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Proximate, powder microscopic, liquide chromatographic and *in-vitro* anti-inflammatory activity of marketed athimadhuram churnas

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Abstract
This article is presented to evaluate proximate, powder microscopy and liquid chromatographic analysis and *in vitro* anti inflammatory activities of marketed Athimadhuram churnas, The raising use of herbal drugs by the human is forcing the driving force to evaluate the health claim of the agents and to develop standards of quality, purity, safety and efficacy of the drug. Mostly the herbal drugs are effective but due to adulteration and lack of standardization, the effectiveness of herbal drugs is decreased. So there is need to develop the standards. It carries the quality, purity and safety of herbal drug and formulation.

Keywords: Athimadhuram churna, proximate analysis, powder microscopy, liquid chromatography, *In-vitro* anti-inflammatory

Introduction
Licorice, the root of *Glycyrrhiza glabra* L. (or) Athimadhuram has been used medically for over 2000 years [1, 2], Indeed, the extract of the root of *Glycyrrhiza glabra* having important medicinal properties, including healing of ulcers and wounds and quenching thirst [3, 4], Also licorice has shown anti-inflammatory [5], antiarthritic [6], anti-arrhythmic [7], anti-bacterial [8], antiviral and expectorant activity [9, 10], A recent animal study indicates that licorice may be useful in treating lupus. It is now known that glycyrrhizic acid and its aglycone glycyrrhetinic acid present in the root extract are responsible for these biological activities [11], The genus *glycyrrhiza* consists of approximately 30 species [12], in which six species produce a sweet saponin glycyrrhizic acid (GA), and they are widely used in Asia countries. These medicinal plants were used as flavorings, sweeteners and as herbal medicine, and they were also used for improving health, detoxification and cures for injury [13].

Materials and Methods
Materials
Athimadhuram churna (from different markets), methanol and ethanol (Merck Pvt. Ltd.), ferric chloride, nitric acid, Fehling's solution, Dragendroff's reagent, Acetic anhydride, (Qualigen chemicals, Mumbai), chloroform (SD-fine, Mumbai).

Determination of Moisture Content (Loss on Drying)
Procedure set forth here determines the amount of volatile matter (i.e., water drying off from the drug). For substances appearing to contain water as the only volatile constituent, the procedure given below, is appropriately used. Place about 10 g of drug (without preliminary drying) after accurately weighing (accurately weighed to within 0.01 g) it in a tarred evaporating dish. For example, for underground or un powdered drug, prepare about 10 g of the sample by cutting shredding so that the parts are about 3 mm in thickness. Seeds and fruits, smaller than 3 mm should be cracked. Avoid the use of high speed mills in preparing the samples, and exercise care that no appreciable amount of moisture is lost during preparation and that the portion taken is representative of the official sample. After placing the above said amount of the drug in the tarred evaporating dish dry at 105º for 5 hours, and weigh. Continue the drying and weighing at one hour interval until difference between two successive weighing corresponds to not more than 0.25 per cent. Constant weight is reached when two consecutive weighing after drying for 30 minutes and cooling for 30 minutes in a desiccator, show not more than 0.01 g difference [14].

Formula: $\frac{(w2-w3)\times 100}{(w2-w1)}$

W1 = weight of crucible; W2= weight of crucible + weight of sample.
W3= weight of crucible + weight of dried content.

Determination of foreign matter
Weigh 100 –500 g of the drug sample to be examined or the minimum quantity prescribed in the monograph, and spread it out in a thin layer. The foreign matter should be detected by inspection with the unaided eye or by the use of a lens (6x). Separate and weigh it and calculate the percentage present.

Determination of Total Ash
Incinerate about 2 to 3 g accurately weighed, of the ground drug in a tared platinum or silica dish at a temperature not exceeding 450º until free from carbon, cool and weigh. If a carbon free ash cannot be obtained in this way, exhaust the charred mass with hot water, collect the residue on an ash less filter paper, incinerate the residue and filter paper, add the filtrate, evaporate to dryness, and ignite at a temperature not exceeding 450º. Calculate the percentage of ash with reference to the air-dried drug.

Formula: $\frac{\text{weight of total ash}}{\text{weight of crude drug taken}} \times 100$

Determination of Acid Insoluble Ash
Boil the ash obtained in for 5 minutes with 25 ml of dilute hydrochloric acid; collect the insoluble matter in a Gooch crucible or on an ash less filter paper, wash with hot water and ignite to constant weight. Calculate the percentage of acid insoluble ash with reference to the air dried drug.

Formula: $\frac{\text{weight of acid insoluble ash}}{\text{weight of crude drug taken}} \times 100$

Determination of Water Soluble Ash
Boil the ash for 5 minutes with 25 ml of water; collect insoluble matter in a Gooch crucible or on an ash less filter paper, wash with hot water, and ignite for 15 minutes at a temperature not exceeding 450º. Substract the weight of the insoluble matter from the weight of the ash; the difference in weight represents the water soluble ash. Calculate the percentage of water-soluble ash with reference to the air dried drug.

Formula: $\frac{\text{weight of water soluble ash}}{\text{weight of crude drug taken}} \times 100$

Determination of Sulfated Ash
A silica crucible was heated to redness for 10 minutes, allowed to cool in desiccators and weighed. 1 g of substance was accurately weighed and transferred to the crucible. It was ignited gently at first, until the substance was thoroughly charred. Then the residue was cooled and moistened with 1 ml concentrated sulfuric acid, heated gently until white fumes are no longer evolved and ignited at 800° ± 25°C until all black particles have disappeared. The ignition was conducted in a place protected from air currents. The crucible was allowed to cool, and a few drops of concentrated sulfuric acid were added and heated. Ignited as before, allowed to cool, and weighed. The operation was repeated until two successive weighing does not differ by more than 0.5 mg. calculate the percentage of Sulphated ash with reference to the air dried drug.

