

STANDARDIZATION OF AN AYURVEDIC FORMULATION: TRIKATU CHURNA USING
BIOANALYTICAL TOOLSShailajan Sunita*, Sayed Neelam, Joshi Harshvardhan, Tiwari Bhavesh
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Email: sunitashailajan@gmail.com**ABSTRACT**

Trikatu Churna (TC) is an ancient traditional Ayurvedic preparation prescribed for a wide range of disorders. Though TC is an age old formulation, there are very few references on its quality control and standardization. In this work, an attempt has been made to standardize TC by qualitatively evaluating the preliminary phytochemicals. Piperine content of TC was determined using HPTLC. Evaluation of safety potential of TC samples and stability evaluation by comparative study of the in house TC formulation with marketed TC formulations with respect to their piperine content is a value addition to the current work.

KEY WORDS: Trikatu Churna, standardization, piperine, HPTLC evaluation, safety evaluation

INTRODUCTION

Trikatu Churna (TC) is an Ayurvedic polyherbal formulation of Maricha (*Piper nigrum* L., fruit), Pippali (*Piper longum* L., fruit) and Sunthi (*Zingiber officinale* Rosc, rhizome)¹. Since ancient times, it is being prescribed for Agnimandya (digestive impairment), Gala roga (throat diseases), Svasa (asthma), Kushtha (skin diseases), Pinasa (sinusitis), Kasa (cough) and Slipada (filariasis).

Modern bioanalytical techniques have been routinely used for assessing the quality of the raw materials and traditional formulations and there are reports on the standardization of some of the herbal preparations widely used in Ayurveda²⁻⁵. Though TC is an age old formulation, there exist meager references on its quality control and standardization. Hence, there is a need to standardize TC using the same.

TC (in house) was prepared in the Herbal Research Laboratory, Mumbai using standardized raw materials as per the classical reference¹. Traditional formula composition of TC is given in Table 1. Preliminary phytochemicals of TC were evaluated as per standard methods⁶.

Piperine (Figure 1), a major alkaloid of Maricha and Pippali (ingredients of TC) is reported as a bioavailability enhancer, anti-inflammatory, anticonvulsant and antiulcer agent⁷⁻⁹. There are reports on extraction of piperine using various extraction techniques and its estimation using analytical tools from single herbs and polyherbal formulations^{2,5,10,11}. But, there are no methods reported for its estimation from the complex matrix of TC. Hence, the piperine content of TC and its ingredients was estimated using HPTLC.

HPTLC method was applied to evaluate the impact of storage periods on the piperine content of stability samples and also to comparatively evaluate marketed samples of TC with the in house TC formulation. Acute toxicity studies were conducted to check the safety potential of TC in mice.

MATERIALS AND METHODS**Materials**

Raw materials used for the preparation of TC and three different marketed brands of TC (M-01, M-02, M-03) were procured from Bharat Aushadhi Bhandar, Pydhonie, Mumbai and authenticated by Herbal Research Lab, Ramnarain Ruia College. They were dried in the incubator at 45° C for a week, powdered and sieved through 85-mesh (BSS) sieve followed by their storage in air tight containers at room temperature.

Standard and reagents

The organic solvents and chemicals of analytical grade were procured from Merck Specialties Private Limited, Mumbai. Standard piperine ($\geq 99\%$ purity) was procured from Sigma Aldrich Chemical Company, Germany.

Proximate analysis of raw materials

The quality of raw materials used in the preparation of TC was assessed by determining the proximate parameters like foreign matter, ash value (total ash, acid insoluble ash and water soluble ash) and loss on drying using the standard pharmacopoeial methods¹².

In house preparation of TC

TC was prepared by mixing the fine powders of Maricha, Pippali and Sunthi in equal proportions as per the classical reference¹ and stored in air tight container at room temperature.

Preliminary phytochemical evaluation

The qualitative phytochemical tests were carried out to evaluate the presence of major phytoconstituents in TC⁶.

Preparation of standard solution of piperine

Stock solution of piperine (1000.0 $\mu\text{g/mL}$) was prepared by dissolving 10.0 mg of accurately weighed standard in small amount of methanol and the volume was made up to 10.0 mL in standard volumetric flask. Aliquots of 20.0-80.0 $\mu\text{g/mL}$ were prepared from this stock solution for calibration curve. Quality control samples [LQC: MQC: HQC (25.0, 40.0 and 65.0 $\mu\text{g/mL}$)] were prepared for precision, accuracy and ruggedness studies.

Extraction of piperine from TC, its ingredients and marketed samples

Extraction of marker components from TC was a daunting task because of its complex polyherbal matrix. Hence, the extraction conditions were optimized to achieve good fingerprinting and to resolve piperine efficiently. Different solvents and varied solvent to sample ratios were tested and finally TC and its ingredients (each 1.0 g) were extracted with 10.0 mL of methanol, vortexed for 1-2 min and kept standing overnight at room temperature. The mixture was filtered through Whatmann filter paper No. 41 (E. Merck, Mumbai, India) and the filtrate was used for further analysis. Similar extraction procedure was followed for marketed samples of TC.

