Toxicology International, *29(3) : 417-428; 2022. DOI: 10.18311/ti/2022/v29i3/29790*

Online ISSN : 0976-5131; Print ISSN : 0971-6580

Toxicology International

Contents available at: www.informaticsjournals.com/index.php/toxi



Comparison of Chemistry and Zebrafish Toxicological Profile of Extract Processed as Per Traditional Method and of its Fraction for Similarity/Dissimilarity – A Case Study of *Terminalia chebula* Fruit

Rojison Koshy[1], D. B. Anantha Narayana[2*], Smriti Yadav[1], Vineet Kumar Singh[1] and Deepak Mundkinajeddu[1]

[1]Research and Development Centre, Natural Remedies Pvt Ltd., Plot 5B, Veerasandra Indl. Area, Electronic City Post, Bangalore – 560100, Karnataka, India

[2]Chief Scientific Officer, Ayurvidye Trust, 15 (Old No. 1101/927), 1F Main Road, 2nd Stage Giri Nagar, Bangalore – 560085, India; dba.narayana@gmail.com

ARTICLE INFO

KEYWORDS: Similarity, *Terminalia chebula*, FET, HoSU, PCA

ARTICLE HISTORY

Received: 12-03-2022

Revised: 30-05-2022

Accepted: 01-06-2022

Abstract

History of safe use, HoSU preparations are formulations mentioned in ancient literature and are according to the Ayurvedic system of medicine. Due to the advent of new technologies, there is a shift in the manufacturing methodologies of Ayurvedic medicines involving extractions and fractionations. This shift, while has helped manufacturers improve their productivity, raises a question on the equivalence to chemistry, and toxicity when compared to traditional products. As a case study, an attempt is made to establish equivalence between HoSU preparation of *Terminalia chebula* with commercially available aqueous and alcoholic extracts and one of its fractions. Chemical analysis of the products using principal component analysis, revealed similar chemistry whereas the LC50 values of HoSU preparation were safest followed by commercial preparations and the reference marker compound when tested on zebrafish. The generated data indicated that chemical composition of all 4 samples tested were similar qualitatively, though variations were observed quantitatively. PCA plot indicated that the HoSU extract, commercial alcoholic extract, and commercial water extract were similar whereas water acetone extract was dissimilar. This emphasizes the need for conducting an equivalence study in terms of chemistry and toxicity between HoSU products and commercially available products.

**Author for correspondence*

1. Introduction

Traditional medicines are gaining traction all over the world due to the long HoSU associated with them. The majority of the population from Asia and Africa rely on traditional medicines when compared with modern medicine[1]. China and India are the largest producers of medicinal plants contributing totraditional Chinese medicine and Ayurvedic, Siddha, and Unani systems of medicine respectively. The Ayurvedic system of medicine in India has a very rich legacy and has provided treatment methods for a wide range of disease conditions. Fifty-fourauthoritative compilations on the Ayurvedic system of medicine are mentioned in the Drugs and Cosmetics Act 1940 and Rules 1945 published by the Ministry of Health and Family Welfare, Government of India[2]. These authoritative compilations include *Ashtanga Hridaya, BhaishajyaRatnavali, Bhava Prakasha, Charaka Samhita, Rasa Tarangini, Sahasrayoga, Sushruta Samhita*, etc. These books provide details of various traditional medicines, the indications for which they may be used, and their method of preparations. A total of 9,038 manufacturers are approved in India by the Department of Ayush of which 7,698 manufacturers are from the Ayurvedic system of medicine[3]. It is estimated that more than 5,12,000 metric tons of herbal raw drugswere consumed annuallyin 2014–15 in India out of which 1,95,000 metric tons were used by the domestic herbal industry and 1,34,500 metric tons were used for exports. The estimated trade value of botanical raw drugs consumed in India in 2014–15 was estimated to exceed one billion USD[4].

HoSU preparations are those formulations that are mentioned in ancient textbooks. The method of preparation, dosage, traditional use, etc. for all HoSU extract products should be as per the ayurvedic literature. Typical HoSU extract preparations may be prepared from fresh herbs (*swaras* (juice) or *lepa* (paste)) or from dried powders (water extract in form of infusions or decoctions), which can be further dried and powdered or the oil extracts in the form of *taila* (extraction using oil) or *ghrita* (extraction using clarified butter). As science and technology have evolved, new methods of preparing ayurvedic preparations have been developed. These modern methods of manufacturing ayurvedic medicines involve the usage of different solvents targeting enrichment of biologically active compounds as well as usage of different extraction methodologies such as rotary solvent extraction, supercritical fluid extraction, ultrasound-assisted extraction, etc. Establishing equivalence between HoSU extract preparations and modern preparations is required to establish similarity in safety. Chemical similarity may be established with the help of chromatographic and spectroscopic techniques, biological similarity and toxicity assessment involve usage of in-vitro or in-vivo methods.

Terminalia chebula Retz. or Myrobalan is a tree found throughout India mainly in deciduous forests. The pericarp of mature fruits of this plant is considered medicinally important. The major compounds present in *Terminalia chebula* include tannins such as gallic acid, chebulic acid, chebulagic acid, chebulinic acid, ellagic acid, etc. It is an important ingredient in the formulation of *Triphala* which is widely known in the world and is indicated for symptoms ranging from stomach ailments to dental cavities and is also believed to promote longevity and overall health. The important formulations incorporating *Terminalia chebula* include *Triphaladi Taila, Abhayarisha, Agastya Haritaki Rasayana, Citraka Haritaki, Danti Haritaki, Dasamula Haritaki, Brahma Rasayana, Abhaya Lavana* and *Pathyadi Lepa*[5].