Determination of Alcohol Soluble Extractive
Macerate 5 g of the air dried drug, coarsely powdered, with 100 ml of Alcohol of the specified strength in a closed flask for twenty-four hours, shaking frequently during six hours and allowing standing for eighteen hours. Filter rapidly, taking precautions against loss of solvent, evaporate 25 ml of the filtrate to dryness in a tared flat bottomed shallow dish, and dry at 105º, to constant weight and weigh. Calculate the percentage of alcohol-soluble extractive with reference to the air-dried drug.

Determination of Water Soluble Extractive
Macerate 5 g of the air dried drug, coarsely powdered, with 100 ml of Water in a closed flask for twenty-four hours, shaking frequently during six hours and allowing standing for eighteen hours. Filter rapidly, taking precautions against loss of solvent, evaporate 25 ml of the filtrate to dryness in a tared flat bottomed shallow dish, and dry at 105º, to constant weight and weigh. Calculate the percentage of alcohol-soluble extractive with reference to the air-dried drug [14].

Determination of heavy metals, pesticide residues and Aflatoxin
The Athimadhuram churnas extracts were analysed to detect the presence of heavy metals (Cd, Pb, As and Hg), pesticides residues and Aflatoxins by HPLC according to official methods of the American Organization of Analytical Chemists (AOAC) [15].

Test for heavy metals
Limit Test for Lead
The following method is based on the extraction of lead by solutions of dithizone. All reagents used for the test should have as low a content of lead as practicable. All reagent solutions should be stored in containers of borosilicate glass. Glassware should be rinsed thoroughly with warm dilute nitric acid, followed by water.

Special Reagents
1. Ammonia-cyanide solution Sp. – Dissolve 2 g of potassium cyanide in 15 ml of strong ammonia solution and dilute with water to 100 ml.
2. Ammonium citrate solution Sp. – Dissolve 40 g of citric acid in 90 ml water.
   Add two drops of phenol red solution then add slowly strong ammonia solution until the solution acquires a reddish colour. Remove any lead present by extracting the solution with 20 ml quantities of dithizone extraction solution until the dithizone solution retains its orange-green colour.
3. Dilute standard lead solution – Dilute 10.0 ml of standard lead solution with sufficient 1 per cent v/v solution of nitric acid to produce 100.0 ml. Each ml of this solution contains 1 µg of lead per ml.
4. Dithizone extraction solution – Dissolve 30 mg of diphenylthiocarbazone in 1000 ml of chloroform and add 5 ml of alcohol. Store the solution in a refrigerator. Before use, shake a suitable volume of the solution with about half its volume of 1 per cent v/v solution of nitric acid and discard the acid.
5. Hydroxylamine hydrochloride solution Sp. – Dissolve 20 g of hydroxylamine hydrochloride in sufficient water to produce about 65 ml. Transfer to separator, add five drops of thymol blue solution, add strong ammonia solution until the solution becomes yellow. Add 10 ml of

a 4 per cent w/v solution of sodium diethyl dithiocarbamate and allow to stand for five minutes. Extract with successive quantities, each of 10 ml, of chloroform until a 5 ml portion of the extract does not assume a yellow colour when shaken with dilute copper sulphate solution. Add dilute hydrochloric acid until the solution is pink and then dilute with sufficient water to produce 100 ml.

6. Potassium cyanide solution Sp. – Dissolve 50 g of potassium cyanide in sufficient water to produce 100 ml. Remove the lead from this solution by extraction with successive quantities, each of 20 ml of dithizone extraction solution until the dithizone solution retains its orange-green colour. Extract any dithizone remaining in the cyanide solution by shaking with chloroform. Dilute this cyanide solution with sufficient water to produce a solution containing 10 g of potassium cyanide in each 100 ml.
7. Standard dithizone solution – Dissolve 10 ml of diphenylthiocarbazone in 1000 ml of chloroform. Store the solution in a glass-stoppered, lead-free bottle, protected from light and in a refrigerator.
8. Citrate-cyanide wash solution – To 50 ml of water add 50 ml of ammonium citrate solution Sp. and 4 ml of potassium cyanide solution Sp., mix, and adjust the pH, if necessary, with strong ammonia solution to 9.0.
9. Buffer solution pH 2.5 – To 25.0 ml of 0.2 M potassium hydrogen phthalate add 37.0 ml of 0.1 N hydrochloric acid, and dilute with sufficient water to produce 100.0 ml.
10. Dithizone-carbon tetrachloride solution –Dissolve 10 mg of Diphenyl thiocarbazone in 1000 ml of carbon tetrachloride. Prepare this solution fresh for each determination.
11. pH 2.5 wash solution – To 500 ml of a 1 per cent v/v nitric acid add strong ammonia solution until the pH of the mixture is 2.5, then add 10 ml of buffer solution pH 2.5 and mix.
12. Ammonia-cyanide wash solution – To 35 ml of pH 2.5 wash solution add 4 ml of ammonia-cyanide solution Sp., and mix.

