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Methodical Analysis of an Absolute Efficacious Ayurveda Medicine-- Amruthotharam

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Abstract: Amruthotharam/Amrutottaram Kashayam is one of such preparation which takes care of metabolic disorder through inflammation. The formulation composed of three ingredients-- *Tinospora cordifolia*, *Terminalia chebula* and *Zingiber officinale*. Amrutottaram acts by treating indigestion and neutralization of AMA, thereby helps reduce inflammation and fever by virtue of its immune-modulatory function providing ingredients. The anti-inflammatory properties prove to be significant in an array of pathologies like fevers, swelling, systemic inflammation or local inflammations, painful pathologies associated with muscular stiffness, neurogenic pain and thereby useful in any pathology pertaining to any system. The metabolic syndrome is a group of risk factors that together enhance chance of developing cardiovascular disease. The dried drugs were extracted separately by soxhlet extraction process. The present work involved the detailed study of drugs in dried form which can be used in routine analysis of drug. As, the formulation contains *Terminalia chebula* in major amount, gallic acid was considered as marker compound. The formulation was quantified for the content of gallic acid by HPLC method.

Keywords: Ayurveda, HPLC, *Tinospora cordifolia*, *Terminalia chebula*, *Zingiber officinale*

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Introduction

The term metabolic syndrome refers to a number of risk factors that increases risk of cardiac disease as well as other health issues including diabetes and stroke. Metabolic is the biochemical process involved in the normal

functioning of body. When this process is disturbed at any point the subsequent alterations are noticed in the body giving rise to various pathologies. These pathologies are usually in-borne and not germ-borne. Many such pathologies

that range from digestive upsets to various kinds of syndromes or carcinogenic alterations in the body are found to be related to the basic process of metabolism in the body. These pathologies are therefore counted as metabolic disorders or collectively as metabolic syndrome (Ahlqvist *et al.*, 2011).

Various kinds of metabolic imbalances are associated with Diabetes mellitus type 2 (DM2), hypertension, cardiovascular disease, atherosclerotic changes in blood vessels and elevated lipids. This can be clubbed under the pathological process associated with Metabolic Syndrome. According to the latest reports, around 440 million worldwide and more than 10 million cases in India, suffer from DM 2. Diabetes type 2 initiates when the body's ability to produce, release or respond to insulin is affected consequently resulting in increased blood sugar levels (Bleich *et al.*, 2008). In the therapy of diabetes mellitus, over 1200 plant species have been identified, many of which may potentially target additional risk factors linked with metabolic syndrome, such as hypertension and hypercholesterolemia (Marles and Farnsworth, 1995).

Traditional herbal medicine and its preparations have been widely utilized in developing and developed countries for thousands of years due to their natural origins or lower adverse reactions or unhappiness with synthetic medication. According to Ayurveda, the 5 elements [prithvi (earth), jala (water), agni (fire), vayu (air), aakash (space or ether)] are the fundamentals on which the body and the universe sustain their harmony. They are the structural units that constitute the universe as well as the human body.

The medication is basically in the forms of various herbal, herbo-mineral, mineral drug formulations aimed at elimination of the root pathology of any kind of disorder in the body (Lad, 1987). Ayurvedic physiology is based on the three humoral pathophysiological elements known as

the Doshas. The three Doshas are termed as Vata, Pitta and Kapha. The type and nature of disease are mostly attributed to the dosha that is imbalanced. For example, inflammatory diseases wherein there is increase in fire element in the body in the form of heat like hyperacidity or putrefaction (Balachandran and Govindarajan, 2005). The vitiated, aggravated Doshas, weak dhatus, and deranged Agni collectively weaken the immune status and make the person fall prey to all kinds of imbalances ranging from small digestive disturbances like hyperacidity, fevers or grave pathologies such as cancer. There are many treatment modalities in Ayurveda, to correct the status of Agni and metabolize or neutralize AMA based on the state and the stage of the disease. In the present work Amrutottaram formulation was prepared by traditional method and modern method and standardization of formulation was done using HPLC method as the standardization of such preparations is a big challenge because of some factors such as variation in the qualitative constitutional analysis of the drug that is sourced from different geographies pertaining to differing climatic inclinations, leading to difference in the overall drug action that what is expected, difference in climate, harvesting and procuring methods, environmental hazards and collection protocols. Therefore, the present work aimed to develop and validate the methods so that we can uplift the classical Ayurvedic drug preparations in global market.

