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Confirmation and optimization of Bhavana Vidhi in Tribhuvankeerti Ras - An Ayurvedic formulation

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Abstract

The present study carried out to analyze the effect of bhavana in standardisation of Tribhuvankeerti Ras. An attempt has also been made to correlate the analytical test results with the efficacy study. The standardization of Tribhuvankeerti Ras was carried out using HPTLC and FT-IR. Also, the therapeutic efficacy of Tribhuvankeerti Ras was carried out on Swiss albino mice. The HPTLC fingerprint profile depicts the presence of all three bhavana dravyas Tulasi Ras, Adrak Ras and Dhatura Ras. The result showed that all the bhavanas in Tribhuvankeerti Ras formulation has exactly same Rf values as they had in alone and FT-IR analysis confirms unique transmittance peak in range of 4000cm^{-1} to 600cm^{-1} . The pharmacological result elaborates the anti-pyretic effect of Tribhuvankeerti Ras in mice. The HPTLC and FT-IR methods developed for confirmation of bhavana dravyas in Tribhuvankeerti Ras will help in establishing the specifications and need for presence of bhavana dravyas.

Keywords: Bhavana, HPTLC, FT-IR, Tribhuvankeerti Ras, Tulasi Ras, Adrak Ras, Dhatura ras etc

1. Introduction

Tribhuvankeerti Ras (TKR) is a herbomineral ayurvedic formulation, well renowned for its usefulness in the treatment of *Jwara* specially Vatakapha Jwara, Vishamjwara as Swedapravartak, Amapachak, Angamardanashak. It helps in reducing excessive nasal discharge, Headache, pain in throat, Galkandu in Pratishaya. Also, it is intended to eradicate Amadosha like Kapha, Ushna and pitta. In preparation of Tribhuvankeerti Ras - Tulsi patra, Adrak and Dhatura pala are sequentially used for bhavana vidhis as per Bharat Bhaishajya Ratnakar 2/2755.

Bhavana is a unique and distinct pharmaceutical trituration process in which a raw form of drugs triturated with sufficient quantity of liquid media [viz. plant extracts (expressed juice, decoction etc.) or animal products (urine, milk, etc.)] till liquid portion gets absorbed completely [1, 2].

Process of bhavana Vidhi can be done in varied time, i.e. In day time, in effluent sunlight or in the night time. But the process like Nivasana (soaking) is done in the night and Mardana is done on the next day. In case where the media are taken as decoction form, the amount of herbs should be taken equal to the amount of material, eight times water should be added to it and should be reduced to one eighth by boiling.

Bhavita Dravya on grinding is converted into sticky paste form and is soft in nature. Any Bhavita Dravya which shows such Lakshana is termed as Subhavita Dravya [4]. Bhavana vidhi has diverse applied roles such as in murchana samskara of parad [5], in hingula shodhan by ardrak bhavana [6], bhavana of nimbu swarasa to shoditha swarnamakshika for Marana [7]. In Nirvisheekarana as in case of ardraka swarasa bhavana to ahiphena [8], bhavana in satwapatana gopitta bhavana in spatika satwapatana [9].

With the process of bhavana Vidhi various benefits are enlisted in the Ayurvedic text. bhavana makes ras oushadhis absolutely non-toxic [10]. Likewise, it make the drug easily digestible and assimilable [11, 12], by converting them into easily absorbable form through the intestinal mucosa [13]. Bhavana has important role in enhancing the therapeutic efficacy of drug and reducing its dose. It is observed that bhavana enhances drug palatability [14], and is known to widen the therapeutic utility [15], bhavana helps in reduction of hardness and particle size for better assimilation and absorption of drug in the body [16].

It is most important that the bhavana vidhis are given in respective manner only i.e. first with Tulsi Ras, second Adrak Ras and last with Dhatura pala ras. Tulsi is useful in cough and cold, Adrak is helpful in throat pain and Dhatura pala is effective against fever. Therefore, we made an attempt to confirm the sequential bhavana vidhis of Tulsi, Adrak and Dhatura pala in Tribhuvankeerti Ras with the help of modern analytical techniques.

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2. Materials and Methods

2.1 Drugs and Chemicals

For efficacy study, Brewer yeast was purchased from Shree Scientific, Mumbai. Paracetamol tablets were purchased from local market and all other chemicals purchased from Sigma Aldrich Chemical Co. St Louis, MO, USA.

For HPTLC, HPLC grade chemical reagents (Chloroform, Hexane, Methanol, Ethyl acetate) were purchased from Merck.

2.2 Preparation of Test Formulation i.e. Tribhuvankeerti Ras

It is prepared following the method mentioned in the Bharat Bhaishajya Ratnakar 2/2755 and list of ingredients used in the preparation is mentioned in Table I. The required quantity of ingredients viz. Suntha, Miri, Pimpali, Pimpalimool as per formula was cleaned and powdered in grinder and passed through 80 mesh. Then remaining ingredients viz. Shuddha Hingul, Shuddha Bachnag, Shuddha Tankankhar were powdered in grinder and passed from 80 mesh. Shuddha Hingul was triturated in Khal with specified quantity of water. Then remaining ingredients Shuddha Bachnag, Shuddha Tankankhar, Suntha, Miri, Pimpali & Pimpalimool were added in Khal and trituration was continued. Juice of bhavana Dravyas viz Tulsi leaf, Adrak and Dhatura Pala was prepared separately in Juicer with Brix of 4-5.