Method

Transfer the volume of the prepared sample directed in the monograph to a separator and unless otherwise directed in monograph, adds 6 ml of ammonium citrate solution Sp., and 2 ml hydroxylamine hydrochloride solution Sp., (For the determination of lead in iron salts use 10 ml of ammonium citrate solution Sp.). Add two drops of phenol red solution and make the solution just alkaline (red in colour) by the addition of strong ammonia solution. Cool the solution if necessary, and add 2 ml of potassium cyanide solution Sp. Immediately extract the solution with several quantities each of 5 ml, of dithizone extraction solution, draining off each extract into another separating funnel, until the dithizone extraction solution retains its green colour. Shake the combine dithizone solutions for 30 seconds with 30 ml of a 1 per cent w/v solution of nitric acid and discard the chloroform layer. Add to the solution exactly 5 ml of standard dithizone solution and 4 ml of ammonia-cyanide solution Sp. and shake for 30 seconds; the colour of the chloroform layer is of no deeper shade of violet than that of a control made with a volume of dilute standard lead solution equivalent to the amount of lead permitted in the sample under examination.

Powder microscopy

Powder microscopic analysis were performed as per the method followed in practical pharmacognosy by K.R. Khandelwal [14].

Preliminary Phytochemical analysis

1. Test for carbohydrates

a. **Molisch's test:** A small quantity of aqueous extract was subjected to Molisch's reagent (α-naphthol in alcohol). Shake and add conc. $H_2SO_4$from sides of the test tube. A violet ring is formed at the junction of two liquids. This indicates the presence of carbohydrates.
b. **Fehling's test:** A small quantity of aqueous extract was subjected to Fehling's A and B reagents ($CuSO_4$ solution), boil on water bath. A brick red precipitate is formed. This indicates the presence of reducing sugars.
c. **Test for Pentose's:** 2 ml of test solution + 2ml of Hydrochloric acid + Phloroglucionol and heat. A red color is produced. This indicates the presence of pentose.
d. **Selivanoff's test** for ketones: Test solution was added to resorcinol crystals, hydrochloric acid and heat on water bath. Rose color is produces. This indicates the presence of keto sugars (Fructoses).

2. Test for amino acids

a. **Ninhydrin test:** Aqueous or alcoholic extract was subjected to ninhydrin solution and boil. A purple or bluish color is appeared. This indicates the presence of amino acids.

3. Test for proteins

a. **Xanthoproteic test:** Test solution was added to conc. HNO_3boil the mixture, cool and add sodium hydroxide (NaOH) solution. A yellow precipitate is formed and after addition of alkali, orange color is formed. This indicates the presence of proteins.

4. Test for fixed oils and fats

a. Extract pressed between two filter papers. Oil stain appears on filter paper. This indicates the presence of fixed oils.

5. Test for alkaloids

a. **Dragendorff's test:** Test extract was subjected to Dragendorff's reagent (Potassium bismuth iodide solution).A orange-brown precipitate is observed. This indicates the presence of alkaloids.

6. Test for saponin glycosides.

a. **Foam test:** Shake the extract vigorously with water. Persistent foam is formed. This indicates the presence of saponin glycosides.
b. **Haemolytic test:** Extract + drop of blood placed on a glass slide. A haemolytic zone is appeared. This indicates the presence of saponin glycosides.

7. Test for triterpinoids aponins

a. **Liebermann's test:** Extract was subject to acetic anhydride, heat, cool and add conc.H_2SO_4. A pink color is appeared. This indicates the presence of triterpinoids aponins.
b. **Trichloroacetic acid test:** Extract + saturated solution of Trichloroacetic acid. Yellow color is formed. This indicates the presence of triterpenes.

8. Test for Flavanoids

a. **Sulphuric acid test:** extract was added to 66% or 80% of sulphuric acid. Deep yellow solution is formed which indicates the presence of flavones and flavonols. Orange to red color solution is formed which indicates the presence of flavones. Red or reddish blue solution is formed which indicates the presence of aurones and chalcones.
b. **Shinoda test:** Extract + 95% ethanol + conc. Hydrochloric acid + Mg turnings. Orange, pink, red to purple color is observed. This indicates the presence of flavonols, dihydro derivatives and xanthenes.
c. **Test for lead acetate:** Test residue was subjected to lead acetate solution. A yellow color precipitate is formed. This indicates the presence of Flavanoids.

9. Test for phenolic compounds.

a. **Ferric chloride test:** Test extract was subjected to ferric chloride solution. Blue color indicates the presence of hydrolysable tannins. Green color indicates the presence of condensed tannins.
b. Alcoholic extract was subjected to dil.HNO_3. Reddish to yellow color is appeared. This indicates the presence of tannins.

10. Test for Phytosterols and Triterpinoid.

a. **Salkowski test:** Extract + chloroform + conc. H_2SO_4, shake well. $CHCl_3$layer shows red color which indicates the presence of steroids, acid layer shows greenish yellow color which indicates the presence of triterpenoids.
b. **Liebermann's test:** Extract + acetic anhydride, heat, cool and add conc. H_2SO_4. Blue color is appeared. This indicates the presence of steroids.

11. Test for resins

a. Alcoholic extract was subjected to ferric chloride solution. Green color is observed. This indicates the presence of resins.

Thin-Layer Chromatography (TLC)

Thin-layer chromatography is a technique in which a solute undergoes distribution between two phases, stationary phase acting through adsorption and a mobile phase in the form of a liquid. The adsorbent is a relatively thin, uniform layer of dry finely powdered material applied to a glass, plastic or metal sheet or plate. Separation may also be achieved on the basis of partition or a combination of partition and adsorption. Identification can be effected by observation of spots of identical R_f value and about equal magnitude obtained, respectively, with an unknown and a reference sample chromatographed on the same plate. A visual comparison of the size and intensity of the spots usually serves for semi-quantitative estimation [16].

