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UNI-Directional Double Run Multi-Marker Based Standardization of "Amruthothram" Ayurvedic Medicine by HPTLC

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ABSTRACT

Ayurveda and Unani Medicine are the most classy and commonly practiced systems in India. In India's AYUSH systems, some 8,000 herbal remedies have been codified. Amruthotharam/Amrutottaram Kashayam is one of such preparation which takes care of metabolic disorders through inflammation. The formulation contains guduchi (*Tinospora cordifolia*), haritaki (*Terminalia chebula*), and shunthi (Zingiber officinale) in the ratio 6:4:2. Natural remedies obtained from wild sources; therefore, sustaining consistent product quality is difficult because of extrinsic variables including soil conditions, light and water availability, temperature changes, nutrients, and geographic location. The present work aimed to develop and validate uni-dimensional double development high-performance thin layer chromatography to standardize the marker-based compounds such as gallic acid, berberine and gingerol-6, because the power of one-dimensional chromatography is often inadequate for complete resolution of the components present in complex samples which can be improved by separating actives through UDDD.

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INTRODUCTION

The term Traditional herbal medicine is a knowledge, skills, and practices relies on indigenous concepts, beliefs, as well as experiences used to maintain health also to prevent, diagnose, improve or cure physical as well as mental illness (World Health Organization, http://www.who.int/topics/traditional medicine/en/). Traditional medicine is divided into many diverse systems, each having its own philosophy and practices inspired by the geographic place as well as environmental conditions in which it first developed (WHO, 2005). Though, a prevalent concept is a holistic approach to life, which emphasizes the body, mind, also environment, as well as a focus on health rather than sickness (WHO 2005). Herb comes from the Latin word "herba" and the old French word "herbe." Together, these two words form the modern English word "herb." Today, the term "herb" can be used to refer to any part of a plant, such as the seed, flower, fruit, stem, leaf, bark, stigma, or root, also the plants that are not woody. According to the evidence, Unani Hakims, Indian Vaids, cultures from Europe and the Mediterranean, and cultures from the rest of the world have all employed plants as medicine for more than 4000 years. Indigenous communities in Rome, Egypt, Iran, Africa, and America practised healing rituals that involved the use of herbs. Other indigenous communities developed traditional medical practises, such as Unani, Ayurveda, and Chinese

Medicine, in which herbal remedies were utilised in a more methodical manner. Ancient civilizations have known India for a very long time to be an extremely rich source of medicinal herbs and plants. There are around 8,000 herbal therapies that have been defined under the AYUSH systems of India (Zahid and Khan, 2016). Ayurveda, Unani, Siddha, and Folk (or Tribal) Medicine are the principal types of indigenous medical systems that are now practised. Ayurveda and Unani Medicine are the two of these traditional medical practises that are the most developed and widely used in India.

Traditional medicine is appealing for a variety of reasons, including the fact that it is less expensive, that it more closely aligns with the patient's ideology, that it allays concerns about the adverse effects of chemical (synthetic) medicines, that it satisfies a desire for more individualised medical care, and that it grants the general public greater access to health information. Herbal medicines are more commonly utilised for the purpose of health promotion and the treatment of chronic conditions rather than acute conditions that threaten life. Furthermore, conventional treatments are typically considered to be safe and non-harmful because of their natural origins.

Amruthotharam/Amrutottaram Kashayam is one of such preparation which takes care of metabolic disorders through inflammation. "Amruthotharam" Kashayam is the decoction prepared from three herbal drugs that have proven to be extremely beneficial in many pathologies composed of three components; guduchi (*Tinospora cordifolia*), haritaki (*Terminalia chebula*), shunthi (Zingiber officinale) in the ratio 6:4:2 (Gupta, 2003). In terms of active ingredients, Guduchi contains berberine, tinosporaside, Tinsosporic acid, caridioside, haritaki contains tannis, gallic acid, chebulagic acid, ellagic acid and shunthi contains volatile oil, gingerols, and shagols (Akintobi *et al.*, 2013). Amrutottaram acts by treating indigestion and neutralization AMA, thereby helps reduce inflammation and fever. Also used in the treatment of rheumatoid arthritis, diabetic foot management and also in the treatment of chikungunya in Kerala (Akintobi *et al.*, 2013).

