

# Chemical Standardization of Thetran Vithai Kutinir Chooranam - An Antidiabetic Siddha Polyherbal Formulation

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## ABSTRACT

**Introduction:** Belief of general public on herbal drugs led to the stable growth of herbal drug industry thereby necessitated the standardization of herbal drugs and pharmacopoeial standards for their quality control. **Aims:** Thetran Vithai Kutinir Chooranam (TVKC) is a Siddha polyherbal formulation used for type II diabetes. Aim of the present study is to standardize the drug chemically. **Methods and Material:** Drug was prepared as per the literature, stored in air tight container and subjected to preliminary phytochemical analysis, physico-chemical, thin layer chromatographic photo documentation, high performance thin layer chromatographic finger printing along with chemical markers such as emodin, chrysophanol and gallic acid, quantitative assay of marker compounds, heavy metal analysis, pesticide residue, aflatoxin and microbial load analysis. **Results:** The results revealed that the drug contains emodin in minor quantity and gallic acid in considerable quantity. The drug is free from microbial, heavy metal contaminations, aflatoxin and pesticide residues. **Conclusions:** The derived results could serve as a ready reference for quality control assessment of the drug.

**Key words:** Thetranvithai, Katukkai, Vilam Pisin Alkaloid, Anthraquinone.

**Key Messages:** The present study of chemical standardization of Thetran Vithai Kutinir Chooranam, an antidiabetic polyherbal Siddha formulation leads to the quality control standards in terms of preliminary phytochemicals, physico-chemical parameters, TLC identification, HPTLC finger print profiles and quantification of gallic acid which may be useful for the quality check of commercial drug in future

## INTRODUCTION

Type II diabetes is an embryonic disease of life style disorders. Many herbal medicines of proprietary nature and classical Siddha/Ayurveda formulations are sold in the market for type II diabetes. It is very inopportune to say that none of these medicines are having pharmacopoeial standards for quality control check and authenticity substantiation. Standardization of any herbal drug has become indispensable for framing out the quality standards of the herbal drug. Physico-chemical parameters, qualitative and quantitative thin layer chromatographic studies play vital role for comparing any geological variation, seasonal variation and maturity of the plants used in the formulation. TVKC comprises of four herbal ingredients (Figure 1), viz., *Strychnos potatorum* Linn. seed (Thetran vithai), *Terminalia chebula* Retz. fruit rind (Katukkai thol), *Cassia auriculata* Linn. seed (Aavaaram vithai) and *Limonia accidisima* L. gum (Vilam pisin) in equal composition. Four gram of the drug is added with 240 ml of water, boiled, filtered and reduced to 60 ml. This 60 ml is prescribed for diabetic adults twice a day.<sup>1</sup> The monographs on *T. chebula*,<sup>2</sup> *Strychnos potatorum*<sup>3</sup> are available. The combinations of above four drugs, TVKC is not reported for standardization, hence authors aimed to standardize.

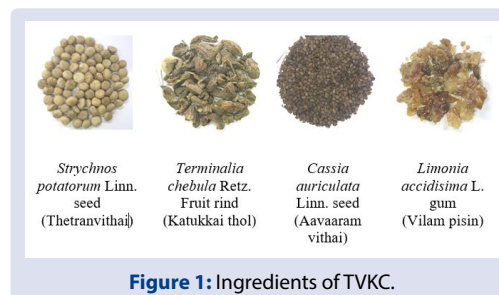


Figure 1: Ingredients of TVKC.

## MATERIALS AND METHODS

**Plants** *Strychnos potatorum* Linn. seed and *Terminalia chebula* Retz. fruit were procured from local crude drug market, authenticated by Research Officer (Pharmacognosy) of this Institute. *Cassia auriculata* Linn. seed and *Limonia accidisima* L. gum were collected and authenticated by Dr. Padma Sorna Subramaniam, Research Officer (Botany), Survey of Medicinal Plants Garden, Mettur, Tamil Nadu. The specimen of all samples were deposited in the Institute.