Apparatus

(a) Flat glass plates of appropriate dimensions which allow the application at specified points of the necessary quantities of the solution being examined and appropriate reference solutions and which allow accommodation of the specified migration path-length. The plates are prepared as described below; alternatively, commercially prepared plates may be used. (b) An aligning tray or a flat surface on which the plates can be aligned and rested when the coating substance is applied. (c) The adsorbent or coating substance consisting of finely divided adsorbent materials, normally 5 µm to 40 µm in diameter is suitable for chromatography. It can be applied directly to the plate or can be bonded to the plate by means of Plaster of Paris (Hydrated Calcium Sulphate) or with any other suitable binders. The adsorbent may contain fluorescing material to help in visualizing spots that absorb ultra-violet light. (d) A spreader which, when moved over the glass plate, will apply a uniform layer of adsorbent of desired thickness over the entire surface of the plate. (e) A storage rack to support the plates during drying and transportation. (f) A developing chamber that can accommodate one or more plates and can be properly closed and sealed. The chamber is fitted with a plate support rack that supports the plates, back to back, with lid of the chamber in place. (g) Graduated micro-pipettes capable of delivering microliter quantities say 10 µl and less. (h) A reagent sprayer that will emit a fine spray and will not itself be attacked by the reagent. (i) An ultra-violet light, suitable for observation at short (254 nm) and long (365 nm) ultra-violet wavelengths.

Preparation of plates-Unless otherwise specified in the monograph, the plates are prepared in the following manner. Prepare a suspension of the coating substance in accordance with the instructions of the supplier and, using the spreading device designed for the purpose, spread a uniform layer of the suspension, 0.25 to 0.30 mm thick, on a flat glass plate 20 cm long. Allow the coated plates to dry in air, heat at 100º to 105º for at least 1 hour (except in the case of plates prepared with cellulose when heating for 10 minutes is normally sufficient) and allow to cool, protected from moisture. Store the plates protected from moisture and use within 3 days of preparation. At the time of use, dry the plates again, if necessary, as prescribed in the monographs.

Preparation of sample (test) solution

Add 10 ml of 70% methanol to 1 gr of dried athimadhuram powder, heat by shaking on water bath for 5 min, cool and filter.

Preparation of reference solution

Add 1 ml of 70% methanol solution to 1 mg of standard Glycyrrhetinic acid solution.

Mobile phase

Toluene: Ethyl acetate: Glacial acetic acid (12.5:7.5:0.2 v/v) is used as the mobile phase. The developed plate was dried under normal air and the spots were visualized or examine under U.V light at 254 & 365 nm and by spraying Anasaldehyde. The (retention factor) values of isolated compounds and standard were calculated and compared.

Fluorescent study of Athimadhuram Churna

0.5gms of Athimadhuram powder were taken into clean and dried test tubes. To each tube 5ml of different organic solvents like distilled water, acetone, ethanol, chloroform, methanol, hydrochloric acid, FeCl3, NaoH, HNO_3, NH_3 were added separately. Then, all the tubes were shaken and they were allowed to stand for about20-25 min. The solutions obtained were observed under the visible light and UV light for their characteristic colour reaction and were compared with a standard colour chart and colours were recorded [17].

Identification of Glycyrrhetinic Acid in Athimadhuram Churnas by Liquid Chromatography

Preparation of Extract

Athimadhuram churna was used in this study. The material is

cleaned and set free from moulds, insects, animal faecal matter and other contaminations such as earth, stones and extraneous materials. The specimen was shade dried and protected from sun light for several days not less than one month. It was ground to a fine powder using mortar and pestle without any loss of powdered drug. Then it was passed through a sieve of 40 mesh and the material passed by the sieve was collected and stored in a well tight amber coloured container and it was used for further study. A coarsely powdered aerial part of the plant (about 5gr) was taken into a neat conical flask. To that 100ml of ethanol is added and heat on the hot plate for 1hr. keeps the conical flask aside for 24 hr. The homogenate was filtered using Whatmann's filter paper and the volume of the filtrate was recorded. About 25ml of filtrate is taken into a clean china dish. Place the china dish on the hot plate to evaporate the solvent totally and collect the extract at the last.

Test sample solution preparation

Accurately weighed 100 mg of herbal alcoholic extract of Licorice or Athimadhuram was transferred to 100mL volumetric flask and dissolved with small amount of ethanol and keep 15 min for sonication for dissolving the extract and final volume was adjusted with same solvent in 100mL volumetric flask to get the solution containing 100µg/mL. The sample solution was then filtered through Whatman's filter paper No.41. Aliquots of working stock solutions of glycyrrhetinic acid was prepared with in the same solvent to get concentration in range of 2-10 µg/ml of glycyrrhetinic acid [18-21].

Chromatographic condition

Table 1: The chromatographic system of the developed method.

Mobile phase	Acetonitrile/phosphoric acid (3/1)
Column	C-18 reversed phase column (VP-DS, (250×4.6 mm, 5 mm))
Temperature	30 °C
Detector	photodiode-array detector (SPD-10A),
Flow rate	1 ml/min
Injection volume	10.0 µl
Run rate	10 min

Anti-Inflammatory activity

Serum albumin protein denaturation method

The reaction mixture contains 1 ml of test extract and 1 ml of 1% w/v aqueous solution of bovine albumin fraction. The pH of the reaction mixture was adjusted by drop by drop small amount of conc. HCL. The sample extract was incubated at 37 ^{o}C for 20 min and the sample mixture was heated at 71 ^{o}C for 5 min. cool the samples and then the colorimetric assay of albumin denaturation was performed. The absorbance was measured at 660nm and all the tests are performed in triplicate [22].

$$\% \text{ inhibition of denaturation} = 100 - \frac{O.D \text{ of test control} - O.D \text{ of product control}}{O.D \text{ of test control}} * 100$$

