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Quality assessment of *Ramabana Rasa*: A comparative study using chromatography, XRD analysis, and physicochemical parameters

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Abstract

Ramabana Rasa, is a classical Ayurvedic herbo-mineral drug formulation. (*Rasaushadhi*) used in traditional Ayurvedic medicine. It is widely used for gastrointestinal disorders, and prepared using purified *Aconitum ferox* rhizome, *Syzygium aromaticum* buds, *Piper nigrum* seeds, *Myristica fragrans* kernels, and purified Mercury–Sulphur complex. Rapid therapeutic action with low dosage and long shelf life are the inherent medicinal properties of *Rasaushadhi*. Since metallic compounds are included as raw materials *Rasaushadha* has a complicated manufacturing process. Lengthy drug formulation procedure, limited supply of raw material, adulteration of raw material, and contamination are common quality control problems in Ayurveda drug manufacturing process. The lack of attention paid in maintaining desired quality parameters directly impact the safety and efficacy of the ethnomedicinal drugs. This study was aimed at comparative analysis of the quality parameters of three commercial samples of *Ramabana Rasa* drawn from different ayurveda drug manufactures in Sri Lanka against a sample prepared in-house under strict quality control conditions. Determination of physicochemical properties, chromatogram analysis using Thin Layer Chromatography (TLC), High-Performance Liquid Chromatography (HPLC), and X-ray diffraction (XRD) analysis was carried out for each drug sample. The results of physicochemical properties revealed deviations between the market samples and the in-house sample. TLC fingerprints of four samples illustrated comparable banding patterns, indicating the presence of similar phytochemical compounds in all analysed samples. HPLC chromatogram showed similar chromatographic profile with different peak concentrations. According to the outcomes of XRD analysis, there were approximately comparable crystalline characters in the four samples. Deviations of processing methods, and quality of the raw materials could be some of the causes for these differences. These findings highlight the need for standardized processing techniques or operating procedures, to ensure the safety, consistency, quality, and therapeutic efficacy of herbo-mineral drugs.

Keywords: *Ramabana Rasa*, comparison analysis, TLC profile, HPLC analysis, XRD analysis

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Introduction

In traditional ayurveda medicine herbo mineral drugs are considered as one of the integral components which plays a crucial role. Metals, minerals, plant materials, and animal origins are used as raw materials to prepare herbo-mineral drugs (Anto et al., 2017), (Savrikar & Ravishankar, 2011). The quality control of *Rasaushadha* is essential for the entire production process. Since metallic compounds are included as raw materials *Rasaushadha* has a complicated and lengthy manufacturing process. Improper and misuse of preparation methods adversely effects on consumers. (Anto et al., 2017)

As a result of commercialization of Ayurveda medicaments, a large amount of raw material suppliers and drug manufacturers have emerged in the industry. Despite large-scale supply and usage of herbo-mineral drugs, there is limited concern about the quality of these drugs which is harmful to the consumers as well as the ethnomedical industry. Some drug manufacturers follow their own formulas and test parameters. Some of these parameters are traditional methods and quality analysis depends on the expertise of the quality checker. Lengthy procedures for drug formulations, difficulties in obtaining the raw materials, adulteration, and contaminations, inferior grades or expired raw materials, high cost of raw materials, and shortcuts followed instead of the standard operating procedures (SOPs) are some common problems in Ayurveda drug manufacturing.

Each herbo-mineral drug is formulated using several plant materials, metals/minerals, components of animal origin. It is an extremely difficult task to identify whether every raw material is present in the final product. Evaluation of organoleptic and physicochemical parameters and comparing them with standard values is a suitable method for authenticating the quality of the drug. Determination of the chemical composition of herbal drugs by chromatographic fingerprinting is an advanced method for appraising the quality with batch-to-batch consistency (Abraham et al., 2020). Therefore, the development of such chromatographic techniques and physico-chemical parameters for standardization of authentication procedures for herbo-mineral drugs are important at this juncture for the herbal industry (Govindarajan et al., 2019).

Ramabana Rasa (RR) is one of the effective drug formulas recommended for gastrointestinal problems. It is recommended for the treatment of *amavata* in Ayurveda. Ingredients for this preparation as mentioned in Ayurveda pharmacopeia are purified rhizomes of *Aconitum ferox*, *Syzygium aromaticum* flower buds, *Piper nigrum L* seeds, *Myristica fragrance* kernel, and a mixture of purified Mercury and purified Sulphur (called as *Kajjali*). (Department of Ayurveda, 1976).

This study carried out a comparative analysis of physicochemical properties in terms of product quality of different market samples of *Ramabana Rasa* against the inhouse prepared samples by using physicochemical properties, chromatographic analysis by High-Performance Liquid Chromatography (HPLC), Thin Layer Chromatography (TLC) and XRD analysis.

Methodology

Authentication of raw materials /QC analysis

All mineral authentication, purification, and quality assessment (smoothness, color, fineness) were done at the Department of Ayurveda Pharmacology Pharmaceutics and Community Medicine, Faculty of Indigenous Medicine, University of Colombo. All plant materials authentication was done at the Ayurveda Research Institute, Nawinna, Maharagama.

Kajjali preparation-

Equal weights of purified Mercury and purified Sulphur were transferred into a mortar and triturated by using a pestle. The procedure continued until the mixture converted into blackish fine powder without shining particles in it.

Plant raw material – Purification of *Aconitum ferox* (Rhizome)

The plant material was mashed into small pieces. *Aconitum ferox* (Rhizome) pieces were placed in a cleaned cloth, and it was prepared as a Pouch. It was hung and submerged in a clay pot (*Dolayanthraya*) which was filled with *cow's milk* and subjected to medium heat for 3 hours. *milk* was re-filled when the liquid level was reduced. After 3 hours pouch was taken out and washed with hot water several times. After drying, the pieces were ground into fine powder.

(a) (b) (c) (d)

Figure 1. *Aconitum ferox* (Rhizome) purification by using cow's milk. (a) *Aconitum ferox* (Rhizome) pieces (b) Pouch hung and submerged in a clay pot filled with cow's milk (c) *Aconitum ferox* Rhizomes after 3 hours drying process (d) Powdered *Aconitum ferox* rhizomes.