In the Khal admixture, specified quantity of Tulsi Ras was added for bhavana vidhi & triturated in it till dryness. This process was repeated 3 times. After completion of first bhavana vidhi, specified quantity of Adrak Ras was added and trituration process completed in Khal till dryness. This process was also repeated 3 times. Then after completion, third and final bhavana dravya of Dhatura Patra Ras was added & trituration carried out as per previous processes. Samples were withdrawn from Khal before bhavana and sequentially after each bhavana processes. The bulk of Tribhuvankeerti Ras prepared was dried at 70 °C. Finally the dried admixture was powdered and passed from 40 mesh (Diagram I).

2.3 Instrumentation

2.3.1 HPTLC

HPTLC Instrument Camag, Linomat 5 fitted with TLC Scanner 4, Wincat Software was used for chromatographic analysis of sample. Twin trough chamber was used for development of HPTLC plate. Photo documentation cabinet fitted with High Resolution camera was used for capturing images at different wavelengths.

2.3.2 FT-IR

ALPHA FT-IR instrument Bruker, with ATR sampling mode, OPUS software was used for analysis of samples.

2.4 Evaluation of Anti-pyretic activity: *In vivo*

Swiss albino male mice weighing about 20-30g obtained from Animal House Facility (AHF) of Shree Dhootapapeshwar Ayurvedic Research Foundation (SDARF) - Panvel were used for study. The animals were housed in polypropylene cages

(47 cm × 34 cm × 18 cm), lined with husk which were renewed every 24 hrs. The animals were fed on a standard pellet diet and water ad libitum throughout the experiment. The experimental animals were maintained in a controlled environment (12:12 hr light and dark cycle) and temperature (24 °C ± 2 °C). The experiments were carried out in accordance with the guidelines of the Committee for Control and Supervision of Experiments on Animals (CPCSEA), New Delhi, India and the experimental protocol was approved by the Ethical Committee for Animal Experimentation (Ethical clearance number: SDARF/PC/2013/01) of SDARF. The animals were acclimatized for one week before starting the experiments.

2.4.1 Study Design

Primarily, the basal rectal temperature of 30 mice were recorded using a digital thermometer. All the 30 mice were injected with 30% (w/v) suspension of brewers yeast in 0.9% NaCl in a dose of 10 mL/kg, s.c. The temperature were recorded 16 h after yeast injection. Animals showed rise in temperature by 0.5 °C were selected for study. Body weight of mice were recorded and they were randomly divided into three experimental groups (n=6).

Group I: Animals were treated with Brewer's yeast (10 mL/Kg) by a subcutaneous injection.

Group II: Animals were administered with Brewer's yeast (10 mL/Kg) and with Standard treatment Paracetamol after 16 hr gap

Group III: Animals were administered with Brewer's yeast (10 mL/Kg) and with Tribhuvankeerti ras after 16 hr gap. Rats were treated as per treatment protocol. Body temperature of each mice were recorded at time interval of 30 min for the period of 3 hrs after the administration of treatment drug.

2.4.2 Statistical analysis

The values were expressed as mean ± SD. The statistical analysis was carried out by one way analysis of variance (ANOVA) followed by Dunnet's 't' - test. P values < 0.05 were considered significant.

Table I : TKR ingredients

Sr. No	Name of Ingredients	Part Used
1	Shuddha Hingul (Purified <i>Cinnabar</i>)	1 Part
2	Shuddha Bachnag (Purified <i>Aconitum ferox</i>)	1 Part
3	Shuddha Tankankhar (Purified Borax)	1 Part
4	Suntha (<i>Zingiber officinale</i>)	1 Part
5	Miri (<i>Piper nigrum</i>)	1 Part
6	Pimpali (<i>Piper longum</i>)	1 Part
7	Pimpalimool (<i>Piper longum</i>)	1 Part
Bhavana:		
8	Adraaka Rasa (<i>Zingiber officinale</i> Juice)	q.s.
9	Tulasi Rasa (<i>Ocimum sanctum</i> Juice)	q.s.
10	Dhatura Rasa (<i>Datura metel</i> Juice)	q.s.