Results and Discussion

This study aimed to evaluate the marketed Athimadhuram churnas with estimation and evaluation of its content and efficacy by UV spectrophotometry and *in-vitro* Bovine albumin denaturation method respectively. Microscopical characters of the powdered churnas were performed for identification of diagnostic features of the known drug powder. This study helps the analyst to know the adulteration of genuine drug. Therefore the results shows Vessels, which are found singly or in small groups; some of the individual vessels are very large and are frequently found fragmented. They are lignified and usually bordered pitted, although in some of the large vessels the pits are very much elongated and the borders are very difficult to discern. These are shown in figure no: 3. Starch grains, The abundant starch granules, most of which are simple; they are rather small, spherical to Ovid and slightly flattened; a slit-shaped hilum is visible in some of the large granules. A few compound granules are present with two, three or four components. These are shown in figure no: 4. Calcium oxalate crystals, The majority of which are fairly uniform in size and occurred in the cells forming the crystal sheath surrounding the fibers. In addition a few large prisms occure; they are present in some of the parenchymateous cells of the medullary rays and pith and may be found in these cells or, more usually, scattered in the powder. These are shown in figure no: 5. Fibers, The very abundant fibers which occurs in group surrounded by the calcium oxalate prism sheath. Individual fibers are very thick-walled, few small pits; the different layers in the walls are sometimes clearly seen and only the middle lamella and primary walls give a reaction for lignin; frequently no lumen is visible. These are shown in figure no: 6. Orange browncork, The fairly abundant fragments of orange brown cork composed of thin-walled cells; in surface view the cells are polygonal and fairly regular in outline. These are shown in figure no: 7.

This study preliminary observed the proximate analysis such as Moisture content, Foreign organic matter, Ash value, Extractive value and fluorescence analysis. The results obtained from the study was depicted in table no (1, 2, 5) and figure no (1, 2, 9). The values are with in the limit prescribed by the CCRAS/WHO monographs.

Through it is a crude form of dosage form the study intended the determination of residues such as metal, pesticides and Aflatoxins. The results are present in table no 3. The residue are complies with the limits prescribed by CCRAS and it is considered as important parameter to evaluate other wise the residual matters will make toxic effect to the end users.

The preliminary phytochemical evaluation is important for any churnas especially churnas with single crude drug. Hence the study reveals the presence of Saponin glycoside, Triterpinoid Saponins, flavonoids, phenolic and tannins. Phytosterols and resins in both the formulation of Ethanolic extract the results were shown in table no: 4.

The preliminary phytochemical screening includes determination of retention factor (R_f) by using thin layer chromatography. This study involved in the optimization of mobile phase to separate the individual compound and to

identify the Glycyrrhetinic acid. The mobile phase of Toluene: ethyl acetate: glacial acetic acid (12.5:7.5:0.2) was taken as mobile phase. This study observes three distinguished spots up on exposed to UV chamber (Short wavelength). The $R_f$values identified spots were taken for calculation of R_f values and standard comparison were also performed to appropriate separation of the Glycyrrhetinic acid from churnas used in this study. The Glycyrrhetinic acid were separated successfully by matching with standard spots as well as 0.53 cm of R_f value. The results were shown in figure no: 8.

The study further extended its aim to confirm the presence of Glycyrrhetinic acid in two marketed formulation. The ethanolic extract of both the formulation were subjected for liquid chromatographic determination with optimum chromatographic conditions of mobile phase of Acetonitrile: phosphoric acid (3:1) column used in is C-18 reversed phase column (VP-ODS, (250×4.6 mm, 5 mm)), temperature maintained in 30 °C. Detector used in the study is photodiode array detector (SPD-10A). flow rate fixed to elute the compound is 1 ml/min. the injection volume 10.0ml with 10 min of run rate. The chromatogram obtained is showing the retention time (Rt) of 1.66 min for Glycyrrhetinic acid. The chromatographic conditions are present in table no: 6 and chromatograms were present in figure no: 10 & 11.

Anti-inflammatory activity of Athimadhuram churnas were evaluated in this study for assessment of efficacy of them. The study took Bovine albumin denaturation method for the evaluation of anti-inflammatory activity by *in-vitro* model. The study reports were presented in table no: 6. The results of both the Churnas paved the linear activity with the concentration increment. The study charts were presented in figure no: 12 &13.

Table 2: proximate analysis of Athimadhuram Churnas

S.no	Constituents	Sample1 gms /%	Sample2 gms /%	Standard values
1.	Moisture content	1%	1%	
2.	Forgein organic matter	0.04 %	0.02%	Not more than 2%
3.	Total ash	0.84 /84%	0.94/94%	Not more than 10%
4.	Acid insoluble ash	0.09 /9%	0.04/4%	Not more than 2%
5.	Water soluble ash	0.03 /3%	0	Not more than 2%
6.	Sulphated ash	0.04/4%	0.05/5%	Not more than 10%

Fig 1: Crucible showing amount of Athimadhuram ashes.

Table 3: Extractive values of Athimadhuram Churnas

Extracts	Sample 1	Sample 2
Alcohol soluble extract	0.22gm	0.28gm
Water soluble extract	0.36gm	0.3gm

Fig 2: Crucible showing water and alcohol soluble extract of Athimadhuram Churnas.

Table 4: Determination of heavy metal, pesticide residues, Aflatoxins (mg/kg).