In-house preparation of *Ramabana Rasa.*

Ramabana Rasa was prepared inhouse according to the following formula

Table 1: Details of raw materials

	English name	Botanical name	QTY	Source
1	Indian aconite Rhizomes	*Aconitum ferox*	12g	Procured from the registered supplier of Department of Ayurveda
2	Clove powder	Syzygium aromaticum	12g	Department of Export Agriculture - Matale
3	Black pepper seeds	*Piper nigrum L.*	24g	
4	Nutmeg	*Myristica fragrans*	6g	
5	Purified Mercury	Hg	12g	

	English name	Botanical name	QTY	Source
6	Purified Sulfur	S	12g	Department of Ayurveda Pharmacology Pharmaceutics and Community Medicine, Faculty of Indigenous Medicine (FIM)
7	Tamarind juice	*Tamarindus indica* juice		Fresh juice

All fine powdered raw materials were sieved through a 180 µm mesh size by using a sieve shaker (AS 200 control Retsch GmbH) and weighed accurately using an analytical balance (AS 220/R/2). After that, all plant materials were mixed with the *Kajjali* until the sample became a homogeneous mixture. Then, *Tamarindus indica* juice was extracted and mixed with the powdered drug mixture. It was triturated until the mixture became a homogeneous paste. The pills were prepared nearly 0.125 g by weight and dried.

(a) (b) (c) (d)

Figure 2. Preparation of *Ramabana Rasa* (a) *Kajjali* preparation by triturating Mercury and Sulphur (b) *Kajjali* mixed with Tamarind juice (c) homogeneous paste (d) prepared pills

Sample collection and Analysis

Three commercial samples for *Ramabana Rasa*, were collected from three different ayurveda manufactures in Sri Lanka and compared with the in-house prepared sample.

Physicochemical analysis

All samples were subjected to comparative analysis to evaluate their physicochemical characteristics according to Ayurveda Pharmacopoeia India, Part II, Vol IV (Ministry of AYUSH,2017) Moisture content, total ash content, water soluble ash content, acid insoluble ash content, water-soluble extractable matter, ethanol soluble extractable matter, and pH were analysed. (Shelar et al., 2021)

Chromatographic analysis

Sample preparation - 2.5 g fine powder of each finished product sample was extracted in 25 mL of methanol (Sigma Aldrich-ACS reagent) using a sonicator (ROCKER SONER 210H) for 30 minutes. The filtrate was evaporated by using rotary evaporator (IKA RV 10). 10 mL of methanol -HPLC grade was added to the dried extract and completely dissolved. The extract was diluted 1:10 and it was filtered using a 0.45 µm disposable filter paper (Abraham et al., 2020), (Banwart et al., 1985)

HPLC analysis

Analysis was performed by Dionex/ Thermo UltiMate 3000 RP-HPLC system, pump- (LPG-3400SD) equipped with online degasser, diode array detector (DAD 3000 detector), column compartment (TCC -3000SD column Thermostat) and column (150 mm X 3.6 mm, i.d., 5 µm pore size) attached to a guard column. HPLC profile was developed to determine the phenolic fraction and flavonoids in poly herbal drug samples. Flow rate was maintained at 1 mL/min. The solvent system was, acetonitrile as solvent A and water: phosphoric acid (99.7: 0.3) as solvent B with a gradient elution in 0-5 min with 85% -50 % of B, 5-10 min with 50%-30% of B, 10-25 min with 30 %-0 % of B. column temperature was maintained at 27 ^{0}C. Fingerprint profile was detected at UV-VIS 254 nm and 280 nm.

TLC analysis

Methanol (Sigma Aldrich-ACS reagent) extract of the sample prepared inhouse and the market samples were separated in precoated silica gel plate. The solvent system was, toluene, ethyl acetate and formic acid. The TLC plate was visualized by UV 254 nm and UV 365 nm. 10 % ethanolic sulfuric acid, 10 mL concentrated sulfuric acid was added to 90 mL of ethanol was used as the spray reagent and the resolved spots were visualized by naked eye. Anisaldehyde solution (85 mL of methanol, 5 mL of concentrated sulfuric acid, 10 mL glacial acetic acid and 0.5 mL Anisaldehyde) was used as the spray reagent and the plate was visualized at UV 365 nm and dried until the plates were visualized by naked eye.

XRD analysis

XRD analyses were done by Model No Regaku Ultima IV XRD analyser, Instrument Centre at University of Sri Jayawardanapura at a scan range of 5.0000-80.0000 degree and a scan speed 2.0000 deg/min. (Dutrow & Clark, 2024)

Results

One Way Analysis of Variances (ANOVA) statistical test showed significant deviation among the moisture % ($F(3,8) = 34.7$, $p < 0.001$) with a value range from 19.03± 0.07 % to 21.97 ± 0.42 %. Total ash content ($F(3,8) = 32.51$, $p < 0.001$) between 4.63± 0.1% to 8.23 ± 0.9%, water insoluble ash content ($F(3,8) = 5.81$, $p < 0.05$) between 0.82±0.25 % to 1.56±0.21%. acid insoluble ash content ($F(3,8) = 79.24$, $p < 0.001$) between 0.26±0.07% to 0.68±0.02%. water soluble extractable matter ($F(3,8) = 302.2$, $p < 0.001$) between 5.98± 0.19% to 14.5 ± 0.33% and ethanol soluble extractable matter % ($F(3,8) = 502$, $p < 0.001$) between 2.81± 0.08 % to 9.44± 0.21 % (Table 2).

