Characterization and antimicrobial study of 

Trinakantamani (Amber) Pishti

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Abstract

Background: Trinakantamani Pishti (TMP) is a cardio-tonic (Hridya), styptic (Rakta Stambhaka), astringent (Kashaya) formulation frequently used in varieties of bleeding disorders such as bloody diarrhea (Raktatisara), Rakta rasha (bleeding piles), and disorders of excessive menstruation (Atyartava). Still, no published data is available regarding its characterization. Aim: To generate a fingerprint for raw and processed TMP using sophisticated instrumental techniques to assess antimicrobial activity of TMP. Materials and methods: Three samples of TMP were prepared using the standard reference method. Characterization of TMP was carried out by Fourier-transform infrared spectroscopy (FTIR), energy dispersive X-ray analysis (EDEX) with scanning electron microscopy, powder X-ray diffraction (XRD). Antibacterial activity was carried out by the well-diffusion method. Results: Analysis by scanning electron microscope revealed maximum particle size <5 μm and <3 μm in the raw sample and TMP, respectively. Minimum particle size in TMP ranges from 1 to 2 μm and 701 nm. EDEX analysis shows carbon and oxygen as major constituents while Na, Mg, Ca, Si, Fe, and S were present in traces. XRD pattern indicates the amorphous nature of the drug, while FTIR analysis reveals the presence of functional groups such as O–H, CO₂, C = O, C–N, N–H. Heavy metals, total microbial count, and microbial limit test were found to be under permissible limits. Anti-microbial study against tested pathogens Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa and Salmonella typhimurium did not show any effect of TMP. Conclusion: The results of EDEX study showed that Pishti samples have the small particle size i.e., 701 nm than the raw i.e., 1-2 μm, which may facilitate absorption of drug into the body. All heavy metals in the samples were within the permissible limit. Carbon, hydrogen and oxygen are the chief elements of drug which confirms similarity to the Amber. Since the present work is the first published literature on characterization and anti-microbial study on TMP, the outcome can be considered as fingerprint for the drug prepared using the mentioned reference method.

Keywords: Amber, Fourier-transform infrared spectroscopy, quality, scanning electron microscope, Trinakantamani Pishti, X-ray diffraction

Introduction

Rasaushadhies, Bhasma; Pishti or other herbo-mineral formulations, are used in clinical practice since hundreds of years. However, the regulatory agencies in most of the countries have banned the sales of these herbo-mineral products in their respective countries quoting safety issues and lack of clinical evidence for their efficacy. Hence, for consumer prospective, scientific validation of Ayurveda, Siddha and Unani systems of medicine in terms of drug standardization is the first and foremost requirement to validate these medicines by using modern tools and techniques.

In the present work, Trinakantamani Pishti (TMP), one of the commonly used formulation was screened for the purpose of standardization and quality control. Trinakanta is a fossil resin which belongs to araucariaceae (conifers/ gymnosperms) and Fabaceae/Leguminoase (flowering plants/angiosperms).[1] Amber (Latin-Succinum) is generally taken as Trinakantamani, that is used since prehistoric period in the manufacture of jewelry and ornaments, and

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also in folk medicine and for amulets. In Ayurveda it is mentioned under various groups like Ratna of herbal origin & Prarthiva Aushadha Varga. Trinakantamani is a powerful cardio-tonic (Hridya), soothing for senses (Indriya-Prasadana), anti-diarrheal (Grahi), styptic (Rakta-Stambhaka) that pacifies Pitta (Pitta-Shamaka). Trinakanta is good for strengthening the heart. It is useful in bloody diarrhea (Raktaaatisara), bleeding piles (Pittarsh), and disorders of excessive menstrual flow (Asrigdara).

In Unani medicine, it is known as Kaharuba – “the straw magnetizer” and comes under group of Mukabbedima and Habishdum drugs, used in the treatment of bleeding disorders and also in certain infectious conditions such as bacillary dysentery, ulcer, and wounds. Since no research work has been done on characteristic study Trinakantamani, the present research work was conducted with the following objectives- (1) To set a standard for preparing good quality of TMP. (2) To know the chemical composition and the microbial contamination (if any) in the finished product (Trinakantamani Pishti). (3) To determine the antimicrobial property of the finished product (Trinakantamani Pishti).