Most of the monographs published for herbal products typically focus on one or more chemical markers for standardization purposes. Monographs published in various pharmacopoeia are focussed on marker based standardization. For example, United StatesPharmacopeia monograph on Terminalia chebula fruit extract mentions standardization using chebulagic acid and chebulinic acid standard markers[6]. Narayana *et al.*[7] proposed an evaluation of a holistic approach beyond the usage of chemical markers in the standardization of botanicals. Zebrafish embryo acute toxicity (FET) test is one of methods reported to assess acute toxicity using embryonic and larval model. This is a suitable model based on the similarities of human and zebra fish genomes and physiology of digestive, nervous and vascular systems[8] and was employed to assess the safety of the test materials in this study. Determining similarities and dissimilarities of processed botanicals based on various chemical constituents in a botanical provides a better alternative to evaluate a botanical processed by different manufacturing methods[9]. Principal Component Analysis (PCA) helps in establishing such chemistry based similarities. A PCA output for a sample, when compared with HoSU preparation, provide data that the sample is similar or not. This approach involves analyzing the botanical by multiple analytical tools/equipment. Similarity to HoSU

would mean extrapolation of the safety assessement to the manufactured/processed botanicals. Such an study can potentially avoid the need to generate exhaustive conventional safety data of the botanical and may lead to generation of only safety data for "concern areas of that botanicals".

In the current study, an attempt is made to establish equivalence by chemical methods and toxicity assessment by zebrafish embryo acute toxicity (FET) test between *Terminalia chebula* water extract prepared as per HoSU, commercially prepared water extract, alcoholic extract, and acetone fraction derived from the water extract and purified chebulinic acid reference material which is a single chemical molecule.

2. Materials and Methods

2.1 Chemicals

Gallic acid, TGG, corilagin, chebulagic acid, ellagic acid, and chebulinic acid standards of purity >95% were provided by M/s. Natural Remedies Pvt. Ltd., Bangalore. Tannic acid was procured from Sigma Aldrich, Germany. Potassium dihydrogen orthophosphate, acetonitrile, and methanol of chromatographic grade were purchased from Rankem (India). HPLC grade water was collected from the Sartorius Arium Pro HPLC water purification system.

2.2 Samples for Testing

Traditionally prepared *Terminalia chebula* extract (HoSU extract) was prepared by extracting 500 g of *Terminalia chebula* dry fruits and boiling with 2.0 L of water till the extract was concentrated to 0.5 L. Filtered the extract and further completely dried under vacuum at 80°C. Chebulinic acid reference standard, commercial aqueous and alcoholic extracts of *Terminalia chebula* and acetone fraction of water extract were provided by M/s. Natural Remedies Pvt. Ltd.

2.3 Chemical Analysis

Chemical analysis was carried out by chromatographic and spectroscopic methods for comparing the constituent levels as well as chemical profile in HoSU extract, commercial aqueous and alcoholic extracts of *Terminalia chebula*.

HPLC analysis was carried out using a validated method for gallic acid, TGG, corilagin, chebulagic acid, ellagic acid, and chebulinic acid. About 100 mg of sample was separately weighed accurately to 100 mL volumetric flask. Added 50 mL of boiling water and dissolved the sample with the aid of an ultrasound sonicator. Cooled the solution to room temperature and made up the volume to 100 mL with water. The solution was filtered with 0.45u PES membrane filtration beforeit was transferred to a 1.5 mL vial for HPLC analysis. Ten mg each of all reference standards wereweighed to 100 mL volumetric flask, added 50 mL of boiling water, and dissolved the sample with the aid of an ultrasound sonicator. Cooled the solution to room temperature and made up the volume to 100 mL with water. Shimadzu Prominence-i quaternary gradient HPLC system equipped with dual-channel UV-visible detector was used for testing. The HPLC column used was a Luna C18 (2), 250 x 4.6 mm, 5 µm (Phenomenex, USA). The UV-visible detector was set at 270 nm and the flow rate was set at 1.5 mL/min. 20 µL sample was injected for testing. Mobile phase gradient from 95 % solvent A (10 mM potassium dihydrogen orthophosphate in water) and 5 % solvent B (acetonitrile) at 0 min to 65 % solvent A and 35 % solvent B at 18 min; to 45 % solvent A and 55 % solvent B at 25 min; maintained 45 % solvent A and 55 % solvent B till 28 min; to 95 % solvent A and 5 % solvent B at 40 min; were maintained till 45 min for equilibration and stopped.

Total tannins content was determined as tannic acid equivalent in the test samples and were performed by the method as described in British Pharmacopoeia with modifications using Shimadzu 1700 series UV-Vis spectrophotometer from Japan[10]. About 0.100g of tannic acid reference material was accurately weighed to a 100mL volumetric flask, added 50 mL of 50 %v/v methanol: water, sonicated for 5-10 minutes, and warmed on a water bath for five minutes avoiding evaporation, cooled, and made up to 100 mL with 50 %v/v methanol. Pipetted out 5 mL of the solution to a 50 mL volumetric flask and made upto 50 mL with water. 200 mg of sample was accurately weighed to a 100 mL volumetric flask, and test solutions were prepared similar to reference standard. The color was developed by pipetting out 2, 5, 7, 10 mL of standard preparation and 5,7 mL of sample preparation to different 100 mL volumetric flasks. Added 40 mL of water, 5 mL of Folins reagent, and 10 mL saturated solution of sodium carbonate. Allowed to stand for 30 minutes. Made up the volume and again allowed to stand for 20 more minutes. The samples were analyzed using a spectrophotometer at 750 nm.

HPTLC system used was from Camag, Switzerland which comprised of Linomat IV sample applicator, TLC scanner III and TLC Visualizer. Twin trough chamber of the dimensions 10 x 10 cm was used to develop the plates. 10 uL of the samples prepared for HPLC analysis were spotted in the HPTLC plates using Linomat IV. The HPTLC plate was eluted using chloroform: acetic acid: methanol: water solvent system in the ratio 60: 32: 12: 8. The eluted plate was observed at 254 nm, 366 nm before derivatization, and 366 nm and visible light after derivatization. Anisaldehyde-sulfuric acid reagent was used for derivatizing the HPTLC plates. HPTLC testing were doen for their "profiles" only and the pure marker compounds were not applied for reference.

Spectroscopic analysis was carried out by taking readings from FT-IR (Shimadzu Prestige spectrometer, Japan) between 600 cm-1 to 4000 cm-1, FT-NIR (Bruker Tango, Germany) between 4,000 cm-1 to 12,000 cm-1, UV-Vis spectra (Shimadzu 1700 series, Japan) from 190nm to 800 nm, 1H and 13C NMR (BrukerAvance II 400, Germany) 1D spectra was generated to evaluate the spectral differences between HoSU extract, commercial aqueous and alcoholic extracts of *Terminalia chebula*.

