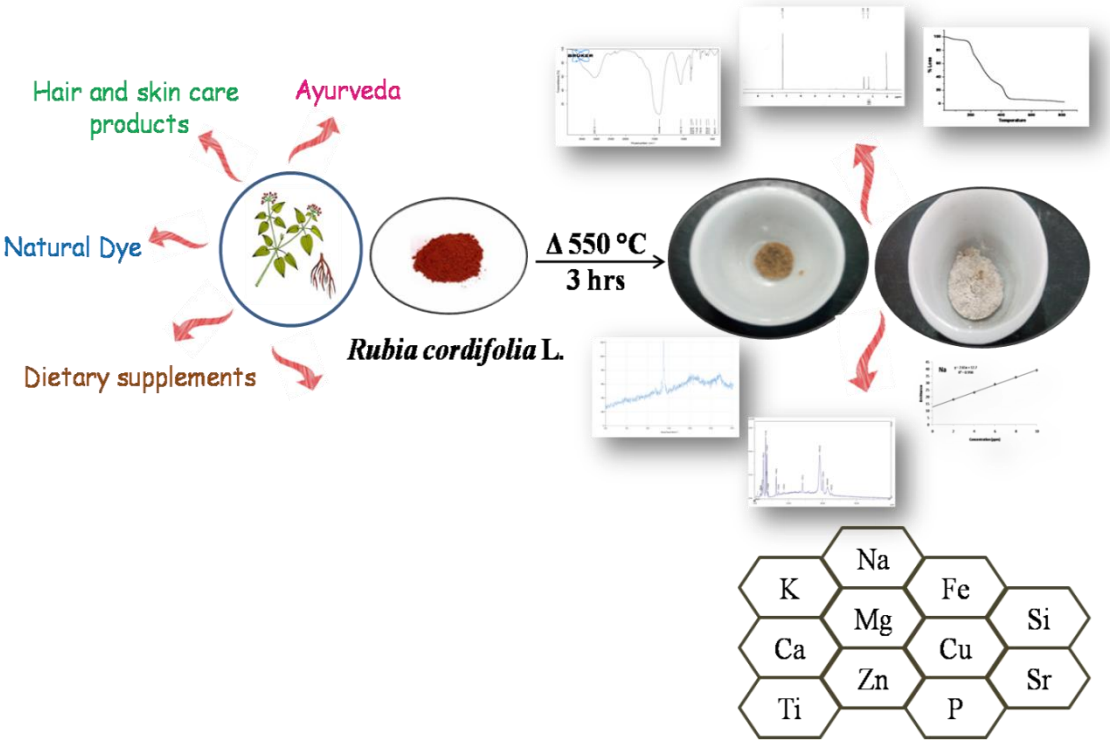
Chapter 2b

Characterization of *Rubia cordifolia* L. root powder and its ash



This chapter describes preparation of ash from dried roots of plant *Rubia cordifolia* L. and optimization of different parameters for ashing. Ashing (dry and wet) is an alternative way of sample preparation, which can help in determination of elemental composition of a sample. As the sample for analysis, after ashing loses its organic framework due to decomposition and thus, remains the ash that is enriched with elemental constituents. Plant extracts find application in Ayurveda for medicinal purposes, as beverages, etc. Both the conventional and sophisticated analytical techniques can be used to determine the elemental composition. Extensive elemental estimation has been performed in root powder of *Rubia cordifolia* L. and its ash in this work.

Chapter 2b: Characterization of *Rubia cordifolia* L. root powder and its ash

2b.1 Introduction

2b.1.1. Role of ash and its significance

Ash represents the matter available after complete combustion or ignition of a particular sample. Matter that even on further combustion remains constant. Ash content or total ash has served as an important parameter to determine nutritional composition or evaluation in food and products [1]. Determination of ash values is an important quality control parameter for allopathic drugs [2]. Ashing is the primary pre-treatment step to remove the organic framework. Ashing serves as a sample preparation step. There are two types of ashing: ***Wet and Dry***. Dry ashing is achieved by heating a sample in an open crucible or evaporating dish in a muffle furnace at elevated heating temperatures of 500 – 600 °C. Wet ashing is performed by treating the material with some reagents, like sulphuric acid, nitric acid, aqua regia followed by heating. Weight of the ash obtained from the known weight of sample serves as the ash value – a parameter used to characterize plant materials.