Materials and methods

Three samples of TMP were prepared using the standard operating procedure mentioned in Rasamritam.

Pharmaceutical processing of Trinakantamani Pishti

Trinakantamani was purchased from the local market of Delhi. The raw sample was identified under UV light (366 nm) at Shriram institute of industrial research, Delhi (JO no307-132-3575, dated 13/08/2013) which was found to be identical with the reference standard of Kaharuba (Trinakantamani) Mani. Analysis was done at Shriram institute of industrial research, Delhi.

Shodhana of Trinakantamani

One thousand milliliters normal-saline solution was taken in stainless steel vessel and heated with the help of LPG gas stove. The vessel was removed from the fire and Trinakantamani stones were put in warm saline water and rubbed with hand till its external impurity was removed. The material was then washed with plain water and then dried under sunlight. The same process was adopted for Shodhana of all three samples named as TM-1, TM-2 and TM-3.

Powdering of Shodhita Trinakantamani

This process is an intermediate process between Shodhana and Pishhirakarana. Although there is no direct reference for this process it is applied in almost all the Pishthi formation processes to bring the material in a powder state to make the processes easier [Table 1].

Preparation of Gulab Arka

Roses were collected from the herbal garden of the college premises and cleaned properly. 7500 ml of water was added to the 750 g rose and was kept overnight for soaking. Next day, it was crushed gently with hand and it was kept in traditional distillation apparatus with mild heating. In the beginning, the vapors consist of only steam and therefore were discarded. The process was completed in approximately 6 h and finally 2500 ml Gulab Arka was obtained.

Impregnation (Bhavana) in Gulab Arka

Powder of processed Trinakantamani obtained after Shodhana was taken in a mortar and subjected to impregnation (Bhavana) in Gulab Arka. The material was subjected to continuous trituration for 8 h a day. In between the process, required quantity of Gulab Arka was added to make the material wet for proper trituration. The material was subjected to continuous trituration for 8 h a day. The same process was repeated for seven times. By the same method, three samples of TMP were prepared named as TMP-1, TMP-2 and TMP-3. Table 2 shows batch-wise observation in the processing of three samples of TMP. Pharmaceutical processing in preparation of TMP is shown in Figure 1.

Organoleptic and analytical study

All samples were subjected to organoleptic tests, Bhasma Pariksha like Rekkapuranta, Varitaratva, as well as Physico-chemical tests, i.e., loss on drying, total ash value, acid insoluble ash, specific gravity, pH value according to “Protocol of testing of Ayurvedic, Siddha and Unani Medicines” Sophisticated techniques such as elemental analysis with energy dispersive X-ray analysis (EDAX), structural study with powder X-ray diffraction (XRD), particle size with the scanning electron microscope (SEM) were also conducted. Elemental analysis with EDAX, was carried out in Carl Zeiss AG-Supra 40 WDS, manufactured by Zeiss Gemini, Carl Zeiss SMT, Oberkochen (Germany). For XRD, Model-D8 advance, manufactured by Bruker Corporation Pvt. Ltd.(Germany) was used. Surface analysis of particles was done using SEM. All tests were conducted at State Ayurvedic Drug Testing Laboratory, Haridwar, Devansh Testing and Research Laboratory Pvt. Ltd., Bhagwanpur, Roorkee and in Institute Instrumental Centre, Indian Institute of Technology (IIT), Roorkee. Fourier-transform infrared spectroscopy (FTIR), analysis was done in Department of Material Science, Indian Institute of Technology (IIT), Banaras Hindu University.

Microbial enumeration test

Total aerobic microbial count

Enrichment technique

Dissolved 10 g or dilute 10 ml which of the preparation was examined in fluid lactose medium showed to have no antimicrobial activity under the conditions of test and adjusted the volume to 100 ml with the same medium.

