug should be extracted with different solvents in order of their increasing polarity to get the correct and dependable values. Generally petroleum ether, alcohol and water extractives are taken into consideration for fixing the standard of a drug. The petroleum ether extract contains fixed oil, resins and volatile substances, but when the extract is heated at 105°C until constant weight, the volatile substances are volatilized leaving only resin, coloring matter and fixed oil. Alcohol can dissolve almost all the substances, but is generally used for determining the extractive index for those drugs which contain glycosides, resins, alkaloids etc. Water is used for the drugs containing water-soluble substances as chief constituents. The extractive values were determined according to the method described in Pharmacopoeia (Indian Pharmacopoeia 1996, Appendix 3.37).

HOT EXTRACTION

The dried and coarsely powdered drug (10gm) was packed in a soxhlet apparatus and was subjected to extraction with solvent like Ethanol, over 72 hours. The total volume of extract was readjusted with the same solvent to 100 ml. The extract was divided into 4 parts each of 25 ml. Then each part of 25 ml of extract was transferred to a tared bottom dish and was evaporated to dryness on a water bath, then weighed without delay and their constant extractive values with different solvents were calculated.

\[
\% \text{ Extractive matter} = \frac{\text{Weight of extractable matter}}{\text{Weight of Drug}} \times 100
\]
2. ASH VALUE (WHO, 1998)

Ash value is an important parameter for the purpose of determination of inorganic materials, such as carbonates, silicates, oxalates and phosphates. Heating causes the loss of organic material in the form of CO$_2$ leaving behind the inorganic components. Ash value is an important characteristic of a drug and with the help of this parameter we can detect the extent of adulteration as well as establish the quality and purity of the drug. There is a considerable difference in the ash values of different drugs but mostly the difference varies within narrow limits in case of the same drug. The ash remaining following ignition of medicinal plant materials is determined by three different methods which measure total ash, acid insoluble ash and water soluble ash. The acid insoluble ash consists mainly of silica and high acid insoluble ash thereby indicating the contamination with earthy material. The water soluble ash is used to estimate the amount of inorganic elements.

Total ash

The total ash is designed to measure the total amount of material remaining after ignition. Indian Pharmacopoeia 1996 and WHO prescribes suitable methods for determination of ash values.

The extract (1 g) was placed in the tared platinum or silica crucible and was incinerated at a temperature not exceeding 450°C until free from carbon. It is then cooled and weighed to get the total ash content.

\[
\text{% Ash content} = \frac{\text{Weight of Ash}}{\text{Weight of extract}} \times 100
\]


pH 1% solution:

An accurately weighed 1 g of the drug was dissolved in accurately measured 100 ml of distilled water, filtered and checked pH of the filtrate with a standardized glass electrode.
4. FOAMING INDEX (WHO, 1998)

Many medicinal plant materials contain saponins that can cause persistent foam when an aqueous decoction is shaken. The foaming ability of an aqueous decoction of plant material and their extract is measured in terms of foaming index.

About 1gm of extract weighed accurately and transferred to 500ml conical flask containing 100ml of boiling water. Maintained at moderate boiling for 30 minutes. Cooled and filtered into 100ml volumetric flask. The decoction was poured into 10ml stopped test tube (height 16cm, diameter 16mm) in successive portions of 1ml, 2ml, 3ml etc. upto 10ml and adjusted the volume of liquid in each tube with water to 10ml. Stoppered the tubes and was shaken them in a lengthwise motion for 15 sec, two shakes per second. Allowed to stand for 15 minutes and the height of foam was measured.

The results assessed as follows:

- If the height of foam in every tube is less than 10m the foaming index is less than 100.

- If the height of foam in any tube is 1cm, the volume of plant material decoction in the tube is used to determine the index. If this tube is the first or second tube in a series, prepare an intermediate dilution in a similar manner to obtain a more precise result.

- If the height of the foam is more than 1cm in every tube, the foaming index is over 1000. In this case repeat the determination using a new series of decoction of detection in order to obtain a result.

