

# Preparation of *Triphala Churna* using the Ingredients Obtained from Local Market and Comparative Standardization

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## History

- Submission Date: 23-07-2018;
- Review completed: 04-10-2018;
- Accepted Date: 20-11-2018

DOI : 10.5530/pj.2019.1.18

## Article Available online

<http://www.phcogj.com/v11/i1>

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## ABSTRACT

**Introduction:** In the recent years there has been rapid growth in the field of herbal medicine most of the tradition systems of medicine are accepted universally after standardization only. It is very important to develop an essential techniques to standardization of herbal related drugs. The present study standardization of *Triphala Churna* majorly focused on that area under WHO guidelines. **Methods:** This polyherbal *Churna* used to treat the constipation and other gastric disorders. In this study a prepared *Triphala Churna* was comparatively standardized with the reference obtained from market. For the standardization of the above formulations were done by evaluating the macroscopical, microscopical, powder flow properties, extractive values, Physicochemical characters, heavy metal content detection, qualitative and quantitative determination of tannins and alkaloids, TLC finger print, *in-vitro* anti-oxidant activity and cytotoxic activity to assess the quality and safety and therapeutic activity of formulation. **Results:** The above parameters for the both formulation complies with the standards. The flow properties are poor. From the preliminary phytochemical test revealed the presence of various bioactive constituents. Majorly the concentration of tannins and flavonoids are high in water extract and also the water extract having the good anti-oxidant and *in vitro* cytotoxic activity. Hence the *Triphala* extracts may be used for various Ayurvedic preparations to chronic diseases like cancer.

**Key words:** *Triphala Churna*, Standardization, TLC finger print, *in-vitro* anti-oxidant activity, Cytotoxic activity.

## INTRODUCTION

Herbal Medicine Plant kingdom had played a vital role in man's existence on this earth. Nature has always been stands as a golden mark to amplify the outstanding phenomenon of symbiosis.<sup>1</sup> Practically every country develops its own medical system, which includes the ancient civilization of China, Egypt and India.<sup>2</sup> Thus, the Indian Medical system Ayurveda came into existence. The WHO estimated that 80% of the population of developing countries relies on traditional medicines, mostly plant drugs, for their primary health care needs. Also, modern Pharmacopoeias still contain at least 25% drugs derived from plants and many others which are synthetic analogues built on prototype compounds isolated from plants. Hence the herbal material used in the practice should be standardized.<sup>3</sup>

Standardization is an essential factor for polyherbal formulation in order to assess the quality of the drugs based on the concentration of their active principle. It is very important to establish a system of standardization for every plant medicine in the market. Plant material when used in bulk quantity may vary in its chemical content and therefore, in its therapeutic effect according to different batches of collection e.g. collection in different seasons and/or from sites with

different environmental surroundings or geographical location. WHO has appreciated the importance of medicinal plants for public health care in developing nations and has evolved guidelines to support the member states in their efforts to formulate national policies on traditional medicine and to study their potential usefulness including evaluation of its quality, safety and efficacy.<sup>4</sup> The process of evaluating the quality and purity of crude drugs by means of various parameters like morphological, microscopically, physical, chemical and biological observation is called standardization.<sup>5</sup>

## Quality control parameters for herbal formulations<sup>6,7</sup>

(i) **Physical parameters:** It includes colour, appearance, Odour, clarity, viscosity, moisture content, ash values, pH, disintegration time, friability, hardness, flow property, flocculation, sedimentation and settling rate. (ii) **Chemical parameters:** It includes limit tests for heavy metal, extractive values, chemical assays for active constituents, etc. (iii) **Chromatographic analysis of herbals:** Chromatographic analysis can be carried out using TLC, HPLC, HPTLC

**Cite this article:** Venkateswarlu G, Ganapaty S, Babu AMSS. Preparation of *Triphala Churna* using the Ingredients Obtained from Local Market and Comparative Standardization. *Pharmacogn J.* 2019;11(1):102-111.

GC, UV, Fluorimetry and GCMS etc. (iv) **Microbiological parameters:** It includes total viable content, total mold count, total entero-bacteria-ceae and their count. Morphology. The in this present study one of the important Ayurvedic *Triphala Churna* was standardized. Churna are preparations comprising of fine powders of drugs and may be simple or compound. Simple *Churna* comprises of only one ingredient while a compound one consists of more than one ingredient. The principle of using *Churna* is due to the fact that therapeutic value of most of the substances greatly increases when they are reduced to very fine state of subdivision.

### *Triphala Churna* and its composition<sup>8</sup>

*Embellica officinalis* is effective in the treatment of hepatotoxicity, amlapitta (peptic ulcer) and in dyspepsia.<sup>9</sup> The fruits exhibit hypolipidaemic and anti-atherosclerotic effects in rabbits and rats.<sup>10</sup> The fruit extract has anti-mutagenic activity on certain directly acting mutagens in some strains of *Salmonella typhimurium*.<sup>11</sup> The extract of alma also has antimicrobial properties.<sup>12</sup> Amlaki is an antioxidant with free radical scavenging properties which may be due to the presence of high levels of super oxide dismutase.<sup>13</sup> Lignin isolated from *Terminalia bellirica* were shown to possess anti-HIV, antimalarial, protective effect on liver and anti-fungal activities.<sup>14</sup> The fruit pericarp of *Terminalia chebula* showed Cytoprotective activity, cardio tonic activity, anti-mutagenic activity and antifungal properties.<sup>15,16</sup>

## MATERIALS AND METHODS

### Collection of herbal material

The ingredients used in the *Triphala Churna* are Amlaki (*Embellica officinalis*), Bibhitaka (*Terminalia bellerica*), Haritaki (*Terminalia chebula*) were purchased from local market. Drugs are cleaned and dried properly. Drugs are kept separately and powdered. They are sieved using 80-mesh sieve and each one of them powdered and weighed separately and then mixed together in a suitable proportion which is showed in Table 1. It is then kept in air tight containers in cool and dry place along with Dabur Triphal Churna also purchased for the reference.

### Chemicals and requirements

Rotary Vacuum Evaporator (Equitron® Roteva), water bath (kokate; sofawora 1993) Automatic Centrifuge (Medica Instrument MFG Co.), Digital Cat cam Camera, Semi-automated Biochemistry analyzer, UV spectrophotometer (1800 Shimadzu Corporation, Kyoto, Japan). (Gallic acid (Sigma-Aldrich), Piperine (Yucca Enterprise.) Reaming chemical like Methanol, Lead nitrate Ammonium Ferrous Sulphide, Hydroxyl amine hydrochloride, Silica gel G were obtained from S. D. Fine-Chem Ltd. Mumbai, India.