## Purification of drugs

*Strychnos potatorum* Linn. seeds was soaked in cow's milk for 24 minutes, washed and shade dried for use. *Terminalia chebula* fruits were broken and seeds were removed.<sup>4</sup>

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## Preparation of TVKC

All the ingredients were powdered separately, taken in the equal ratio and mixed well, stored in airtight container for all the studies.

## Chemicals and solvents

All the solvents used for this study were of Analytical Grade (Merck). Standards chrysophanol, emodin and gallic acid were procured from Sigma Aldrich, Bangalore.

## Preliminary phytochemical analysis

The preliminary phytochemical tests were carried out to check the presence or absence of steroids, triterpenes, flavonoids, alkaloids, coumarins, quinones, anthraquinones, glycosides, saponins, proteins, phenols and tannins as per the standard test methods.<sup>5</sup>

## Physicochemical parameters

The drug was analyzed for loss on drying at 105°C, total ash, acid insoluble ash, water soluble extractive, ethanol soluble extractive and pH as per the PLIM guidelines.<sup>6</sup> Alkaloid content was determined by soaking 10 g of TVKC with 100 ml of 5% acetic acid solution for 24 hrs., filtered, pH was altered to 8 by adding KOH solution and extracted with chloroform. The process was repeated for complete extraction. Finally the chloroform was evaporated and the total alkaloid extracted was calculated.

## Preparation of extracts

One gram of TVKC drug was extracted successively with hexane, chloroform, ethanol and made up to 10 ml in standard flasks separately for qualitative TLC and finger printing. Similarly one gram of TVKC drug was extracted directly with ethanol and made up to 10 ml in a standard flask for quantitative estimation of gallic acid. In the same manner, one gram of TVKC drug was added with 25 ml of water, boiled to one fourth, filtered and freed from moisture. Then extracted with ethanol and made up to 10 ml in a standard flask.

## Preparation of standards

10 mg of each of chrysophanol, emodin and gallic acid was dissolved in ethanol and made up to 10 ml in standard flasks. The stock gallic acid standard was diluted further by taking 1 ml and making up to 10 ml in ethanol.

## Derivatizing agent

The ethanolic potassium hydroxide (10% solution) was used as derivatizing agent which is specific reagent for anthraquinones.

## HPTLC instrument

For developing the TLC plate, CAMAG's twin chamber was used. For the application of the extract, Linomat IV (CAMAG, Muttens, Switzerland) applicator was used. Aluminium plate precoated with silica gel 60F<sub>254</sub> of 0.2 mm thickness (Merck) was used as TLC plate. For qualitative and quantitative scanning, CAMAG's scanner 030618 attached with WINCATS software were used. For photo documentation at UV 254 nm, 366 nm and in visible lights after dipping in vanillin-sulphuric acid reagent followed by heating in an air circulated oven till the development of colored spots, CAMAG's visualizer was used.

## TLC identification

5 µl of chrysophanol, emodin and 10 µl of successive hexane, chloroform and ethanol extracts of TVKC were applied on a TLC aluminium plate (10 cm x 10 cm) precoated with Silicagel 60F<sub>254</sub> of 0.2 mm thickness as 10 mm bands. The plate was developed in *toluene:ethyl acetate:formic acid* (8:1.5:0.5, v/v/v) up to a height of 8 cm from the

point of application in a presaturated twin trough chamber. The plate was dried and viewed under UV 254 nm, 366 nm and the respective images were captured using the visualizer. Then the plate was dipped in vanillin sulphuric acid reagent, heated at 105°C till the appearance of coloured spots which was also captured and documented as above.

## HPTLC finger printing

Both the above TLC plates were scanned under UV 254 nm using deuterium lamp in the absorbance/reflectance mode. The finger print profiles of emodin, chrysophanol, hexane, successive chloroform and successive ethanol extracts of TVKC and the 3D chromatogram was documented.