Examination of the sample by plate count for bacteria by pour-plate technique

Using Petri dishes 90-100 mm in diameter, and about 15 ml of liquefied soyabean casein digest agar (SCDA) at not more than 45°C was added to each dish. If necessary, pre-treated preparation was diluted as described above so that a colony
count of not more than 300 may be expected. At least two such Petri dishes were prepared using the same dilution and incubated at 30°C to 35°C for 5 days, unless a more reliable count was obtained in a shorter time. The number of colonies that are formed were counted. The results was based on using of colony counter considering the greatest number of colonies but taking 300 colonies per plate for the maximum consistent with good evaluation.

Plate count for fungi by pour-plate technique
As described in the test for bacteria using SCDA and the plates were incubated at 25°C for 5 days, unless a more reliable count was obtained in a shorter time. The result calculated using Colony Counter with not more than 100 colonies.

Validity of the tests for total aerobic microbial count
The following test strains were grown separately in tubes containing soyabean-casein digest medium at 30°C to 35°C for 18–24 h or, for Candida albicans, at 20°C for 48 h. Staphylococcus aureus (ATCC 6538), Salmonella typhimurium (ATCC 14028), E. coli (ATCC 8739), Pseudomonas aeruginosa (ATCC 27853) and Candida albicans (ATCC 2091).

Anti-microbial study
The in vitro antibacterial activities of test samples were determined by the agar-well plate diffusion method. Stock solutions of samples were prepared in ethanol and were filtered using 0.45 um sterile filters. A well was made in the plates amended with test pathogens namely, S. aureus, E. coli, P. aeruginosa and S. typhimurium, with sterile borer (5 mm). The test sample (50 μl) was introduced into the well and plates were incubated at 37°C for 72 h. Ampicillin was used as a standard drug for comparison as a positive control. Mueller Hinton agar plate without amended bacteria was taken as a negative control. After incubation plates were observed for the carried out at zone of inhibition around the wells. The study has been Gurukul Kangri University, Haridwar and Himalayan Institute of Medical Sciences (HIMS), Jollygrant.

Results
Raw Trinakantamani was tasteless, yellowish-brown color fossil resin which produces lemon odor on rubbing with silk or woolen cloth. Processed Trinakantamani had similar organoleptic characteristics as that of unprocessed Trinakantamani. The Shodhana process marginally increased its luster only because of the removal of dust or foreign particles. TMP was tasteless, odorless, soft to touch and solemn yellow [Table 3].
Table 3: Organoleptic characters of raw sample and Trinakantamani Pishti

| Character          | TM     | TMP-1         | TMP-2         | TMP-3         |
|--------------------|--------|---------------|---------------|---------------|
| Colour             | Yellowish Brown | Solemn yellow | Solemn yellow | Solemn yellow |
| Taste              | Lemon odour on rubbing | Tasteless | Tasteless | Tasteless |
| Odour              | Not specific | Odourless | Odourless | Odourless |
| Touch              | Hard | Soft | Soft | Soft |
| Rekhapurnatva      | Negative | Positive | Positive | Positive |
| Varitaratva        | Negative | Positive | Positive | Positive |
| Shlakshanatva      | Negative | Positive | Positive | Positive |

TM: Trinakantamani, TMP: Trinakantamani Pishti

Evaluation of classical parameters

All samples of TMP passed through classical parameters as namely, Rekhapurnatva, Mriudutva and Varitaratva. The floating of Pishti on stagnant water surface justifies the Varitaratva as well as its light weight, whereas smoothness on touch reveals the Mriudutva nature of Pishti. Micro fineness of the Pishti is depicted by its Rekhapurnatva [Table 3].

Physico-chemical parameters

The pH of raw Trinakanta (RT) and Trinakantamani Pishti (TMP) was 6.08 and 6.65 respectively, predicting its weak acidic nature. Gulab Arka, the levigating Dravya for TMP with pH 4.5 (moderate acidic) did not show any significant effect on the pH of the formulation. Loss of drying of both the raw sample (1.38) as well as prepared TMP (1.19) was found to be low. Lower the moisture content better will be the shelf life of the substance. The total ash value for RT and TMP was 0.54 and 0.663 respectively indicative of the presence of less inorganic contents in the drug.

