



Original Research Article (Experimental)

Phytochemical analysis of *Pathyashadangam kwath* and its standardization by HPLC and HPTLC

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ABSTRACT

Background: *Pathyashadangam kwath*, a classical ayurvedic polyherbal formulation is used for the treatment of cluster head ache, migraine, upper respiratory diseases, ear ache and night blindness. Review of literature suggested that characterization parameters of *Pathyashadangam kwath* are not reported.

Objective: To report characteristic parameters of *Pathyashadangam kwath* to confirm quality and purity. **Materials and methods:** The fruit pericarps of *Haritaki*, *Bibhitaki* and *Amalaki*, aerial parts of *Bhunimba*, rhizome of *Haridra*, stem bark of *Nimba* and stem of *Guduchi* were the ingredients of *Pathyashadangam kwath*. Three batches of the *kwath* were prepared as per standard procedures. The *kwath* was evaluated for organoleptic, physical, phytochemical and chromatographic parameters as per standard methods.

Results: HPTLC analysis revealed that Toluene: Ethyl Acetate: Formic acid (2.5: 2.0: 0.5) was a suitable mobile phase for characterization of the *kwath*. HPLC analysis revealed that andrographolide was a suitable marker for standardization of the *kwath*.

Conclusion: The characterization parameters presented in this paper may serve as standard reference for quality control analysis of *Pathyashadangam kwath*.

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1. Introduction

More than 75% of world's population relies on traditional medicine for their basic health issues. India with its quintessence in ayurvedic medicine, is the coffers of more than 45,000 plant species. Of these 7500 finds praxis in convalesce. *Ayurveda*, a holistic science which accentuate on maintaining fitness in addition to treatment of diseases, relies mainly on healing potential of plants. The focus of *Ayurveda* is to restore balance by eradicating the root cause of disease using a blend of natural elements and prevent the recurrence of imbalance by creating a healthy life style. *Ayurveda* treatises like *Sarngadhara samhita* highlights the importance of combining different herbs in a particular proportion to lessen toxicity and augment therapeutic efficacy [1]. According to *kwatha vidhi* of *S. samhita*, *kashayam* or *kwath* is the filtered decoction obtained after boiling a mixture of herbs with 16 times water under

low flame for a prolonged period till the volume is reduced to one eighth [2]. It is used as a starter for the preparation of different dosage forms or employed directly as drug.

On account of its natural origin and lack of side effects, the demand for ayurvedic drugs have aggrandized across the world. The commercialization of traditional medicines has led to the widespread use of adulterants and low cost substitutes, because of non-availability and high cost of standard authentic drugs. Hence it has now become imperative to testify polyherbal formulations as per modern research parameters to standardize and evaluate its quality [3].

Quality assurance which guarantees the delivery of apt quantity of standard medicament is an integral part of traditional medicine [4]. Organoleptic and physical evaluation of drugs and its comparison with standard values is helpful to authenticate top quality drugs, free of adulteration. Chromatographic fingerprinting with its ability to characterize chemical composition of herbal drug products can serve as a useful tool for appraising batch to batch consistency [5]. Physicochemical parameters, biochemical analysis, microbiological features and HPTLC fingerprint profile may be used as marker parameters for quality evaluation and standardization of polyherbal formulations [6].

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Pathyashadangam kwath (*Pathyadi shadangam kashayam*) is a classical ayurvedic polyherbal formulation prescribed by ayurvedic physicians for the treatment of upper respiratory tract infections and different types of head ache. There is dearth of information regarding the scientific analysis of *Pathyashadangam kwath*. Hence characterization of *Pathyashadangam kwath* was attempted to confirm its quality and purity.

2. Materials and methods

2.1. Plant materials

Pathyashadangam kwath was prepared from seven ingredients, viz., *Haritaki* (*Terminalia chebula* Retz.), *Bibhitaki* (*Terminalia bellirica* (Gaertn.) Roxb.), *Amalaki* (*Phyllanthus emblica* L.), *Bhunimba* (*Andrographis paniculata* (Burm. f.) Wall. ex Nees), *Haridra* (*Curcuma longa* L.), *Nimba* (*Azadirachta indica* A. Juss.) and *Guduchi* (*Tinospora cordifolia* (Willd.) Miers.). The fruit pericarps of *Haritaki*, *Bibhitaki* and *Amalaki*, aerial parts of *Bhunimba*, rhizome of *Haridra*, stem bark of *Nimba* and stem of *Guduchi* were employed for preparation of the formulation [7]. Voucher specimens of all these ingredients were deposited in the herbarium of KFRI, Peechi for future reference (Accession numbers 13037–13043).