### Preparation of aqueous and Ethanolic extracts of *Triphala Churna*

A weighed quantity of powder (500 gm.) was passed into sieve no. 40 and subjected to aqueous and Ethanolic extraction (maceration) with distilled water and 90% ethanol and kept at room temperature for 7 days with occasional stirring. The extract was filtered. The aqueous and Ethanolic extracts were concentrated in water bath (kokate; sofawora 1993) extraction was dried under reduced pressure using Rotary Vacuum Evaporator (Equitron® Roteva). Then the both dried extracts are preserved in the desiccator and used for further purpose.

## Pharmacognostical evaluation of powdered crude drug<sup>17-24</sup>

### Macroscopical Evaluation

Macroscopic study was carried out by color, odour and taste for samples in the form of *Churna*.

### Determination of Powder Flow Property

Physical properties of lab and market formulations were determined by the parameters described below:

#### [A] Bulk and Tap density

Both bulk density (BD) and tapped density (TD) was determined as per USP. A quantity of 10 gm of powder blend was introduced in to 25 ml measuring cylinder. After that the initial volume was noted and the cylinder was allowed to fall under its own weight on to a hard surface from the height of 2.5 cm at second intervals. Tapping was continued until no further change in volume was noted. BD and TD were calculated using the following equations.

$$\text{BD} = \text{Weight of the powder blend} / \text{Untapped Volume of the packing}$$

$$\text{TD} = \text{Weight of the powder blend} / \text{Tapped Volume of the packing}$$

#### [B] Carr's Index (Compressibility index)

The Compressibility Index of the powder blend was determined by Carr's compressibility index. The formula for Carr's Index is as below:

$$\text{Carr's Index (\%)} = [(TD - BD) \times 100] / BD$$

#### [C] Housner's ratio

The formula for Housner's ratio is as below: Housner's ratio = Tape density/Bulk density.

#### [D] Angle of Repose

The angle of repose of powder blend was determined by the funnel method. The accurately weight powder blend were taken in the funnel. The height of the funnel was adjusted in such a way the tip of the funnel just touched the apex of the powder blend. The powder blend was allowed to flow through the funnel freely on to the surface. The diameter of the powder cone was measured and angle of repose was calculated using the following equation.

$\tan \alpha = h/r$ , Where, h and r are the height and radius of the powder cone.

### Microscopical examination

Powder characteristics of the drug were studied under the microscope. The stained and unstained slide was prepared and the characters were examined and photographed using digital CCD camera.

**Procedure:** Powder was boiled with clarifying reagent chloral hydrate for few minutes. After boiling, the powder was mounted on the slide with lactophenol 50% glycerin for the unstained slide preparation and covered with the cover slip. While for the stained slide preparation, powder was stained with the phloroglucinol and Conc HCl and mounts with 50% glycerin and covered with the cover slip. The stained slide was also prepared using iodine solution for starch grains. The slides were examined under the microscope.

## Physico-Chemical Parameters

### Ash Values

#### 1) Total ash value

3 gm of the powdered drug was accurately weighed and taken in a silica crucible which was previously ignited and weighed. The powdered drug was spread as a fine even layer on the bottom of the crucible. The crucible was incinerated gradually by increasing the temperature to make it dull red hot until free from carbon. The crucible was cooled and weighed, repeated for constant value. The percentage of the total ash was calculated with reference to the air dried drug.

#### 2) Acid insoluble ash

The ash obtained as described in total ash was boiled with 25 ml of 2 N HCl for 5 min. The insoluble ash was collected on an ash less filter paper and washed with hot water. The insoluble ash transferred into a silica crucible was ignited and weighed. Repeat experiment to get a constant weight. The percentage of acid insoluble ash was calculated with reference to the air dried drug.

#### 3) Determination of Sulphated Ash value

About 3 gm. of accurately weighed air dried powdered drug taken in silica crucible which has previously ignited and weighed. Then ignited gently at first until the drug was thoroughly charred. The crucible was cooled, residue was moistened with 1 ml of concentrated Sulphuric acid heated gently until the white fumes were no longer evolved and ignited at  $800^{\circ}\text{C} \pm 25^{\circ}\text{C}$  until all the black particles has disappeared. The crucible was allowed to cool. Few drops of Sulphuric acid were added again heated. The ignition was carried out as before, allowed cooling and weighed to get a constant weight (difference is not more than 0.5 gm. between two consecutive readings). The percentage of Sulphated ash was calculated with reference to the air dried drug. All the ash values were calculated and recorded.

### Loss on drying

2 gm of powdered drug was taken in tarred china dish. Dried in the oven at  $100^{\circ}\text{C}$  or  $105^{\circ}\text{C}$ , cooled in a desiccator and watch. After that the loss was recorded as moisture. The procedure was continued for at least two common readings.

### Extractive values

#### 1) Determination of alcohol-soluble extractive

Macerated 5 g of shade dried coarse powder of leaves with 100 ml of alcohol (90%) in two separate closed flasks for 24 hrs, shaking frequently during first 6h and allowed to stand for another 18 hrs. Filtered rapidly, taking precautions against loss of alcohol. Evaporated 25 ml of the filtrate to dryness in a tarred flat bottomed shallow dish. Dried at  $105^{\circ}\text{C}$  and weighed. Calculated the percentage of alcohol soluble extract with reference to the shade dried drug.

#### 2) Determination of water soluble extractive

The same procedure was followed as directed for the determination of the alcohol soluble extractive by using chloroform water instead of alcohol. The extractive values of drugs were calculated and recorded.

### Limit test for Heavy metal of *Triphala Churna*

**Preparation of Lead nitrate stock solution** – Accurately weighed 0.1598 g of lead nitrate was diluted in 100 ml of water to which had been added 1 ml of nitric acid, then diluted with water to 1000 ml.

**Standard lead solution**–10.0 ml of lead nitrate stock solution was diluted with water to 100ml. Each ml of standard lead solution contains the equivalent of 10 µg of lead.

**Preparation of Standard solution**- 2 ml of standard lead solution was pipetted out into a 50 ml Nessler cylinder and diluted with water to 25ml. then pH Adjusted with dilute acetic acid or dilute ammonia solution between 3.0 and 4.0, then diluted with water to about 35 ml and mixed.

**Preparation of Test solution**- Accurately weighted 2 gm of *Triphala Churna* (lab and market *Churna*) was added in to suitable crucible. Sufficient Sulphuric acid was added to wet the sample and ignited carefully at a low temperature until thoroughly charred. 2 ml of nitric acid was added to charred the mass and then five drops sulphuric acid was added. It was heated cautiously until white fumes are no longer evolved Ignited, in a muffle furnace, at  $500^{\circ}\text{C}$  to  $600^{\circ}\text{C}$  until the carbon is completely burnt off. It was cooled and 4 ml of hydrochloric acid was added, covered and digested on a water bath for 15 min. The residue was moisten with one drop of hydrochloric acid. 10 ml of hot water was added and digested for two minutes. It was cooled and ammonia solution was added, drop wise, until the solution is just alkaline to litmus paper, it was diluted with water to 25 ml and pH was adjusted with dilute acetic acid between 3.0 and 4.0. The Solution was filtered. The crucible was rinsed with 10 ml of water, Filtrate were combined in a 50 ml Nessler cylinder, diluted with water, to about 35 ml and mixed.