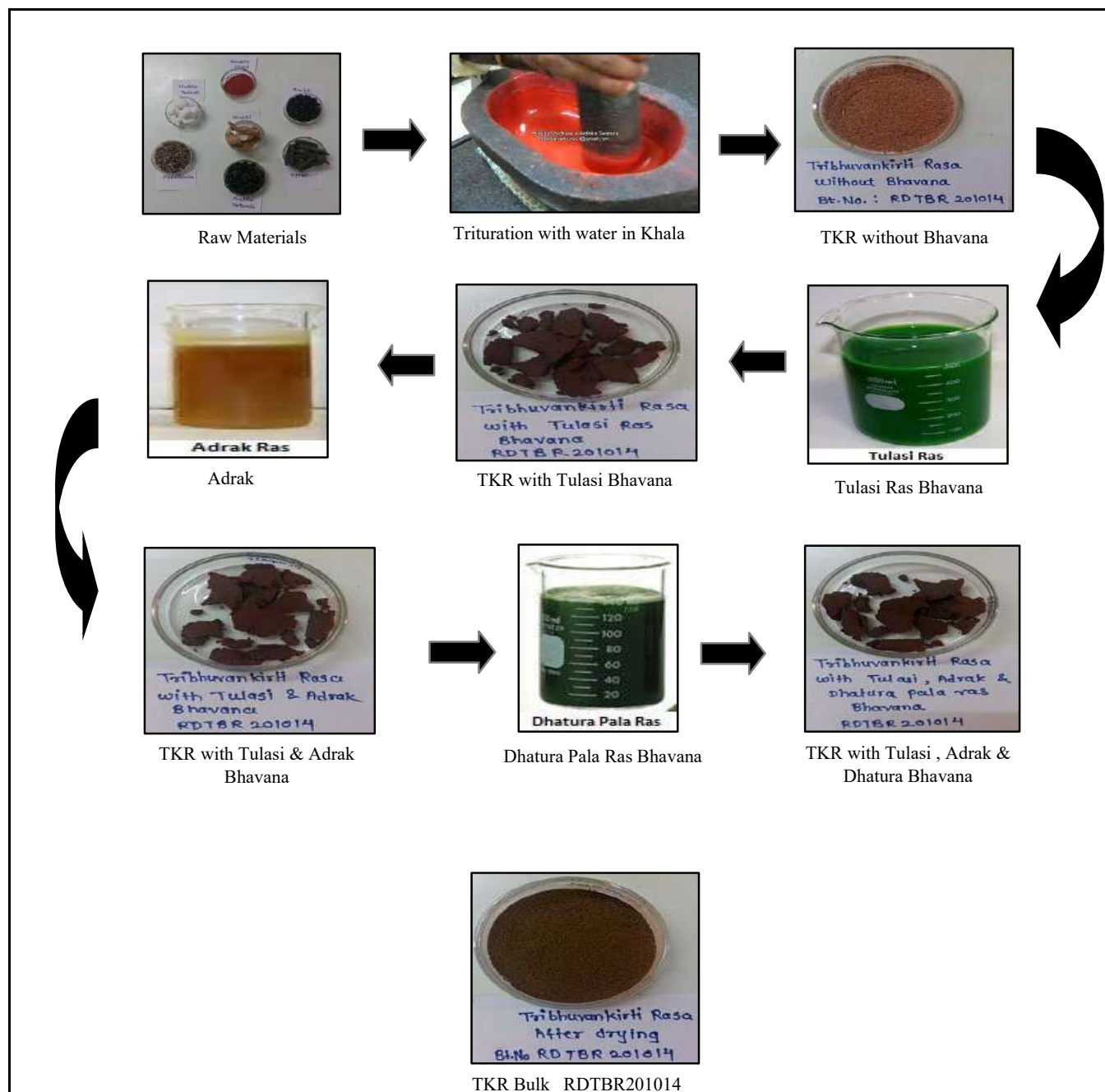


Diagram I: Sequential bhavana Vidhi in Tribhuvankeerti ras

3. Results

3.1 HPTLC Analysis

The chloroform extracts of Tribhuvankeerti Ras without bhavana (WB), Tribhuvankeerti Ras after Tulsi ras bhavana (TB), Tribhuvankeerti Ras after Tulsi & Adrak bhavana (TAB), Tribhuvankeerti Ras after Tulsi, Adrak & Dhatura pala ras bhavana (TADB), Tribhuvankeerti Ras dry Final sample-RDTBR201014 (Final), Tulsi ras, Adrak ras and Dhatura pala ras were prepared. To achieve good resolution, double development was done using Chloroform : Hexane : Methanol : ethyl acetate (5 : 4.8 : 0.2 : 0.2) as solvent system. The bands of bhavana dravyas after bhavana vidhi was observed with good resolution in 366nm than in 254nm and visual light after derivatisation.

3.1.1 Tribhuvankeerti Ras with Tulsi bhavana

3.0 µl of Tulsi ras extracted sample was applied in the form of band on silica gel 60 F254 TLC plate of thickness 0.2 mm and developed in chamber with Chloroform : Hexane : Methanol : ethyl acetate (5:4.8:0.2:0.2) as solvent system. The HPTLC fingerprint profile of Tulsi ras showed four Major Bands of Red colour under 366 nm at R_f value of 0.23, 0.56, 0.66 & 0.77. Simultaneously, HPTLC developed for extracted sample of Tribhuvankeerti Ras after Tulsi ras bhavana (TB). The fingerprint profile showed 11 bands with R_f Values 0.16 to 0.87. TB plate when visualized under 366nm shows 3 Red bands at R_f value of 0.23, 0.56 & 0.77 which indicates the presence of Tulsi ingredients in the sample (Fig.I). The chromatogram developed also showed peaks of Tulsi ras at R_f value of 0.23, 0.56 & 0.77.