Table 2: Physicochemical analysis of *Ramabana Rasa* (n=3)

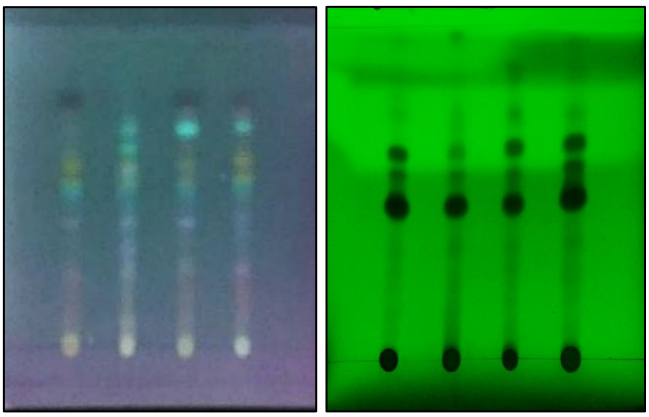
Sample Test parameter	Inhouse sample	Market sample 01	Market sample 02	Market sample 03
Loss on drying at 105 ^{0}C	21.97 ± 0.42	19.47± 0.50	19.49 ± 0.46	19.03± 0.07
Total ash%	8.23 ± 0.9	4.63± 0.1	6.29± 0.12	5.26± 0.28
Water insoluble ash %	1.03±0.29	1.56±0.21	0.93±0.18	1.03±0.29
Acid insoluble ash %	0.257±0.07	0.677±0.023	0.55±0.018	0.32±0.01

Sample Test parameter	Inhouse sample	Market sample 01	Market sample 02	Market sample 03
Water soluble extractable matter %	14.5 ± 0.33	6.99± 0.13	7.42 ± 0.19	5.98± 0.19
Ethanol soluble extractable matter %	9.44± 0.21	3.95± 0.27	3.19± 0.11	2.81± 0.08
pH at room temperature	2.52	3.31	3.58	4.16

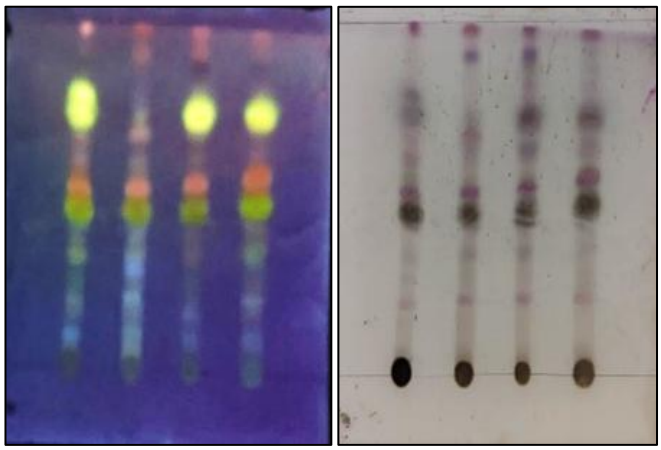
TLC analysis of *Ramabana Rasa*

TLC fingerprint for methanolic extract of *Ramabana Rasa* detected at UV-365 nm and UV-254 nm are illustrated in Figure 3(a). TLC fingerprint after spraying 10% ethanolic sulfuric acid reagent at UV-365 nm and under visible light is given in Figure 3(b). The TLC fingerprint after spraying Anisaldehyde reagent at UV 365 nm and under visible light is shown in Figure 3(c).

a)



b)



c)

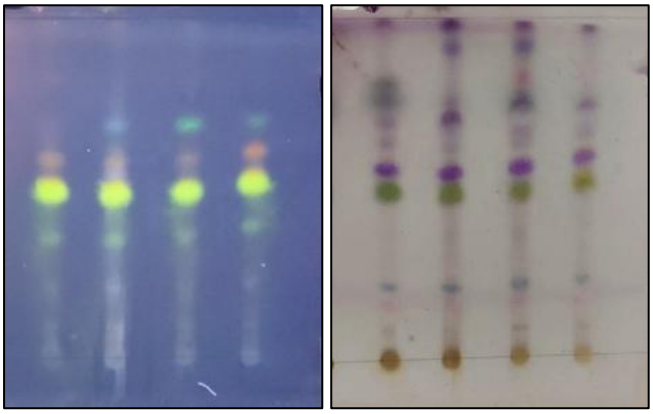
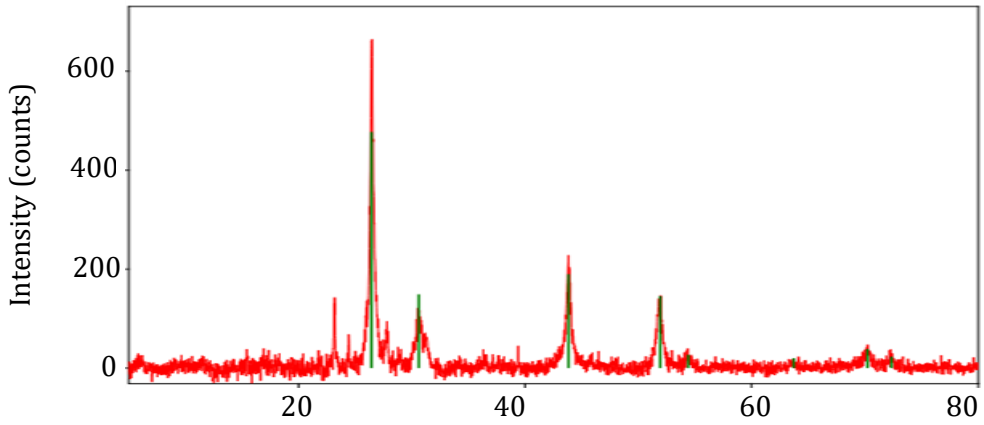


Figure 3.TLC fingerprint for methanolic extract of *Ramabana Rasa* a): without using spray reagent, at UV-365 nm (Left) and UV- 254 nm (Right), b) after spraying 10% ethanolic sulfuric acid reagent at UV 365 nm (Left) and under the visible light (right), c) after spraying Anisaldehyde reagent at UV 365 nm (Left) and under the visible light (Right). S_{in}, S_1, S_2 and S_3 in lanes left to right in a), b) and c) respectively.