S. No	Test parameters	Athimadhuram churnaextract		Permissible limit
		Sample 1	Sample 2	
1	Cadmium (Cd)	0.28 ± 0.03	0.26 ± 0.01	0.30 mg/kg
2	Lead (Pb)	0.48 ± 0.12	0.45 ± 0.11	10 mg/kg.
3	Arsenic (As)	0.47 ± 0.05	0.43 ± 0.02	0.5 mg/kg
4	Mercury (Hg)	0.33 ± 0.08	0.31 ± 0.06	1.0 mg/kg.
5	Pesticide residue	Absent	Absent	0.01%
6	Aflatoxins	Absent	Absent	Absent

Microscopic evaluation

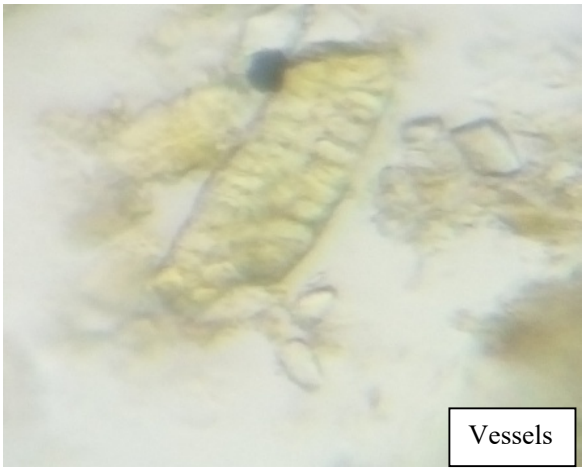


Fig 3: Vessels present in Athimadhuram churna.

Vessels: which are found singly or in small groups; some of the individual vessels are very large and are frequently found fragmented. They are lignified and usaually borderded pitted, although in some of the large vessels the pits are very much elongated and the borders are very difficult to discern. Many of the smaller, narrow vessels show a single perforation in the somewhat oblique end walls. The larger vessels are usually accompained by lignified xylem parenchyma composed of moderatly thin walled cells, square to enlongated rectangular in outline with variably pitted walls.

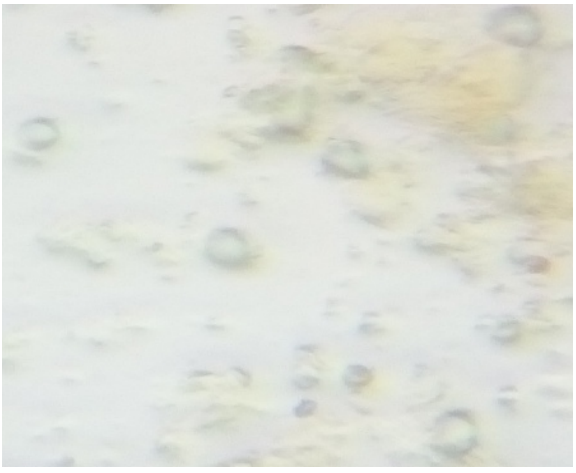


Fig 4: Calcium oxalate crystals present in Athimadhuram churna.

Calcium oxalate crystals: The majority of which are fairely uniform in size and occurred in the cells forming the crystal sheath surrounding the fibers. In addition a few large prisms occure; they are present in some of the parenchymateous cells of the medullary rays and pith and may be found in these cells or, more usually, scattered in the powder

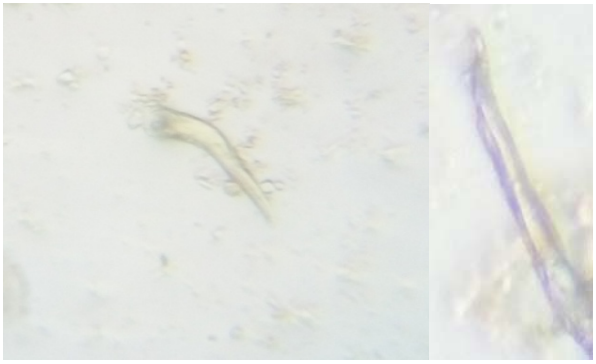


Fig 5: Fibers present in Athimadhuram churna.

Fibers: The very abundant fibers which occurs in group surrounded by the calcium oxalate prism sheath. Individual fibers are very thick-walled, few small pits; the different layers in the walls are sometimes clearly seen and only the middle lamella and primary walls give a reaction for lignin; frequently no lumen is visible.

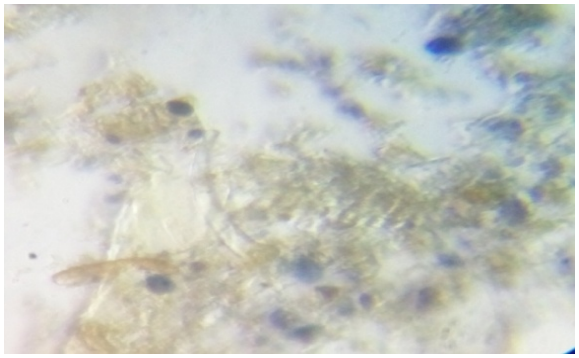


Fig 6: Starch grainspresent in Athimadhuram churna.

Starch grains: The abundant starch granules, most of which are simple; they are rather small, spherical to Ovid and slightly flattened; a slit-shaped hilum is visible in some of the large granules. A few compound granules are present with two, three or four components.

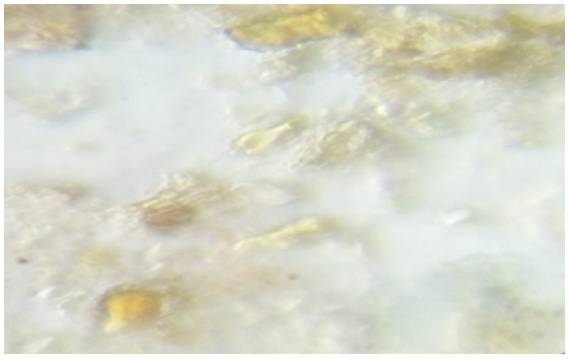


Fig 7: Orange brown corkpresent in Athimadhuram churna.

Orange brown cork: The fairly abundant fragments of orange brown cork composed of thin-walled cells; in surface view the cells are polygonal and fairly regular in outline.