## Quantitative estimation of gallic acid

1 to 6 µl of TVKC ethanol extracts were applied on a TLC aluminium plate (20 cm x 10 cm) precoated with Silicagel 60F<sub>254</sub> of 0.2 mm thickness as 8 mm bands in the tracks 1, 2, 3, 10, 11 and 12. The gallic acid standard was applied on tracks 4 to 9 with the volumes of 1, 2, 4, 6, 8 and 10 µl. The plate was developed using the mobile phase, *toluene:ethyl acetate:formic acid* (3.5:3.5:0.5, v/v/v). The developed plate was scanned densitometrically under UV 254 nm in quantitative mode using deuterium lamp.

## Heavy metal analysis

The lead, cadmium, arsenic and mercury are considered as heavy metals and their concentration in the drug were estimated using Inductively Coupled Plasma – Optical Emission Spectroscopy (ICP-OES) by following the methods of AOAC 20<sup>th</sup> Edition.

## Pesticide residue analysis

Pesticide residue was analyzed using GC-MS (Agilent-7890 A, MS 5975) with DB 5 MS column of 30 m x 0.25 mm ID x 0.25 µm film thickness. 1 µl was injected at a injection temperature of 150°C and 1 ml/min flow rate was maintained. Helium gas of 99.9995% purity was the carrier gas. The ion source was Electron Impact in the MS and the mass from 50 to 550 was scanned at a fixed electron energy of -70 eV.

## Microbial load and pathogens

*Enterobacteriaceae*, *E. coli*, *Salmonella spp.* *Pseudomonas aeruginosa*, *Staphylococcus aureus*, total bacterial count and total fungal count were determined as per the WHO methods.<sup>7</sup>

## Aflatoxin analysis

For aflatoxin analysis was carried out in HPLC (Agilent-1260 Infinity System) with - fluorescence detector and C-18 (Zorbax Eclipse Plus) column of 4.6 mm x 150 mm x 5 µm inner diameter. Injection volume was 20 µl. Eluted the column isocratically with 1:1 mixture of methanol and water containing 238 mg of KBr and 700 µl of 4 M HNO<sub>3</sub> in one litre of water. The hold time was 12 min. and the flow rate was 1 ml/min. KOBRA cell was used for electrochemical derivatization.

## RESULTS

### Preliminary phytochemical analysis

The preliminary phytochemical tests showed the presence of steroids, triterpenes, flavonoids, alkaloids, coumarins, quinones, anthraquinones, glycosides, proteins, phenols and tannins.

### Physicochemical parameters

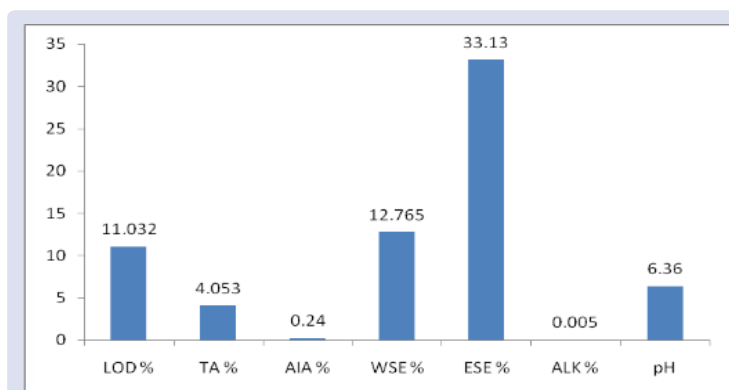
The physicochemical parameters of TVKC is shown in Figure 2. The mean calculated value of loss on drying was 11.032%; total ash value

was arrived as 4.053%; acid insoluble ash was 0.240%; water soluble extractive was calculated as 12.765%; the ethanol soluble extractive was determined as 33.130%; pH value of 10% solution was measured as 6.37.