Low value of acid-insoluble ash i.e., 0.035 and 0.037 for raw and prepared sample, may be postulated as probably good dissolution in gastric secretions, leading to better bioavailability. Water soluble extract indicates that the bioavailability of the drug would be more in a media other than water. The value of water-soluble extractives for RT and TMP sample was 0.83 and 2.90 respectively. It shows that the raw sample as well Pishti, both are less soluble in water which proves the resinous nature of the sample.

Alcohol soluble extractives

Alcohol soluble extract indicates that the bioavailability of the drug would be more in a media other than alcohol. The value of alcohol-soluble extractives for RT, TMP were 10.47 and 14.79, respectively [Table 4].

Phase identification of Trinakantamani Pishti by X-ray powder diffraction

XRD is an essential tool for the rapid identification and quantification of minerals, compounds and other crystalline phases. As Trinakantamani comes under group of either precious or semiprecious gems (Ratna or Upratna Varga) XRD study was carried out. XRD of raw Trinakantamani and TMP, is shown in Figures 2 and 3. The XRD patterns exhibited definite amorphous character. II XRD patterns indicates amorphous humps between 2 θ =10 and 70°, reaching maximum height around 15° with RT showing peaks at d = 5.82829, θ = 15.190° d = 6.21016, 2 θ = 14.250° d = 3.39351, 2 θ = 26.240° and TMP at d = 5.85957, 2 θ = 15.108° d = 2.19605 2 θ = 41.068° d = 2.41803 2 θ = 37.152°. This pattern indicates the amorphous nature of the drug which means it does not have an ordered structure. Comparison of diffraction pattern 2theta, space lattice parameters were studied by version 4.9 of High Score X'Pert software analysis. XRD pattern showed the presence of a structure containing elements silica, calcium, sodium, oxygen, aluminum of the chemical name wairakite (98-009-8200), mesolite (98-016-8088) with tetragonal, orthorhombic crystal system in both the samples. Impregnation (Bhavana) of RT in Gulab Arka 7 times titration makes additional peaks in TMP. Average crystallite size was found to be RT (90.20 nm) and TMP (79.53 nm) as calculated by Scherer’s formula. Each sample contains maximum concentration of carbon and oxygen. Amorphous nature and presence of a high concentration of carbon and oxygen strongly correlate it with Amber.

Particle size with scanning electron microscopy

Scanning electron microscopy with energy-dispersive X-ray spectroscopy (SEM/EDX) is the best known and most widely used of surface analytical techniques. SEM/EDX can be used to simultaneously determine a particle’s morphology and elemental composition. SEM photographs of raw Trinkanta and TMP at different magnifications are shown in Figures 4 and 5, respectively. SEM photographs of the raw and Pishti samples show the heterogeneous structure of the sample, i.e., all the particles are not of equal size and shape. The SEM images of RT and TMP sample reveal the maximum particle size <5 μm and <3 μm respectively whereas the minimum particle size ranges 1–2

Table 4: Value of physico-chemical parameters of samples of raw sample and Trinakantamani Pishti

| Parameter                                        | TMP1 | TMP2 | TMP3 |
|--------------------------------------------------|------|------|------|
| Loss on drying (%w/w)                            | 1.20 | 1.12 | 1.24 |
| Total ash value (%w/w)                           | 0.64 | 0.68 | 0.67 |
| Water soluble ash (%w/w)                         |      |      |      |
| Acid insoluble ash (%w/w)                        | 0.031| 0.041| 0.039|
| Water soluble extractive (%w/w)                  | 2.63 | 2.79 | 3.29 |
| Alcohol soluble extractive (%w/w)                | 15.12| 14.37| 14.89|
| Petroleum ether soluble extractive value         | 12.16| 13.56| 13.27|
| pH                                               | 6.67 | 6.60 | 6.69 |