Standardization is an important measurement for assuring herbal medication quality control (Patel *et al.*, 2006). All procedures implemented during the production process and quality control that contribute to a reproducible quality are referred to as "standardization." It also includes the full area of research, from plant development to therapeutic use. It also entails adding excipients or combining herbal medications or herbal drug preparations to achieve a specific content of a component or a set of compounds with established therapeutic action in the herbal drug preparation (Bhutani, 2003).

Natural remedies generated from botanicals are typically obtained from wild sources; therefore, sustaining consistent product quality is difficult. Extrinsic variables, including soil conditions, light and water availability, temperature changes, nutrients, and geographic location, influence the accumulation or proportion of phytochemicals or phytoconstituents in plants. Cultivation and harvesting practices, as well as postharvest processing and storage processes, all have an impact on the plant's physical appearance and chemical properties. According to the Natural Health Product Directorate of Canada, "marker compounds are a component that exists naturally in the material and is picked for special attention (for example, for identification or standardisation) by a researcher or manufacturer." Markers play a crucial function in identifying or authenticating the source of the item. They may be used in a variety of ways to assess the quality and assure that natural health products are effective and safe (NHPs).

Marker chemicals are not always pharmacologically active, but their existence in products with distinctive chemical properties is widely recognized. Active principles, active markers, analytical makers, and negative markers are all types of marker components, whereas biomarkers are defined as indicators with established pharmacological activity (Mukherjee *et al.*, 2011). The use of marker chemicals to standardize conventional pharmaceutical formulations is gaining traction. Marker testing, on the other hand, is not a replacement for physicochemical, chemical, macroscopic, or microscopic examinations. However, it is a practical method for ensuring the identification and purity of herbal medications (Mukherjee, 2002).

In marker profiling of herbal medications, many analytical methods such as HPLC, HPTLC, and LC–MS were utilised. TLC is a multi-applicative, fast, accurate, and robust approach among several analytical instruments. However, no HPTLC technique for estimating markers in Amruthotharam kashayam formulation has been found in the literature. The polarity difference between the three markers might be the cause for this. However, because TLC applications are so diverse, it provides a number of different development modes, including multidimensional development (MDD), uni-dimensional double development (UDDD), multiple incremental developments (IMD), and gradient multiple development (GMD) (Szabady, 2001).

All of these approaches increase component resolution since each successive development leads to band re-concentration, which improves separation efficiency (Szabady, 2001; Poole *et al.*, 1989).

The aim of the present work was to separate compounds like gallic acid, gingerol-6 and berberine by uni-dimensional double development HPTLC method because of their difference in polarities. This method could be used to control the quality of formulation as well as compounds.

MATERIALS AND METHODS

Material

Amruthotharam kashaya marketed formulation was purchased from the local market of Ratnagiri. The standard gallic acid was procured from Sigma Aldrich Pvt. Ltd. Mumbai, India. The other standards, berberine and gingerol- 6, were procured from Yucca Enterprises, Mumbai, India. The analytical grade chemicals and organic solvents were purchased S.D Fine chemicals Pvt. Ltd. Mumbai, India.

Method

Markers standardization was performed by Uni-dimensional double development high-performance thin layer chromatography (UDDD-HPTLC) method.

Chromatographic conditions

A CAMAG HPTLC system equipped with CAMAG Linomat V with CAMAG microlitre syringe, TLC scanner 3, and WinCATS 1.2.2 software (CAMAG, Muttens, Switzerland) was employed. Chromatography was performed on precoated silica gel 60 F_{254} TLC plates (20 cm $\times$ 10 cm with 250 μm thickness; E. Merck, Darmstadt, Germany, supplied by Anchrom Technologists, Mumbai) using mobile phase including toluene: ethyl acetate: methanol: formic acid (3:5:4:0.5 v/v/v/v) for gallic acid and berberine and n-hexane: diethyl ether: formic acid (6.5:4.8:0.2 v/v/v) for gingerol-6. The development was carried out on twin trough glass chamber saturation for 10 min at 25 $\pm$ 2°C for gallic acid and berberine and 5 min for gingerol-6. Ascending development to a distance of 95 mm was performed on a 20$\times$10 cm twin trough chamber (CAMAG). The samples on the HPTLC plates were scanned over the wavelength of 200 – 400 nm. The source of radiation used was a UV Spectrophotometer.

Preparation of solutions

Standard Stock Solution

The standard stock solutions of gallic acid and gingerol-6 of 1 mg/ml concentration and berberine of 0.1 mg/ml concentration were prepared separately.