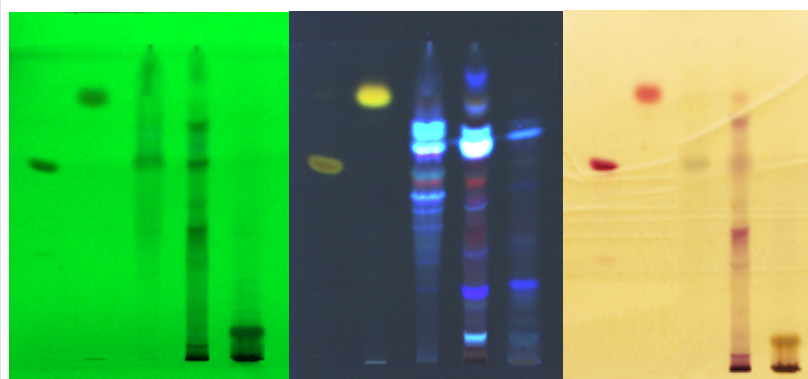
### TLC identification

The TLC photodocumentation of successive hexane, chloroform and ethanol along with emodin and chrysophanol under UV 254 nm,

366 nm and after dipping in alcoholic KOH is shown in Figure 3. The HPTLC finger print profile of successive hexane (A), chloroform (B), ethanol (C) extracts separated with the solvent system of *toluene:ethyl acetate:formic acid* (8:1.5:0.5, v/v/v) and the total ethanol extract (D) separated with the solvent system of *toluene : ethyl acetate : formic acid* (3.5:3.5:0.5, v/v/v) are shown in Figure 4. The TLC chromatogram developed for the quantification of gallic acid in total ethanol extract is shown in Figure 5. The linear regression curve obtained for the standard gallic acid is produced in Figure 6.