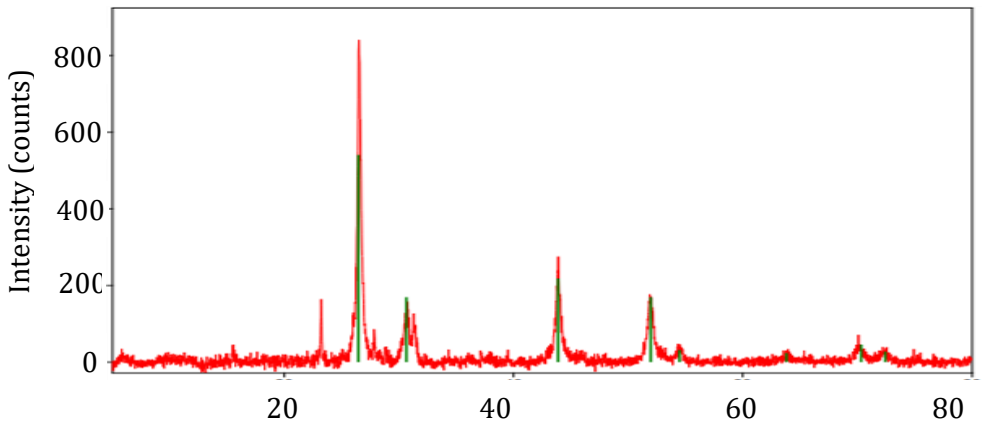
XRD analysis

XRD analysis of *Ramabana Rasa* is illustrated in Figure 04 for the four samples. XRD spectrum patterns illustrated diffraction peaks corresponding to metacinnabar. ICDD (PDF) card number 01-089-0432 database was used for phase identification.

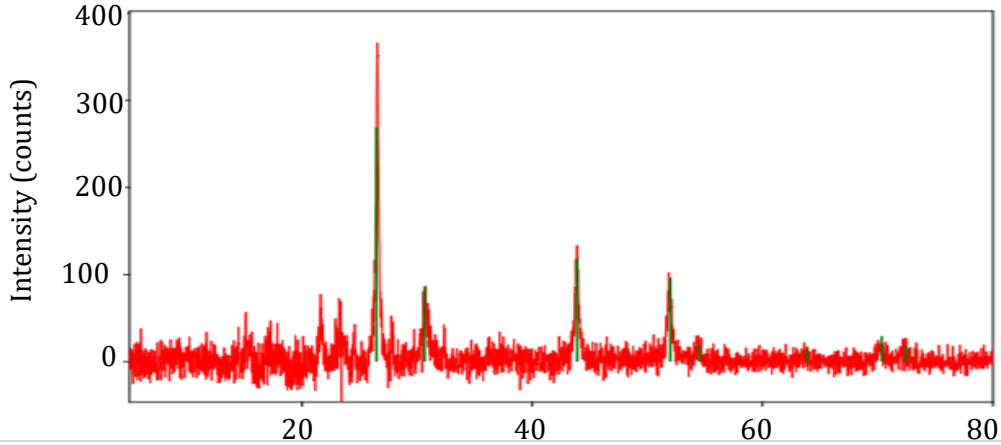
a)



b)



c)



d)

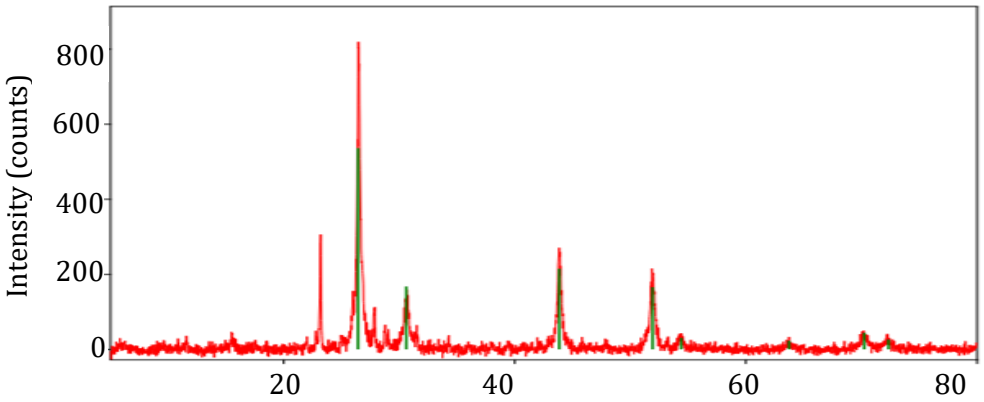


Figure 4. XRD analysis of *Ramabana Rasa* samples, a) $S_{in,}$ b) S_1, c) S_2, and d) S_3

Table 3: XRD analysis of *Ramabana Rasa* In house sample (S_{in}). Space group F-43m.

No 1	2θ (⁰) 2-theta(deg)	d (Å)	Relative Intensity (%)	hkl	Phase	Lattice structure
1	26.453	3.3666	100.0 %	(111)	Metacinnabar	Cubic Zinc blende
2	30.608	2.9184	11.0 %	(200)	Metacinnabar	Cubic Zinc blende
3	43.801	2.0651	30.3 %	(220)	Metacinnabar	Cubic Zinc blende
4	51.93	1.7594	19.0 %	(311)	Metacinnabar	Cubic Zinc blende
5	54.32	1.6874	4.1%	(222)	Metacinnabar	Cubic Zinc blende
6	70.11	1.3412	4.4 %	(330)	Metacinnabar	Cubic Zinc blende
7	72.23	1.3069	3.1 %	(331)	Metacinnabar	Cubic Zinc blende

Table 4: XRD analysis of *Ramabana Rasa* Market sample 1 (S_1) Space group F-43m.

No 2	2θ(⁰) 2-theta(deg)	d (Å)	Relative Intensity (%)	hkl	Phase	Lattice structure
1	26.488	3.3623	100.0 %	(111)	Metacinnabar	Cubic Zinc blende
2	30.68	2.912	14.79 %	(200)	Metacinnabar	Cubic Zinc blende
3	43.893	2.0610	30.70 %	(220)	Metacinnabar	Cubic Zinc blende
4	51.93	1.7593	19.38 %	(311)	Metacinnabar	Cubic Zinc blende
5	54.44	1.6840	3.43%	(222)	Metacinnabar	Cubic Zinc blende
6	63.79	1.4580	1.37 %	(400)	Metacinnabar	Cubic Zinc blende
7	70.03	1.3424	4.45 %	(330)	Metacinnabar	Cubic Zinc blende
8	72.24	1.3067	2.40 %	(331)	Metacinnabar	Cubic Zinc blende