**Procedure:** To each of the cylinders containing the standard solution and test solution respectively 10 ml of freshly prepared hydrogen sulphide solution was added, mixed and diluted with water to 50 ml, allowed to stand for five min. It was observed downwards over a white surface, the color produced in the test solution and standard solution were observed and compared.

### Preliminary Phytochemical Studies

**Introduction:** Plants may be considered as biosynthetic food laboratories in which various compounds are synthesized such as carbohydrates, proteins, lipids, flavonoids, alkaloids, volatile oils, tannins etc that exerts physiological effects. The medicinal value of any drug depends on the nature of chemical constituents present in it, which is referred as active constituent. In order to detection of active constituents plants are needed to be subject to phytochemical screening according to the standard procedures.

### Qualitative Phytochemical Analysis

#### Test for alkaloids

A small portion of the solvent free extract was stirred separately with a few drops of dilute HCL and filtered. The filtrate was tested with various reagents

**Dragendrof's Test:** To the 1 ml of extract add 1 ml of reagent (potassium bismuth iodide). An orange red precipitate indicates the presence of alkaloids.

**Mayer's Test:** To the 1 ml of extract add 1 ml of reagent (potassium mercuric iodide). Whitish yellow or cream color precipitate indicates the presence of alkaloids.

**Hager's Test:** To the 1 ml of extract add 1 ml of reagent (saturated aqueous solution of picric acid). Yellow color precipitate indicates the presence of alkaloids.

**Wagner's Test:** To the 1 ml of extract add 1 ml of reagent (iodine in potassium iodide). Reddish brown precipitate indicates the presence of alkaloids.

#### Test for flavonoids

**i) Shinoda's test:** the alcoholic extract was treated with magnesium foil and conc. HCL gives intense cherry red color, indicates the presence of flavones.

**ii) Alkaline Ammonium Test:** The Ethanolic extract is treated with 10% sodium hydroxide solution and ammonium was added. Dark yellow color indicates the presence of flavonoids.

### Test for proteins and amino acids

**i) Biurets test:** Add 1 ml of 40% sodium hydroxide solution and 2 drops of 1%  $\text{CuSO}_4$  solution till a blue color is produced and then add to the 1ml of the extract. Formation of pink or purple violet color indicates the presence of proteins.

**ii) Ninhydrin test:** Add two drops of freshly prepared 0.2% Ninhydrin reagent (0.1% solution in n-Butanol) to the small quantity of extract and heat. Development of blue color reveals the presence of proteins and amino acids.

**iii) Xanthoproteic test:** To 1 ml extract, add 1ml of concentrated nitric acid, a white precipitate is formed, it is boiled and cooled. Then 20% sodium hydroxide or ammonia is added. Orange color indicates the absence of aromatic amino acids.

### Test for tannins

About 0.5 g of the dried powdered samples was boiled in 20 ml of water in a test tube and then filtered. A few drops of 0.1% ferric chloride was added and observed for brownish green or a blue-black coloration.

### Test for steroids

Two ml of acetic anhydride was added to 0.5 g Ethanolic extract of each sample with 2 ml  $\text{H}_2\text{SO}_4$ . The color changed from violet to blue or green in some samples indicated the presence of steroids.

### Test for Terpenoids (Salkowski test)

Five ml of extract was mixed in 2 ml of chloroform and concentrated  $\text{H}_2\text{SO}_4$  (3 ml) was carefully added to form a layer. A reddish brown coloration of the interface was formed to show positive results for the presence of terpenoids.

### Test for Fats and Fixed Oils

Press a small quantity of extract and powder separately between two filter papers. Oil stains on the paper indicates the presence of fixed oil.

### Test for Volatile oils

The extract gives pink cooler with Sudan red III solution

## Quantitative phytochemical estimation of *Triphala Churna*

### Estimation of Total Tannins

Total tannin content in water extract of Amla, Baheda, Harde and *Triphala Churna* were measured by redox titration method as per reported method.

**Reagents:** 0.1 N  $\text{KMnO}_4$ : It was prepared by 3.16 gm  $\text{KMnO}_4$  was dissolved in 1000 ml distilled water. 0.1 N Oxalic acid: it was prepared by 0.63 gm oxalic acid dissolved in 100 ml distilled water.

**Preparation of *Triphala Churna* and its ingredients extract:** 1 gm powder of Amla, Baheda, Harde and *Triphala Churna* were extracted with 100 ml distilled water by heating at 70-80°C for 1 hr separately. Extract was filtered and volume adjusted to 100 ml in volumetric flask.

**Standardization of  $\text{KMnO}_4$ :** 25 ml of 0.1 N Oxalic acid was transferred in 100 ml conical flask. 25 ml of water, 5 ml of concentrated  $\text{H}_2\text{SO}_4$  was added in to conical flask and heated at 70°C. 0.1 N Oxalic acid was titrated with 0.1 N  $\text{KMnO}_4$ . End point was characterized by change color of solution from colorless to pink.

**Procedure:** 10 ml of extract from stock solution was transferred into 500 ml conical flask. 10 ml indigo carmine was added in flask as indicator then

volume was adjusted up to 300 ml. Solution was heated at 70°C for 20min. Solution was titrated with 0.1 N  $\text{KMnO}_4$ . End point was characterized by change color of solution from colorless to pink.

**Factor:** 1 ml of 0.1 N  $\text{KMnO}_4 = 0.004157$  gm. of total tannin calculated as tannic acid.

### Estimation of Total Phenolic Content

The phenolic content in the water extract of Amla, Baheda, Harde and *Triphala Churna* was measured according to reported method.

**Preparation standard stock solution:** 100 µg/ml Gallic acid standard stock solution was prepared by 10mg Gallic acid dissolved in methanol and makeup volume up to 100 ml with methanol in volumetric flask.

#### Preparation of reagent:

[A] Folin ciocalteu reagent: Folin ciocalteu reagent: distilled water (1: 2) solution was prepared.

[B] 20% sodium carbonate solution: 20 gm of anhydrous sodium carbonate was dissolved in 100 ml of distilled water.

**Preparation of *Triphala Churna* and its ingredients extract:** 1gm. powder of *Triphala Churna* and ingredients were extracted with 100 ml distilled water by heating at 70-80°C for 1h separately. Extract was filtered and volume adjusted to 100 ml in volumetric flask.