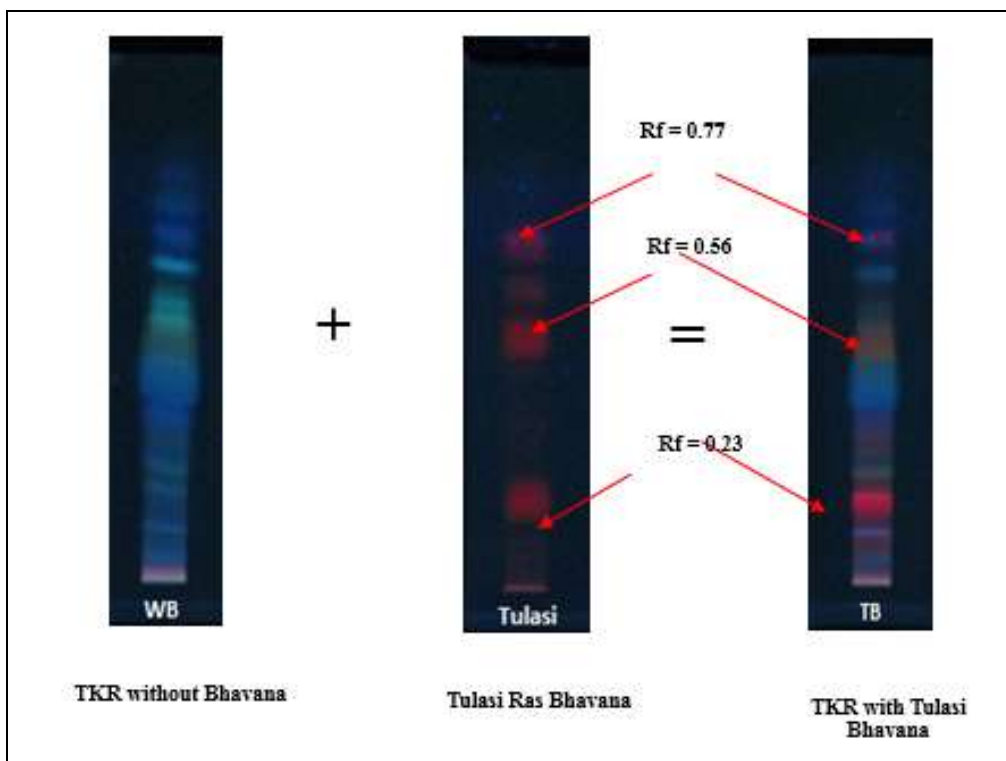
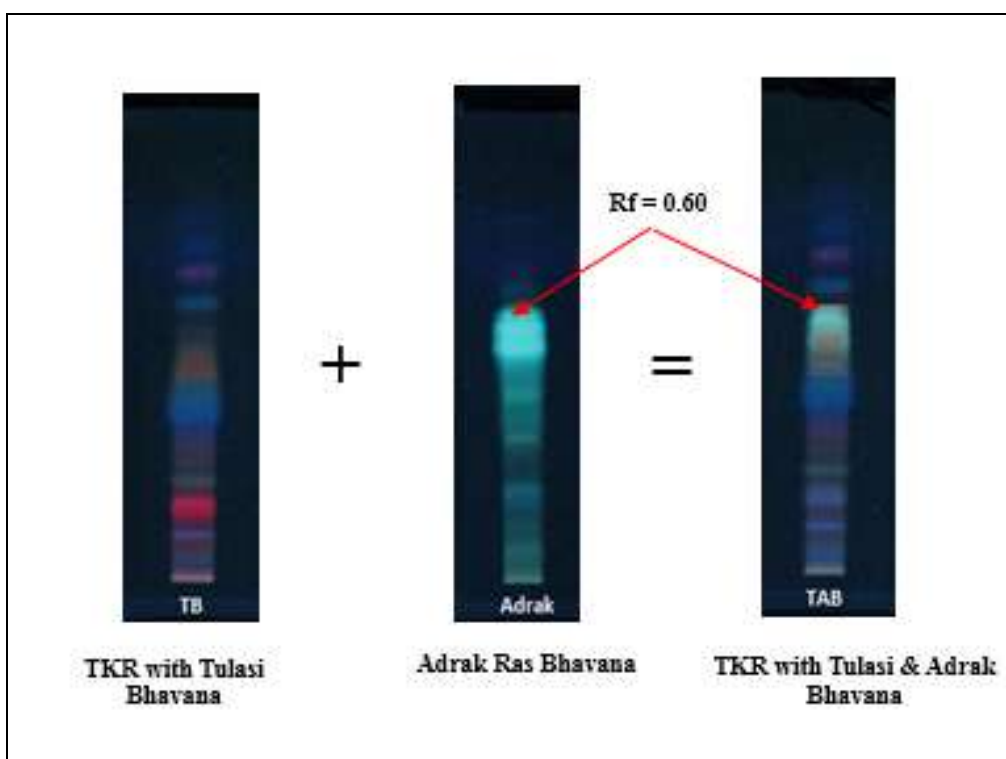


Fig I: HPTLC fingerprint profile under 366 nm after Tulasi Ras bhavana

3.1.2 Tribhuvankeerti Ras after Tulsi & Adrak bhavana (TAB):

3.0 μ l of Ardrak ras sample was applied on silica gel 60 F254 TLC plate and developed in chamber with Chloroform: Hexane:Methanol:Ethyl acetate (5:4.8:0.2:0.2) as solvent system. The HPTLC fingerprint profile of Ardrak Ras showed 6 Bands of Fluorescent Blue colour at 366 nm at Rf value of 0.30, 0.39, 0.47, 0.60, 0.64 & 0.71. Simultaneously,