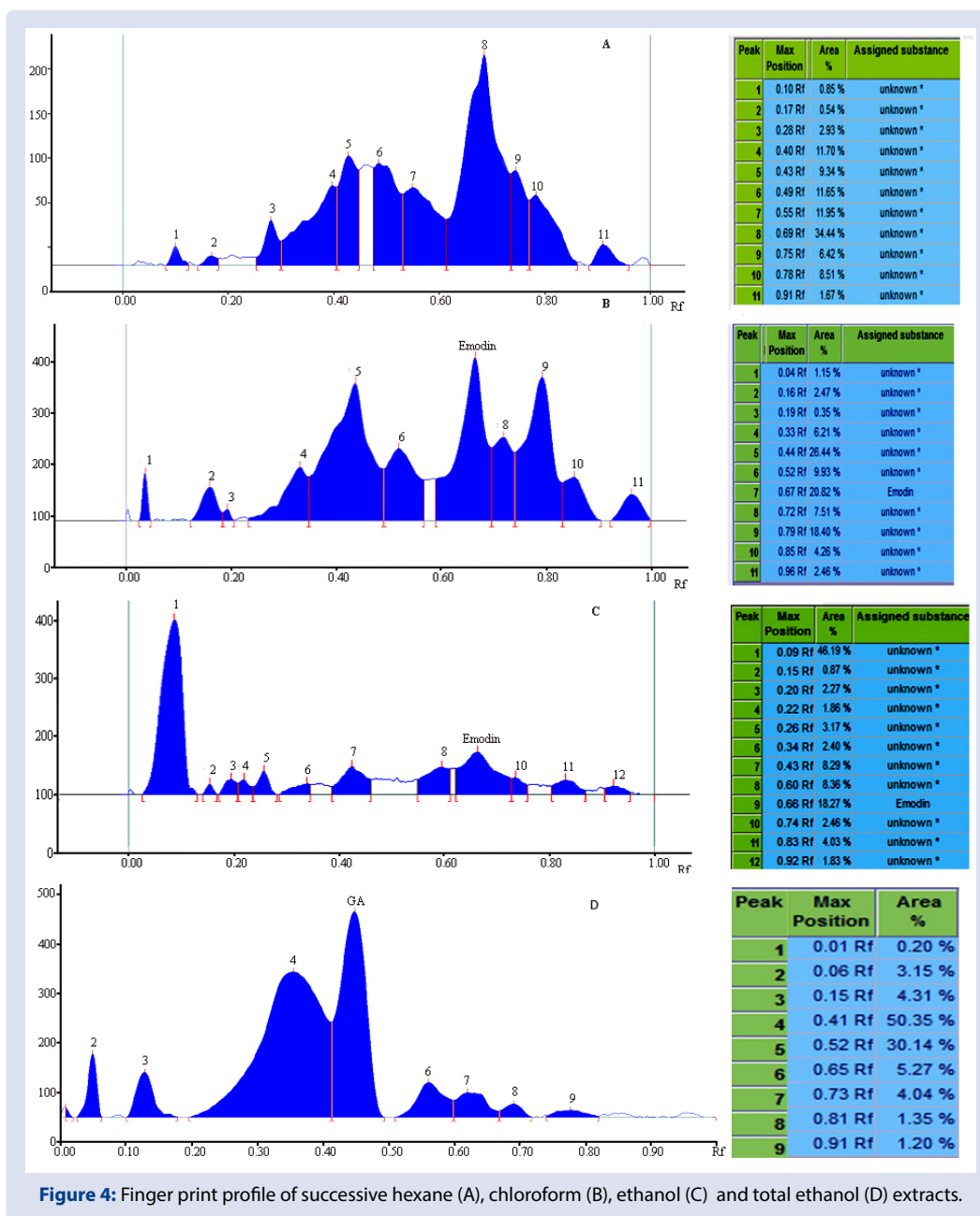


**Figure 2:** Graphical representation of physico-chemical values of TVKC (LOD: Loss on drying at 105°C; TA: Total ash; AIA: Acid insoluble ash; WSE: Water soluble extractive; ESE: Ethanol soluble extractive; ALK: Alkaloid content; pH: pH of 10% solution).

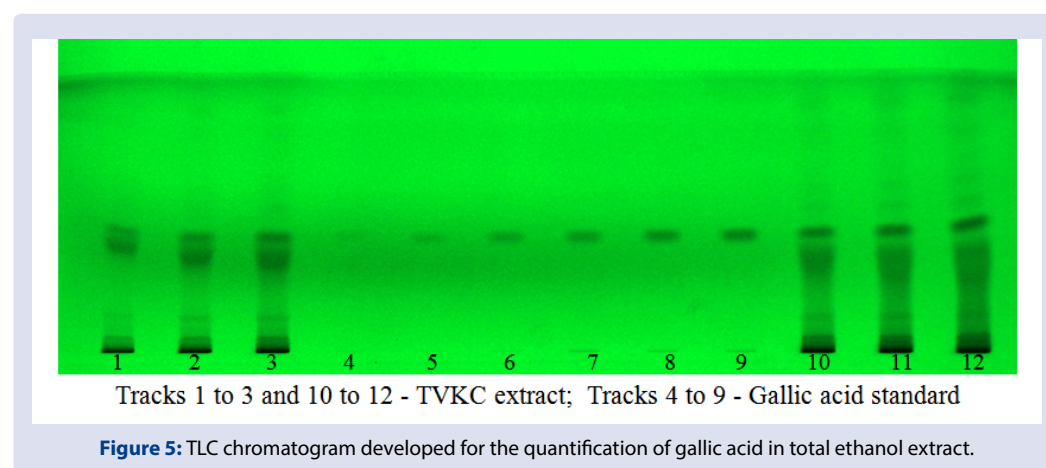


UV 254 nm			UV 366 nm		White light after dipping in ethanolic KOH	
Track	R <sub>f</sub>	Colour	R <sub>f</sub>	Colour	R <sub>f</sub>	Colour
1- Emodin	0.62	Green	0.62	Yellow	0.62	Red
2- Chrysophanol	0.83	Green	0.83	Yellow	0.83	Red
3-TVKC hexane extract	0.28	Green	0.42	Blue	0.62	Grey
	0.41	Green	0.47	Blue	-	-
	0.64	Green	0.52	Blue	-	-
	-	-	0.56	Red	-	-
	-	-	0.67	Fluorescent Blue	-	-
	-	-	0.71	Fluorescent Blue	-	-
4- TVKC chloro form extract	0.32	Green	0.07	Light blue	0.04	Purple
	0.42	Green	0.22	Blue	0.31	Purple
	0.50	Green	0.34	blue	0.42	Purple
	0.63	Green	0.49	blue	0.63	Purple
	0.75	Green	0.56	Red	0.75	Purple
	-	-	0.66	Fluorescent blue	-	-
5- TVKC ethanol extract	0.09	Green	0.08	Blue	0.09	Grey
	0.25	Green	0.24	Blue	-	-
	-	-	0.70	Blue	-	-