2.4 Preparation of Test Solutions for Zebrafish Embryo Acute Toxicity Test

The test organism used for the study was the embryo/larva of wild-type shortfin zebrafish (*Danio rerio*). The study was conducted at the Council of Scientific and Industrial research's Institute of Himalayan Bioresources and technology, (CSIR-IHBT). The study was approved by IAEC on 16th December 2020 vide CPCSEA Registration Number: 1381/GO/ReBiBt/S/10/CPCSEA. The test sample was dissolved in fish system water (Zebtec, Tecniplast, Italy) to achieve different concentrations as described below. Initially, a limit test at 100 mg/L was performed according to the OECD TG 236 guidelines[11]. Briefly, newly fertilized zebrafish eggs (20/observation) were incubated with 100 mg/L of the sample (prescribed threshold concentration) for 96 h at 26 °C in a BOD incubator. The standard water conditions were maintained during incubation (OECD, 236; S. No. 12). Four apical observations were performed on each tested embryo after every 24 h intervals as an index of toxicity, (A) coagulation of the fertilized embryos; (B) absence of somite formation; (C) absence of tail-bud detachment from the yolk sac; and (D) absence of heartbeat.

Based on the observations, the test was carried out at different concentrations of 200-1000 µg/mL for HoSU extract, 200-800 µg/mL each for acetone fraction of *Terminalia chebula* water extract, commercial aqueous extract, commercial alcoholic extract, and chebulinic acid, to calculate LC50. The test chambers were conditioned for 24 h with the test solutions before the initiation of the test. At each concentration of the test compound, 20 embryos were incubated in standard inert chambers (OECD, 2013) for 96 h. During the incubation period, regular changes were made by a semi-static renewal approach. Care was taken so that the embryos remained covered, with previous test solutions to prevent drying. Further care was taken to minimize any type of stress on the embryos during handling and observations. All the embryos were observed at the end of 24 h till 96 h for any sign of toxicity. Mortality of embryos/larvae during the 96 h of incubation was recorded at all the tested concentrations. The mortality data was used to calculate LC50 using the Probit analysis method[12] with slight modifications. The mortality data at each concentration was converted to the respective probit using the probit table[13]. The percentage of dead larvae during 96 h of incubation for 0 and 100 were corrected to determine probit as, 0% dead: 100 (0.25/n) and 100 % dead: 100 (n-0.25/n)[14]. The probit values were graphically plotted on the Y-axis with log concentrations on X-axis. A concentration representing 50 % mortality on the logscale was recorded and its antilog provided LC50 value.

2.5 Principal Component Analysis (PCA)

PCA was performed using the online tool of ClustVis, a web tool for visualizing clustering of multivariate data using PCA and heatmap[15]. The data was uploaded as a delimited text file and the PCA plot was generated for interpretation ofthe similarity and dissimilarity of the samples under test. Heatmap data matrix visually values in cells by color gradient was generated showing the similarities and dissimilarities for various variables and test samples. Diverging palette fix color was used to better depict the range of data both on the positive side and negative side. The numbers were also added to the heatmap for better understanding.

3. Results and Discussion

3.1 Chemical Analysis

The content of gallic acid, Trigalloyl Glucose (TGG), corilagin, chebulagic acid, ellagic acid, and chebulinic acid in all the samples is summarized in Table 1. It is observed that all the compounds were present in the samples tested. The content of each chemical constituent varied based on the manufacturing process. Correspondingly the High-Performance Liquid Chromatography (HPLC) profile intensities varied for each sample.

Table 1. Chemical constituents present in the samples tested

Tests conducted / Sample details	HoSU extract	Commercial water extract	Commercial alcoholic extract	Acetone fraction of water extract
Total tannins content (%w/w)	36.23	50.21	59.01	63.98
Gallic acid (%w/w)	5.34	9.81	1.37	2.20
Corilagin (%w/w)	5.26	0.51	2.55	4.44
TGG (%w/w)	8.90	0.69	1.79	8.22
Chebulagic acid (%w/w)	0.78	0.56	0.99	7.96
Ellagic acid (%w/w)	1.97	1.70	2.45	0.44
Chebulinic acid (%w/w)	1.30	0.39	0.38	15.25

High-performance thin-layer chromatographic (HPTLC) analysis revealed a difference in the chromatographic profile between HoSU extract, acetone fraction of commercial aqueous extract, commercial aqueous extract, and alcoholic extract. Though all bands were present, the intensities varied indicating the there was a difference in concentration levels of individual compounds in the extract. One major difference observed was bluish bands from Rf 0.8 in the commercial aqueous and alcoholic extracts which was not present in HoSU extracts. The chromatographic profile is summarized in supplementary Figure 1.

The UV-visible, FT-IR, FT-NIR and NMR spectra for HoSU extract, commercial aqueous extract, and alcoholic extract had similarities exceeding 95 % indicating chemical equivalence. However intensities of the response varied due to difference in concentration of compounds present (Figure 2).

3.2 Risk Assessment Based on Fish Embryo Acute Toxicity (FET) Test

The test revealed no sign of toxicity in the embryos/larvae during the initial limit test for all samples tested. *Terminalia chebula* extracts acetone fraction was considered to evaluate the contribution to toxicity by relatively non-polar constituents present in the extract.

All the embryos/larvae incubated at a 100 µg/mL concentration of HoSU extract, *Terminalia chebula* water extract acetone fraction, commercial aqueous extract, alcoholic extract, and chebulinic acid showed normal growth without mortality and the developmental stages of zebrafish embryo and Probit analysis graph for the calculation of LC50 value are shown in Figure 3. The calculated LC50 value of all the samples tested is tabulated in Table 2.