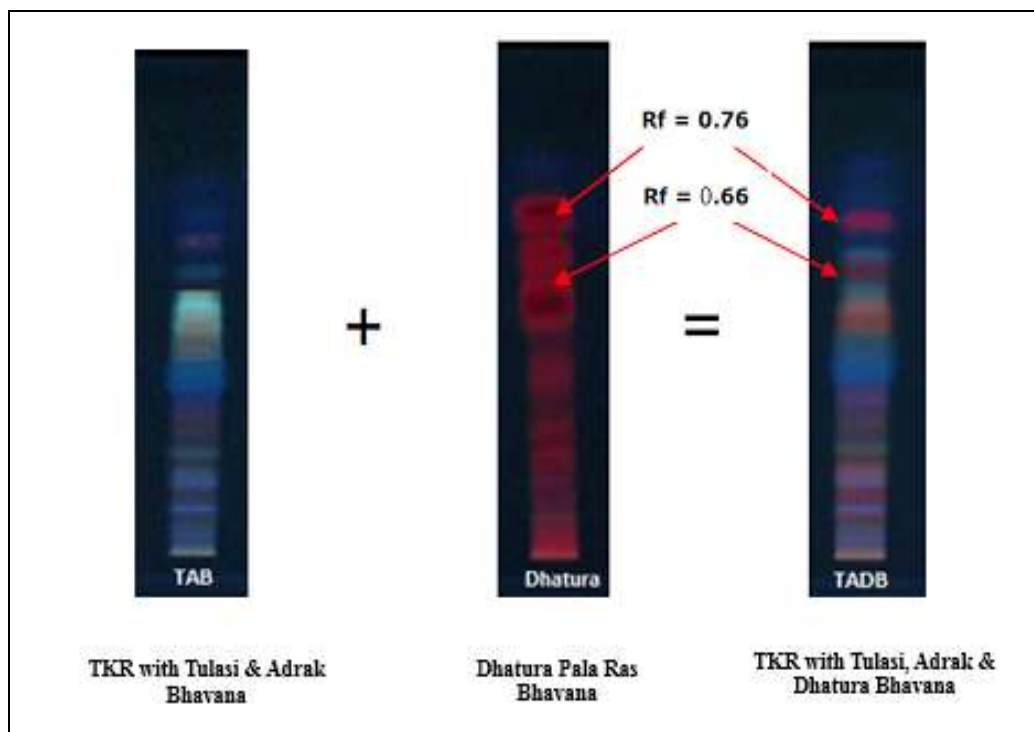
HPTLC developed for extracted sample of Tribhuvankeerti Ras after Tulsi & Ardrak bhavana (TAB). The fingerprint profile showed 11 bands with Rf Values 0.18 to 0.87. At Rf Value 0.60, TAB plate shows Fluorescent Blue bands which is in consonance of Ardrak ras band at 0.60 Rf value. This confirms the presence of Ardrak ras bhavana ingredients in the sample TAB (Fig.II).



3.1.3 Tribhuvankeerti Ras with Dhatura ras bhavana (TADB):

3.0 μ l of Dhatura ras Extracted sample was applied in the form of band on silica gel 60 F254 TLC plate of thickness 0.2 mm and developed in chamber with Chloroform : Hexane : Methanol : ethyl acetate (5:4.8:0.2:0.2) as solvent system. The HPTLC fingerprint profile of Dhatura Ras showed Eight Major Bands of Red colour under 366 nm at Rf value of 0.13,

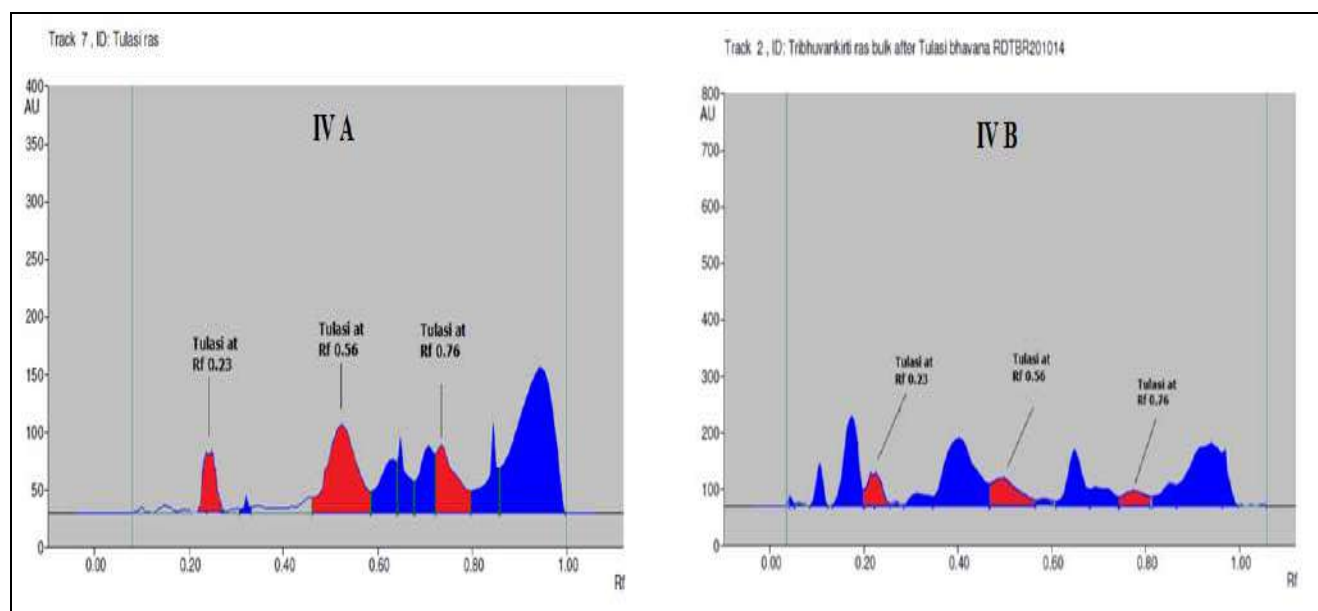
0.16, 0.28, 0.33, 0.44, 0.56, 0.66 & 0.76. Simultaneously, HPTLC developed for extracted sample of Tribhuvankeerti Ras after Dhatura ras bhavana (TADB). The fingerprint profile showed 15 bands with Rf Values 0.16 to 0.87. TADB plate when visualized under 366nm shows 2 Red bands at Rf value of 0.66 & 0.76 which indicates the presence of Dhatura ras ingredients in the sample (Fig.III).