**Procedure:** 1ml of the samples solution was transferred into 25 ml volumetric flask separately. From the standard stock solution of 0.5, 0.75, 1.0, 1.25, 1.5 and 2.0 ml were transferred into 25 ml volumetric flask which gives 2, 3, 4, 5, 6 and 8 µg/ml concentrations respectively. 10 ml of water and 1.5 ml of Folin-ciocalteu reagent was added into each volumetric flask. The above mixture was kept for 5min and then 4 ml of 20% sodium carbonate solution was added. Volume was made up to 25 ml with distilled water. These mixtures were kept for 30 min and absorbance of blue color was measured at 765nm. Percentage of total phenolic was calculated from calibration curve of Gallic acid plotted by using the above procedure and total phenolic were expressed as % Gallic acid.

### Estimation of Total Flavonoid content

Total flavonoid content in *Triphala Churna* was measured by the aluminum chloride colorimetric assay.

**Preparation of Drug Extract:** 1g of *Triphala Churna* was weighed accurately and extracted with ethanol and volume is made up to 100 ml.

**Preparation of Standard solution:** Standard stock solution of Quercetin 1000 µg/ml was prepared by dissolving 10 mg in 10 ml ethanol. From this standard solution were prepared of concentration ranging 20 µg/ml, 40 µg/ml, 60 µg/ml, 80 µg/ml, 100 µg/ml.

**Procedure:** An aliquot (1 ml) of extracts and standard solution was added to 10 ml volumetric flask containing 4 ml of distilled water. To this 0.3 ml 5 %  $\text{NaNO}_2$  were added. After 5 min, 0.3 ml 10 %  $\text{AlCl}_3$  was added. Then after 1 min, 2ml of 1 M NaOH was added and the total volume was made up to 10 ml with distilled water. The solution was mixed well and the absorbance was measured against prepared reagent blank at 510nm. Total flavonoid content mg quercetin equivalents (CE)/100 G fresh weights. All samples were analyzed in triplicates.

### Estimation of Total Alkaloid content

**Preparation of std. calibration curve:** Dissolved 100 mg Piperine in 10 ml chloroform and make up volume up to 100 ml with methanol. The spectrophotometric analysis carried out by followed 10 ml of each 5, 10, 15, 20, 25, 30 and 35 µg/ml concentration of Piperine was made proper dilution and taken into separating funnel in which added 5 ml acetate buffer and 3ml 0.05% methyl orange solution and shake well. The complex formed was extracted thrice (10+10+5 ml) with chloroform.

The complex formed was transferred to another separating funnel containing 25 ml of 1 M HCL. The dye liberated into hydrochloric acid from the complex was measured against a blank at 530nm using spectrophotometer (UV1800). Blank was prepared by same method without addition of standard.

**Preparation of sample solution:** Accurately Weighed 1 gm. *Triphala Churna* powder lab and marketed *Churna* were taken, moistened with 10% ammonia (2ml), dried and refluxed and refluxed with chloroform (50ml) for 1hr. This mixture was filtered, filtrate concentrated volume was adjusted to 25 ml with chloroform. Measured volume 0.5 ml of this extracted was taken and diluted to 10 ml with chloroform in volumetric flask and further treated with reagents as described above.

### Estimation of Iron content (%)

The % Iron content in *Triphala Churna* powder lab and marketed *Churna* was measured by the colorimetric assay.

**Preparation of Standard stock solution:** Accurately weighed 25 mg Ammonium Ferrous Sulphate was dissolved in 100 ml distilled water.

**Preparation of Test solution of Churna (lab and market sample):** Accurately weighed 100 mg of material was dissolved by heating with 100 ml dilute hydrochloric acid. The temperature was not allowed to exceed 50°C. After half an hour sample was filtered. 10 ml from this filtered solution was taken and diluted up to 250 ml with distilled water.

**Procedure:** An aliquot (2, 4, 6, 8 and 10 ml of standard solution and 25 ml of test solution) was taken in 50 ml volumetric flask each. To this 4 ml of 10 % Hydroxylamine hydrochloride solution and 4 ml of 0.3 % O-Phenanthroline solution were added in to each volumetric flask. It was swirled and allowed to stand for 10 min. The total volume was made up to 50 ml with distilled water. The solution was mixed well and the absorbance was measured against prepared reagent blank at 515nm. Percentage of Iron was calculated from calibration curve of Standard Iron solution plotted by using the above procedure.

### TLC fingerprinting of Triphala Churna

#### TLC fingerprinting for Tannins

**Stationary phase:** Aluminum-backed silica gel 60 F254 plates (E. Merck)  
Solvent system: Toluene: Ethyl acetate: formic acid: methanol (3:3:0.8:0.2)  
Chamber saturation: 30 min.

**Standard solution:** Standard Gallic acid dissolved in methanol.

**Test solution:** *Triphala Churna* (laboratory and market samples) and its ingredients (Amla, Baheda, Harde) were extracted with methanol by cold maceration and methanol extracts were used for TLC fingerprinting.

**Detection:** detection carried with these wavelengths UV 254nm UV 366nm before and After Spraying with 5% FeCl<sub>3</sub> reagent.

#### TLC fingerprinting for alkaloids

**Stationary phase:** Aluminum-backed silica gel 60 F254 plates (E. Merck)

**Solvent system:** Benzene: Ethyl acetate (2:1)

**Chamber saturation:** 30 min

**Standard solution:** Standard Piperine dissolved in methanol.

**Test solution:** *Triphala Churna* (laboratory and market samples) and its ingredients were extracted with methanol by cold maceration and methanolic extracts were used for TLC fingerprinting.

**Detection:** detection carried with these wavelengths UV 254nm UV 366nm before and after Spraying with Dragendrof's reagent.

### *In vitro* Antioxidant activity of Triphala Churna<sup>25, 26</sup>

#### Method: 1, 1-Diphenyl-2-picryl hydrazyl (DPPH) radicals scavenging activity

**Preparation of Test and STD sample:** STD solution: Ascorbic acid was used as standard. Aliquots of 5- 30 mcg/ml in DMSO Test solution: Ethanolic extract of Amla, Baheda, Harde, were taken in range of 5- 30 mcg/ml in DMSO. Ethanolic extract of *Triphala Churna* (lab and market sample) were taken in range of 5- 50 mcg/ml in DMSO were prepared.

**Procedure:** 2ml of DPPH solution was added to 2ml DMSO and absorbance was taken after 30 minute at 517 nm for control reading. 2ml of DPPH were mixed with 0.6 ml of Different concentrations of sample and standard and add 2ml of DMSO. The mixture was kept in dark for 30 minutes and absorbance was measured at 517 nm after 30min.

**The % reduction:** % scavenging = (A control - A test / A control) × 100

Where, A test is the absorbance of the tested sample after 30minutes. A control is the absorbance of Control sample. IC<sub>50</sub> is the concentration required to reduce % reduction by 50%.