Though none of the samples tested showed toxicity in the FET test, the relative toxicity of the samples was calculated using HoSU extract and chebulinic acid as base values since both samples were at extremes of the LC50 values observed. When we kept LC50 of HoSU extract as 100% (based on the exact LC50), then, the LC50 of the fraction was found to be 76.96 %, LC50 of the commercial alcoholic extract was 85.55 %, LC50 of commercial aqueous extract was 79.11 % and LC50 of chebulinic acid was 52.99 % when compared to LC50 value of HoSU extract. The lower percentage represented more toxicity to HoSU extract. A value less than 100 % means the sample was more toxic than HoSU extract. Further, when we kept LC50 of chebulinic acid (pure compound) as 100 % (based on the exact LC50), then, LC50 of HoSU extract was found to be 187.58 %, LC50 of the fraction was found to be 144.94 %, LC50 of the commercial alcoholic extract was 161.13 % and LC50 of commercial aqueous extract was 149.00 % when compared to LC50 value of chebulinic acid. The lower percentage represented more toxicity to chebulinic acid. A value of more than 100 % means the sample was less toxic than chebulinic acid. On a relative scale of toxicity, this data showed that HoSU extract was

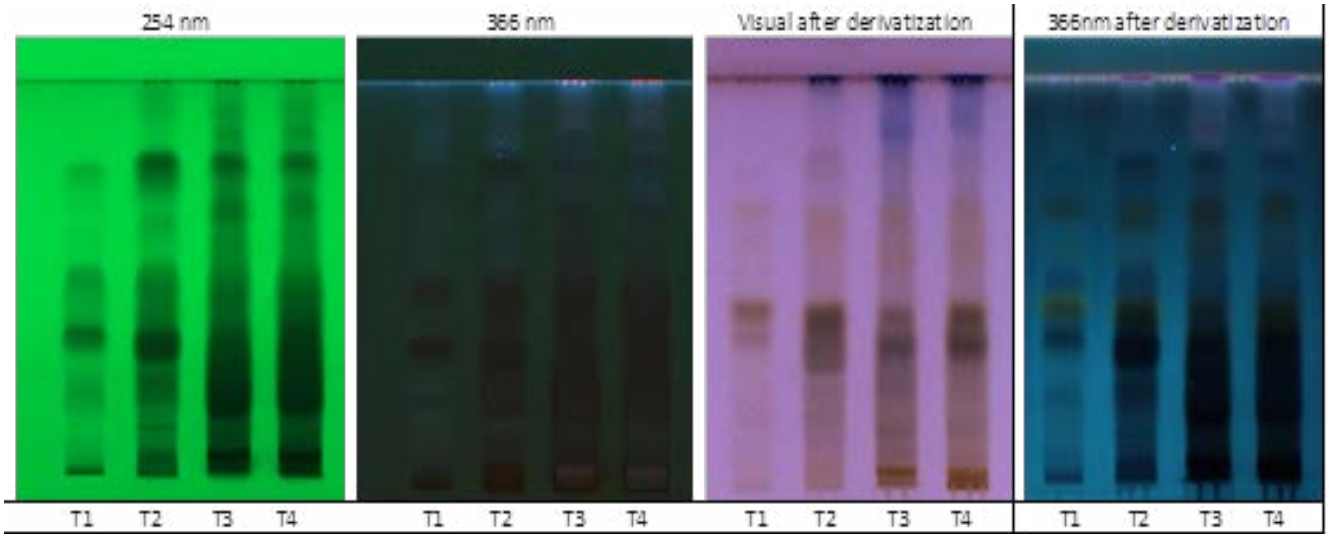
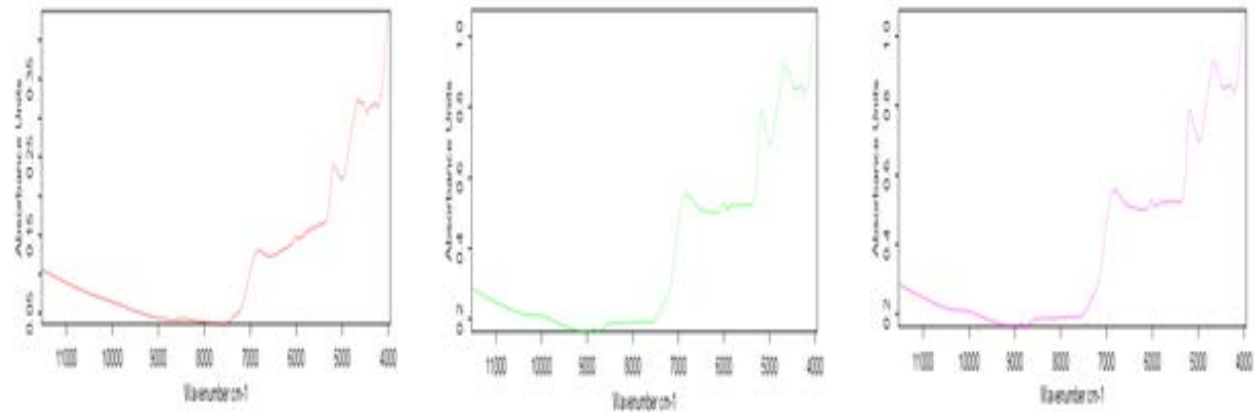
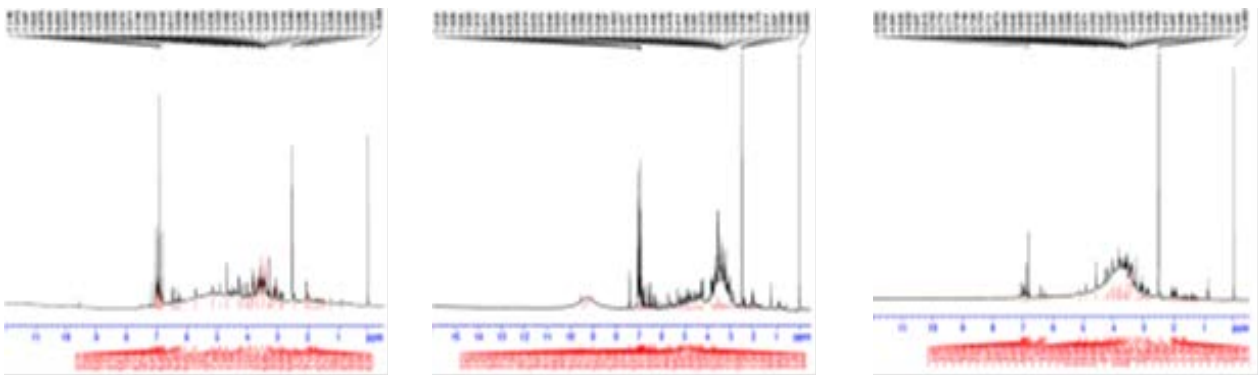


Figure 1. HPTLC profile for HoSU extract (T1), acetone fraction of commercial aqueous extract (T2), commercial aqueous extract (T3), and commercial alcoholic extract (T4).