Materials and Methods

Materials:

Drugs: The crude forms of drugs *Tinospora cordifolia*, *Terminalia chebula*, *Zingiber officinale* were procured from authentic supplier in dried form.

Chemicals: Chloroform, HCl, ethyl alcohol, phloroglucinol, were procured from SD fine.

Apparatus and Instruments: Magnetic stirrer, silica crucible, Hot air Oven, ashless Whatman filter

paper, electric shaker, petridish, TLC, HPLC.

Method of preparation:

Preparation of extracts:

Preparation of all three extracts such as *Terminalia chebula* extract, *Tinospora cordifolia* extract and *Zingiber officinalis* extract were carried out by dissolving Hareda Dry Powder in water in the ratio of 2:1, Guduchi dry powder in water in the ratio of 7:1 and Ginger powder in methanol in the ratio of 5:1, respectively. The extraction was done by soxhlet extraction method.

Method of preparation of Amruthotharam Kashaya:

Amruthotharam Kashaya was formulated by mixing 2 parts of aqueous *Tinospora Cordifolia*, 4 parts of aqueous *Terminalia Chebula* and 1 part of methanolic *Zingiber officinale*. The prepared formulation was then studied for their phytochemical studies.

Evaluation of raw material and formulations:

Pharmacognostical Evaluation of raw materials:

Macroscopic Examination:

Evaluation of macroscopic and organoleptic characters have been based on shape, size, colour, surface characteristics, texture, fracture, odour and taste according to WHO guidelines (WHO, 1998).

Microscopic Examination:

The microscopic evaluation was carried out on stem part of *Tinospora cordifolia*, Stem part of *Tinospora chebula* and root part of *Zingiber officinale*. The study involved the determination of cork, cortex, vasculature, epicarp, mesocarp and stem of the species. The research microscope was used for the anatomical studies.

Powder Microscopy:

The investigation of powder characteristics for all three powders including *Tinospora cordifolia*, *Tinospora chebula* and *Zingiber officinale* were done by pulverizing to 80#. The staining of the pulverized powder of all three drugs was done with phloroglucinol and concentrated

hydrochloric acid in 1:1 ratio to study fibres, crystal fibres containing prisms of calcium oxalate, tracheids, polygonal epidermal cells, stone cells and parenchyma containing starch grains under the microscope.

Phytochemical Evaluation of raw materials:

Examination of phytochemical properties of the powdered drugs including loss on drying, ash value, moisture content and extractive value was done according to Trease and Evans (1983) and Evans (2008).

Phytochemical Evaluation of all three prepared extracts:

Examination of phytochemical properties such as pH, heavy metals and microbial contamination was done for the prepared extracts of three crude drug samples.

pH:

The pH of sample was determined by taking 1 g of powdered drug blended with 10 ml of distilled water and left in a magnetic stirrer for 10 min and measured with a digital pH meter that had been calibrated and stabilized with buffer tablets. Steady readings in pH were documented.

Test for heavy metal:

Sample digestion:

Accurately weighed 1 g of each powdered plant sample was taken in 250 ml beaker. 80 ml water was added to it and the solution in the beaker was boiled on hotplate till the volume reduced to 5-10 ml. This was then cooled and transferred into 100 ml volumetric flask. The beaker was washed 3- 4 times with distilled water and the washings were transferred to the volumetric flask. Finally the volume adjusted up to the mark with distilled water. From this stock solution suitable amount of filtrate was pipetted out in another 100 ml volumetric flask and diluted with distilled water. The sample was used for the analysis of copper, zinc, iron and lead.