**Cell Culture:** Human colorectal carcinoma (HCT116) cell line obtained from the American Tissue Culture Collection (ATCC) was used for the *in-vitro* assay and grown in Roswell Park Memorial Institute medium (RPMI-1640) supplemented with 2gm of sodium bicarbonate. The pH 7.4 was maintained and the cells were incubated at 37°C with 5% CO<sub>2</sub> in humidified incubator.

**Thawing and Revival:** Cryovials containing the frozen cells from liquid nitrogen storage were quickly thawed (< 1 min) by gently swirling the vial in the 37°C water bath. Thawed cells were transferred to a sterile tube containing required amount of medium corresponding to the cell lines and inverted for uniform distribution. The cell suspension was centrifuged at 1200g for 5 min. Clear supernatant was checked for visibility of the complete pellet, re suspended in complete growth medium and transferred to T-25 flask under the recommended culture environment (5% CO<sub>2</sub> at 37°C). Growth was monitored and cells were trypsinized and sub cultured once they reached a confluence of 70-80%.

### *In vitro* Cytotoxic Activity by MTT assay<sup>28-31</sup>

The cell lines were prepared and cryopreserved using reagents such as DMSO which preserve the cell during freezing. DMSO is toxic at room temperature. The frozen ampoule is brought to room temperature by slow agitation (thawing). The frozen cryo-vials plunged into the water bath and is rapidly thawed until it gets liquefied. Solution, centrifuged with saline for 10 mins to remove the DMSO. The saline is discarded and aliquot is taken for cell counting, cell viability and for sub-culturing. MTT assay is a quantitative colorimetric assay for measuring cellular growth, cell survival and cell proliferation based on the ability of living cells. The assay was carried out using (3-(4, 5- dimethyl thiazol-2yl) - 2, 5-diphenyl tetrazolium bromide (MTT)). MTT is cleaved by mitochondrial enzyme dehydrogenase of viable cells, yielding a measurable purple product formazan. This formazan production is directly proportional to the viable cell number and inversely proportional to the degree of cytotoxicity. The result of *In vitro* cytotoxic activity of both extracts of *Triphala Churna* on Breast cancer cell lines (MCF 7 cell line) is tabulated in Table 2 and Figure 2.

#### Preparation of Herbal extract for the assay

0.5 ml of stock (100 mg/ ml) herbal extract was dissolved in 4.5 ml of DMSO for a concentration of 10 mg/ml. The fresh working suspension was filtered through 0.45µm membrane filter prior to the assay. Using the 1 mg/ ml concentration herbal extract, nine serial doubling dilutions of the extract of 500µl each was prepared in DMSO to get the concentration

of the extract as indicated and the diluted extracts will be transferred to 10 wells of a 12 well culture plate. 500 µl of 48h culture of MCF 7 cell lines at a concentration of 105 cells/ ml was added to each well. Two control wells received only cell suspensions without plant extract. The plate was incubated in a humidified CO<sub>2</sub> incubator at 37°C for 4-6h. The plate was microscopically examined for confluent monolayer of cells, turbidity and toxicity.

#### Assay Process

After incubation, the medium from the well was aspirated carefully and then discarded. Each well was washed with Eagle's Minimum Essential Medium (EMEM) without Fetal Calf Serum (FCS). 200 µl of MTT solution (5mg MTT/ ml of PBS, pH 7.2) will be added to each well. The plate was incubated for 6-7 h at 37°C in a CO<sub>2</sub> incubator with 5% CO<sub>2</sub>. After incubation 1 ml of DMSO was added to each well and mixed with pipette and left for 45s at room temperature. Purple formazan was formed in the wells. Cell control and solvent controls were included in each assay to compare the full cell viability in cytotoxicity and antitumor activity assessments. The suspension was transferred to a spectrophotometer cuvette and the optical density (OD) was measured at 540nm using DMSO as blank. The % cell viability was calculated with the following formula

$$\text{Cell viability \%} = \frac{\text{Mean OD of wells receiving each plant extract dilution}}{\text{Mean OD of control wells}} \times 100.$$

**Determination of IC<sub>50</sub>:** IC<sub>50</sub>, the concentration of compound required to inhibit 50 % cell growth, was determined by plotting a graph of Log (concentration of Extract) vs % cell inhibition. A line drawn from the 50 % value on the Y axis meets the curve and interpolate to the X axis. The X axis value gives the Log (concentration of the compound). The antilog of that value gives the IC<sub>50</sub> value. Percentage inhibition of novel compounds against all cell lines was calculated using the following formula:

$$(\text{At} - \text{Ab}) \div \text{Cell survival} = \text{-----} \times 100(\text{Ac} - \text{Ab})$$

Where, At = Absorbance of Test, Ab= Absorbance of Blank (Media), Ac= Absorbance of control (cells) % cell inhibition = 100 – % cell survival

**Statistical analysis:** The results were expressed as mean ± SEM. The Statistical comparison made by Students t-test. Only those mean values showing statistical difference  $p < 0.05$  will be considered as statistically significant.

## RESULTS AND DISCUSSION

### Macroscopical examination

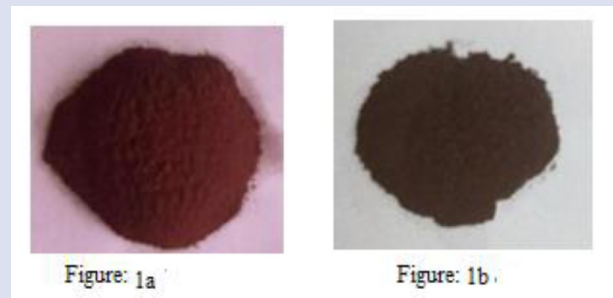
Laboratory *Churna* is reddish- brown in color with pungent odor and spicy, pungent taste, while market *Churna* is dark brown in color may be due to change in quality of raw materials the results are showed in Figure 1. All pass through sieve number 44 and not less than 50% pass through sieve number 85.

### Powder Flow Properties

For checking their flow properties such as Bulk density, Tape density, Carr's index, Angle of repose, Housner's ratio were performed for laboratory as well as market samples. The result is shown in Table 2 there was no significant difference between results of Lab as well as Market samples. From this values both samples we understand *Triphala Churna* powder having poor flow properties.