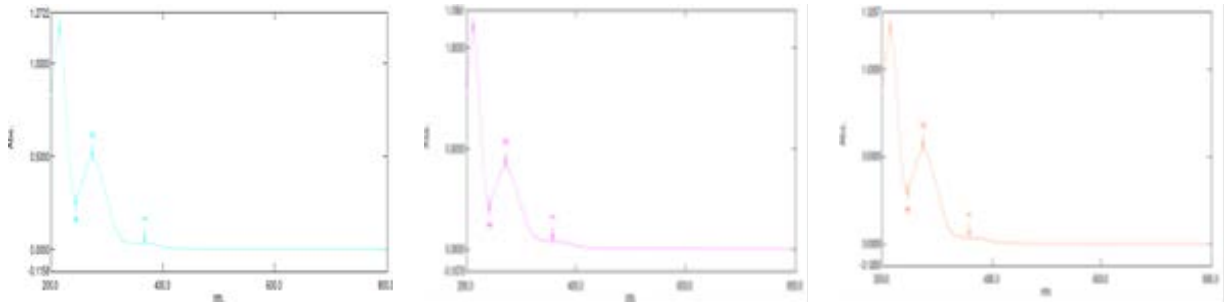
FT-NIR Spectra



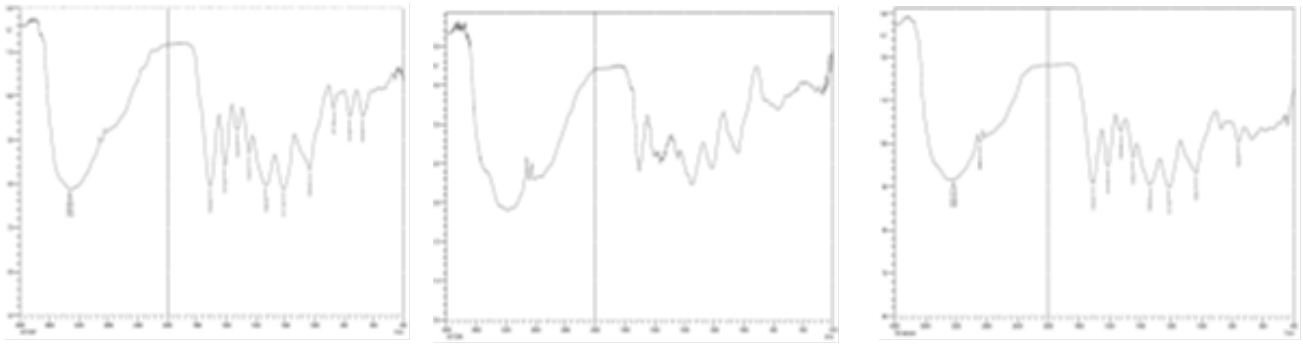
NMR Spectra



UV Spectra



IR Spectra



HoSU preparation | Commercial alcoholic extract | Commercial aqueous extract

Figure 2. FT-NIR, UV-Visible, NMR and FT-IR spectra for HoSU extract, commercial aqueous extract and commercial alcoholic extract.

the safest and chebulinic acid was the least when consumed.

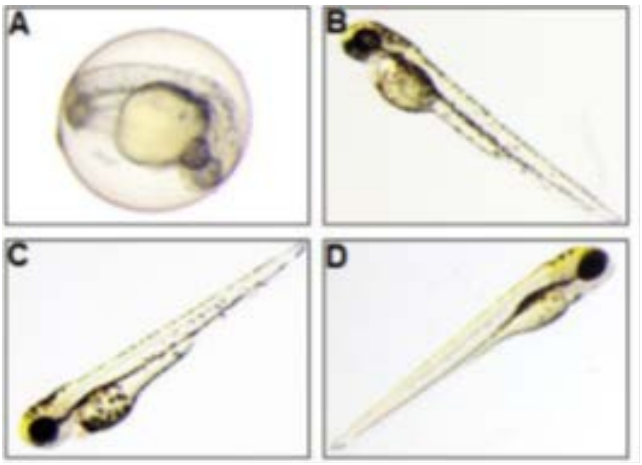
3.3 Principal Component Analysis

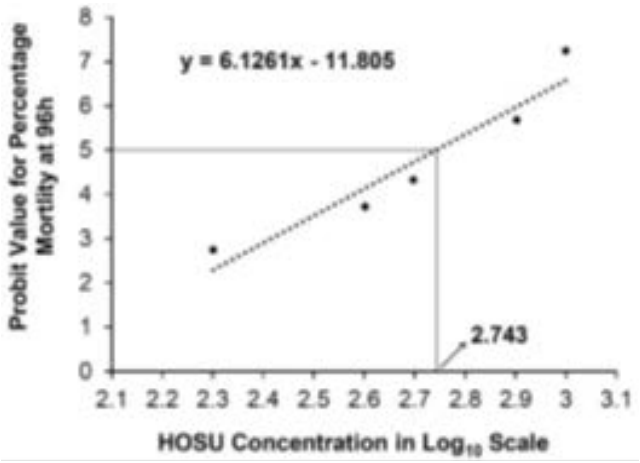
The PCA plot revealed that all the samples tested were having qualitative similarities to the chemical constituents whereas the FET results were not similar (Figure 4). The HoSU extract, commercial alcoholic extract, and commercial water extract were in the same half of the plot whereas water acetone extract was very dissimilar. When PCA was plotted without considering the FET test as a variable (Figure 5), it was observed that HoSU extract and the commercial alcoholic extract was grouped in the same quadrant indicating high similarity, whereas commercial aqueous extract was in the same half as HoSU extract and acetone fraction was distant from all these samples indicating that the sample was dissimilar. In the samples, all the chemical constituents except the content of total tannins were grouped in the PCA plot indicating similarity. The heat map generated (Figure 6) showed the similarities of the variables in the samples and was a convenient tool for visual understanding of the variance.