HPTLC conditions

Chromatographic separation was achieved on HPTLC plates precoated with silica gel 60 F₂₅₄ (E. Merck) of 0.2 mm thickness with aluminum sheet support. Samples were spotted using CAMAG Linomat IV Automatic Sample Spotter (Camag Muttentz, Switzerland) equipped with syringe (Hamilton, 100 μL). Plates were

developed in a glass twin trough chamber (CAMAG) pre-saturated with mobile phase. Scanning device used was CAMAG TLC Scanner II equipped with CATS 3 software. The experimental condition was maintained at $20 \pm 2^\circ\text{C}$. Detection of piperine was possible after derivatizing the plates with anisaldehyde sulphuric acid reagent and photo documentation with CAMAG Reprstar 3 at 550 nm. ICH guidelines were followed for the validation of the developed HPTLC method¹³.

Solvent system

Solvent system consisting of toluene-ethyl acetate-glacial acetic acid (8: 2: 0.1, v/v/v) was used to resolve and quantify piperine from the matrix of TC, its ingredients and marketed samples.

Estimation of piperine from TC

Samples [10.0 μL , filtrates (obtained as per section "extraction of piperine from TC, its ingredients and marketed samples)] were applied in triplicate to a pre-coated silica gel 60 F₂₅₄ HPTLC plate (E. Merck) with the Camag Linomat IV sample spotter. The plate was developed and analysed as per the optimized chromatographic condition (as per section "HPTLC conditions").

Method application

The developed HPTLC method was applied to study the effect of storage on the stability of TC samples stored at different time periods (at room temperature) in terms of piperine content. Three different marketed samples of TC (M-01, M-02 and M-03) were individually compared with the in-house formulation with respect to their piperine content.

Safety evaluation

As a safety parameter, acute oral toxicity of TC was conducted on Albino Swiss female mice weighing 18-22 g. Animals were purchased from Haffkine Institute, Mumbai and after seven days of acclimatization, animals were divided into two groups containing three mice per group for the evaluation of toxicity. First group (test) received aqueous slurry of TC orally at the dose of 2.0 g/kg body weight of the animal while the second group (control) received 2.0 mL of distilled water. Study was conducted as per the methodology laid down in the OECD guideline 425 viz., fixed dose procedure (evident toxicity). Toxicity was evaluated in terms of mortality, daily food, water intake, body weight and general behavioral changes¹⁴.

RESULTS AND DISCUSSION

Standardization is an essential factor for ASU preparations in order to assess their quality based on the concentration of chemical or bioactive marker³. Modern bioanalytical techniques like HPTLC and HPLC are being used to achieve the aforesaid objectives. In the current work, an attempt has been made to standardize TC using HPTLC.

The results of proximate analysis TC and its ingredients for the parameters like ash values (total ash, acid insoluble ash and water soluble ash), loss on drying and foreign matter are represented in (Table 2). It was observed that the values obtained were in compliance with the limits documented in the Pharmacopoeia¹⁵⁻¹⁷. In house preparation of TC was carried out as per the classical reference (Table 1).

The preliminary phytochemical evaluation of TC revealed the presence of alkaloids, tannins and essential oils, flavonoids, glycosides and resins as major phytochemicals. Piperine content of TC and its ingredients (Maricha and Pippali) was determined using HPTLC technique.

The HPTLC method for estimation of piperine was validated in terms of specificity, precision, sensitivity, recovery and ruggedness as per ICH guidelines. Response for piperine was found to be linear in the range of 20-80 $\mu\text{g/mL}$ ($r^2 = 0.991$) which resulted as a regression equation $y = 11.838x - 98.064$. This equation was used to determine piperine content of TC, its ingredients and marketed samples. Developed HPTLC method was found to be precise with %

RSD < 2 % for intra-day and inter-day precision. LOD and LOQ value for piperine was found to be 2.0 and 6.0 $\mu\text{g/mL}$ respectively. Average recovery at three different levels of piperine for formulation was found to be 99.16 %. Method was found rugged for the parameters like change in analyst, change in mobile phase composition and change in spotting volume etc.

Among the various solvent systems tested, the mixture containing toluene-ethyl acetate-glacial acetic acid (8: 2: 0.1, v/v/v) gave the best resolution for piperine ($R_f = 0.43$) from the formulation matrix which enabled its quantification as well as phytochemical fingerprint (Figure 2). The identity of the band of piperine in TC was confirmed by comparing its UV absorption spectra with that of the standard.