### Microscopical Examination

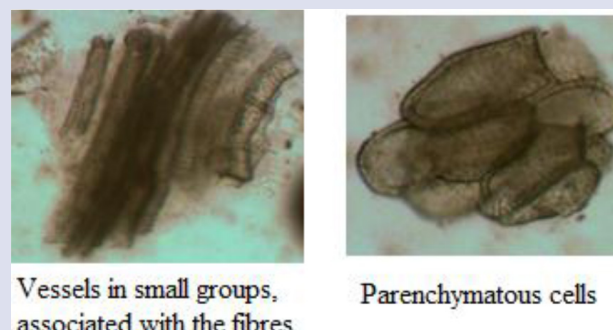
Study confirms the presence of the diagnostic characters of raw materials in *Triphala Churna*. The Microscopical characters of the plant drugs



**Figure 1:** Figure 1a and 1b Morphological characters of lab *Churna* and marketed *Churna*.



**Figure 2:** Microscopical characters of *Triphala Churna*.



**Figure 3:** Microscopical characters of *Amla* (fr.p powder).

**Table 1: Ingredients of *Triphala Churna*.**

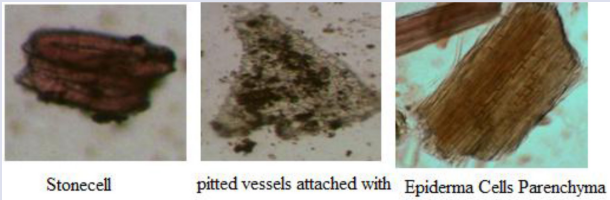
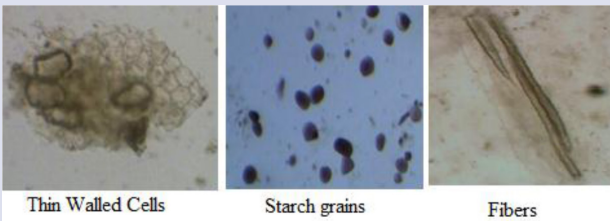
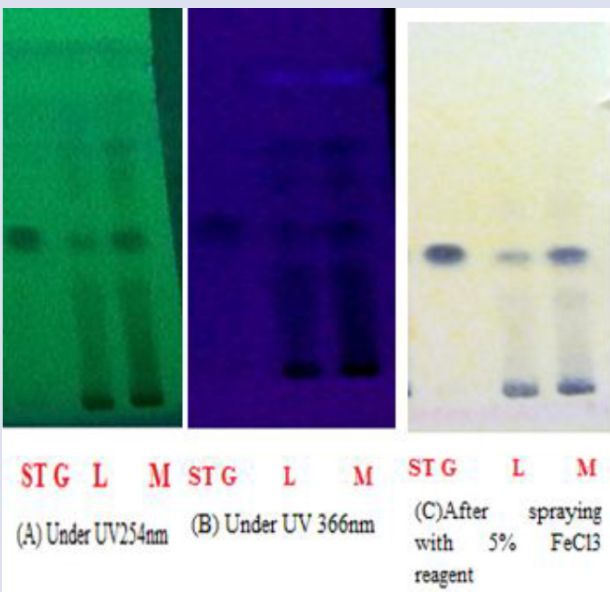
Constitute	B.S	Plant part	Quantity
Amlaki	<i>Embellica officinalis</i> .	Fr.p	1 part
Bibhitaka	<i>Terminalia bellerica</i> .	Fr.p	1 part
Haritaki	<i>Terminalia chebula</i> .	Fr.p	1 part

**Table 2: Results of powder Micromeritics of *Triphala Churna*.**

Powder flow Property	Laboratory Churna	Marketed Churna
Bulk Density*(gm/ml)	0.94±0.12	0.86±0.36
Tap Density*(gm/ml)	1.176±0.18	1.2±0.15
Hausners ratio*	1.25±0.34	1.39±0.56
Carr's index *(%)	20.06%±0.34	28.33%±0.45
Angle of Repose*	31.39±0.13	31.80±0.17

**Table 3: Ash value of *Triphala Churna*.**

Sr. no	Samples	Total Ash*(%±S.D)		Acid Insoluble*(%±S.D) (%±S.D) Ash*(%±S.D)		Water soluble *(%±S.D) (%±S.D)***Ash*(%±S.D)	
		%	STD Limit	%	STD Limit	%	STD Limit
1	Marketed	4.56±0.04	NMT 7	1.5±0.05	NMT 2	1.87±0.08	2
2	Laboratory	4.45± 0.56	NMT 7	0.75±0.003	NMT 1	1.45±0.07	2

**Figure 4:** Microscopical characters of Baheda (fr.p powder).**Figure 5:** Microscopical characters of Harde (fr.p powder).**Figure 6:** TLC fingerprinting for Tannins.**Table 4: Extractive value of *Triphala Churna*.**

Sr. no	Sample	Water Extractive value*(%±S.D)		Alcohol Extractive value* (%±S.D)	
		%	Std Limit	%	Std Limit
1	Marketed	60.5±0.05	NLT 50	46.84±0.79	NLT 40
2	Laboratory	39.78±0.67	NLT 35	9.14±1.67	NLT 8

**Table 5: Results of LOD *Triphala Churna*.**

Sr. no	Sample	Loss on drying* (%)
1	Marketed	4.05±0.56
2	laboratory	3.25±0.24

Amla, Baheda, Harde and Powders were shown in Figures. (Figure 3, Figure 4 and Figure 5) While laboratory as well as market formulations of *Triphala Churna* shows some of similar characters. So that the locally available material can be used for Ayurvedic preparations (Figure 2).