**Figure 3:** TLC Photo documentation of TVKC.



**Figure 4:** Finger print profile of successive hexane (A), chloroform (B), ethanol (C) and total ethanol (D) extracts.



**Figure 5:** TLC chromatogram developed for the quantification of gallic acid in total ethanol extract.



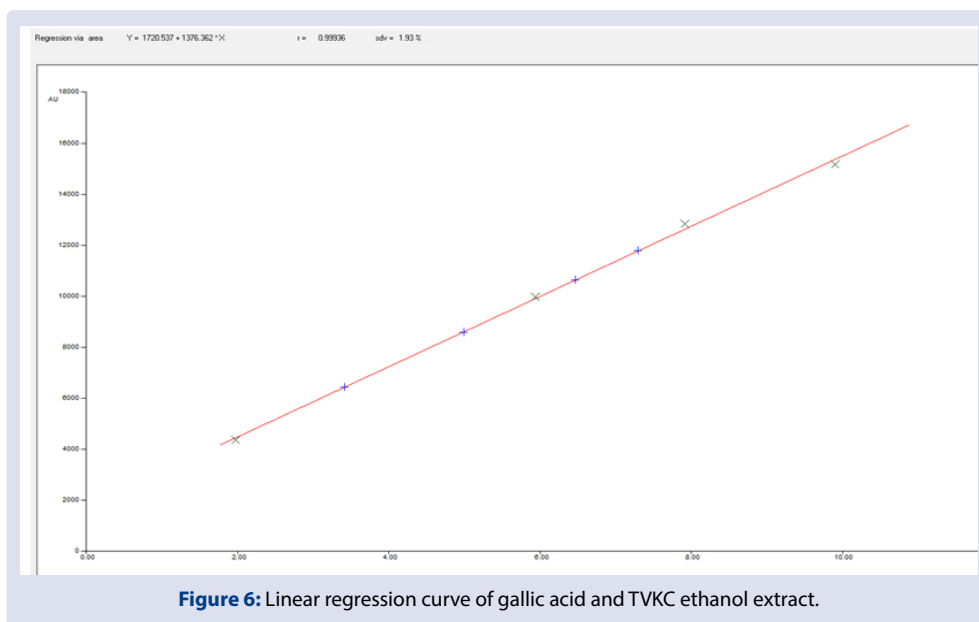


Figure 6: Linear regression curve of gallic acid and TVKC ethanol extract.

### HPTLC finger printing

The HPTLC finger print profile of hexane extract showed a total of 11 peaks in which the peak at  $R_f$  0.69 (34.44%), 0.59 (11.95%), 0.55 (11.65%), 0.40 (11.70%), 0.43 (9.34%), 0.78 (8.51%) and 0.65 (6.42%) were the major and all other peaks were minor. In the HPTLC finger print profile of chloroform extract also, there were 11 peaks in total in which the peaks at  $R_f$  0.44 (26.44%), 0.67 (20.82%), 0.79 (18.40%), 0.52 (9.93%) and 0.72 (7.51%) were the major peaks and all other peaks were minor peaks. In the HPTLC finger print profile of ethanol extract, there were 12 number of peaks separated but the major peak appeared at  $R_f$  0.09 (46.19%), 0.66 (18.27%), 0.43 (8.29%) and 0.60 (8.36%) and all other peaks were minor peaks. The 3D chromatogram confirmed the presence of emodin in all the extracts as distinct peaks whereas presence of chrysophanol in chloroform as a minor peak.

### Quantification of gallic acid

The linear regression curve for gallic acid ( $Y = 1720.532 + 1376.362 \cdot X$ ) was obtained for the concentration range 0.1 to 1.0  $\mu\text{g}$  with standard deviation of 1.93% and the value of correlation coefficient was 0.99936 showing the best peak purity. The percentage of gallic acid was determined as 1.61%. The limit of detection was 0.0046  $\mu\text{g}$  and the limit of quantification was 0.014  $\mu\text{g}$ .