Sample Stock Solution

The 100 mg of amruthotharam kashayam formulation was extracted with 100 ml of acetone for 1 hr on the sonicator with the aid of heat. The extract was filtered and allowed to complete dryness.

The 50 mg of residue was weighed and dissolved in 10 ml of acetone. The resulting concentration of the solution was 250μg per spot used for qualitative analysis of gallic acid, berberine and gingerol-6.

Validation of the method

Linearity

The standard stock solutions of gallic acid and gingerol-6 of 1 mg/ml concentration and berberine of 0.1 mg/ml concentration were prepared. Different volumes of each solution were applied to the HPTLC plate to deliver 12- 60 μg/μl of gallic acid and gingerol-6, 1.2- 6 μg/μl of berberine. Each concentration was analyzed in triplicate. (FDA, 1996)

Precision

Intra-day and inter-day precision tests were used to assess the precision of the developed method. Triplicates of three different quantities of each gallic acid, gingerol-6, and berberine were spotted and analysed on the same day for intra-day study and two separate days for inter-day study with corresponding chromatographic conditions to assess intra-day and inter-day precision. The findings of all three criteria were reported as percentage RSD.

Accuracy

The accuracy of the method was evaluated by adding known quantities of gallic acid, berberine, and gingerol- 6 to the procedure at three different concentrations (80%, 100%, and 120%). The findings of all three criteria were reported as percentage RSD.

Limit of detection and limit of quantitation

The lowest quantity of analyte in a sample that can be detected but not necessarily quantified as an exact number is the detection limit of an individual analytical method.

The lowest quantity of analyte in a sample that can be quantitatively measured with sufficient precision and accuracy is the quantitation limit of a particular analytical process.

The signal-to-noise ratio (S/N) of 3 and 10 was determined for triplicate measurements of each drug to estimate the limit of detection (LOD) and limit of quantitation (LOQ).