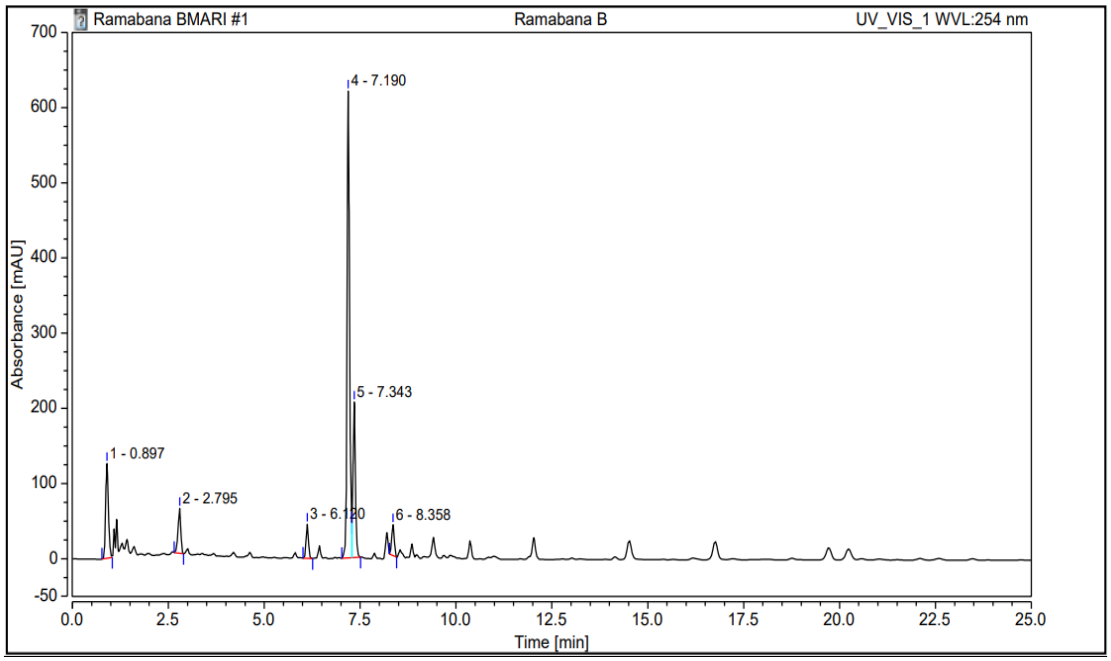
Table 5: XRD analysis of *Ramabana Rasa* Market sample 3 (S_3) Space group F-43m.

No 4	2θ(⁰) 2-theta(deg)	d (Å)	Relative Intensity (%)	hkl	Phase	Lattice structure
1	26.482	3.3630	100.0 %	(111)	Metacinnabar	Cubic Zinc blende
2	30.585	2.905	16.96 %	(200)	Metacinnabar	Cubic Zinc blende
3	43.83	2.0639	34.10 %	(220)	Metacinnabar	Cubic Zinc blende
4	51.84	1.7622	25.71 %	(311)	Metacinnabar	Cubic Zinc blende
5	54.45	1.6840	3.92%	(222)	Metacinnabar	Cubic Zinc blende
7	70.11	1.3411	5.89 %	(330)	Metacinnabar	Cubic Zinc blende
8	72.31	1.3057	3.21 %	(331)	Metacinnabar	Cubic Zinc blende

HPLC Analysis

HPLC analysis of *Ramabana Rasa* at UV- 254 nm and UV- 280 nm for the four samples are illustrated in Figures 5,6,7,8.

(a)



(b)

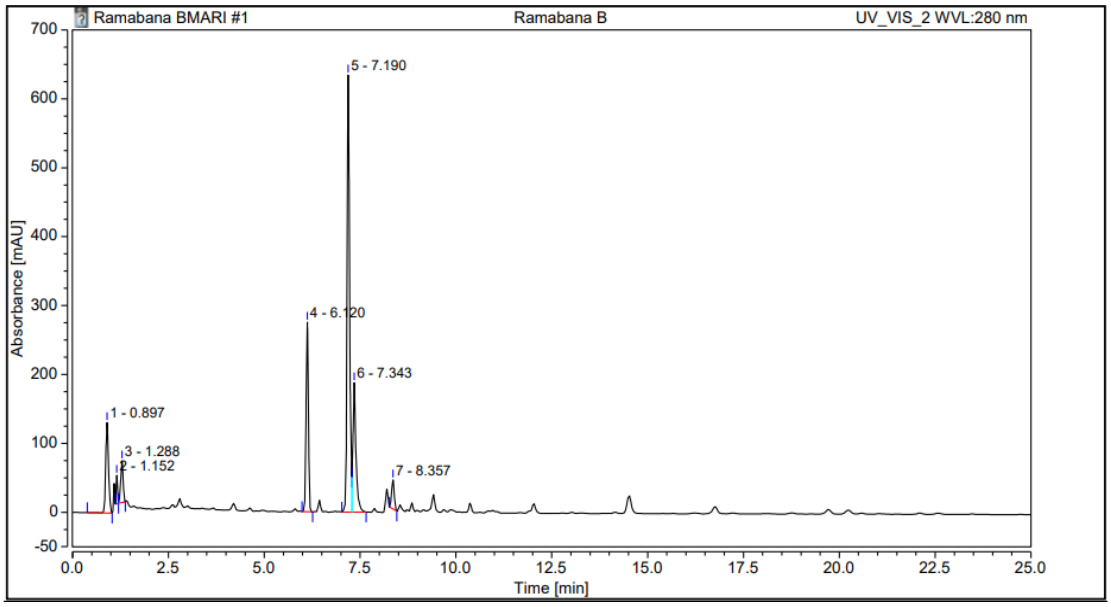
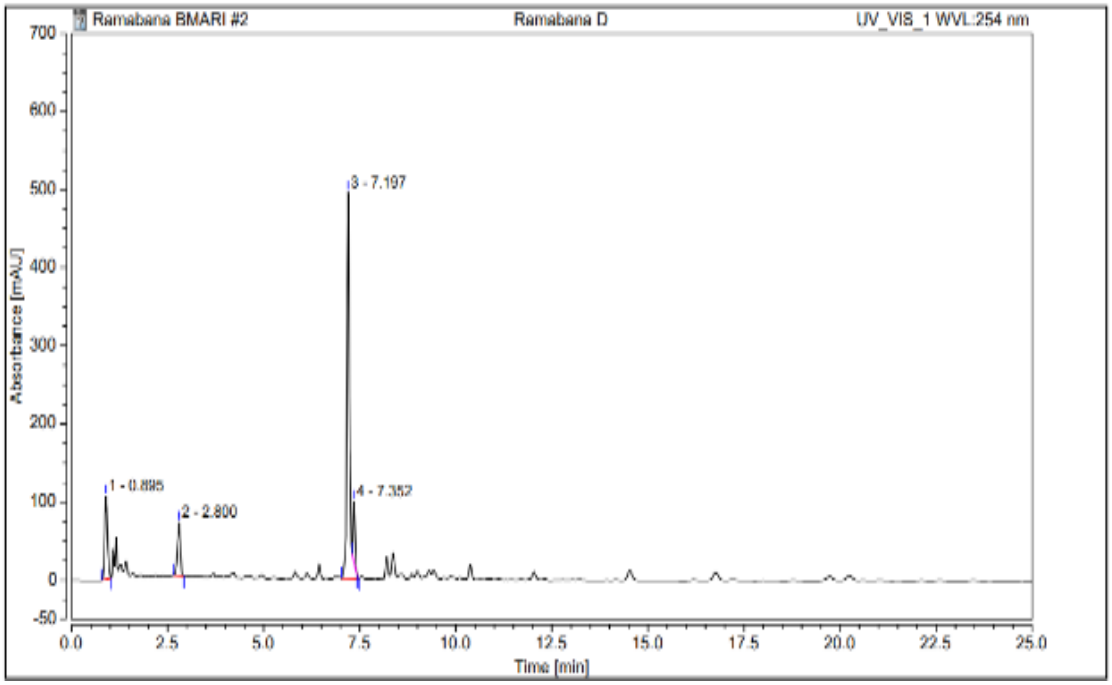