Table 5: Preliminary Phytochemical screening of Athimadhuram churnas

Name of the phytoconstituents	Sample 1	Sample 2
Carbohydrates	+	+
Amino acids	_	_
Proteins	+	+
Fixed Oils	_	_
Alkaloids	_	_
Saponin glycosides	+	+
Triterpenoids Saponins	+	+
Flavonoids	+	+
Phenol/Tannins	+	+
Pentose's	+	+
Ketones	+	+
Phytosterols &Triterpenoids	+	+
Resins	+	+

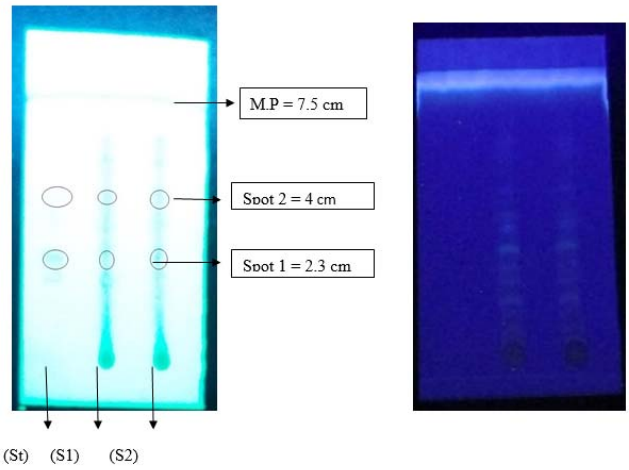
Thin layer chromatography (TLC)

Calculation of R_f values:

Solvent front travelled = 7.5cm

R_f value of spot 1 = $\frac{2.3}{7.5}$ = 0.306cm.

R_f value of spot 2 = $\frac{4}{7.5}$ = 0.53cm.



a. Short wavelength (254nm) b. Under long wavelength (365nm)

Fig 8: showing the equal R_f (0.306, 0.53) for standard, sample 1, sample 2.

R_f value of about 0.3 (β-glycyrrhetinic acid) and smaller spot with an R_f value of about 0.5 ($^{\alpha}$-glycyrrhetinic acid).

Fluorescence analysis of Athimadhuram Churna

Table 6: Fluorescence analysis of Athimadhuram churnas

S. No	Organic solvents	Visible/Day light	254 nm	365nm
1	Distilled water	Pale brown	greenish	Light greenish
2	Acetone	Pale brown	Light greenish	Light yellowish brown
3	Ethanol	Light brownish	greenish	Brownish green
4	Chloroform	Brownish	Thick greenish	Pale green
5	Methanol	Light brownish	greenish	Pale brown
6	Hydrochloric acid	Clay colour	Thick greenish	Thick brownish
7	Ferric chloride	Dark green	Light greenish	Brownish
8	Sodium hydroxide	Dark brown	Light greenish	Brownish
9	Nitric acid	Brownish yellow	Light greenish	Brownish yellow
10	Ammonia	Dark brownish	Brownish green	Brownish yellow

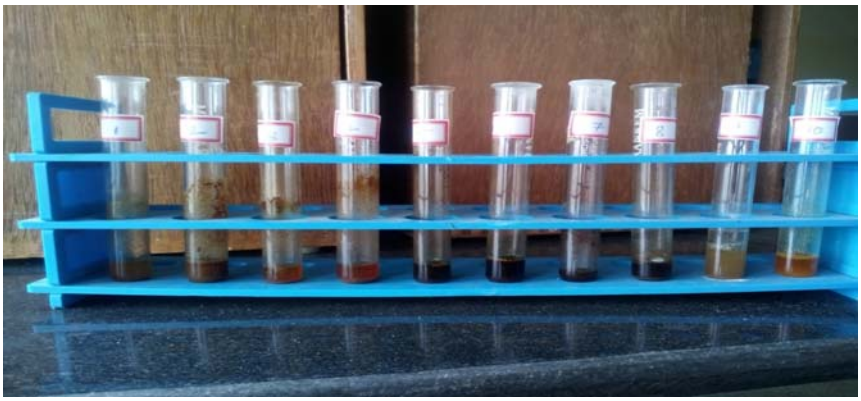


Fig 9: Fluorescent analysis of athimadhuram Churna. Powder + organic solvents (1.acetone, 2.Chloroform, 3.Methanol, 4 Ethanol, 5.Ferric chloride, 6.Sodium hydroxide, 7.Hydrochloric acid, 8.Ammonia, 9.Water, 10.Nitric acid).

Determination of Glycyrrhetinic Acid in Athimadhuram Churna by Liquid chromatography

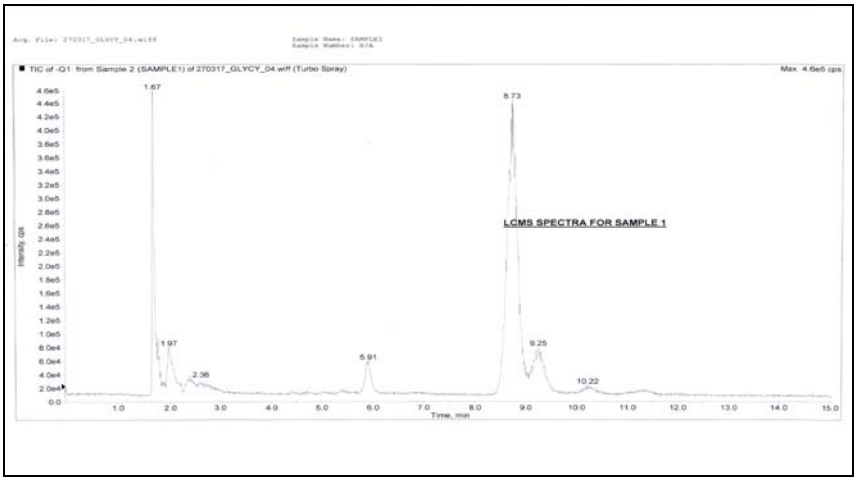


Fig 10: Chromatogram of glycyrrhetinic acid in marketed sample 1.