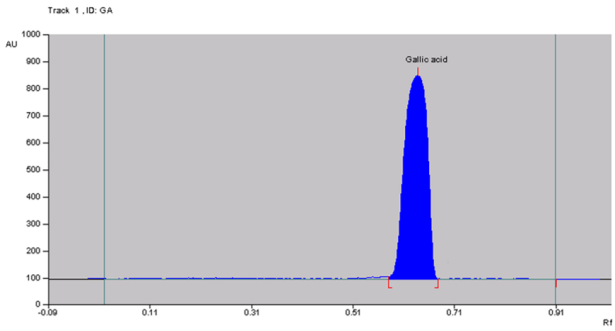

Figure 1: Densitogram of standard gallic acid

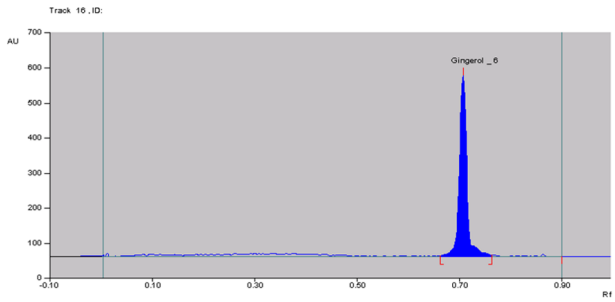

Figure 2: Densitogram of standard gingerol-6

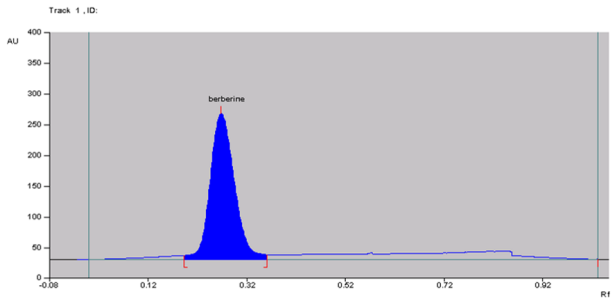

Figure 3: Densitogram of Standard berberine

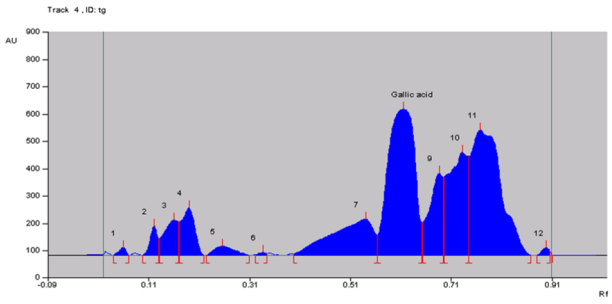

Figure 4: Densitogram of gallic acid in marketed formulation

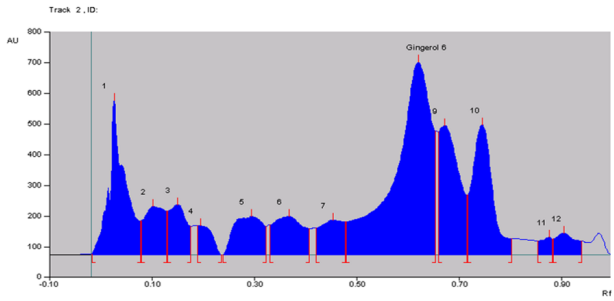

Figure 5: Densitogram of gingerol-6 in marketed formulation

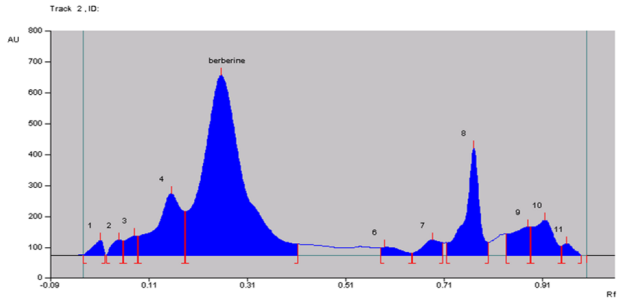

Figure 6: Densitogram berberine in marketed formulation

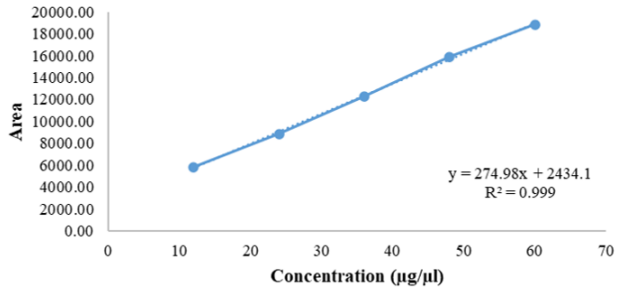

Figure 7: Linearity graph of standard gallic acid

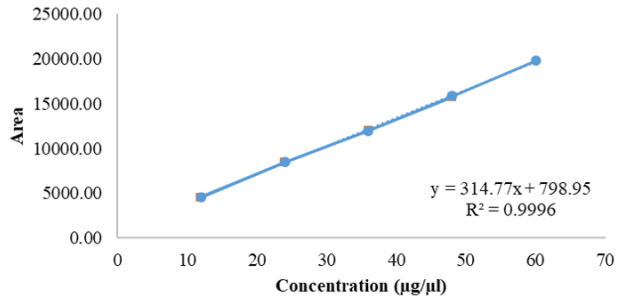

Figure 8: Linearity graph of standard gingerol-6

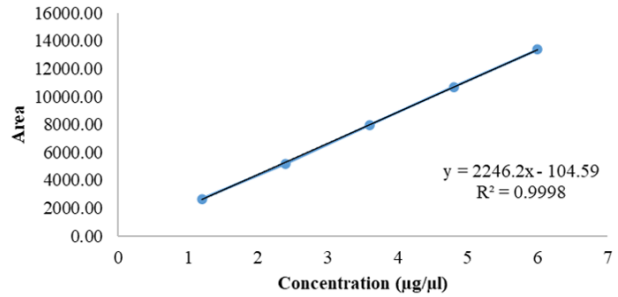

Figure 9: Linearity graph of standard berberine

Robustness

The robustness was investigated by determining the effect of small alterations in the chromatographic parameters, such as chamber saturation time and wavelength, on the retention factor and performing quantitative analysis.

By computing the % RSD for each parameter, the impact of these adjustments on Rf values and peak regions was investigated.