Table 2. LC50 values of from FET test

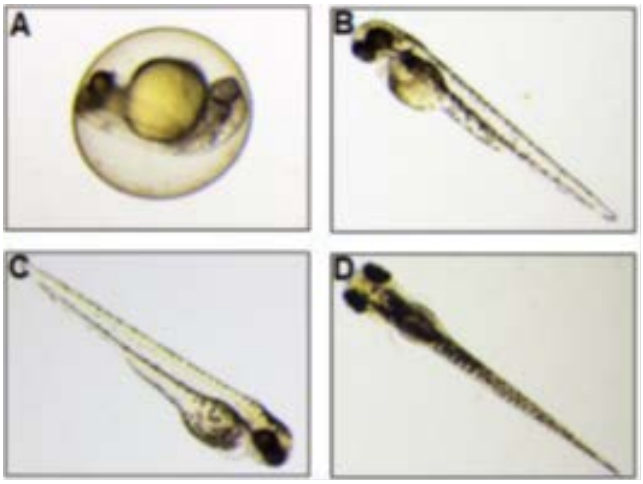
Tests conducted/Sample details	LC50 value
HoSU extract	553.35 µg/mL
Commercial water extract	439.54 µg/mL
Commercial alcoholic extract	475.33 µg/mL
Water extract acetone fraction	427.56 µg/mL
Chebulinic acid	294.44 µg/mL