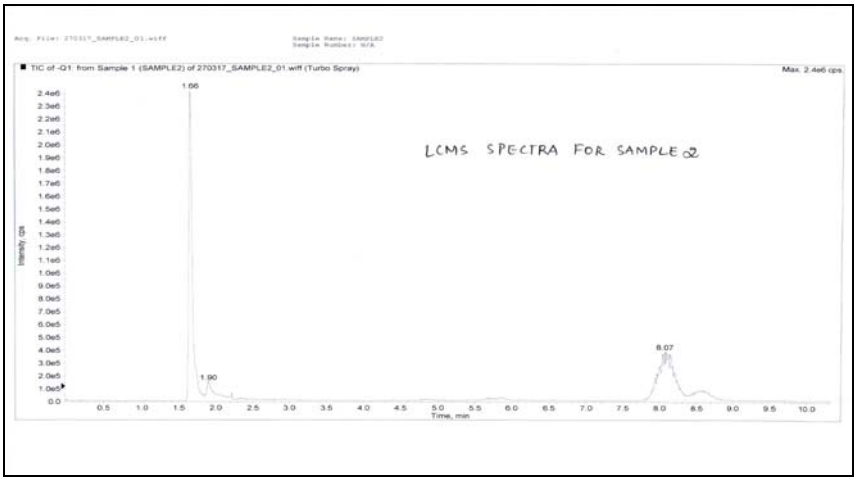


Fig 11: Chromatogram of glycyrrhetinic acid in marketed sample 2.

In-vitro Anti-inflammatory activity
Bovine albumin denaturation method

Table 7: Anti-inflammatory activity of ethanolic extract of athimadhuram churna by serum albumin denaturation method.

S. No.	Concentration of standard Glycyrrhetinic acid (µg/ml)	Sample 1Absorbance at 660 nm	Sample 2Absorbance at 660 nm
1	0	0	0
2	250	0.0233	0.0195
3	500	0.0498	0.0339
4	1000	0.0913	0.0618

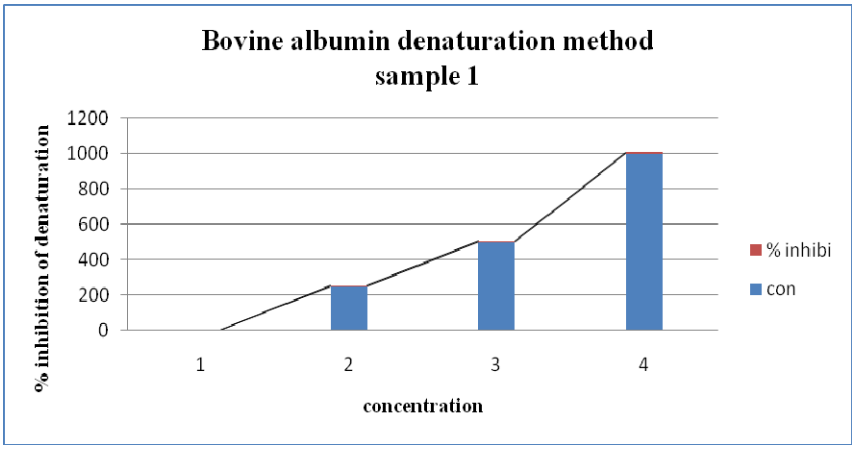


Fig 12: Anti-inflammatory activity elicited by ethanolic extract of marketed athimadhuram churna 1.

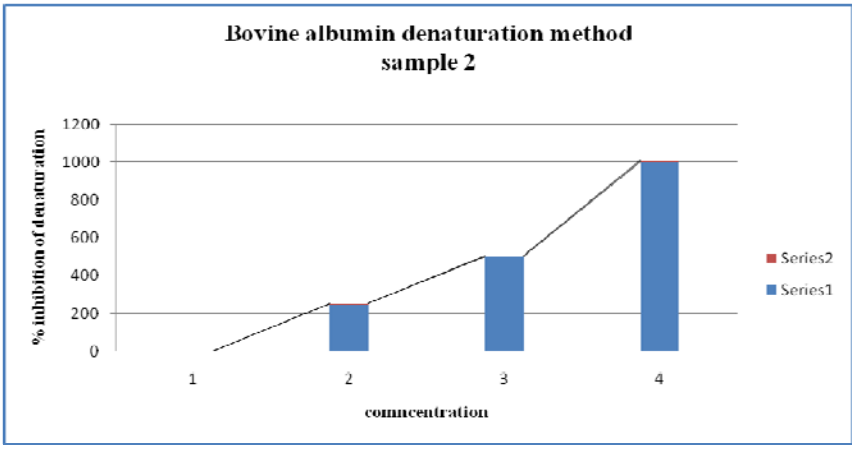


Fig 13: Anti-inflammatory activity elicited by ethanolic extract of marketed Athimadhuram churna 2.

Conclusion

The study concluded with the procured athimadhuram Churnas are fulfilling the required standards mentioned in WHO (World health organization) and CCRAS (Central council for research in ayurveda and siddha) guidelines and hence the proprietary medicines sold in market also carrying the qualities to the end user.

Acknowledgement

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Conflict of interest

The authors are not showing any conflict of interest to publish this work and they were worked equally.

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