3.1.4 Final Tribhuvankeerti Ras Bulk:

3.0 μ l of Final Tribhuvankeerti ras Extracted sample was applied in the form of band on silica gel 60 F254 TLC plate of thickness 0.2 mm and developed in chamber with Chloroform: Hexane: Methanol: ethyl acetate (5:4.8:0.2:0.2)

as solvent system. The HPTLC fingerprint profile depicts the presence of all the three bhavana dravyas viz Tulsi ras, Ardrak ras and Dhatura ras in the tested sample. The chromatogram developed also showed peaks of these bhavana dravyas at respective Rf Values. (Fig. IV)



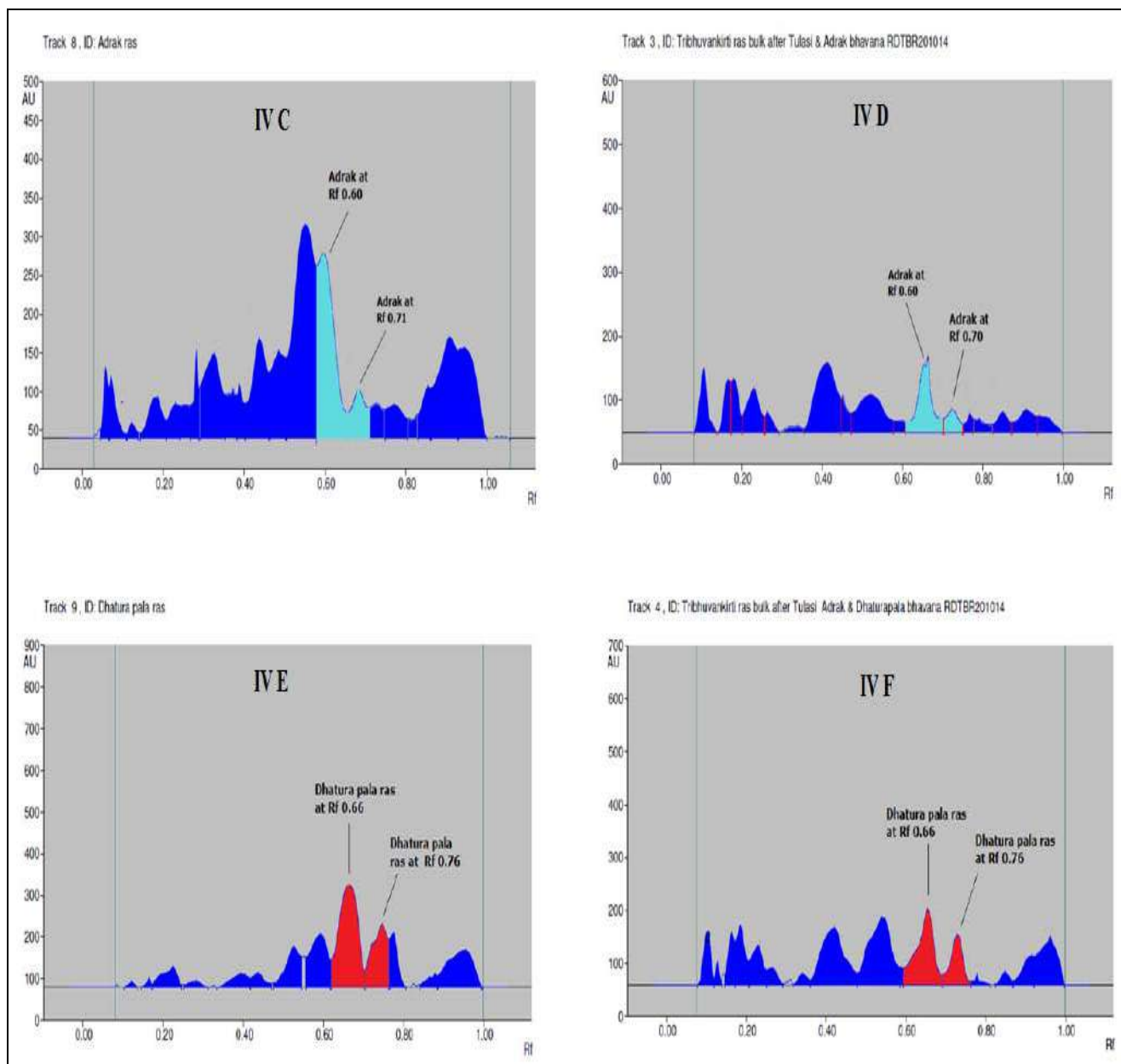


Fig IV: Peak display of Tribhuvankeerti Ras after sequential bhavana vidhi

IV A – Peak display of Tulasi ras

IV B – Peak display of TKR after Tulasi ras bhavana

IV C – Peak display of Adrak ras alone

IV D – Peak display of TKR after Tulasi and Adrak bhavana,

IV E – Peak display of Dhatura Pala ras alone

IV F – Peak display of TKR after Tulasi, Adrak and Dhatura Pala ras bhavana.

3.2 FT-IR Analysis

The samples of Tribhuvankeerti Ras without bhavana (WB), Tribhuvankeerti Ras after Tulsi ras bhavana (TB), Tribhuvankeerti Ras after Tulsi & Adrak bhavana (TAB), Tribhuvankeerti Ras after Tulsi, Adrak & Dhatura pala ras bhavana (TADB), Tribhuvankeerti Ras dry Final sample – RDTBR201014 (Final), Tulsi ras, Adrak ras and Dhatura pala ras were analysed in range of 4000 cm^{-1} to 600 cm^{-1} using FT-IR, Bruker.