Figure 5. HPLC analysis of *Ramabana Rasa* sample S_{in} at a): UV-254 nm and b) UV-280 nm.

(a)



(b)

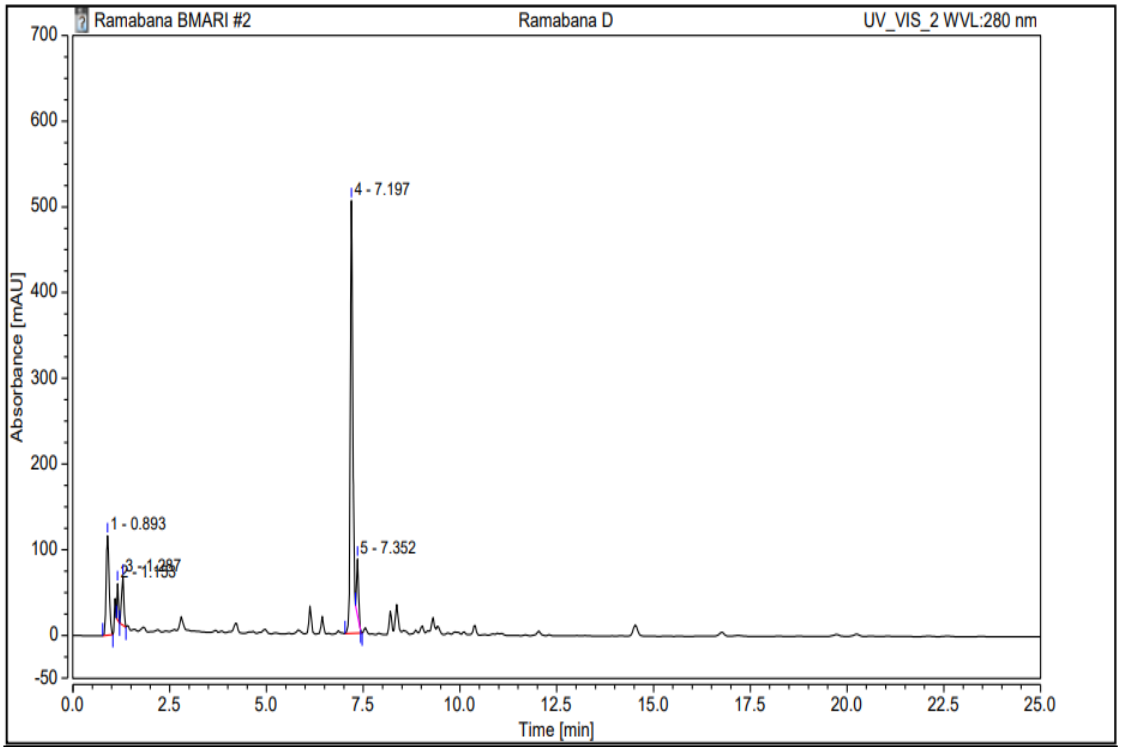
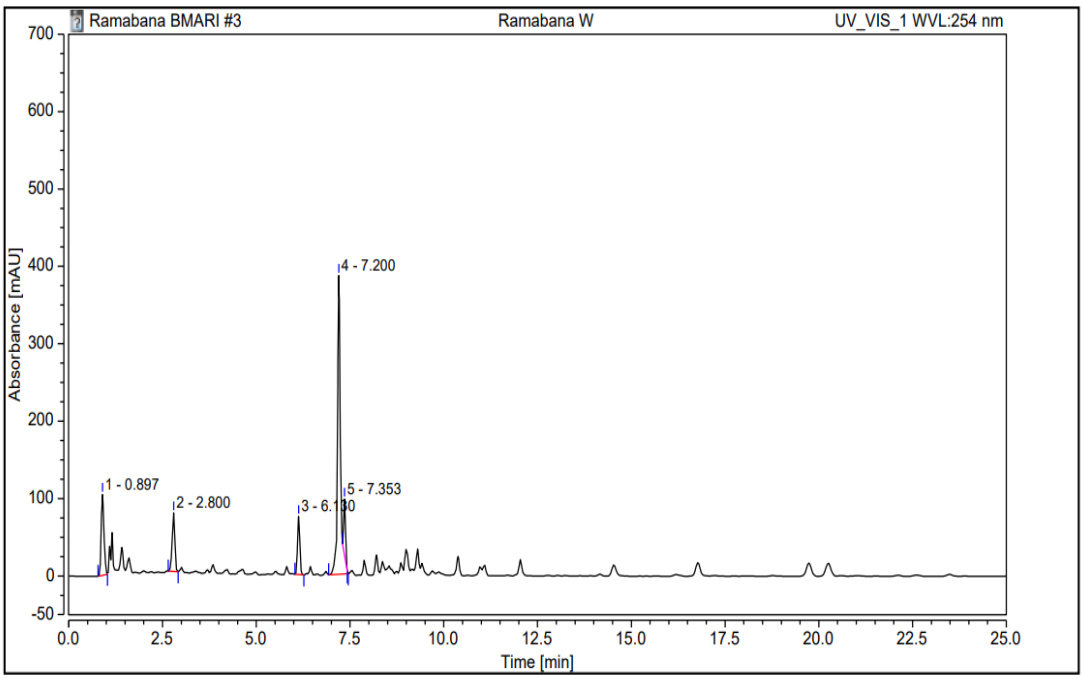