Calculate the foaming index using the following formula:

\[
\frac{1000}{a}
\]

Where \( a \) = the volume in ml of decoction used for preparing the dilution in the tube where foaming to a height of 1cm is observed.
PRELIMINARY PHYTOCHEMICAL SCREENING

The preliminary phytochemical screening was carried out using plant extracts for their content of different classes of compounds. The extract obtained then subjected to qualitative chemical tests for identification of various plant constituents present in the crude drug. The extracts should be subjected to preliminary phytochemical investigation for detection of following (Ali, 1998; Evans, 2002):

I. Alkaloids
II. Carbohydrates
III. Glycosides
IV. Phenolic Compounds
V. Flavonoids
VI. Protein & amino acids
VII. Saponins
VIII. Sterols
IX. Acidic Compounds
X. Resins
XI. Lipids/Fats

I. Tests for Alkaloids

Each of the extract was taken separately in 5ml of 1.5% v/v hydrochloric acid and filtered. The filtrate was then tested with following reagents:

a. Dragendorff’s Reagent

It was prepared by mixing solution A (17 gm of Bismuth subnitrate + 200 gm Tartaric acid + 800 ml distilled water) and solution B (160 gm potassium iodide + 400 ml distilled water) in 1:1 v/v proportion. From this solution, working standard was prepared by taking 50 ml of this solution and adding 100 gm of tartaric acid and making upto 500 ml with distilled water.

The above Dragendorff’s Reagent was sprayed on whatmann No.1 filter paper and the paper was dried. The test filtrate after basification with dilute ammonia was extracted with chloroform and the chloroform extract was applied on the filter paper,
impregnated with Dragendorff’s Reagent, with the help of a capillary tube. Development of an orange red color on the paper indicated the presence of alkaloids.

b. Hager’s Reagent

A saturated aqueous solution of picric acid in cold water was added in each of the extracts and observed for the formation of yellow precipitate.

c. Wagner’s Reagent

1.27 g of iodine and 2 g of potassium iodide were dissolved in 5 ml of water and the solution was diluted to 100 ml with water. When few drops of this solution were added to the test filtrate, a brown flocculent precipitate was formed indicating the presence of alkaloids.

d. Mayer’s Reagent

The Mayer’s reagent was prepared as follows: 1.36 gm of mercuric chloride was dissolved in 60 ml of distilled water. Both the solutions were mixed and diluted to 100 ml with distilled water.

Few drops of Mayer’s Reagent were added in each of the extracts and observed for formation of white or cream colored precipitate.

II. Tests for Carbohydrate

a. Molisch Test

The Molisch’s reagent was prepared by dissolving 10 gm of α-naphthol in 100 ml of 95% alcohol. A few mgs of the test residue was placed in a test-tube containing 0.5 ml of water, and it was mixed with 2 drops of Molisch’s reagent. To this solution, 1 ml of concentrated sulphuric acid was added from the side of the inclined test-tube, so that the acid formed a layer beneath the aqueous solution without mixing with it. If a red brown ring appears at the common surface of the liquids, sugars are present.

b. Fehling Reagent (Detection of reducing sugar)

The Fehling’s solution was prepared as follows:

Solution A:

<table>
<thead>
<tr>
<th>Copper sulphate</th>
<th>34.64 g</th>
</tr>
</thead>
</table>
The two solutions were mixed in equal volumes immediately before use.

A little of the test residue was dissolved in water, and a few ml of the Fehling’s solution was added to it. This mixture on warming gives a red precipitate of cuprous oxide.

**III. Tests for Glycosides**

About 2ml of extract was taken and subjected to following tests:

**a. Keller-Killiani Test**

One ml of glacial acetic acid containing traces of ferric chloride and one ml of concentrated sulphuric acid were added to the extract and observed for formation of reddish brown color at the junction of two layers and the upper layer turned bluish green in presence of glycoside.

**b. Borntrager’s Test**

One ml of benzene and 0.5ml of dilute ammonia solution was added to the extract and observed for formation of reddish pink color.

**c. Legal Test**

Concentrated extracts were made alkaline with few drops of 10% sodium hydroxide solution and then freshly prepared sodium nitroprusside solution was added to the solution and observed for the formation of pink or red color.

**d. Baljet Test**

To the concentrated extracts, sodium picrate reagent was added and observed for the formation of orange and yellow color.
IV. Tests for Phenolic Compounds

a. Ferric Chloride Solution

The extracts were taken in water and warmed; to this 2ml of ferric chloride solution (5% w/v solution of ferric Chloride in 90% alcohol) was added and observed for the formation of green and blue color.

b. Lead Acetate Solution

To the extract (2ml) lead acetate solution (10% w/v of Basic lead acetate in distilled water) was added and observed for the formation of precipitates.

V. Tests for Flavonoids

a. Ammonia Test

Filter paper strips were dipped in solution of the extracts, ammoniated and observed for color change from white to yellow.

b. To 2ml of extract ferric chloride solution was added by the sides of test tube and observed for reddish brown color.

c. To aqueous & ethanolic extract sodium hydroxide solution was added and observed for pale yellow color.

d. Shinoda Test

A small quantity of the test residue was dissolved in 5 ml ethanol (95% v/v) and reacted with a few drops of concentrated hydrochloric acid and 0.5 g of magnesium metal. The pink, crimson or magenta color is developed within a minute, if flavonoids are present.