TMP: Trinakantamani Pishti
μm and 701 nm, shown in figure 6. The particle size range is shown in Table 5. It is also true that when the particles are broken down into very smaller size, strong cohesive forces act between them. Hence due to this cohesive force and binding nature of the Bhavana Dravya, nanoparticles aggregate and give the particle size in micron range. The aggregation of particles is clear in all the SEM images. From the image, it is clear that several crystallites are agglomerated in a particle giving rise to microcrystalline structure as shown in Figures 4 and 5. It suggests that Pishti shows the presence of few nanoparticles. However, individual particle size cannot be estimated due to the aggregation of particles. Pishti containing nanoparticles in comparison to the raw are very easily absorbed through ion channels into the cells of the body making it very fast effective absorption without taking large doses. It is significant reduction of size, that allows the phenomenon of Rekhapurnatva and Varitaratva to develop in particle size that facilitates absorption and assimilation of the Bhasma in the system. Hence, it becomes clear that particles become finest by extensive grinding and pounding in the wet state with Gulab-Arka using mortar and pestle to increase the dissolution as well as the absorption of drug. This trituration process significantly reduced the particle size from 10 microns to <1 microns. Reduction in particle size influences bioavailability, being one of the key factors for the same. The rate of diffusion is proportional to the surface area and the particle size is inversely proportional to the rate of absorption.

Figure 2: X-ray diffraction pattern Trinakantamani

Figure 3: X-ray diffraction pattern of Trinakantamani Pishti

Figure 4: Stick pattern of TM and Trinakantamani Pishti
Chemical composition analysis by energy dispersive X-ray analysis
With the help EDAX, information about the distribution of different elements was carried out. On analysis, it was found that TMP is chiefly composed of carbon and oxygen while other elements such as Na, Mg, Ca, Si, Fe, S were found to be in traces as shown in Tables 6 and 7. The organic structure of the drug confirms its similarity to Amber which also have the same elemental composition in major part while trace elements are supposed to be trapped in the drug during the process of fossilization of Trinakanta. Heavy metals such as As, Hg, Cd, Pd were detected below the permissible limits\[13\] [Table 8], The Government of India, Department of Health and Family Welfare, Ministry of Ayush, has issued new safety standards for the Ayurvedic drugs. The permissible limits of the heavy metals in Ayurvedic drugs with herbal ingredients as per WHO (World Health Organization) and FDA (Federal Drug Organization) are shown in Table 9. So, Pishti prepared by the method followed in the study is not only quite safe but also a natural source that provides essential micronutrients on internal administration.

Figure 5: Scanning electron microscope photographs of Trinakantamani

Figure 6: Scanning electron microscope photographs of Trinakantamani Pishti

Figure 7: Fourier-transform infrared spectroscopy pattern
Fourier-transform infrared spectroscopy for identification of functional groups

The board peak around 3500-3000 cm⁻¹, 3300-2500 cm⁻¹, 2500-2000 cm⁻¹, 1710-1665 cm⁻¹, 1400-1000 cm⁻¹, 1250-1020 cm⁻¹, 1000-650 cm⁻¹, 830-790 cm⁻¹,730-665 cm⁻¹, 600-500 cm⁻¹ corresponds to O–H, O–H, CO₂, C = O, O–H, C–N, N–H, stretching vibrations [Figure 7]. Peak at 3443 cm⁻¹, 2931 cm⁻¹ assigned to carboxylic acids, Alkyl have role in the pathogenesis of inflammatory disorders within the CNS and possibly other organs. A sharp peak at 1649 cm⁻¹ assigned to vibrations of the Alpha, beta–unsaturated aldehydes, ketones (C = O), while another peak at 1452 cm⁻¹ is recognized as stretching vibration of carboxylic acid group (O–H). A peak around 1161 cm⁻¹ identified as aliphatic amines (C–N) stretching vibrations. Peaks lying in 879 cm⁻¹ is identified as Alkene stretching vibrations [Table 10]. Although the FTIR spectra of RT and TMP are identical, a well-defined carboxylic acids, alkyl peak around 3300-2500 cm⁻¹, 2500-2000, cm⁻¹ was not observed in TMP spectra may be due to the weak intermolecular bonding between alpha carbon and an-OH or hydroxyl group. Alpha carbon is aromatic may be due to the addition of Gulab Arka, the carboxylic acid forms an aromatic ring.