Perpetration of Standard solution:

Copper:

Cupric acetate of about 286 mg was accurately weighed; added to 1 ml concentrated hydrochloric acid and warmed on water bath. The dilution was made up to 100 ml in volumetric flask with distilled water and filtered. The reading was taken by using copper lamp at 324.8 nm.

Iron:

Ferric ammonium sulphate (864 mg) was accurately weighed; added to 1 ml concentrated hydrochloric acid and warmed on water bath. The dilution was made up to 100 ml in volumetric flask with distilled water and filtered. The reading was taken by using Fe-lamp at 248.3 nm.

Lead:

Lead (1 mg) was dissolved in 6 N HNO₃ and was diluted with 1000 ml distilled water. The reading was taken by using 1 amp lamp at 405.8 nm.

Zinc:

Zinc of about 100 mg was accurately weighed; added to 1 ml concentrated hydrochloric acid and warmed on water bath. The dilution was made up to 100 ml in volumetric flask with distilled water and filtered. The reading was taken by using copper lamp at 213.9 nm.

Microbial contamination:

Standard method was used to study Microbial contamination in herbal drugs (Mukherjee, 2002).

Total viable count:

Powdered plant sample (each of 10 g) was dissolved in 100 ml of sterile nutrient broth, kept in incubator at 37°C for 24 h separately. After 24 h incubation if culture shows growth of organism, then serial dilution was done. 1 ml sample (plant material + nutrient broth) was added into 9 ml sterile saline. Similarly, dilutions were carried up to 10¹⁰ and 1 ml of 10⁶ dilutions were pipetted into each of two sterile nutrient agars. Similarly 10⁷, 10⁸, 10⁹ and 10¹⁰ dilutions were pipetted into duplicate plates. Samples were spread on nutrient agar by use of spreader in sterile condition. Plates were further incubated at 37°C for 5 days. Positive and negative control was run. The numbers of

colonies were counted and the average for 3 plates was expressed in terms of microorganism per g of plant sample (colony forming units - cfu per g of plant sample).

Total Yeast and Mould count:

Powdered plant sample (10 g each) was dissolved in 100 ml of sterile nutrient broth kept in incubator at 37°C for 24 h separately. After 24 h incubation if culture shows growth of organism, then serial dilution was done. 1 ml of each sample (plant material + nutrient broth) was added into 9 ml sterile saline. Similarly, dilution was carried up to 10¹⁰ and 1 ml of 10⁶ dilutions was pipetted into each of two sterile Sabouraud chloramphenicol agar. Similarly 10⁷, 10⁸, 10⁹ and 10¹⁰ dilution were pipetted into duplicate plates. Samples were spread on nutrient agar by use of spreader in sterile condition. Plates were further incubated at 20- 25°C for 5 days. Positive and negative control was run. The numbers of colonies were counted and the average for 3 plates were expressed in terms of microorganism per g of plant sample (colony forming units - cfu per g of plant sample).

Quantitative analysis of bitters in Tinospora cordifolia:

Weighed the sample of 3 g and extracted with 50 ml of methanol for 30 min. Filtered the extracts and extracted the residue two times with 50 ml of methanol for 15 min each. Evaporated all the mixed filtrate in a steel dish to dryness on water bath. Distilled water of about 10 ml was added to the residue in steel dish and extracted it with 25 ml of ethyl acetate (four times) in a separating flask. The upper organic layer was combine and evaporated to dryness in a previously weighed beaker on a water bath. Weighed the beaker previously heated in an oven at 105°C for 1 h.

Qualitative analysis of tannins in harida extract:

The sample of about 1 g was weighed taken in a conical flask and add 100 ml of distilled water. Shake the prepared solution for 2 h and then allowed to stand for overnight. Filtered the solution and pipette out 10 ml of filtrate in a conical flask. Distilled water of about 750 ml and

25 ml of indigo sulphonic acid solution was added to the above filtrate and titrated against 0.1N potassium permanganate solution. By excluding the amount of sample, blank reading was performed.