Table 1: LOD and LOQ of compounds

Sr. No.	Compounds	Parameters	
		LOD (ng/spot)	LOQ (ng/spot)
1.	Gallic acid	1.65	5.01
2.	Gingerol-6	0.67	2.03
3.	Berberine	0.20	0.62

Table 2: Analysis of Formulation for Determination of % Content of Bioactives

Sr. No.	Bioactives	% Content in Formulations	
		F1	F2
1.	Gallic acid	1.06	1.38
2.	Gingerol-6	0.85	0.87
3.	Berberine	2.12	2.42

Analysis of percent content

The prepared formulations were evaluated for the percent content of bioactives present in them. The developed and validated method was utilized for the determination of percent content.

RESULTS AND DISCUSSION

Method Optimization

The optimized HPTLC separation was achieved on the TLC plate by employing a CAMAG HPTLC system equipped with TLC scanner 3, and WinCATS 1.2.2 software (CAMAG, Muttens, Switzerland). During the stage of method development, different ratios of mobile phases were tried and the mobile phase comprising of toluene: ethyl acetate: methanol: formic acid (3:5:4:0.5 v/v/v/v) for separation of gallic acid and berberine and n-hexane: diethyl ether: formic acid (6.5:4.8:0.2 v/v/v) for gingerol were confirmed.

The separation of compounds carried out by the UDDD method involves two different mobile phase as stated above. The detection of samples were obtained at 254 nm and 366 nm on a single TLC plate. The importance of the uni-dimensional double development method was to separate both polar as well as non-polar compounds. The chromatographic conditions confirmed for the analysis gave well-resolved peaks for each standard and sample solution (Figure 1, Figure 2, Figure 3, Figure 4, Figure 5 and Figure 6). The optimized chromatographic method gives good separation and resolution of the standard gallic acid, gingerol-6 and berberine with Rf values at 0.62, 0.74 and 0.28, respectively.

Method Validation

Linearity

Under the chromatographic conditions stated above, the linear correlation between the peak area and the concentration (Figure 7, Figure 8 and Figure 9) was obtained in the range of 12- 60 μg/μl of gallic acid and gingerol-6, 1.2- 6 μg/μl of berberine.

Precision

Intraday and interday precision was done in triplicate at 3 distinct concentration levels. Data on repeatability and instrumental variation were obtained. The % RSD values for both intraday and interday precision were found within acceptable limit proved that the method was highly precise.

Accuracy

This was accomplished using the recovery method at three different concentrations: 80%, 100%, and 120%. For gallic acid recoveries were ranges from 99.56- 101.58 %, for gingerol-6 98.57- 101.58 %, and for berberine 99.71- 101.58 %.

The HPTLC technique was confirmed to be accurate for the measurement of gallic acid, gingerol-6, and berberine, according to the findings.

LOD and LOQ

The LOD and LOQ were estimated given in Table 1.

Robustness

The wavelength was altered from 252 nm to 256 nm for gallic acid and gingerol-6, and 364-368 for berberine, while the saturation period was modified from 8 to 12 minutes for berberine and gallic acid and from 3 to 7 minutes for gingerol-6.

In both cases, the measured area under the curve was found in the limit. For both, there was no change in peak area or retention time. The method was shown to be robust since resolution and separation did not alter.

Analysis of Percent Content

The developed and validated method was further used to evaluate the % content of bioactives in the prepared formulation given in Table 2.

CONCLUSIONS

The newly developed UDDD-HPTLC technique is novel, time-saving, precise, and accurate. The developed method analyzed actives including berberine in guduchi, gallic acid in haritaki and gingerol-6 in shunthi. That is the method useful for the estimation of polar and non-polar compounds simultaneously. It is stated that the new technique has various advantages, including a quick, low-cost mobile phase, simple sample preparation processes, and enhanced sensitivity, making it reliable and reproducible in quality control setups. The studied formulation contains different polar and non-polar compounds. It is difficult to separate the polar and non-polar compounds together on the same TLC plate. The utilization of the UDDD technique resolved the problem by separating both compounds at the same time by using different mobile phases. The method was also used to estimate the % content of bioactives. The % content of gallic acid, gingerol- 6, and Berberine was found to be 1.06, 0.85, and 2.12 respectively in F1, whereas 1.38, 0.87, and 2.42 found in F2 formulation, concluding that the amount of bioactives was found to be significantly higher in F2 formulation prepared by modern techniques. The suggested UDDD-HPTLC approach is better than HPLC method because of its low cost, simpler, faster, and more versatile. This approach may be used for routine quality control examination formulation as it allows simultaneous estimation of all the markers.

Conflict of interest

The authors declare that they have no conflict of interest.

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