Microbial limit test

Microbial and fungal contamination not only affects the chemical composition but also decreases the therapeutic potency of herbal drugs. Microbial contamination of herbal drugs is a major impediment that prevents India from becoming an herbal giant. Therefore, fungal contamination of drugs, especially raw materials, should be prevented during the manufacture of these preparations. Under Microbial limit test, all samples of Trinakantamani processed for the quantitative determination of microbial load, in which total bacterial count and total fungal count of sample I having 28 colonies forming unit (cfu) followed by sample II and III having 27 and 26 cfu/ml/gm, respectively [Table 11 and Graph 1]. Above microbial count satisfies the microbiological quality of given samples and defined the least probability of contamination during processing shown in Figure 8. Conclusively the samples are having microbial count under the limits and samples are microbiological quality assured. The microbial limit confirmatory test for

| Sample | Particle size range |
|--------|---------------------|
| TM     | 4.592 µm-1.069 µm   |
| TMP    | 2.275 µm-701 nm     |

| Element | TMP-1 | TMP-2 | TMP-3 |
|---------|-------|-------|-------|
| C       | 78.78 | 84.65 | 78.27 |
| O       | 15.25 | 15.47 | 15.63 |

| Element | TMP-1 | TMP-2 | TMP-3 |
|---------|-------|-------|-------|
| Na      | 0.16  | 0.07  | -0.04 |
| Mg      | 0.11  | 0.04  | 0.04  |
| Ca      | 0.05  | 0.16  | 0.12  |
| Si      | 0.16  | 0.18  | 0.14  |
| Fe      | 0.18  | 0.17  | 0.20  |
| S       | 0.15  | 0.44  | 0.22  |

| Element | TMP-1 | TMP-2 | TMP-3 |
|---------|-------|-------|-------|
| Hg      | -0.14 | -0.23 | 0.01  |
| As      | -0.06 | -0.17 | 0.00  |
| Cd      | -0.19 | -0.54 | 0.10  |
| Pb      | -0.49 | 0.04  | -0.08 |

Table 5: Average particle size range in different samples

Table 6: Major elements in all the samples of Trinakantamani Pishti in energy dispersive x-ray analysis

Table 7: Minor elements in all the samples of Trinakantamani Pishti in Energy dispersive x-ray analysis

Table 8: Heavy elements in all the samples of Trinakantamani Pishti

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pathogens confirmed the absence of any of the pathogens in the test samples. None of the tested mediums showed the characteristic features for any pathogens. The result of the present study of microbial limit count test in TMP shows that microbial load of the preparation was under acceptance limit [Table 12]. E. coli, S. typhimurium, S. aureus and P. aeruginosa, all were absent. Total aerobic microbial count and total yeast and mould count of TMP was within the limit [Table 13] and indicating that TMP is safe Ayurvedic preparation for oral administration.

Antimicrobial activity of test sample

According to Ayurveda, TMP is efficacious in the treatment of bloody diarrhea (Raktatisara), diarrhea (Atisara), and infected chronic wounds (Dushtavrana).\textsuperscript{[24,25]} Unani medicine too recommends Kaharuba Pishti in the treatment of infective conditions like wound, ulcer, etc. From the modern point of view, Amber is also thought to inhibit bacterial infection.\textsuperscript{[26]} It is used as snuff to treat the flu and in the tender gums of infants. So in addition to standardization, three samples of TMP had also undergone anti-microbial study against S. aureus, Escherichia coli, P. aeruginosa and S. typhimurium. All three samples were found to be ineffective as evident by no inhibition zone around the wells as compared to that of control [Figure 7a and b]. Hence, to establish role of TMP in infective conditions more advanced studies are still required.