Quantitative analysis of Volatile oil in Zingiber officinale:

The sample of 10 g was weighed and added in a round bottom flask. Glycerin and distilled water of about 75 ml and 175 ml, respectively were added to the above round bottom flask. Few glass beads and 6 stripes of filter paper (7 cm x1 cm) were also added to it. Attached the flask to the Dean stark apparatus. The oil was collected from the mixture after heating.

Marker based standardization of the formulation using HPLC:

HPLC Instrumentation, method development and method validation:

The study was carried out on Agilent (1100) system HPLC. The column used was Cosmosil C18 (4.6 x 250 mm, 5 μ m). Various solvent systems were tried for the development of suitable HPLC methods for the standardization of gallic acid. The suitability of the solvent system was decided on the basis of the retention time and tailing factor. The amount of standard gallic acid was transferred in the volumetric flask to produce 1000 μ g/ml of concentration. Further dilutions were made to produce 10- 50 μ g/ml of concentrations. The sample solution of 1 mg/ml concentration was prepared by dissolving amount of sample in the solvent to produced sample stock solution. The dilutions were carried out to obtain the 0.5 mg/ml of concentration used as injection solution. The solutions were scanned between the wavelength range 400-200 nm using the UV spectrophotometer. The method was validated for linearity parameter as per ICH guidelines (ICH Q2, 2005). The linearity was analyzed for different concentrations ranges from 10- 50 μ g/ml. Least-square regression analysis was used to treat the data of the peak areas plotted against the corresponding concentrations.

Results

Evaluation of raw material and formulation:

Pharmacognostical Evaluation of raw materials:

Macroscopic Examination:

Macroscopic evaluation of Tinospora cordifolia:

The drug's young stems are green and smooth, with swelling at the nodes; the older stems are light brown and marked with warty protuberances caused by circular lenticels; a transversely smoothed surface reveals a given the high level with obvious medullary rays traversing porous tissues; and the drug does have a bitter taste (Fig. 1).

Macroscopic evaluation of Terminalia chebula:

The intact fruit has an astringent taste and is oval in shape. It is 20-35 mm in length and 13-25 mm in width. Thickness-wise, the pericarp averages 3-4 mm and is fibrous (Fig. 2).

Macroscopic evaluation of Zingiber officinale:

Rhizome is laterally compressed and bears short, flattish, ovate, oblique branches above; fracture is short and smooth; transverse surface has a thin cortex (approximately one-third of the radius), a well-marked endodermis, and a broad stele with many dispersed fibro-v structures (Fig. 3).

Microscopic Examination:

Microscopic evaluation of Tinospora cordifolia:

Rhizome is laterally compressed and bears short, flattish, ovate, oblique branches above; fracture is short and smooth; transverse surface has a thin cortex (approximately one-third of the radius), a well-marked endodermis, and a broad stele with many dispersed fibro-v structures (Fig. 4).

Microscopic evaluation of Terminalia chebula:

Transverse section of the pericarp reveals the epicarp, which is made up of one layer of epidermal cells and the inner tangential and upper portions of the radial wall thick, the mesocarp, which has two to three layers of collenchyma, followed by a broad zone of parenchyma, which



Fig. 1: Dried material of *Tinospora cordifolia*.



Fig. 2: Dried material of *Terminalia chebula*.



Fig. 3: Dried material of *Zingiber officinale*.

has fibres and sclereids grouped together and vascular bundles dispersed, fibres with peg-like outgrowth and simple (Fig. 5).

Microscopic evaluation of Zingiber officinale:

Rhizome cortex is seen in the transverse slice of isodiametric thin-walled parenchyma with scattered vascular strands and numerous isodiametric idioblasts, about 40-80 in diameter, containing a yellowish to reddish-brown oleo-resin, endodermis slightly thick walled, free of starch, and immediately inside endodermis a row of nearly continuous collateral bundles, typically without fibres, stele of thin-walled, parenchyma cells (Figs. 6A- E).