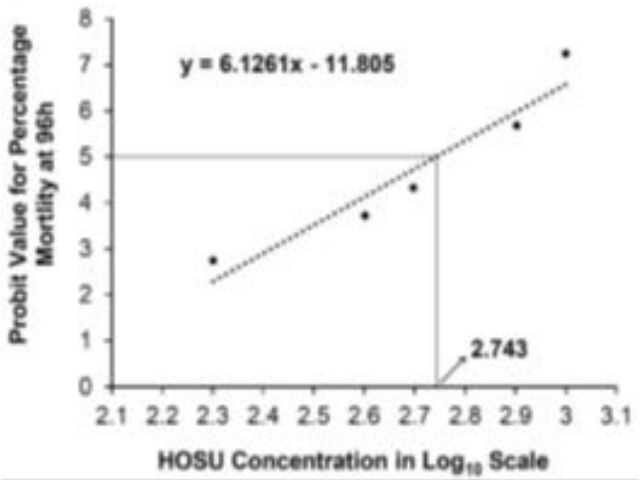
HoSU extract



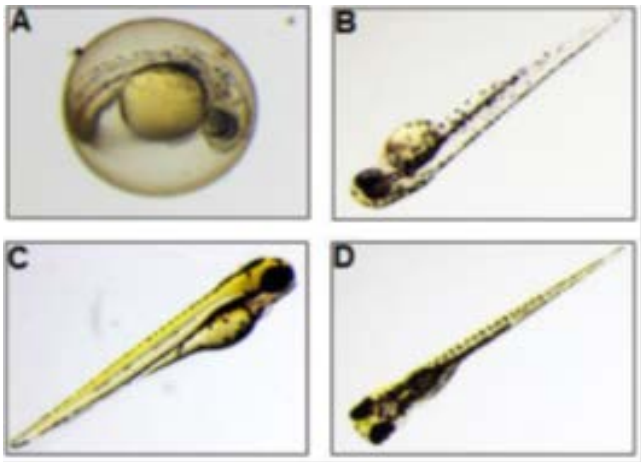


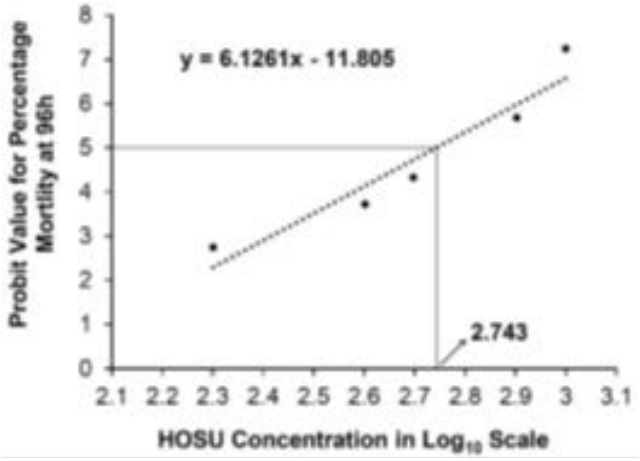
Acetone fraction of aqueous extract



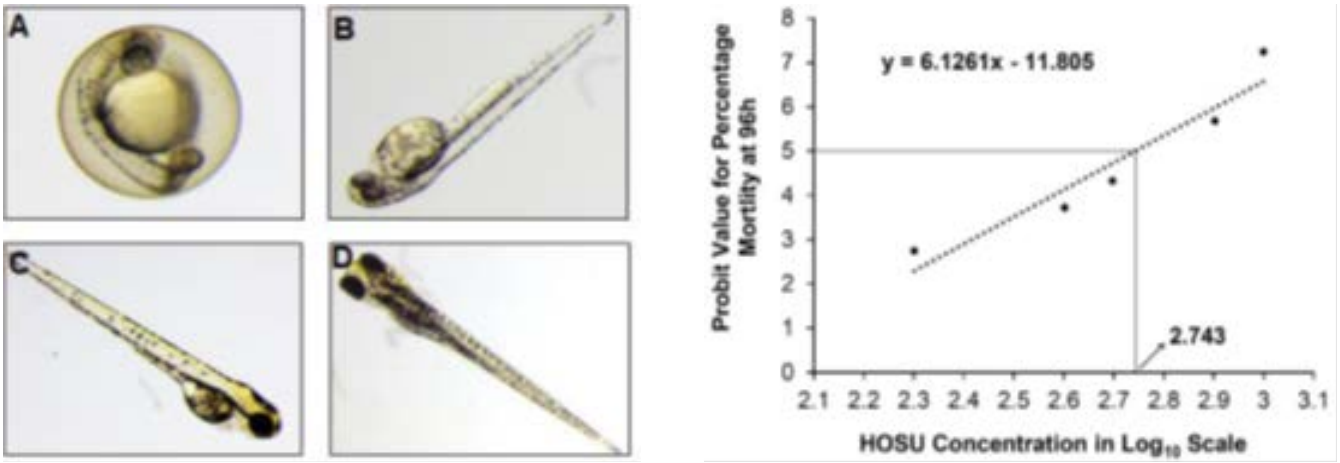


Commercial aqueous extract





Commercial alcoholic extract



Chebulinic acid

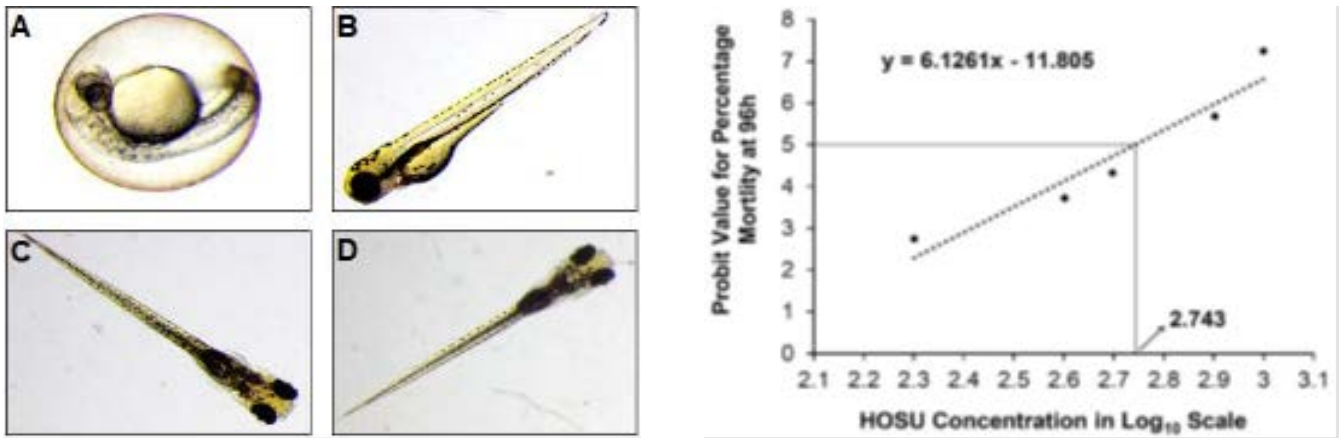


Figure 3. Developmental stages of zebrafish embryos/larvae during a limit test carried out at 100 mg/L of all test samples at 24 h **(A),** 48 h **(B)**, 72 h **(C)**, and 96 h **(D)** and Probit analysis for the calculation of LC50 value.

Variables

Observation

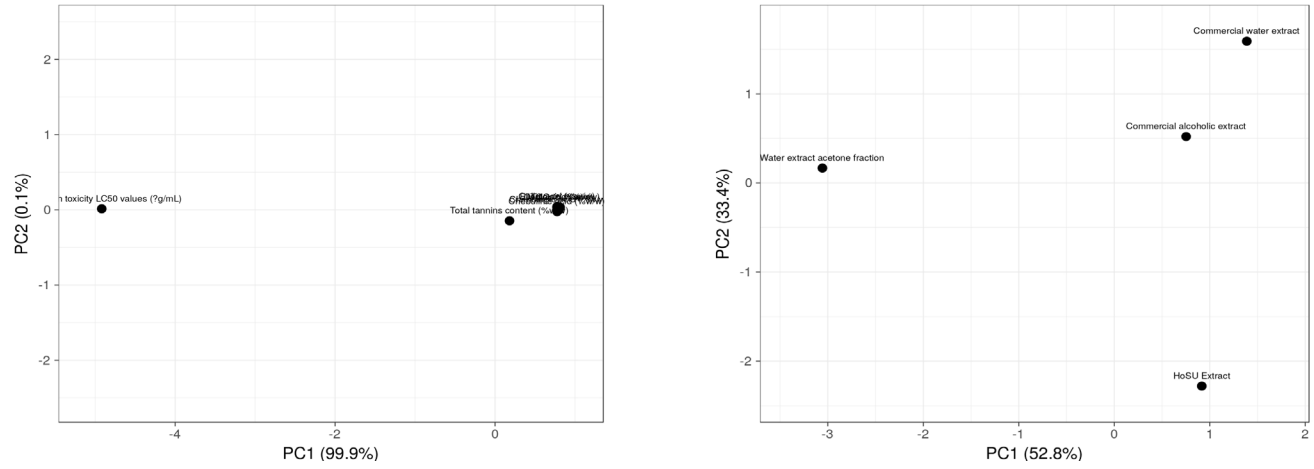


Figure 4. Principal component analysis plot.

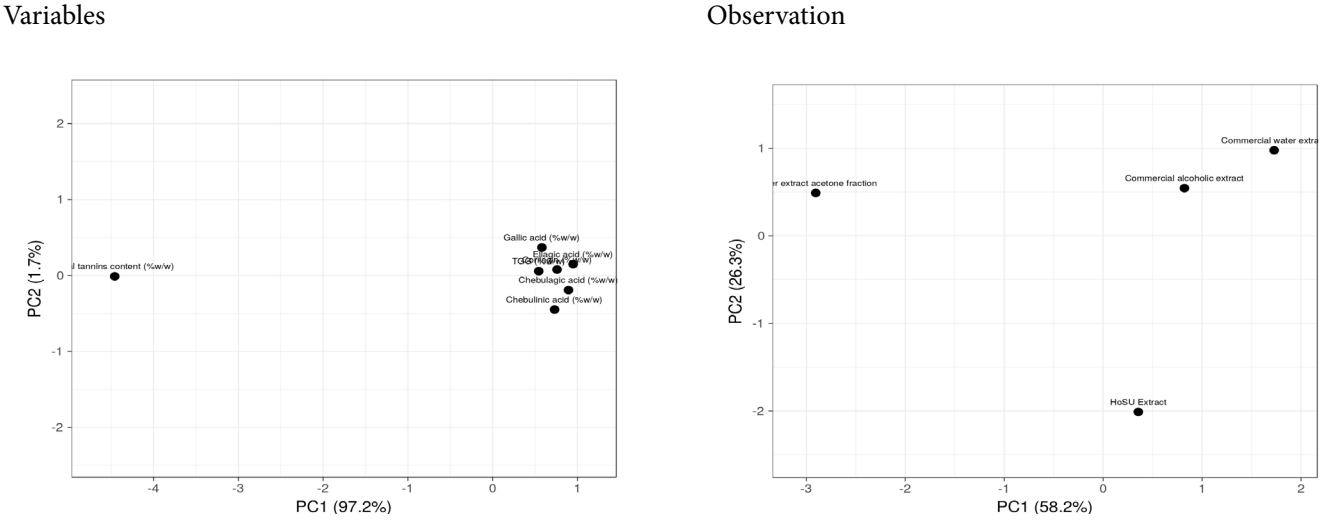


Figure 5. Principal component analysis plot without considering the FET test.