Table 9: Permissible limits of heavy metals in Ayurvedic drugs

| Heavy metal | Maximum permissible limit |
|-------------|---------------------------|
| As          | $10^3$ ng/g               |
| Cd          | 0.3 µg/g                  |
| Pb          | 10 µg/g                   |
| Hg          | 1 µg/g                    |

Hg: Mercury, As: Arsenic, Cd: Cadmium, Pb: Lead

Graph 1: Showing result after microbial study

Table 10: Functional group

| Peak   | Actual peak in RT | Actual peak in TMP | Bond | Functional group | Appearance             |
|--------|-------------------|--------------------|------|------------------|------------------------|
| 3500-3000 | 3443.51           | 3443.51            | O-H  | Carboxylic acids, alkyl | Medium to strong       |
| 3300-2500 | 2931.24           | 2931.24            | O-H  | Carboxylic acids, alkyl | Medium to strong       |
| 3300-2500 | 2853.77           | -                  | O-H  | Carboxylic acids, alkyl | Short and broad        |
| 2500-2000 | 2357.97           | -                  | O-H  | Carboxylic acids, alkyl | Short and broad        |
| 1710-1665 | 1743.06           | 1734.34            | C=O  | Alpha, beta-unsaturated Aldehydes, ketones | Strong |
| 1710-1665 | 1649.12           | Peak not observed  | C=O  | Alpha, beta-unsaturated Aldehydes, ketones | Short and broad        |
| 1400-1000 | 1452.55           | 1469.01            | O-H  | Carboxylic acid     | Short and broad        |
| 1400-1000 | 1375.08           | 1383.79            | O-H  | Carboxylic acid     | Short and broad        |
| 1250-1020 | 1161.07           | 1161.07            | C- N | Aliphatic amines    | Short and broad        |
| 1250-1020 | 1161.07           | 1161.07            | C- N | Aliphatic amines    | Short and broad        |
| 1250-1020 | 1024.53           | 1015.81            | C- N | Aliphatic amines    | Short and broad        |
| 1000-650  | 879.27            | 887.99             | N-H  | Primary Amines, secondary amines | Short and broad        |

Graph 1: Showing result after microbial study

Table 11: Drug samples give the following results after microbial study

| Days | TFC (cfu/ml) | TBC (cfu/ml) | TFC (cfu/ml) | TBC (cfu/ml) | TFC (cfu/ml) | TBC (cfu/ml) |
|------|--------------|--------------|--------------|--------------|--------------|--------------|
| 1    | Nil          | 20           | Nil          | 24           | Nil          | 25           |
| 2    | Nil          | 20           | Nil          | 24           | Nil          | 25           |
| 3    | Nil          | 22           | Nil          | 25           | Nil          | 25           |
| 4    | Nil          | 28           | Nil          | 27           | Nil          | 26           |
| 5    | Nil          | 28           | Nil          | 27           | Nil          | 26           |

TMP: Trinakantamani Pishti, TBC: Total bacterial count, TFC: Total fungal count

Graph 1: Showing result after microbial study
Conclusion

The adopted method for preparation of Trinakantamani Pishti (TMP) can be considered standardized procedure for preparation of Pishti. It may be considered as bio-medicine that contains carbon and oxygen as major constituents in similarity with Amber. It also contains certain trace elements such as, sodium, magnesium, calcium, silica, iron, sulphur which act as micronutrients and thereby help in the therapeutic applicability of Trinakantamani Pishti (TMP) in variety of disorders.

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Conflicts of interest
There are no conflicts of interest.

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Table 12: Microbial contamination limits according to World Health Organization

| Parameters                  | Permissible limits |
|-----------------------------|--------------------|
| *Staphylococcus aureus/g.   | Absent             |
| *Salmonella typhimurium/g.  | Absent             |
| *Pseudomonas aeruginosa/g.  | Absent             |
| *Escherichia coli           | Absent             |
| TPC                         | 105/g*             |
| Total yeast and mould       | 103/g              |

Table 13: Microbial contamination limits in samples of Trinakantamani Pishti

| Product name | *Escherichia coli | *Pseudomonas aeruginosa | *Staphylococcus aureus | *Salmonella typhimurium | *Aspergillus brasiliensis |
|--------------|-------------------|------------------------|-----------------------|-------------------------|--------------------------|
| TMP-1        | Absent            | Absent                 | Absent                | Absent                  | Absent                   |
| TMP-2        | Absent            | Absent                 | Absent                | Absent                  | Absent                   |
| TMP-3        | Absent            | Absent                 | Absent                | Absent                  | Absent                   |

Figure 8: (a) Bacterial colony forming units in microbial limit test. (b) No inhibition zone against test pathogens in antimicrobial activity test.
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