Powder Microscopy:

Powdered microscopy of Tinospora cordifolia:

It is a cream-brown substance with starch grains, tracheids, fibres, crystal fibres containing calcium oxalate prisms, and vessel components with bordered pits visible under a microscope (Fig. 7).

Powdered microscopy of Terminalia chebula:

Under a microscope, it appears yellowish brown in colour, bitter and astringent with a sweetish aftertaste, and has parenchyma that contains starch granules in addition to a few fibres and polygonal epidermal cells (Fig. 8).

Powdered microscopy of Zingiber officinale:

Fibers, polygonal epidermal cells, stone cells, and parenchyma containing starch grains make up the yellowish brown, bitter, and astringent substance.

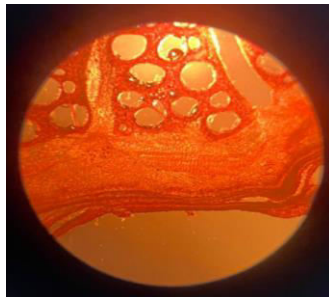
Phytochemical Evaluation of raw materials:

Following the examination of raw materials for microscopic features, phytochemical parameters were examined, including foreign matter, total ash, acid-insoluble ash, extractives that were alcohol soluble and those that were water soluble, as shown in Tables 1, 2 and 3. The outcomes were found to be within the acceptable requirements.

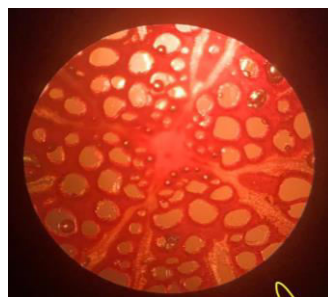
Phytochemical Evaluation of all three prepared extracts:



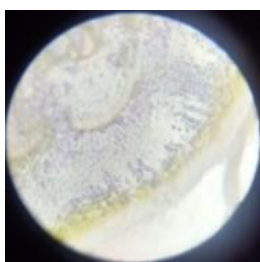
4A



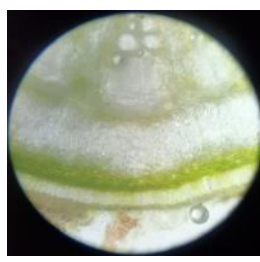
4B



4C



Iodine solution



Dil.sulphuric acid



Phloroglucinol + HCl

4D

Fig. 4A- D: T.S. of *Tinospora cordifolia*.



Fig. 5: T.S. of *Terminalia chebula*.



6A



6B



6C



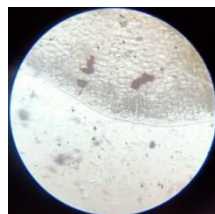
6D



Phloroglucinol + HCl



Alcoholic picric acid



Sudan red



Dil. Iodine solution

6E

Fig. 6A- E: T. S. of *Zingiber officinale*.

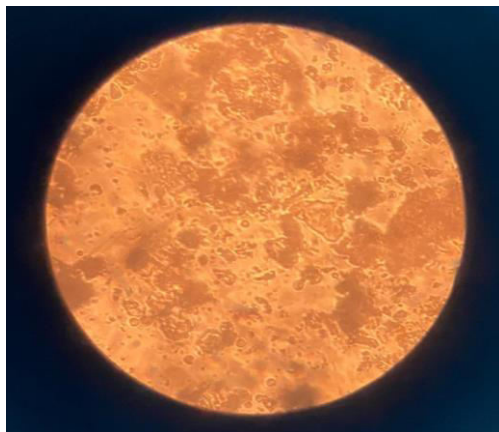
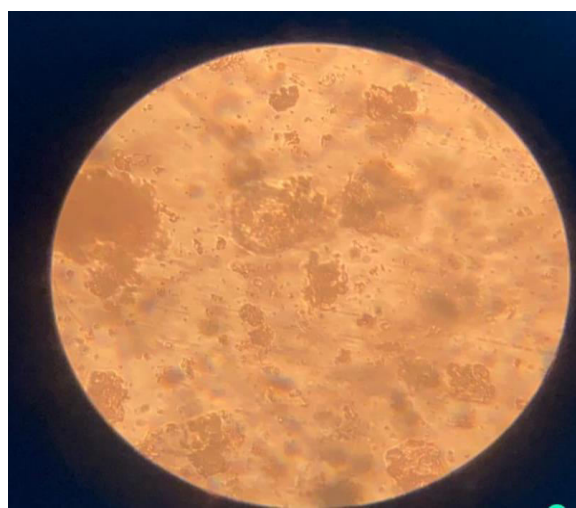