2.2. Methods

Pathyashadangam kashayam was prepared as per standard method described in *kwatha vidhi* of *S. samhita*. Three batches of *kwath* viz PS 15, PS 23 and PS 25 were prepared in the same manner at different times in the months of April and May.

2.2.1. Organoleptic evaluation

Organoleptic characters like colour, odor, taste and consistency of the *kashayam* were evaluated based on the method described by Siddiqui et al. [8].

2.2.2. Physical evaluation

Different physical parameters like total solids, specific gravity, water soluble extractive value, alcohol soluble extractive values and pH were evaluated using standard pharmacopoeial methods [9].

2.2.3. Microbial load

Total aerobic microbial count (bacterial and fungal) was determined by plate count method [10]. Test for specific organisms was carried out as per standard procedures [11].

2.2.4. Heavy metal analysis

The *kashayam* was digested in HNO₃ and HClO₄ (5:2). The volume of the digested samples was then made up to 100 ml with deionized water and analyzed with ICP-AES system (Thermo Electron, IRIS INTREPID II XSP DUO, Munich, Germany). The limit of detection was Cd 0.01 ppm; Pb 0.05 ppm; Hg 0.1 ppm and As 0.04 ppm.

2.2.5. Phytochemical investigation

The *kashayam* was evaporated on a water bath for the removal of water, the dried mass was refluxed with methanol for three times and filtered. The methanol extract was subjected to preliminary phytochemical analysis. Ethanol extract was prepared in the same manner and phytochemical analysis was carried out. Dragendorff's test for alkaloids, Alkaline reagent test for flavonoids, Ferric chloride test for tannins, Froth formation test for saponins, Keller-killiani test for glycosides, Libermann Burchard test for steroids and Salkowski test for triterpenoids were conducted [12].

2.2.5.1. Quantification of total bitter. To estimate total bitter of the *kashayam*, 5 g of the *kashayam* was refluxed with 50 ml ethanol for 30 min and filtered. The process was repeated thrice. The alcohol was evaporated from the filtrate and the residue was shaken with 25 ml hot water. The extract was shaken with 25 ml ethyl acetate and the solution was transferred to a separating funnel and the ethyl acetate layer was collected in a previously weighed flask (W₁). The process was repeated with 20 ml and 15 ml hot water and ethyl acetate and continued till the ethyl acetate layer turned colourless. The ethyl acetate was distilled off and the final residue was weighed (W₂). The percentage of total bitter was calculated from the formula $(W_2 - W_1) \times 100 / \text{Weight of the sample}$ and was expressed as mean \pm standard deviation of three values [13].

2.2.5.2. Quantification of andrographolide. Andrographolide in the three batches of the *kwath* was quantified. 3 g of the *kwath* was refluxed with 50 ml ethanol for 30 min and filtered. The process was repeated thrice. The alcohol was evaporated from the filtrate and the residue was shaken with 25 ml hot water. The above extract was shaken with 25, 15 and 10 ml petroleum ether till the ether layer turned colourless. The above solution was transferred to a separating funnel and the ether layer was discarded. The aqueous phase was shaken with 25, 20, 15 and 10 ml ethyl acetate. The ethyl acetate layer was collected in a previously weighed flask (W₁). The ethyl acetate was distilled off and evaporated to dryness. Weight of the final residue was taken (W₂). Percentage of andrographolide was calculated by the formula $(W_2 - W_1) \times 100 / \text{Weight of sample}$ and was expressed as mean \pm standard deviation of three values [13].

2.2.6. HPTLC

The methanol extracts of three batches of *kashayam* were subjected to HPTLC analysis. The *kashayam* was dried in a water bath at a fixed temperature and was extracted with methanol. 4 micro litres of the extract was spotted on HPTLC silica gel 60F 254 (Merck) plate as bands of length 6 mm at a distance of 10 mm. The plates were developed using Toluene: Ethyl Acetate: Formic acid (2.5: 2.0: 0.5) in the CAMAG twin-trough glass chamber, previously saturated with the solvent for 30 min. The mobile phase was chosen after testing different solvent systems of varying polarity. After development, the plates were dried in an oven at 60 °C and scanned using a CAMAG TLC Scanner in absorbance mode. Data processing was performed with winCATS planar chromatography manager software. The compounds were scanned at 254 and 366 nm.