### Heavy metal analysis

The heavy metals, viz., lead, cadmium mercury and arsenic were lower than the limit of quantifications, the LOQ of which are 5, 0.2, 1 and 1 ppm respectively.

### Pesticide residue analysis

The organo phosphorus pesticides and organo chloro pesticides such as phorate, fenitrothion, phorate sulfone, fenthion chlorfenvinphos fenthion sulfone, fenthion sulfoxide, ethion,  $\beta$ -BHC,  $\gamma$ -BHC,  $\delta$ -BHC,  $o,p'$ -DDE  $p,p'$ -DDE,  $o,p'$ -DDD,  $p,p'$ -DDD,  $p,p'$ -DDT and  $o,p'$ -DDT were found to be below the limit of quantification (LOQ 0.01 ppm).

### Microbial load and pathogens

The *Enterobacteriaceae*, *Escheria coli*, *Salmonella spp.*, *Staphylococcus aureus* were found to be absent in the drug, the total bacterial count and total fungal count were within the permissible limit for internal use as per WHO.

### Aflatoxin analysis

The aflatoxin  $B_1$ ,  $G_1$ ,  $B_2$  and  $G_2$  were found to be below the limit of quantification (LOQ 0.1 ppm).

## DISCUSSION

The presence of phytochemicals such as steroids, triterpenes, flavonoids, alkaloids, coumarins, quinones, anthraquinones, glycosides, proteins, phenols and tannins would enhance the therapeutic efficacy of the drug. The *C. auriculata* seed is rich in fatty acids, sterols,<sup>8</sup> many flavonoids and flavonoid glycosides.<sup>9</sup> Flavonoids are known for their antioxidant, antibacterial and anticancer activities.<sup>10</sup> The alkaloids, diaboline and its acetate,<sup>11</sup> brucine<sup>12</sup> were reported from *S. potatorum* seeds. Many phenols reported in *T. chebula* include ellagic acid, punicalagin, chebulanin, neochebulinic acid, chebulinic acid, 1,2,3,4,6-penta-*O*-galloyl-*H*-*D*-glucose, 1,6-di-*O*-galloyl-*D*-glucose, casuarinin, 3,4,6-tri-*O*-galloyl-*D*-glucose and terchebulin.<sup>13-15</sup> These phenols are very good antibacterial agents and they facilitate in extending the shelf life of the drug.<sup>16</sup>

The 11.032% of loss on drying indicates that the moisture content is slightly high which may be due to the fixed oil present in the *C. auriculata* seed and the resinous matter present in the *T. chebula* fruit rind. The total ash value of 4.053% represents the presence of lesser inorganic minerals in the TVKC. Similarly, the acid insoluble ash was very less 0.240% which infers the absence of siliceous matter. The water soluble extractive was calculated as 12.765% and the ethanol soluble extractive was determined as 33.130%. When compared to water soluble extractive, the ethanol soluble extractive is higher indicating the presence of high polar compounds viz., glycosides, sugars, tannins, saponins, alkaloids, etc. The total alkaloid content was determined as 0.005% which is very less and is not detectable by Dragendorff reagent on TLC plate. The pH value of 6.37, indicates the acidic nature which could reduce the opportunity for microbial attack thereby extending the shelf life of TVKC. With the standardized the physico-chemical parameters, the drug could be subjected to routine analysis quality control in bulk production in batches<sup>17</sup> and serves as the prerequisite for toxicity<sup>18</sup> and clinical<sup>19</sup> evaluations.

The TLC plate under UV 254 nm showed the presence of spot with the  $R_f$  of emodin in hexane, chloroform and ethanol extracts where as the presence of spot with the  $R_f$  of chrosophanol was not visible. However, the finger print profile under UV 254 nm, the peak present in