Fig. 7: Powdered microscopy of *Tinospora cordifolia*.



A



B

Fig. 8A- B: Powdered microscopy of *Terminalia chebula*.

Table 1: Phytochemical Evaluation of *Tinospora cordifolia*

S. No.	Parameters	Standard Values	Actual Values
1.	Foreign matter	NMT 2%	0.1
2.	Total ash	NMT 16 %	5.2
3.	Acid-insoluble ash	NMT 3%	1.2
4.	Alcohol-soluble extractive	Not less than 3%	4.20
5.	Water-soluble extractive	Not less than 11%	11.50

Table 2: Phytochemical Evaluation of *Terminalia chebula*

S. No.	Parameters	Standard Values	Actual Values
1.	Foreign matter	NMT 1%	0.1
2.	Total ash	NMT 5 %	2.1
3.	Acid-insoluble ash	NMT 5 %	0.45
4.	Alcohol-soluble extractive	Not less than 40%	42.55
5.	Water-soluble extractive	Not less than 60%	65.84

Table 3: Phytochemical Evaluation of *Zingiber officinale*

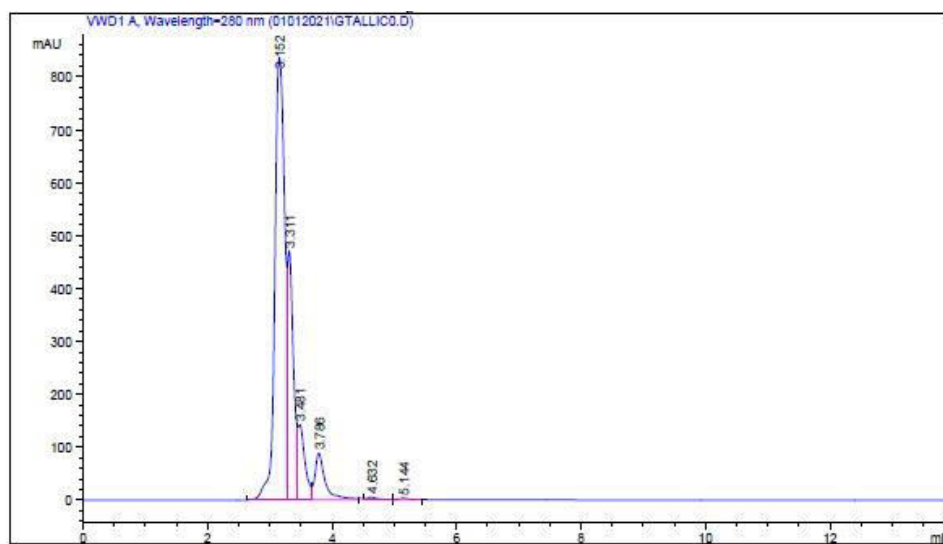
S. No.	Parameters	Standard Values	Actual Values
1.	Foreign matter	NMT 1%	0.1
2.	Total ash	NMT 6 %	3.4
3.	Acid-insoluble ash	NMT 1.5 %	0.3
4.	Alcohol-soluble extractive	Not less than 3%	3.54
5.	Water-soluble extractive	Not less than 10%	11.86