TC samples subjected to storage up to 4 months, showed variation in piperine content. It was observed that the content of piperine decreased on prolonged storage (Table 4, Figure 3) when compared to freshly prepared formulation. The results of stability studies are supported by the frequent references in classical Ayurvedic texts regarding use of Churna¹⁸. Thus, it can be recommended that this formulation should be consumed when fresh. The comparative study of in-house TC formulation with other marketed formulations revealed that the piperine content was more in the freshly prepared in-house formulation (Table 5, Figure 4).

In acute toxicity studies, no significant change in body weight, food intake and water intake of the animals was observed compared to animals of control group and also no mortality was recorded. Thus, at the dose empirically used in traditional medicine, the formulation in the form of an aqueous slurry (2.0 g/kg body weight of animals), can be considered with a wide margin of safety for oral use.

Such reproducible modern techniques can make the traditional Ayurvedic medicines more acceptable in the local and global market. Thus, rationally designed, carefully standardized, synergistic traditional formulations and botanical drug products with robust scientific evidence can be used as an alternative to modern medicine.

CONCLUSION

Results of the present study can be used to characterize the samples in industry to check their uniformity. The obtained values of physical, chemical and biological parameters for TC can be adopted to lay down new pharmacopoeial standards to be followed in its preparation with batch to batch consistency.

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Table 1: Traditional formula composition of TC

Ingredients		Quantity
Sanskrit name	Botanical identity	
Maricha	Fruit of <i>Piper nigrum</i> L.	1 part
Pippali	Fruit of <i>Piper longum</i> L.	1 part
Sunthi	Rhizome of <i>Zingiber officinale</i> Rosc.	1 part

Table 2: Proximate analysis of raw materials and TC

Samples	Foreign matter	Total ash	Acid insoluble ash	Water soluble ash	Loss on drying
	% Mean (n = 3) ± S. D.				
Maricha	0.463 ± 0.036	4.276 ± 0.080	0.176 ± 0.023	2.360 ± 0.051	14.856 ± 0.427
Pippali	1.557 ± 0.030	3.987 ± 0.060	0.108 ± 0.036	2.093 ± 0.034	12.076 ± 0.308
Sunthi	0.350 ± 0.037	5.176 ± 0.036	0.684 ± 0.053	3.433 ± 0.043	9.596 ± 0.355
TC	Not applicable	5.765 ± 0.200	0.337 ± 0.012	3.216 ± 0.037	9.273 ± 0.012

Table 3: Piperine content in TC and its ingredients using HPTLC

Sample	Piperine content (mg/g) [Mean ± S. D., n=3]
Maricha	3.53 ± 0.12
Pippali	1.65 ± 0.24
Sunthi	---
TC	3.14 ± 0.55

Table 4: Stability study of TC in terms of piperine content using HPTLC

Storage period (month)	Piperine content (mg/g) [Mean ± S. D., n=3]
0	3.14 ± 0.55
2	2.48 ± 0.06
4	0.50 ± 0.10

Table 5: Comparative study of in-house TC and marketed formulations in terms of piperine content using HPTLC

Name of formulation	Piperine content (mg/g) [Mean ± S. D., n=3]
In-house TC	3.14 ± 0.55
M-01	2.24 ± 0.11
M-02	2.67 ± 0.04
M-03	2.88 ± 0.17

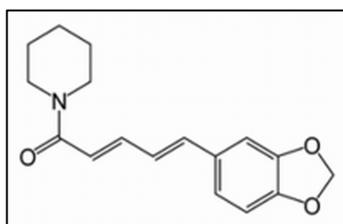


Figure 1: Structure of piperine

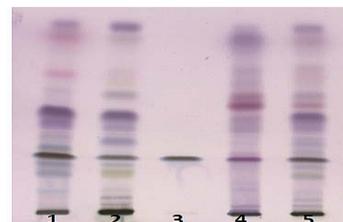


Figure 2: HPTLC detection of piperine from TC and its ingredients at 550 nm, Track details - 1: Maricha, 2: Pippali, 3: Piperine, 4: Sunthi, 5: TC

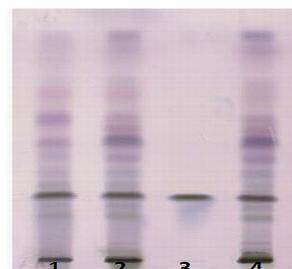


Figure 3: Stability samples of TC on HPTLC at 550 nm, Track details - 1: 0 month sample, 2: 2 month sample, 3: piperine, 4: 4 month sample

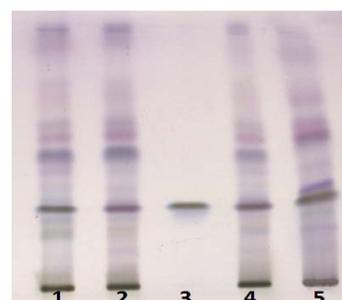


Figure 4: In house and marketed samples of TC on HPTLC at 550 nm, Track details - 1: TC, 2: M-01, 3: piperine, 4: M-02, 5: M-03

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