VI Tests for Protein and Amino acids

a. Millon’s Test

Millon’s reagent was prepared by dissolving 3 ml of mercury in 27 ml of fuming nitric acid, keeping the mixture well cooled, this solution was then diluted with equal quantity of distilled water.
To few ml of extract, 5ml of distilled water was added and filtered. To two ml of this filtrate, 5-6 drops of Millon’s reagent were added and observed for the formation of red precipitate.

b. Xanthoprotein Test

To 2ml of extract few drops of concentrated nitric acid were added by the sides of test tube and observed for formation of yellow color.

c. Biuret Test

To the ammoniated alkaline filtrate of the extract 2-4 drops of 0.02% copper sulphate solution were added and observed for the formation of red and violet color.

d. Ninhydrin Test

To the extract, Lead acetate solution was added to precipitate tannins. The precipitate was spotted on a paper chromatogram, sprayed with ninhydrin reagent and heated at 110 °C for 5 minutes and observed for the formation of red and violet color.

VII Tests for Saponins

a. Foam Test

Few mg of residue was taken in test tube with small amount of water and shaken vigorously for one minute and observed for formation of rich lather which was stable for more than ten minutes.

b. To the alcoholic extract few drops of sodium bicarbonate were added, shaken well and observed for formation of Honey comb like frothing.

VIII Tests for Sterols

The extracts were evaporated to dryness and the residue was extracted with petroleum ether and acetone. The insoluble residue left after extraction with petroleum ether & acetone was tested for sterols as:

a. Salkowski Reaction

To the extracts, two ml of concentrated sulphuric acid were added & observed for formation of yellow ring at the junction which turns red after one minute.
b. Hecht's Reaction

To the residue, 2-3 ml of trichloroacetic acid was added, heated and observed for the formation of red and violet color.

IX. Tests for Acidic Compounds

a. To the alcoholic extracts, sodium bicarbonate solution was added and observed for the production of effervescence.

b. A small amount of alcoholic extract was taken in warm water and filtered. The filtrate was then tested with litmus paper and methyl orange and observed for appearance of blue color.

X. Tests for Resins

a. Distilled water (5.0ml) was added to the extract and observed for turbidity.

b. A mixture of extract in acetone (3ml) and HCl (3ml) was heated on a water bath for 30 minutes and observed for pink color.

XI. Test for Lipids/fats

Rub a small quantity of extract on a paper and observed for a permanent translucent stain.

5. MICROBIAL LOAD DETERMINATION (Anantnarayan et al; 1992)

Medicinal plant materials generally carry a great number of bacteria and moulds, often originating in the soil, while a large range of bacteria and fungi form the naturally occurring microflora of herbs, aerobic spore forming bacteria frequently predominate. Current practice of harvesting, handling and production may cause additional contamination and microbial growth. The determination of Escherichia coli and moulds can indicate the quality of production and harvesting practices. There are some microbes seen in the plant materials that are pathogenic to the human beings, e.g. Escherichia coli, Salmonella, Pseudomonas, Staphylococcus aureus and other type of yeast and moulds. Determination of microbial counts explained as per WHO guidelines.
One gram of extract was taken and suspended in 50ml of sterile distilled water. The suspension was shaken for sufficient period of time so as to allow maximum mixing. After this the suspension was filtered by using a disposable sterilized filter paper. The filtrate was used as stock solution. Series dilution (1:1, 1:10, 1:100) of this stock solution were made and 1ml of different diluted solution was separately inoculated (with spreading method) on a nutrient agar medium and incubated at 37°C for 24 hours. After 24 hours, the Petri plates with most clearly visible colonies were taken and number of colonies determined by using colony counter.

The microbial load per gram of sample was then calculated by using dilution factor.

Composition of nutrient agar medium

- Agar 15.0%
- Peptic Digest of Animal Tissue 5.0%
- Sodium Chloride 5.0%
- Beef Extract 1.5%
- Yeast Extract 1.5%
- pH 7.4±0.2 at 25°C
- Distilled Water 1000 ml

The medium was autoclaved at 15lbs per square inch pressure at 121°C.