Table 4: Phytochemical evaluation of all three extracts

S. No.	Parameters	<i>Tinospora cordifolia</i>	<i>Terminalia chebula</i>	<i>Zingiber officinale</i>
1.	pH	3.82@ 23°C	3.98@23°C	4.12@24°C
2.	Tests for heavy metals			
	Copper (mg/kg)	Absent	Absent	Absent
	Iron (mg/kg)	Absent	Absent	Absent
	Lead (mg/kg)	Absent	Absent	Absent
	Zinc (mg/kg)	Absent	Absent	Absent
3.	Microbial contamination			
	Total viable count	Absent	Absent	Absent
	Total Yeast and Mould count	<10 CPU	<10 CPU	<10 CPU

Table 5: Quantitative analysis of bitters, tannins and volatile oil

S. No.	Parameters	Amount in percent	
		Extract mixture	Amrutottaram Kashaya
1.	Quantitative analysis of bitters	3.25	2.48
2.	Quantitative analysis of tannins	41.05	38.02
3.	Quantitative analysis of volatile oil	1.91	1.45

Fig. 9: HPLC profile of gallic acid in *Terminalia chebula*.

The produced extracts were tested for the phytochemical traits listed in Table 4 (pH, presence of heavy metals, and microbial contamination), as well as other features.

Quantitative analysis of bitters, tannins and volatile oil:

The quantity of bitters, tannins, and volatile oil present in the combination of extracts and kashaya made using traditional and contemporary methods (Table 5). It was discovered that the extract combination included more bitters, tannins, and volatile oil than the kashayam.

Marker based standardization of the formulation using HPLC:

Instrumentation and Method development:

The chromatographic conditions were established by trial and error and were kept constant throughout the experiments. The desired separation was obtained on column Cosmosil C18 (4.6 x 250 mm, 5 μ m) using Acetonitrile: 0.1% Orthophosphoric acid water (20:80 v/v) as a mobile phase. The flow rate was maintained at 0.7 ml/min and detection was done at 280 nm. The retention factor was found to be 3.311 (Figs. 9, 10).

Under the chromatographic conditions mentioned above, a linear correlation between the peak area and the applied concentration was

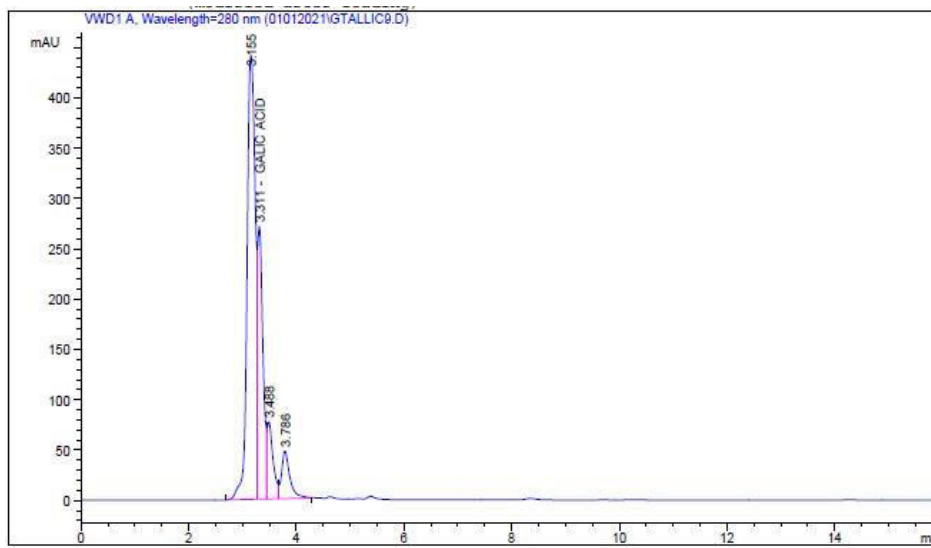


Fig. 10: HPLC profile of gallic acid in mixture.