hexane extract did not match with the peak of emodin and identified as a unknown peak, ie., emodin was not present in hexane extract and very very less in ethanol extract. The plate under UV 366 nm showed the presence of chrysophanol in hexane and chloroform extracts. The derivatized plate showed the presence of emodin in chloroform only and chrysophanol in none of the extract. The successive ethanol extract showed the presence of high polar compounds. Since emodin and chrysophanol were present in traces, the estimation of these anthraquinones was not done. These two anthraquinones were from the seed of *C. auriculata* and the gallic acid was from the *T. chebula*. Though the presence of gallic acid was not qualitatively run for TLC, it was quantitatively estimated in the drug TVKC. The gallic acid has been reported for anti-inflammatory,<sup>20</sup> cardioprotective effects in diabetes induced myocardial dysfunction,<sup>21</sup> antibacterial,<sup>22</sup> cytotoxic activity,<sup>23</sup> antioxidant<sup>24</sup> and anti-diabetic<sup>24,25</sup> activities. As the content of gallic acid as determined by HPTLC is 1.61% (w/w), TVKC will exhibit the similar activities of gallic acid. The standardization of any herbal formulation would be complete only if done along with ingredients and chemical markers.<sup>26</sup> The quantification of chemical markers for the standardization of herbal drugs using HPTLC/HPLC has been reported.<sup>27,28</sup>

The heavy metals, viz., lead, cadmium mercury and arsenic were well within the WHO prescribed permissible limits 10, 0.3, 1 and 3 ppm respectively. The trepidation of herbal drug consumers with respect to heavy metal poisoning<sup>29,30</sup> is ruled out and the drug is safe. As the aflatoxin B<sub>1</sub>, G<sub>1</sub> (< 0.5 ppm), B<sub>2</sub> and G<sub>2</sub> (< 0.1 ppm) were found to be below the limit of and the drug could be administered internally. The organo phosphorus pesticides and organo chloro pesticides were also found to be below the limit of quantification indicating the collection of wild plants as per good collection practices. The drug was free from *Enterobacteriaceae*, *Escheria coli*, *Salmonella spp.*, *Staphylococcus aureus* and the total bacterial count and total fungal count were within the safety limit for internal medicine.

## CONCLUSION

The antidiabetic siddha formulation TVKC has been standardized for physicochemical parameters, screened the presence of different secondary metabolites, showed the presence of quinones & polyphenol by TLC and estimated the gallic acid. Also the drug was ensured for the absence of heavy metals, pesticide residues, microbial load and aflatoxins. These standardization parameters could be considered as a reference standard of this drug for quality control assessment in future.

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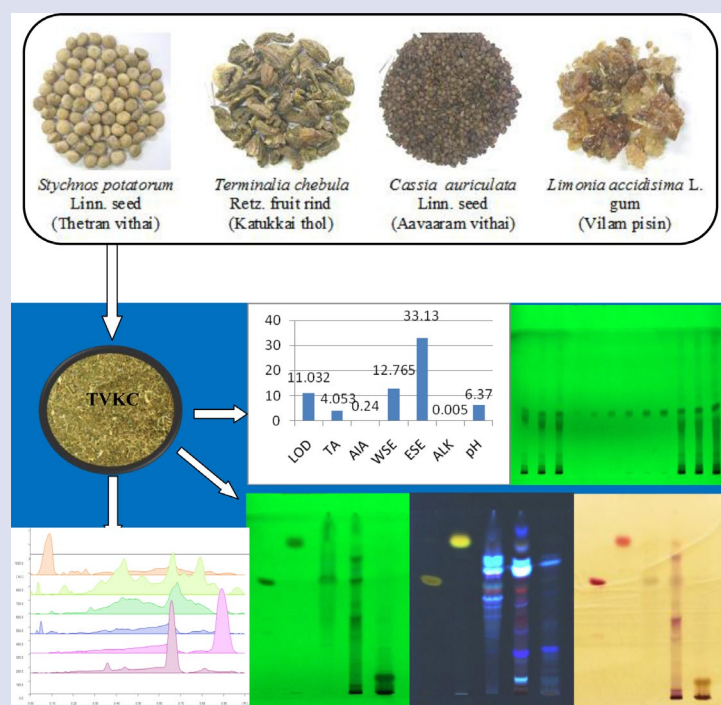
## CONFLICTS OF INTEREST

None.

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## GRAPHICAL ABSTRACT



## ABOUT AUTHORS



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