6. THIN LAYER CHROMATOGRAPHY (TLC PROFILE)

The extract obtained from the crude drugs was subjected to thin layer chromatography to find out the number of compounds present in them. The details of the procedure followed were as follows:

Preparation of the plates

The adsorbent used for thin layer chromatography was silica gel G. About 25 g of silica gel G was taken in a glass mortar and about 35 ml of distilled water was added to it. The mixture was stirred with glass rod until it became homogeneous. This mixture was stirred with glass rod until it became homogeneous. This mixture was then allowed to swell for about 15 minutes. Then an additional 15 ml of distilled water was added to it with stirring. The suspension was then transferred to a 150 ml flask fitted with a plastic stopper, and was shaken vigorously for about 2 minutes.
This suspension was then spread immediately on thin layer chromatographic plates with the help of a thin layer chromatography (TLC) applicator (SUPERFIT), of Continental Instruments, Bombay was used.

**Drying and storage of the plates**

The freshly coated plates were then air dried until the transparence of the layer had disappeared. The plates were then stacked in a drying rack and were heated in an oven for 30 min. at 121°C. The activated plates were kept in a desiccator, till required for further use.

**Application of the sample**

For applying test samples on TLC plate, glass capillaries were used. The spots were applied with the help of a transparent template, keeping a minimum distance of 1 cm between the two adjacent spots. The spots of the samples were marked on the top of the plate to know their identity.

**Chromatographic chamber, conditions of saturation and the development of TLC plates**

Chromatographic rectangular glass chamber (16.5cm x 29.5cm) was used in the experiments. To avoid insufficient chamber saturation and the undesirable edge effect, a smooth sheet of filter paper approximately of 15 x 40 cm size was placed in the chromatographic chamber in a ‘U’ shape and was allowed to be soaked in the developing solvent. After being thus moistened, the paper was then pressed against the walls for the chamber, so that it adhered to the walls. The chamber was allowed to saturate for 24 hours before use. The experiments were carried out at room temperature in diffused day light.

**Developing Solvent System**

A number of developing solvent systems were tried.

**7. HIGH PERFORMANCE THIN LAYER CHROMATOGRAPHY (HPTLC PROFILE)**

Thin layer chromatography is a technique where the components of mixtures separate by differential migration through a planar bed of a stationary phase, the mobile phase
flowing by virtue of capillary forces. The solutes are detected in-situ on the surface of the thin layer plates by visualizing reagents after the chromatography has been completed. High Performance Thin Layer Chromatography is a technique of simultaneous processing of the sample under similar conditions leading to better analytical precision and accuracy. For development of HPTLC, extracts (20 mg) were suspended in 2ml of methanol separately. The solution was filtered through whatman No. 1 filter paper. Chromatograms were viewed under long wavelength UV light. The Rf values for different spots were recorded by HPTLC Apparatus (Reprostar Chromatography Documentation Apparatus i.e. Camag Switzerland).

High performance thin layer liquid chromatography

TLC Identity Test

Test solution

20 mg of water soluble fraction of Gymnema sylvestre ethanolic extract was dissolved in 2 ml of ethanol respectively and apply 4 & 8 µl of these solutions on TLC plate.

Standard solution

Dissolve 10 mg of reference compound Gymnemic acid in 1 ml of methanol and apply (0.1, 0.2, 0.4 and 0.8 µl) of this solution on TLC plate as reference marker.

Solvent system

Chloroform: Methanol (9: 1) for water soluble fraction of Gymnema sylvestre ethanolic extract

Procedure

Sample solutions (water soluble fraction of Gymnema sylvestre extract in methanol) were applied onto the plates with automated TLC sampler Linomat V (Camag, Muttenz, Switzerland) and were controlled by Win CATS software 1.3.3 (Camag, Muttenz, Switzerland). Plates were developed in 10 x 10 cm twin trough glass chambers (Camag, Muttenz, Switzerland). A TLC scanner III with WinCATS software was used for scanning the TLC plates. Pre-coated silica gel aluminum plates
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60 F254 (10 x 10) with 250 μm thicknesses (Merck, Darmstadt, Germany), were used for all determinations. The plates were pre-washed with methanol and activated at 60° C for 5 minutes prior to chromatography. Four different aliquots (0.1, 0.2, 0.4 and 0.8 μl) of standard solution were applied in duplicates on 10 x 10 cm TLC plates for the preparation of calibration curve.

The mobile phase consisted of chloroform: methanol (9:1) v/v. 15 ml mobile phase was used per plate. The optimized chamber saturation time for mobile phase was 30 min at room temperature (25°C ± 2) at relative humidity of 60% ± 5. The length of chromatogram run was 8.0 cm. After development, chromatographic plates were air-dried to remove the solvent and then scanned at 293 nm in the UV reflectance mode. Visualization TLC plates were observed in UV-light (293 nm).