2.2.7. HPLC

The methanol extract of the *kashayam* was subjected to HPLC using andrographolide as marker. A *kashayam* was prepared excluding all ingredients of *Pathyashadangam kashayam* other than *A. paniculata* and was also subjected to HPLC. The HPLC analysis was carried out using Luna 5u C18 analytical column (250 × 4.6 mm), Shimadzu LC-10 AT vp binary pump and SPD-M10A vp photo diode array detector (PDA). The mobile phase consisted of water (A) and acetonitrile (B). The elution condition was 0–25 min, 20%–55% B [14]. The injection volume was 20 µl and flow rate was 1 ml/min. UV spectral range was 190 nm–400 nm. The auto sampler and column compartment were maintained at 25 °C and 35 °C respectively.

3. Results

3.1. Organoleptic evaluation

Organoleptic evaluation of the *kashayam* revealed that it had bitter taste, brown colour, and characteristic odour and was a colloidal suspension.

3.2. Physical evaluation

The specific gravity of the *kashayam* was found to be 1.075 for all the three batches. The pH was found to be 3.46, 3.39 and 3.48 and total solids was 18.72, 17.95 and 17.34% for PS 15, PS 23 and PS 25 respectively. Water soluble extractive value of PS 15, PS 23 and PS 25 was 14.79, 15.43 and 12.50% respectively. The methanol soluble extractive value was 13.13, 11.89 and 10.43% and ethanol soluble extractive value was 9.15, 9.66 and 9.18% respectively for PS 15, PS 23 and PS 25. Thus it was found that the extractive value was highest for water followed by methanol and ethanol.

3.3. Microbial load

Analysis of the *kwath* for aerobic microbial count revealed that the *kwath* was free from bacterial and fungal contamination. Test for specific organisms revealed that pathogenic bacteria like *Escherichia coli*, *Salmonella*, *Staphylococcus aureus* and *Pseudomonas aeruginosa* were absent in the *kwath*.

3.4. Heavy metal analysis

Heavy metal analysis of the formulation revealed that the formulation lacked heavy metals or if present their concentration was below the permissible limits.

3.5. Phytochemical investigation

Preliminary phytochemical analysis of methanol and ethanol extracts of *kashayam* revealed the presence of alkaloids, flavonoids, tannins, glycosides, sterols, triterpenoids and saponins in both the extracts.

The percentage of total bitter and andrographolide in three batches of the *kwath* were similar. PS 15, PS 23 and PS 25 were found to contain $4.24 \pm 0.11\%$, $4.82 \pm 0.10\%$, $4.18 \pm 0.41\%$ total bitter and $3.89 \pm 0.42\%$, $3.87 \pm 0.18\%$ and $3.78 \pm 0.35\%$ andrographolide respectively.

3.6. HPTLC analysis

HPTLC analysis of three batches of *kashayam*, PS 15, PS 23 and PS 25 gave similar finger print patterns. 13 bands of similar R_f value were observed for all the three batches. The intensity and colour of bands in the three batches were remarkably similar (Fig. 1). The total area under the peak was 31,359.5 for PS 15, 29,102.7 for PS 23 and 26,264.7 for PS 25 (Supplementary Table 1).

3.7. HPLC analysis

HPLC analysis of methanol extracts of three batches of *Pathyashadangam kwath* and *Andrographis kwath* was carried out along with andrographolide standard (Fig. 2a). The analysis revealed that all the three batches of *pathyashadangam kwath* and *A. kwath* showed characteristic peak corresponding to andrographolide. The RT for andrographolide was 16.46, 16.508, 16.542, 16.526 and 16.522 for standard, *A. kwath*, PS 15, PS 23 and PS 25 respectively.

The spectral index confirmed that the peak obtained was that of andrographolide (Fig. 2b,c,d,e).