3.2.1 Tribhuvankeerti Ras without bhavana (WB): The sample of Tribhuvankeerti Ras without bhavana (WB) was analysed in range of 4000 cm^{-1} to 600 cm^{-1} . The FT-IR

spectra showed unique transmittance peaks at wave number 988.04 cm^{-1} , 1130 cm^{-1} , 1342 cm^{-1} . (Fig. V – V-A)

3.2.2 Tribhuvankeerti Ras with Tulsi bhavana (TB): The sample of Tribhuvankeerti Ras with Tulsi bhavana (TB) was analysed in range of 4000 cm^{-1} to 600 cm^{-1} . The FT-IR spectra showed unique transmittance peaks at wavenumbers 1149 , 1076 & 1015 cm^{-1} were found common in Tulsi Ras & Tribhuvankeerti Ras after Tulsi bhavana. It confirms bhavana vidhi of Tulsi in Tribhuvankeerti Ras (Fig. V – V-B). A shift in peak 988.04 cm^{-1} (before bhavana) to 1015 cm^{-1} (after bhavana) has been observed which can be understood on account of bhavana vidhi process by trituration.

3.2.3 Tribhuvankeerti Ras with Ardrak ras bhavana (TAB): The sample of Tribhuvankeerti Ras with Ardrak ras bhavana (TAB) was analysed in range of 4000 cm^{-1} to 600 cm^{-1} . The FT-IR spectra showed unique transmittance peaks at wavenumbers 2929 & 1359 cm^{-1} were found common in Ardrak Ras & Tribhuvankeerti Ras after Ardrak ras bhavana (Fig. V – V-C). It confirms bhavana vidhi of Ardrak in Tribhuvankeerti Ras.

3.2.4 Tribhuvankeerti Ras with Dhatura ras bhavana (TADB): The sample of Tribhuvankeerti Ras with Dhatura ras bhavana (TAB) was analysed in range of 4000 cm^{-1} to 600 cm^{-1} . The FT-IR spectra showed unique transmittance peaks at wavenumbers 2192, 1636, 1405 & 1024 cm^{-1} were found common in Dhatura Ras & Tribhuvankeerti Ras after Dhatura ras bhavana (Fig. V – V-D). It confirms bhavana vidhi of Dhatura in Tribhuvankeerti Ras.