RESULTS

1. EXTRACTIVE VALUES

Extractive value of ethanololnic extract of Gymnema sylvestre in ethanol (70%) was: 10.8% (w/w).

2. Total Ash value:

<table>
<thead>
<tr>
<th>Wt of tared crucible (gm)</th>
<th>Wt of tared crucible + extract (gm)</th>
<th>Wt of extract (gm)</th>
<th>Wt of tared crucible + Ash (gm)</th>
<th>Wt of Ash (gm)</th>
<th>% (w/w) Ash</th>
</tr>
</thead>
<tbody>
<tr>
<td>15.425</td>
<td>16.439</td>
<td>1.013</td>
<td>15.443</td>
<td>0.018</td>
<td>1.77%</td>
</tr>
</tbody>
</table>

The total ash content (% w/w) in the ethanolic extract of G. sylvestre was 1.77%.

3. pH of 1% w/v aqueous solution

pH of G. sylvestre ethanolic extract (1% w/v aqueous solution) with standardized glass electrode -6.85.
4. Foaming index:

In all test tubes foam height was less than 1 cm. So the foaming index of ethanolic extract of *Gymema sylvestre* was less than 100.

5. Phytochemical constituent testing:

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Tests for the presence of</th>
<th>Test performed</th>
<th>Extract</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Saponins</td>
<td>Foam test</td>
<td>+</td>
</tr>
<tr>
<td>2.</td>
<td>Proteins &amp; amino acids</td>
<td>Xanthoprotein test</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Millon’s reagent test</td>
<td>+</td>
</tr>
<tr>
<td>3.</td>
<td>Reducing Sugar</td>
<td>Fehling test</td>
<td>+</td>
</tr>
<tr>
<td>4.</td>
<td>Alkaloids</td>
<td>Dragendorff test</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Mayers reagent test</td>
<td>+</td>
</tr>
<tr>
<td>5.</td>
<td>Carbohydrate</td>
<td>Molisch’s reagent test</td>
<td>+</td>
</tr>
<tr>
<td>6.</td>
<td>Tannins</td>
<td>Lead acetate test</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ferric chloride test</td>
<td>-</td>
</tr>
<tr>
<td>7.</td>
<td>Mucilage</td>
<td>Ruthenium red solution test</td>
<td>-</td>
</tr>
<tr>
<td>8.</td>
<td>Glycosides</td>
<td>Keller Killiani test</td>
<td>+</td>
</tr>
<tr>
<td>9.</td>
<td>Steroid/Triterpenes</td>
<td>Salkowski reaction test</td>
<td>+</td>
</tr>
</tbody>
</table>

(+) - Present, (-) - Absent

6. Microbial load determination:

<table>
<thead>
<tr>
<th>Dilutions of extract</th>
<th>No. of colonies</th>
<th>Characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Colour</td>
</tr>
<tr>
<td>1:1</td>
<td>Uncountable</td>
<td>White</td>
</tr>
<tr>
<td>1:10</td>
<td>Uncountable</td>
<td>White</td>
</tr>
<tr>
<td>1:100</td>
<td>82</td>
<td>White</td>
</tr>
</tbody>
</table>

Using dilution factor, the number of colonies forming unit/gm of ethanolic extract of *Gymema sylvestre* was found to be: 41×10⁴.

7. THIN LAYER CHROMATOGRAPHY (TLC)

WATER SOLUBLE FRACTION OF ETHANOLIC EXTRACT

<table>
<thead>
<tr>
<th>Solvent system</th>
<th>Visualizing agent</th>
<th>No. of spots</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chloroform: Methanol (9:1)</td>
<td>UV-293</td>
<td>2</td>
</tr>
</tbody>
</table>
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8. HIGH PERFORMANCE THIN LAYER CHROMATOGRAPHY (HPTLC PROFILE)

HPTLC chromatograms of standard gymnemic acid (Rf 0.33) and of a water soluble fraction of Gymnema sylvestre ethanolic extract, showing a sharp and symmetrical peak that was identical to that of standard gymnemic acid. The Rf value of the sample was 0.33.

<table>
<thead>
<tr>
<th>Solvent system</th>
<th>No. of Spots</th>
<th>Rf values of spots</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chloroform: Methanol (9:1)</td>
<td>2</td>
<td>0.33 and 0.85</td>
</tr>
</tbody>
</table>

Figure 4: HPTLC Chromatogram of standard Gymnemic acid
Figure 5: HPTLC Chromatogram of water soluble fraction of *Gymnema sylvestre* ethanolic extract