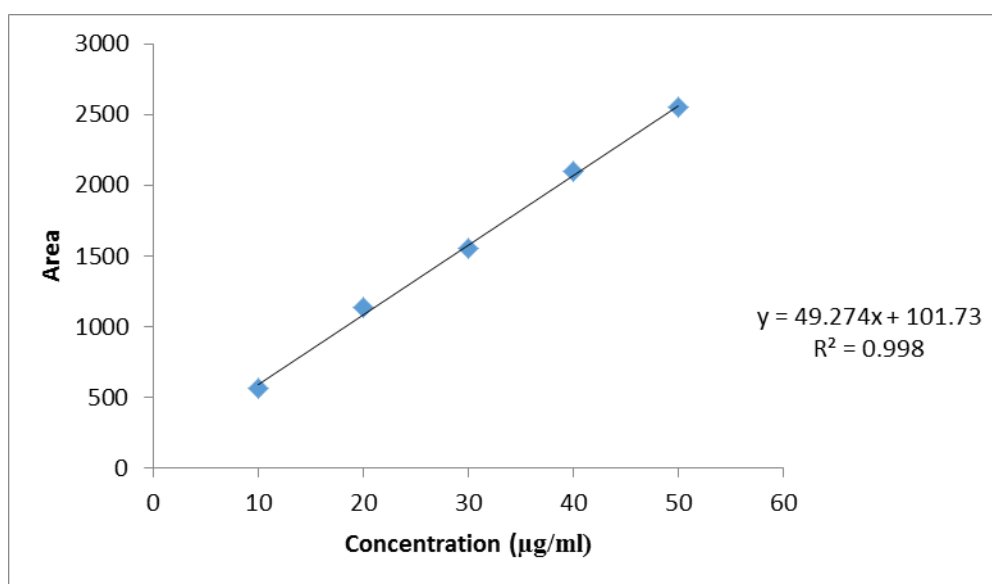


Fig. 11: Linearity graph of Standard Gallic acid.

observed in the concentration range 10- 50 µg/ml for gallic acid given in Figure 11.

Discussion

T. cordifolia has also been found to improve learning and memory in normal rats and to counteract the memory loss caused by cyclosporine. *T. cordifolia* extracts, both alcoholic and aqueous, decreased learning scores in the Hebb William maze and retention memory tests, showing improvement in these areas. *T. cordifolia* prevented neurodegenerative alterations seen on histological analysis of the hippocampus in

cyclosporine-treated rats. Beneficial effects on the immune system, as documented by clinical trials, have been shown to be induced by *T. cordifolia* extracts in both alcoholic and aqueous media (Upadhyay *et al.*, 2010). *T. chebula* methanol extract has shown promise as a potential replacement for currently used antibiotics in the treatment of respiratory tract infections caused by bacteria. Haritaki (*T. chebula*) is a plant that, in general, cleans the blood and stops bleeding by preventing haemorrhaging. Additionally, it is used as an internal cleanser, assisting in the elimination of harmful poisons as well as excess fats from the

body. It is able to strengthen the roots of the hair and improves the colour of the hair (Radharaman *et al.*, 2011). Ginger and the chemicals derived from it, have a number of important pharmacological effects, including immunomodulatory, anti-tumorigenic, anti-inflammatory, anti-apoptotic, anti-hyperglycemic, anti-lipidemic, and anti-emetic effects. Ginger is a powerful antioxidant ingredient that may either reduce the effects of free radicals or stop their production altogether. It is generally accepted as a risk-free herbal treatment, with only a small number of negligible unfavourable or side effects (Ali *et al.*, 2008).

Conclusion

Amruthotharam/Amrutottaram kashayam is one of such preparation which takes care of metabolic disorder through inflammation. It is also proven to be extremely beneficial in many pathologies of ranging from indigestion to deeper cellular inflammation wherein cellular neutralization of AMA is expected. It is also used in the treatment of rheumatoid arthritis, diabetic foot management and also in treatment of chikungunya in Kerala. The present work involved detailed study of herbal drug. The method evaluated in the present study used as a protocol to do analysis of the compounds and formulation in routine analysis. Standardization of mixtures prepared through traditional and modern method indicated the improved global acceptance of the modern method.

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