Figure 6. HPLC analysis of *Ramabana Rasa* sample S_1 at a) UV- 254 nm and b) UV- 280 nm.

(a)



(b)

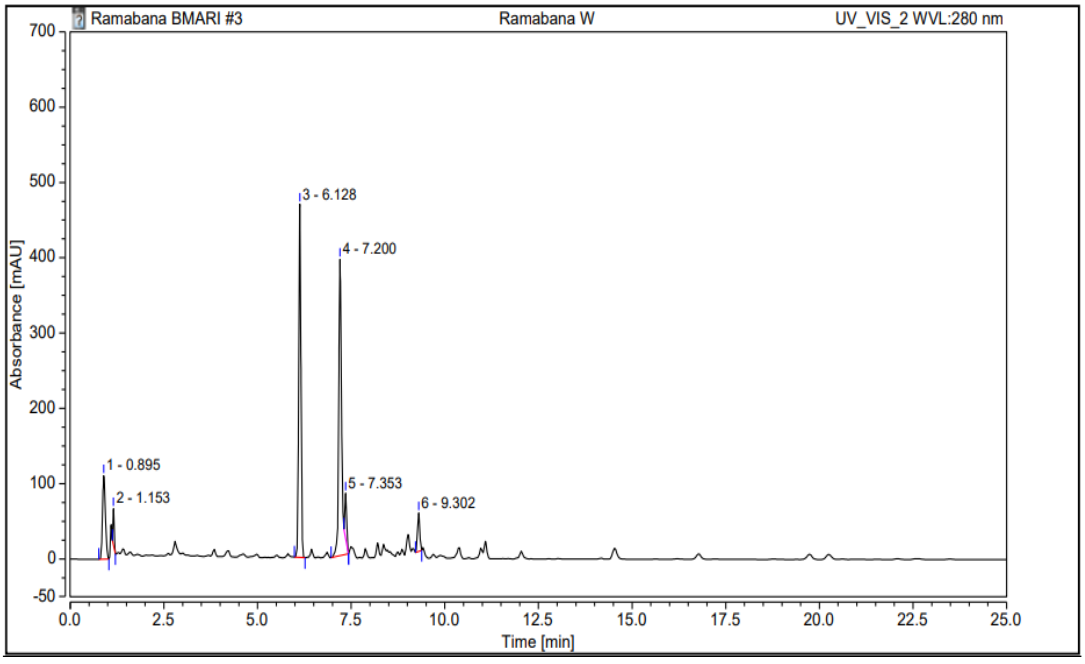
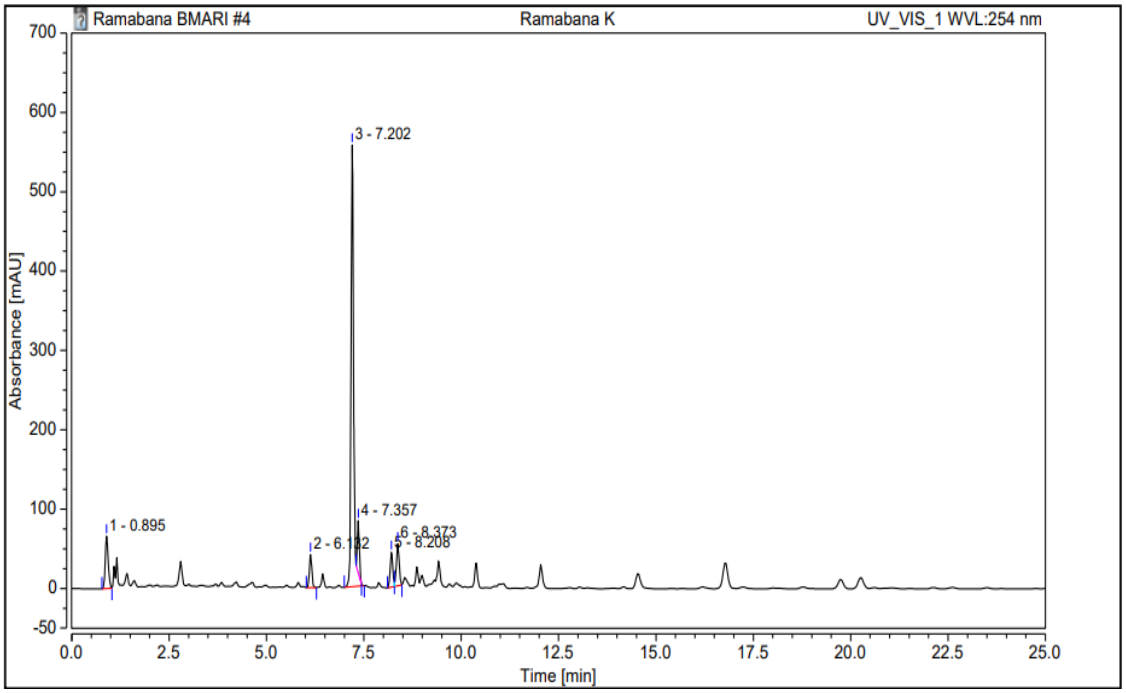