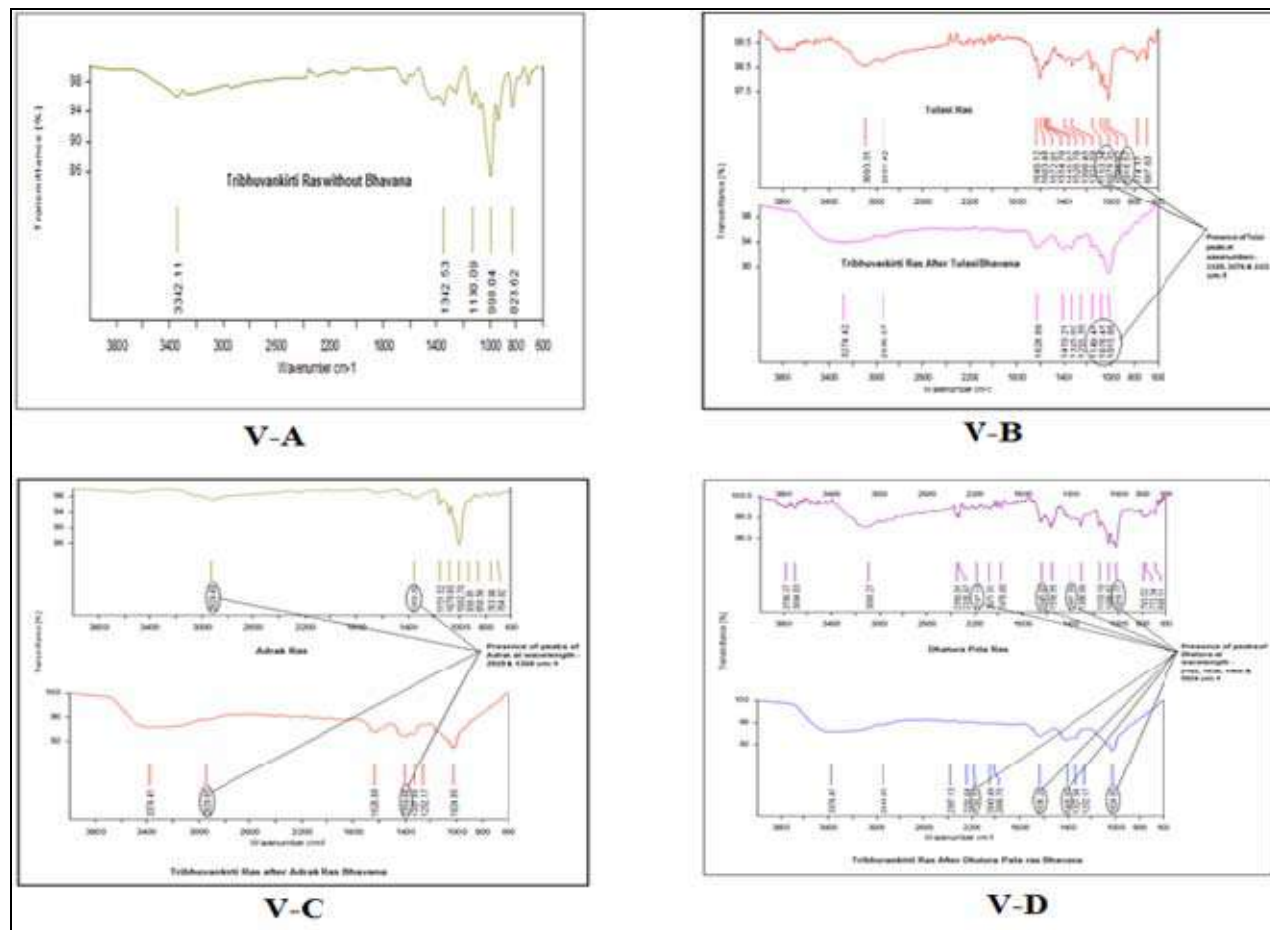


Fig V: FT-IR Spectra of Tribhuvankeerti Ras after Sequential bhavana

3.3 Antipyretic evaluation of Tribhuvankeerti Ras *in vivo*

In present study, pyrexia was induced by a single dose subcutaneous injection of Brewer's yeast. Pyrexia was induced 16 hrs after the administration of yeast. Treatment with the Tribhuvankeerti ras Tablet at the doses of 26 mg/kg significantly ($P < 0.01$) decreased the rectal temperature of the rats in a dose-dependent manner. The antipyretic effect

started as from the first hour and the effect was maintained upto 3 h, after administration of the TKR Tablet. The result obtained from both the standard i.e. Paracetamol and TKR Tablet treated rats were compared with that of control and a significant reduction in the yeast-induced elevated rectal temperature was observed. (Table II)

Table II : Anti-pyretic activity of Tribhuvankeerti ras in mice

Groups	Initial	After administration of Brewer yeast	After administration Treatment doses					
			Half hour	I hour	1:30 hour	2 hour	2:30hour	3 hour
Normal Control	37.75 ± 0.22	38.45 ± 0.06	38.38 ± 0.09	38.28 ± 0.11	38.12 ± 0.08	38.08 ± 0.06	37.97 ± 0.06	37.90 ± 0.04
TKR	37.35 ± 0.18	38.70 ± 0.31	38.58 ± 0.31	38.27 ± 0.40	37.60 ± 0.20*a	37.31 ± 0.20*a	37.49 ± 0.16*a	37.32 ± 0.12**a
Paracetamol Treated	37.42 ± 0.19	38.03 ± 0.1	38.20 ± 0.16	38.00 ± 0.14	37.44 ± 0.11**a	37.40 ± 0.12**a	37.41 ± 0.14*a	37.25 ± 0.14**a

All Data were expressed in Mean \pm SEM, where, $^*p < 0.05$, $^{**}p < 0.01$ with comparison to normal control animals.

4. Discussion

Bhavana plays a significant role in effective drug delivery and thus contributing in efficacy of drug. Various factors need to be monitored while process of bhavana vidhi takes place. Grinding should be done continuously for specific period like in case of Nimba bhavana in arogyavardhini for 2 days [17]. Wherever repetition of bhavana is mentioned, proceeding bhavana is given after complete drying of the previous one [18]. The Mortar and Pestle should be inert, must not react with the material. The bhavana given with specific liquid helps in impregnating bhavana dravya in the formulation. Liquid media facilitates in easy and smooth grinding [19]. In Tribhuvankeerti ras, bhavana vidhi of Tulsi Ras, Adrak Ras and Dhatura pala Ras is given in sequential manner. These bhavana dravyas impregnates their active ingredients in the formulation- Tribhuvankeerti Ras. Tulsi is useful in cough and cold, Adrak is helpful in throat pain and Dhatura pala is effective against fever.

It is also found interestingly in practice that the finer particle size can be achieved by wet grinding than by dry grinding. In case of *Kharaliya Rasayana* liquid media helps in preparation of pills. It is observed that the liquid media is selected according to the therapeutic application of the drug, particularly its application in different system. The liquid bhavana dravyas also serves as source of trace elements which are mixed with drug and is useful for our body [20].

A validated HPTLC and FT-IR method has been developed to determine the quantity of Bhavana's in alone and after it incorporates in to TKR test formulation. As we mentioned above each bhavana has its own mechanism in prevention of disease. The HPTLC fingerprint profile depicts the presence of all the three bhavana dravyas viz Tulsi ras, Ardrak ras and Dhatura ras in the tested sample at Rf values. The extracted TKR after Tulsi bhavana showed band at Rf value of 0.23, 0.56 & 0.77 which is exactly as shown in Tulsi ras alone. The extracted TKR after Adrak ras showed band at Rf value of 0.60 & 0.77 which is exactly as shown in Adrak ras alone. Likewise, the extracted TKR after Dhatura ras showed band at Rf value of 0.60 & 0.77 which is exactly as shown in Dhatura ras alone. Where, FT-IR analysis showed, unique transmittance peaks of the presence of all the three bhavana dravyas viz Tulsi ras, Ardrak ras and Dhatura ras in the tested sample in range of 4000 cm^{-1} to 600 cm^{-1} using FT-IR, Bruker. It is important to mention here that the presence or the absence of these bhavana in test formulation affect the quality and efficacy of a particular formulation. The proposed HPTLC and FT-IR method can be used for quantitative monitoring of Bhavanas in test formulation without interference. Its use for standardization and quality control of present TKR of traditional medicine containing sequential Bhavanas as an ingredient can be explored. In meantime, *in vivo* study also showed that TKR significantly reduced the elevated body temperature as compare to control animals.

5. Conclusion

Bhavana plays a vital role in enhancing the efficacy of drug by making desirable changes while processing through various types of bhavana dravyas which act as catalyst. The HPTLC and FT-IR methods developed for confirmation of sequential bhavana Dravyas in Tribhuvankeerti Ras will help in establishing the specifications and need for the presence of

bhavana Dravyas. These methods were found to be accurate and useful for routine Quality control analysis.

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