4. Discussion

Organoleptic analysis is the appraisal of a product as it is perceived by sense organs and helps in the preliminary quality evaluation. Hence the organoleptic parameters of three batches of *kwath* were analysed as preliminary quality check which revealed that brown colour and bitter taste were characteristic of *Pathyashadangam kashayam*. Physicochemical analysis can be employed for routine evaluation at the sites of manufacture and established physicochemical standards can furnish information for further investigation and facilitate identification of formulations. Hence the specific gravity, pH, total solids and extractive values of the *kwath* were determined, which will serve as reference for future analysis. Plants are nature's drug stores. The enormous chemical compounds present in plants are to be explored for solving present day health problems. Phytochemical analysis of plants and plant products can open the keys to the goldmine of chemicals present therein. Plant derived secondary metabolites are valuable source of new drugs while some are drug precursors [15]. Flavonoids are low molecular weight secondary metabolites which possess antiaging, antibacterial and antioxidant properties and with their ability to inhibit carcinogenesis at different stages, they can emerge as effective cancer chemotherapeutic agents [16]. Tannins are water soluble polyphenols most of which have antimicrobial properties and hence can enhance the shelf life of preparations [17]. Phytochemical analysis revealed that the *kashayam* contained secondary metabolites like alkaloids, flavonoids, tannins, sterols, triterpenoids, and glycosides conferring cumulative pharmacological properties to the formulation.

The fear of presence of detrimental elements including heavy metals is a major apprehension causing scepticism in minds of common man regarding the safety and quality of *ayurvedic* formulations. Hence it is indispensable to rule out the occurrence of undesirable elements to augment the acceptance of formulations. Cadmium and Lead are reported to be of great concern since they cause adverse health issues in humans [18]. Elemental analysis of three batches of *Pathyashadangam kashayam* by ICP-AES revealed that toxic heavy metals like Lead and Cadmium were below detectable limits in all the three batches. In two batches of *kashayam*, mercury was below detectable limit and in the other batch it was below permissible limit. Arsenic was below detectable limit in two batches and very much below permissible limit in the third batch. The permissible limits for heavy metals in *ayurvedic* formulations is 10 ppm for lead, 1 ppm for mercury, 3 ppm for arsenic, and 0.3 ppm for cadmium [19]. So it was found that all the three batches of *Pathyashadangam kashayam* are safe to consume as the concentration of heavy metals was either absent or very much below permissible limits.

Herbal drugs may be contaminated with microorganisms from soil, air or water. Some of these can cause health problems to their consumers. The therapeutic activity of herbal products may be reduced or even nullified by the presence of microbial contaminants causing ill health to patients taking the drug [20]. Hence it is vital to ensure that the finished product is free from microbial contamination. Microbial load of the *kwath* was determined with this objective in mind. The absence of microbial growth in the *kwath* assures safety of its consumption.

Polyherbal formulation is a complex mixture of more than one herb in a particular proportion. The absence of any ingredient or the presence of an undesirable ingredient knowingly or unknowingly can lead to reduction of therapeutic value or even serious health problems to the consumers. Hence it is essential to ensure that only