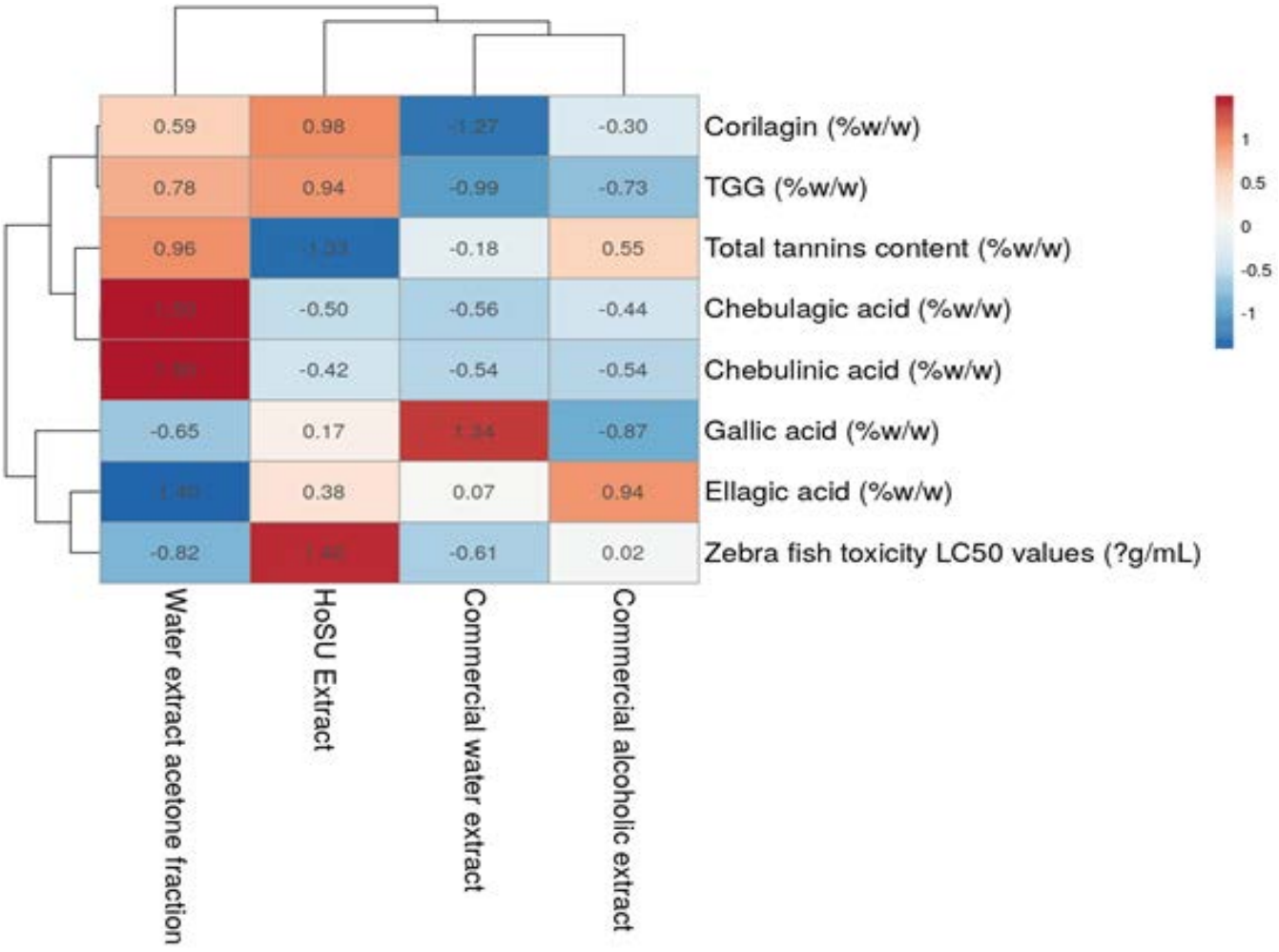


Figure 6. Heatmap between samples and chemical composition and toxicity.

4. Conclusion

This present study shows the importance of establishing equivalence (similarity/ dissimilarity) between HoSU preparations and commercial preparations of herbal products. Chemical analysis of the products revealed similarities between HoSU extract, acetone fraction of commercial aqueous extract, commercial aqueous extract, and alcoholic extract., and dissimilar to the pure Chebulinic acid. The chromatographic fingerprint and spectral data generated also indicated the presence of similar compounds. Though all extracts were demonstrated safe in the FET test against the embryo of zebrafish, it was observed that HoSU extracts were the safest products followed by commercial preparations (both aqueous extracts and alcoholic extracts), acetone fraction, and chebulinic acid from the LC50 values. PCA plot indicated commercial alcoholic extract being similar to HoSU extract followed by commercial aqueous extract and acetone fraction of aqueous extract. This study indicates and emphasizes the need for conducting an equivalence study in terms of chemistry and toxicity between HoSU products and commercially available products which are manufactured by modern technologies. This is to ensure that traditional wisdom is not lost during modernizations implemented to improve productivity. PCA is one of the important tools in conducting such an equivalence study between various herbal preparations. Zebrafish toxicity studies are economic, rapid, and useful in assessing product safety equivalence instead of the conventional toxicity studies involving animals, which are expensive and time-consuming. Studies with more botanical on similar lines are suggested.

5. Acknowledgements

The authors are thankful to the Director of the IHBT, Dr Dinesh Kumar, Test Facility Manager, and Dr Damanpreet Singh, Senior Scientist at Institute of Himalayan Bioresource Technology (Division of Council of Scientific and Industrial Research, Govt of India), Palampur, Himachal Pradesh forpermitting and arranging to carry out zebrafish toxicity studies and providing the results and inputs for their interpretations. The authors are also thankful to M/s. Natural Remedies Pvt. Ltd. Bangalore for providing resources to carry out this study. Acknowledgments are due to the Ayurvidye Trust, Bangalore for funding the zebrafish toxicity studies and partially sponsoring the cost of marker compounds and related expenses.

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