### Physico Chemical properties

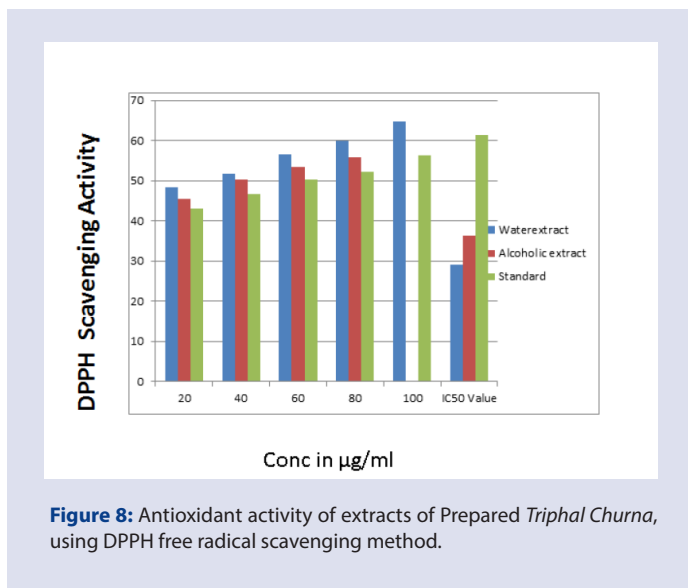
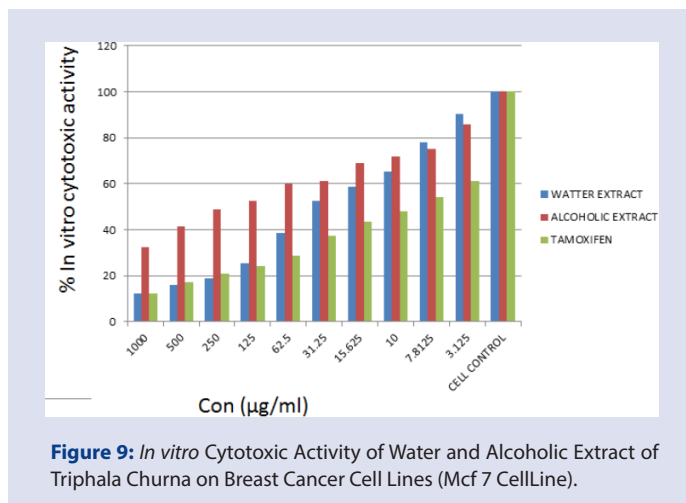
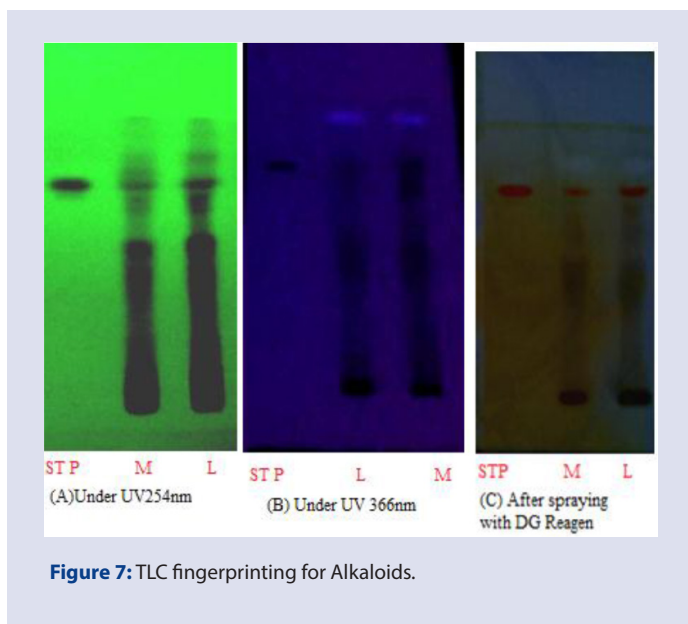
Results of the experiment on the ash values of *Triphala Churna* (laboratory and market samples) and its ingredients are given in Table 3. And that reveals all ingredients having the results in agreement with those mentioned in pharmacopoeia. *Triphala Churna* (laboratory and market sample) has mainly water soluble ash, Amount of acid insoluble ash suggesting the acceptable range of undesired heavy metal impurities. The results of alcohol soluble extractives and water soluble extractives of *Triphala Churna* (laboratory and market samples) and its ingredients are mentioned in Table 4. It appears from the results that results of extractive values of all ingredients are in agreement with those mentioned in pharmacopoeia and other references. And the results of loss on drying (laboratory and market samples) and its ingredients are mentioned in Table 5. All ingredients and *Churna* have shown loss on drying within the Pharmacopoeial limit. This indicates that these samples contain moisture content within the acceptable range.

### Limit test for Heavy metals of *Triphala Churna*

The color produced in the test solution is not darker than that produced in the standard solution. Therefore *Triphala Churna* (lab and market sample) complies with the limit of heavy metals.

### Preliminary phytochemical studies

Various Phytochemicals present in all ingredients and *Triphala Churna* are shown in Table 6 and respectively *Triphala Churna* contains Tannin, alkaloid, flavonoid and volatile oils.



**Table 6: Results of Phytochemical investigation of *Triphala Churna*.**

Sr.No	Test For	Water extract	Ethanolic
1	Phenolics and Tannins	+	+
2	Alkaloids	+	+
3	Saponins	-	-
4	Flavanoids	+	+
5	Volatile oil	+	+
6	Proteins	-	-

**Table 7: Quantitative phytochemical analysis of *Triphala Churna*.**

Sr no	Sample	Total Tannins	Total Phenolics	Total Alkaloid	Total Flavonoid	% Iron
1	Lab Churna	6.34±1.32	5.26±1.67	1.56±1.56	0.48±1.23	43.3±0.24
2	Market Churna	4.93±2.45	4.25±1.34	1.07±1.73	0.33±2.45	34.1±1.45

**Table 8: Antioxidant activity of extracts of Prepared *Triphal Churna*, using DPPH free radical scavenging method.**

Conc in µg/ml	Water extract	Alcoholic extract	Standard
20	48.42 ± 1.03	45.4 ± 1.03	43.19 ± 1.112
40	51.78 ± 0.9	50.18 ± 0.9	46.63 ± 1.10
60	56.66 ± 0.72	53.54 ± 0.72	50.4 ± 0.83
80	59.9 ± 1.12	55.8 ± 1.12	52.29 ± 0.53
100	64.71 ± 0.69	59.35 ± 0.69	56.32 ± 0.44
IC <sub>50</sub> Value	29.065	36.2789	61.46

## Quantitative phytochemical estimation of *Triphala Churna*

Results of the quantitative phytochemical showed in the Table 7. By observing the values the *Triphala Churna* having Significantly Higher Contents and this phytochemical are having good biological importance.

## TLC fingerprinting of *Triphala Churna*

### TLC fingerprinting for Tannins, Alkaloids

TLC fingerprinting of *Triphala Churna* (Lab and Market sample) for Tannins give the spot corresponds to Gallic acid with the same R<sub>f</sub> (0.57). It gives dark blue colored spot after spraying with 5 % FeCl<sub>3</sub> Methanol Solution (Figure 6). For the alkaloids *Triphala Churna* gives the spot corresponds to the standard piperine with the same R<sub>f</sub> (0.83). It gives yellow colored spot after spraying with Dragndroff’s Reagen (Figure 7). *Triphala Churna* also gives the spot at the same R<sub>f</sub> as embelin (0.93). It gives blue fluorescence under U.V. light (366 nm).

## Pharmacological Evaluation of *Triphala Churna*

### *In vitro* Antioxidant activity of *Triphala Churna*

By the 1-Diphenyl-2-picryl hydrazyl (DPPH) radicals scavenging activity the conducted for the both extracts and anti-oxidant activity or the above extracts is good. The results are showed in Table 8 and Figure 8.



**Table 9: In vitro Cytotoxic Activity of Water And Alcoholic Extract Of Triphala Churna On Breast Cancer Cell Lines (Mcf 7 Cell Line).**

S. No	Con (µg/ml)	Dilutions	In vitro cytotoxic activity		Tamoxifen
			Water extract	Alcoholic extract	
1	1000	NEAT	12.32 ±0.25	32.56 ±0.59	12.33 ± 0.51
2	500	1:01	15.96 ±0.89	41.23 ±0.32	17.18 ±1.58
3	250	1:02	18.96 ±1.23	48.92 ±0.57	20.98 ±1.47
4	125	1:04	25.36 ±0.89	52.35 ±0.44	24.13 ±0.55
5	62.5	1:08	38.59 ±1.05	59.87 ±0.14	28.78 ±0.87
6	31.25	1:16	52.55 ±1.47	61.23 ±0.57	37.17 ±0.69
7	15.625	1:32	58.79 ±1.69	68.77 ±0.22	43.46 ±0.54
8	10	1:64	65.32 ±0.58	72.02 ±1.06	48.19 ±1.25
9	7.8125	1:128	78.19 ±0.47	75.12 ±1.08	54.33 ±1.06
10	3.125	1:256	90.23 ±0.55	85.64 ±1.89	61.22 ±0.88
11	Cell Control	-	100	100	100

### In-vitro Cytotoxic Activity of Water and Alcoholic Extract of *Triphala Churna* (Breast Cancer Cell Lines Mcf7)

In vitro cytotoxic studies were conducted for the aqueous and ethanol extracts and results are showed in Table 9 and Figure 9.