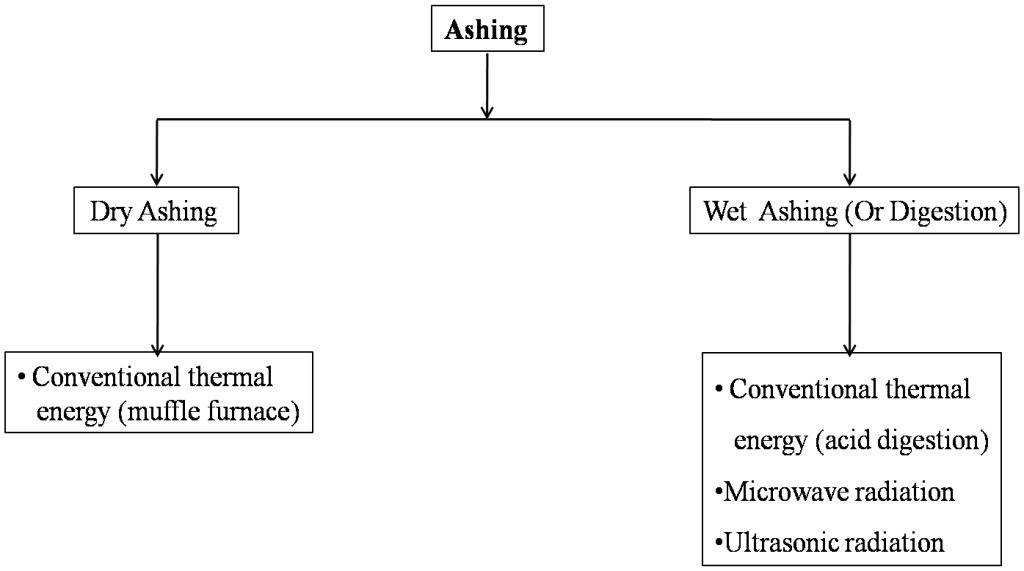


Figure 1 Classification for ashing [ref 3]

The samples are subjected to dry ashing either in a crucible or an open vessel in a muffle furnace [4] for combustion of the organic matter such that the residue remaining after it is the inorganic matter comprising of minerals. All the volatiles are lost and organic matter burned (or oxidized in presence of O_2) to achieve a residue enriched with the mineral content of the sample. All the mineral content after the combustion is now available as their oxides, sulphates, carbonate and silicates. In short, the steps of dry ashing include: evaporation of water and volatiles and oxidation of non-volatile material to remove organics until the organic matter is destroyed. Dry

ashing offers the following advantages: it requires only sample, utilizes fewer apparatus and reagents. It is used due to its easy operation and effective destruction of organic material [5]. Dry ashing is often used for food, meat and biological samples such as algae. The other type of ashing is wet ashing (or digestion). It is primarily used as a sample preparation for mineral analysis in medicinal plants, fly ash samples and such other samples. It requires reagents such as hydrochloric acid (HCl), nitric acid (HNO_3), hydrofluoric acid (HF), etc. This is based on the organic matter's oxidation [3, 6] in the sample using these inorganic acids individually or in combination.

In short, ashing can prove as a pretreatment step to determine the elemental composition in case of medicinal plants. Sample can be subjected to dry ashing such that the organic framework doesn't become a hindrance or observed as interference in the analysis and thus the elemental composition can be determined by conventional analytical techniques such as Flame emission spectrophotometry, Atomic absorption spectrometry. It can prove to be a cost-effective way as less concentrated reagents are used and employed to dissolve the sample and thus, determine the elements in the sample.

Without any such preparation step, determination of essential and trace elements in medicinal plants have been achieved using very sophisticated analytical techniques such as Proton induced X-ray emission (PIXE) and Proton induced γ-ray emission (PIGE) wherein pellets (thick targets) are prepared using powders of specimen and thus analysis is performed [7]. PIXE technique has been used in determination of elemental composition in various parts of medicinal plants [8, 9 and 10]. Elemental compositions of commonly used medicinal plants of north-east India were determined using PIXE-PIGE technique by group of Sarma and co-workers [11]. A group of R Devi and co-workers determined trace and major elements in anti-asthamatic medicinal plants of North-eastern region of India using PIXE and PIGE techniques [12]. Rihawy et al determined and performed elemental analysis using PIXE technique on Syrian medicinal plants traditionally used to enhance body immunity [13]. The use of ion beam analysis (IBA) techniques such as that of PIXE, though the sample preparation involved in the technique is easy, the use of the technique was not extensive in the past due to its expensive operation [13]. Activation analytical techniques such as Instrumental neutron activation analysis (INAA) ion or neutron activation analysis (NAA) [14] have been used for the determination of elemental