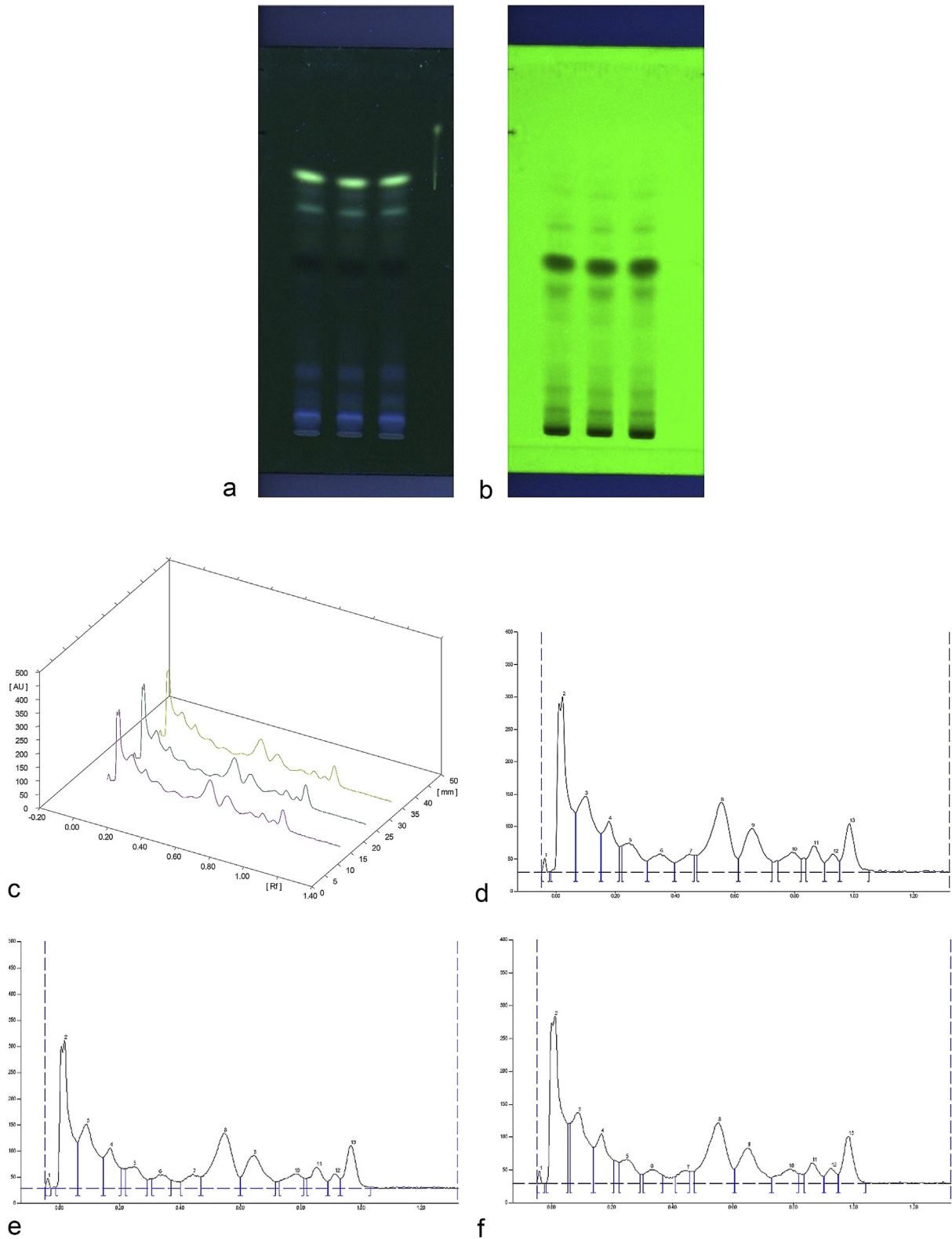


Fig. 1. HPTLC plate of three batches of *kwath* under (a) UV 366 nm (b) UV 254 nm (c) Chromatogram of three batches of *Pathyashadangam kwath* at 366 nm (d) HPTLC chromatogram of PS 15 at 366 nm (e) HPTLC chromatogram of PS 23 at 366 nm (f) HPTLC chromatogram of PS 25 at 366 nm.