### CONCLUSION

Standardization of *Triphala Churna* was done using pharmacognostical and physicochemical parameters, preliminary photochemical investigation; TLC fingerprinting and proximate analysis of active constituents (tannins, phenolic, alkaloids, flavanoids and iron) was also done by UV spectrophotometer. Market sample was also evaluated and compared with laboratory sample. Limit test of heavy metals (lead) was done as for lab *churna* and Marketed *churna*. Heavy metals are found in limit in *Triphala Churna* (lab and market sample).

There was variation between market and laboratory *Churna* regarding ash values, extractive values, total tannins, phenolic and iron content. These variations may be due to change in the quality of raw materials. Result of the analysis of the laboratory sample of *Triphala Churna* gave higher amount of tannins, phenolic, flavonoids, alkaloids and iron compared to market *Triphala* sample. It is also concluded that from the pharmacognostical and physicochemical parameters, preliminary photochemical investigation, TLC fingerprinting and proximate analysis of active constituent that all raw materials are genuine.

The antioxidant activity of the plant extract was then characterized using the DPPH radical scavenging method. Antioxidant activity using DPPH was found to increase in concentration dependent manner. All the extracts of aqueous and methanol exhibited potential antioxidant activity with an IC<sub>50</sub> value of 25 µg/ml when compared to the standard Ascorbic Acid with an IC<sub>50</sub> value of the 7.5 µg/ml.

The cytotoxic activity of the extracts of *Triphala Churna* extraction MCF-7 cells from human breast cancer was investigated *in vitro* 3-(4) 5-Dimethyl-thiazol-Zyl) - 2,5 biphenyl tetrazolium bromide (MTT). The results showed decreased cell viability and cell growth inhibition in a dose dependent manner. The IC<sub>50</sub> value of standard Tamoxifen, Ethanolic extract were 9.3, 39.06 µg/ml respectively. Ethanolic extracts of *Triphala* extract demonstrated strong antioxidant and anti-proliferative activities. Accumulating evidence clearly indicates that apoptosis is a critical molecular target by dietary bioactive agents, in the prevention of cancer. Since the phytochemical analysis has shown the presence of potent phytochemicals like alkaloids, phenols, flavonoids, terpenoids, glycosides, saponins, steroids, tannin and sugars, etc. Several authors reported that

phenolic, acids, flavonoids, steroids, terpenoids are known to be bioactive principles.

### ACKNOWLEDGEMENT

The authors would like to thank Management, Principal, Teaching, Non-teaching Staff of GITAM University, Visakhapatnam and AM Reddy Memorial College of pharmacy Narasaraopet Andhrapradesh, India for providing support for successful completion of research work.

### CONFLICT OF INTEREST

The authors declare no conflict of interest.

### ABBREVIATIONS

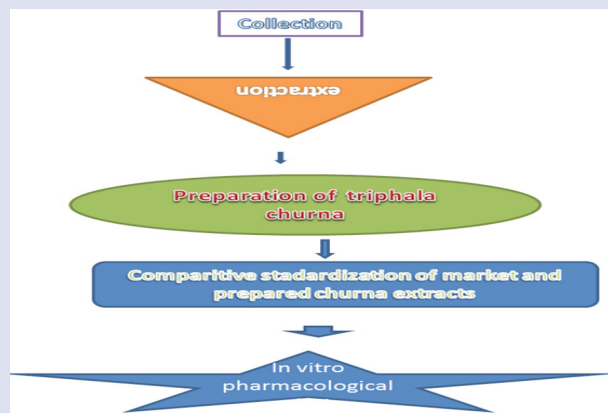
**WHO:** World Health Organization; **TLC:** Thin Layer Chromatography; **HPLC:** High Performance Liquid Chromatography; **GC:** Gas Chromatography; **Bd:** Bulk density; **TD:** Taped Density; **LPTC:** Laboratory prepared *Triphala churna*; **MTL:** Marketed *Triphala churna*; **AELPTC:** Aqueous extract of Laboratory prepared *Triphala churna*; **EELPTC:** Ethanolic extract of Laboratory prepared *Triphala churna*; **DPPH:** 1-Diphenyl-2-picryl hydrazyl and 3 MTT:3-(4) 5-Dimethyl-thiazol-Zyl) - 2,5 biphenyl tetrazolium bromide.

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## GRAPHICAL ABSTRACT



## SUMMARY

- Collection Triphala Churna ingredients from local market.
- Preparation and extraction using aqueous and ethanol.
- Evaluation of flow properties of powder.
- Pharmacognostical Evaluation of both extracts using standard guidelines.
- Evaluation of *in-vitro* anti-oxidant and *in-vitro* cytotoxic activities of both extraction cell lines breast cancer cell lines MCF7.

## ABOUT AUTHORS



**Mr. Gunji Venkateswarlu:** Currently working as Asst. Prof, Department of Pharmacognosy, A M Reddy Memorial College of Pharmacy, Narasaraopet, He Is Research scholar In GITAM university and Published Many Article In National and International peer reviewed journal .His interest is to develop a New Herbal Formulation and standardization using WHO Guidelines In the present article he focused on *In Vitro* Evaluation of Triphala Powder Extract.



**Dr. Seru Ganapaty:** Professor and Principal in Gitam Institute of Pharmacy, Vizag and previously he worked As Rector as Well as Principal In Andhra University. His teaching experience (UG&PG)- (34years) and Research experience (34years). He Supervised 43 doctorates (Ph.D. degree) And Supervised 60 M.Pharm projects till to date Apart from that he was Successfully completed 3 Major Research projects and 3 Minor Research projects on Indian Medicinal Plants funded by UGC and AICTE, New delhi published more than 145 Research papers in National and International Journals and 2 Patents



**Dr. A M S Sudhakar Babu:** Currently working professor Cum Principal, Department of Pharmaceutics, A.M. Reddy Memorial College of Pharmacy. His Main Contribution Is development various new formulation Regarding the Herbal as well as Allopathic Drugs.

**Cite this article:** Venkateswarlu G, Ganapaty S, Babu AMSS. Preparation of *Triphala Churna* using the Ingredients Obtained from Local Market and Comparative Standardization. Pharmacog J. 2019;11(1):102-11.