Figure 7. HPLC analysis of *Ramabana Rasa* sample S_2 at a): UV- 254 nm and, b) UV-280 nm

(a)



(b)

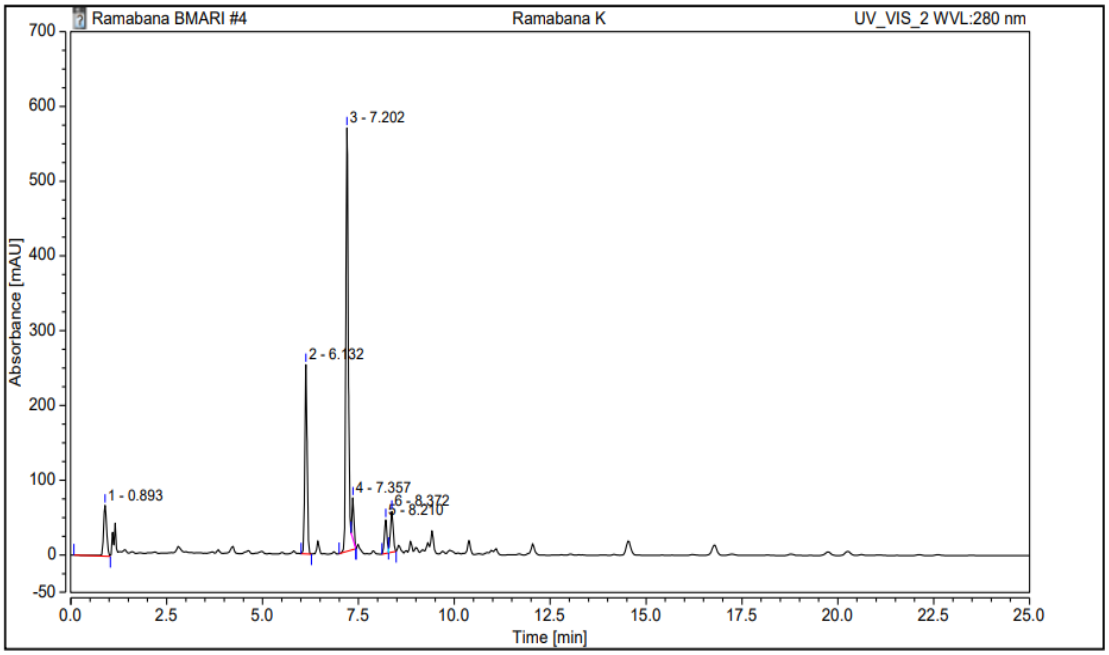


Figure 8. HPLC analysis of *Ramabana Rasa* sample S_3 at a): UV- 254 nm b) UV- 280 nm

Discussion

The comparative evaluation of quality assessment of *Ramabana Rasa* revealed notable differences in physicochemical characteristics between the inhouse prepared formulation (S_{in}) and the three commercial samples (S_1, S_2, and S_3).

The statistical analysis of quality parameters clearly illustrates the quality deviations of the drug formulations. Higher water-soluble extractable matter %, ethanol soluble extractable matter % indicates the higher availability of bioactive compounds. The high value of total ash % indicates the high mineral content and the lower water insoluble Ash % and, acid insoluble Ash % reflect the higher quality of raw materials and its purification process. pH of in-house prepared sample was lower than the three market samples. This difference could influence both stability and the bioavailability of the formulation. The deviation in the physicochemical properties may suggest the differences in raw material selection, or manufacturing techniques.

The TLC profile of S_{in} and the commercial samples (S_1, S_2, and S_3) exhibited comparable banding patterns, indicating the presence of similar phytochemical compounds.

HPLC chromatogram of S_{in} showed a higher number of peaks and higher concentrations than the market samples. All four samples were illustrated highest peaks at 7.190±0.015 min retention time with different concentrations

The sample S_{in}, at 2θ, 26.45 (111), 30.60 (200), 43.80 (220), 51.93 (311), 54.32(222), 70.11 (330) and 72.23 (331) major peaks were identified. For sample S_1, at 2θ, 26.48 (111), 30.68 (200), 43.89 (220), 51.93 (311), 54.44(222), 63.79(400), (70.03 (330) and 72.24 (331) major peaks were identified. However, the sample S_2 illustrated broad XRD diffraction but crystalline phases were not identified by ICDD (PDF) database while 26.48 (111), 30.58 (200), 43.83 (220), 51.84 (311), 54.45(222), 70.11 (330) and 72.31 (331) major peaks were identified in sample S_3, at 2θ. Sample S_{in}, S_1 and S_3 were mostly comparable with each other indicating changes to the physicochemical parameters of the drug under the different preparation conditions.

Conclusion

This research was carried out to assess the quality of *Ramabana Rasa* available in the Sri Lankan Ayurvedic drug market by using chromatography, XRD analysis, and physicochemical parameters.

When considering the physicochemical analysis of *Ramabana Rasa*, moisture content, total ash content, water-soluble extractable matter, and ethanol-soluble extractable matter there are statistically significant deviations among the samples indicating the need for quality control in the production processes to ensure consistency and likely efficacy of these herbomineral drugs. Although the TLC profile of all four samples showed a similar pattern suggestive of similar chemical composition, the RP-HPLC chromatogram of the inhouse prepared sample showed several additional peaks and higher concentrations compared to the market samples indicating the influence of the different production processes on the composition of the herbo-mineral formulations. In addition, XRD analysis of *Ramabana Rasa* revealed differences among the spectrum patterns among the analysed samples further highlighting the need for quality control in the production process.

Taken together, this study indicated similarities in qualitative analysis of chemical properties and mineral characteristics, but notable deviations in quantitative analysis. Therefore, the findings reveal the importance of adopting proper manufacturing methodologies and defining standard

specifications for the quality parameters, when determining the quality and consistency of herbo-mineral drugs. Hence, the standardization of herbo-mineral drugs through standard manufacturing procedures is needed to ensure the consistency, safety and efficacy of herbal drugs and to ensure community health and safety.

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Declaration of Funding Sources

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Conflict of interest statement

The authors declare that there are no conflicts of interest.

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