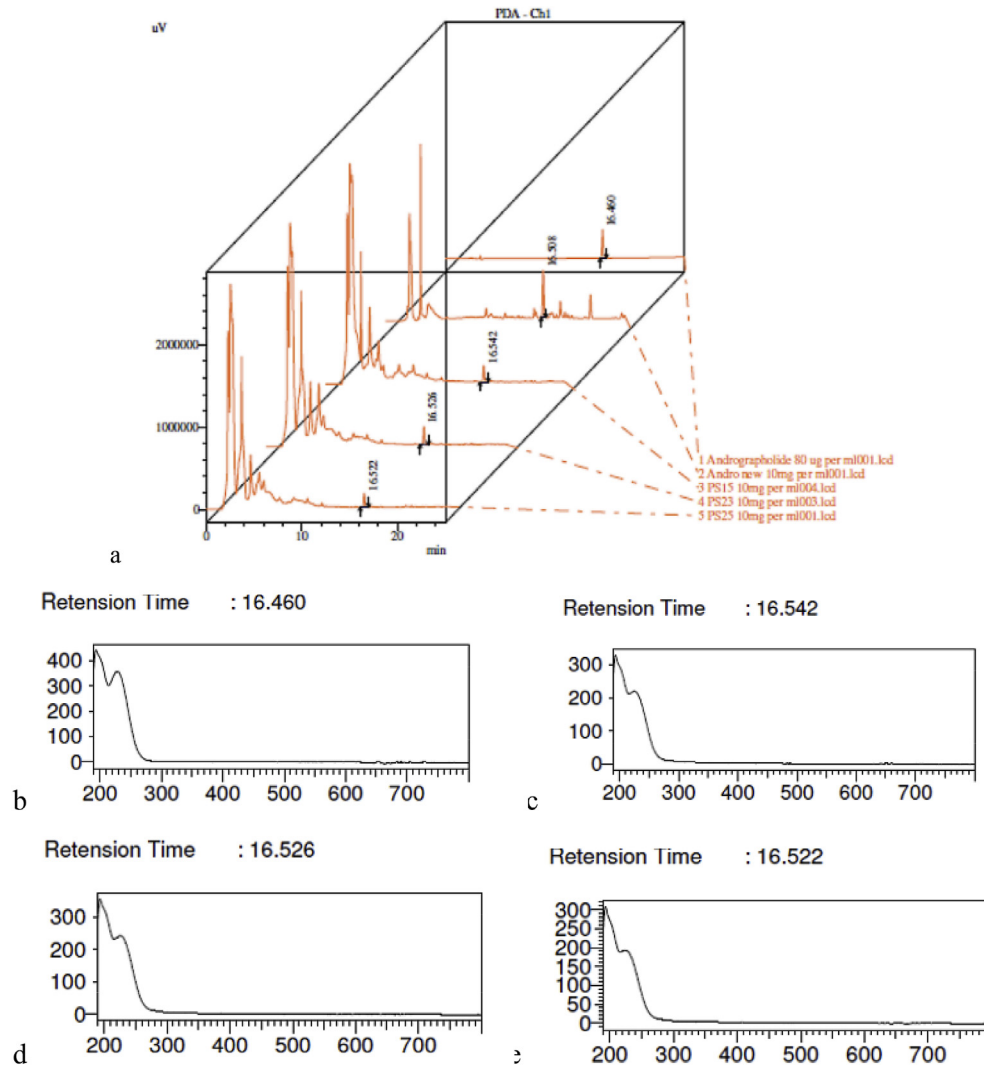


Fig. 2. (a) HPLC profile of three batches of *kwath*, *Andrographis kwath* and andrographolide standard. (b) Spectral index of andrographolide standard and of peak corresponding to andrographolide of (c) PS 15 (d) PS 23 and (e) PS 25 at 225 nm.

desirable drugs in precise proportion are included in the preparation of polyherbal drugs. This can be assured by quality checks and standardized manufacturing practices. For finished products quality can be ensured through fingerprinting by HPLC and HPTLC with suitable markers. HPLC/HPTLC method has been used for the estimation of markers and standardization of different *ayurvedic* formulations [21,22]. Three batches of the *kwath* were subjected to HPTLC analysis which generated a fingerprint, wherein similarity in number, R_f , intensity and colour of bands were obtained, showing that the active constituents present in all the three batches were similar and helped to establish batch to batch consistency of the formulation.

Standardization of formulation and its ingredients based on specific marker compounds and its validation is very important [23]. Andrographolide is a diterpene lactone conferring bitter taste to *A. paniculata*, commonly known as the king of bitters. Standardization of market preparations containing *A. paniculata* was achieved by RP HPLC using andrographolide as marker [24]. Methanol is a better solvent for extraction of andrographolide and dehydroandrographolide than ethanol and aqueous acetone [25]. The fingerprint of a plant, its extracts and products will be the same under identical conditions which makes chromatographic fingerprinting an ideal method for authentication and identification of

herbal drugs [26]. Co-chromatography followed by comparison of RT and absorption spectra of formulation and standards of marker compounds by which the presence of marker can be ascertained is an important parameter for quality evaluation of polyherbal formulations [27]. HPLC analysis of methanol extracts of three batches of *kwath* along with andrographolide standard could confirm the presence of andrographolide in the formulation. The similarity in retention time and spectral index in all the three batches of *kashayam* and standard andrographolide showed that andrographolide could be used as marker for the standardization of *Pathyashadangam kwath*.

5. Conclusion

Pathyashadangam kwath was characterized on the basis of organoleptic, physical, microbiological, heavy metal, phytochemical and chromatographic analysis. Bitter taste, brown colour, absence of heavy metal and microbial contamination, pH of 3.44 ± 0.047 , specific gravity of 1.075, water soluble extractive value of $14.24 \pm 1.54\%$, methanol soluble extractive value of $11.82 \pm 1.35\%$, ethanol soluble extractive value of $9.33 \pm 0.29\%$, presence of alkaloids, flavonoids, tannins, sterols, triterpenoids, saponins, glycosides and the marker compound andrographolide were found to be

characteristic of the *kwath*. HPLC analysis using Toluene: Ethyl acetate: Formic acid (2.5:2:0.5) mobile phase was found suitable for consistency evaluation of the *kashayam*. All these parameters may be employed as standard reference for quality control analysis of the formulation.

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Conflict of interest

None.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <https://doi.org/10.1016/j.jaim.2017.10.011>.

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