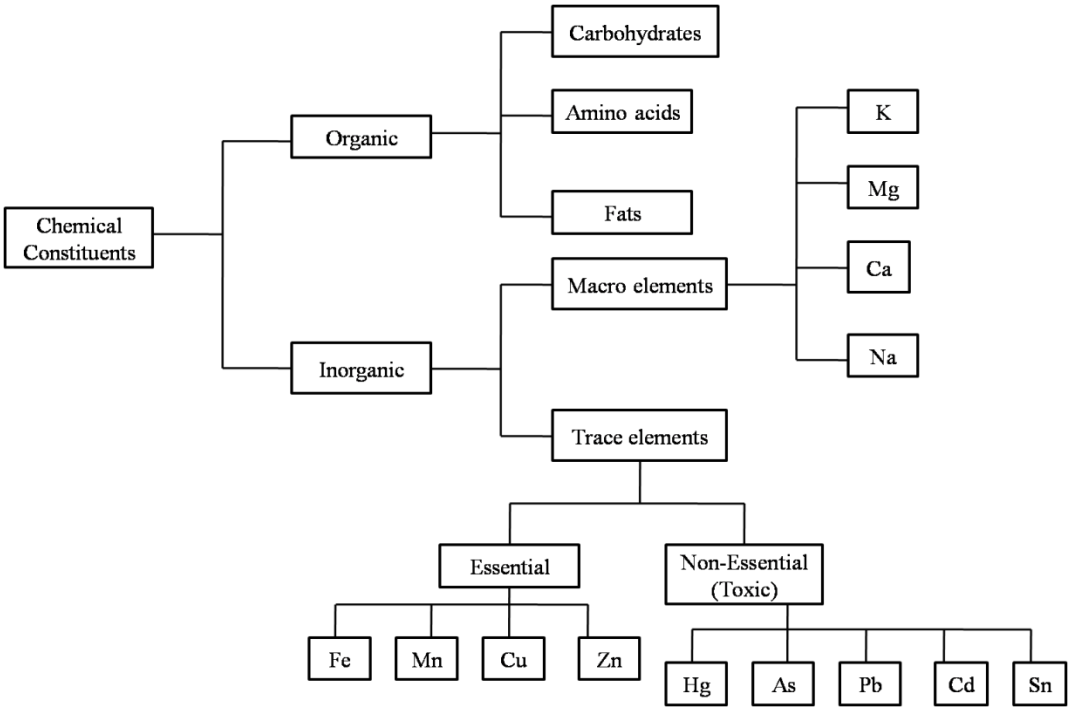
composition in plants at trace levels [14]. In these techniques the plant material is directly subjected for the determination of elemental composition in them.

2b.1.2 Minerals in biological system

Minerals, elements, play a vital role in all living systems though found in trace quantities. These are known to have very serious and major roles in terms of biological activities in growth and development. They are responsible in various metabolic processes. They tend to combine with organic molecules so as to yield an active chemical constituent in the form of a chelate which has biological application. Trace elements have importance in various biochemical processes that occur in living systems. For instance, role of "Fe" in blood complexing with the porphyrin ring and thus forming haemoglobin which acts as O_2 carrier in human system is classic case. Similar is the case with chlorophyll which has a very important role in photosynthesis in plants, which turns out to be a source of food for all the living systems. Plants have been the natural source for all the major and trace elements required by living beings. Plants and plant materials have been extensively used not only as food but as therapeutic for number of diseases. They are the backbone of Ayurvedic medicinal practice. As numbers of drugs are prepared that uses these plant materials or the active ingredient being extracted from them. Nowadays, the awareness amongst people has made herbal medicines more popular because of the advantages that these medicines offer and the less side-effect they cause. Therefore, it becomes necessary to investigate and judge the efficacy and medicinal properties of these plants.

The role of these trace elements in the human system is very crucial; their deficiency can lead to disorders. Not only can that but their excess lead to disorders. Presence of certain toxic elements in very low concentrations can prove to be hazardous. For this reason, reliable techniques need to be developed which can accurately analyse and determine their quantities.

These nutrients have been classified based on their concentration found in them and other biological system (soil, water, etc). Chemical constituents or compounds those are essential for all the living systems for the functioning of their metabolic processes, growth and development are classified as:



According to the classification, when the concentration of the elements is found below 100 mg d^{-1}, is defined as trace elements, which is required by an adult person [15]. Definition for an essential element has been given as "an element is considered essential if the plant fails to grow normally and tocomplete its life cycle, if grown in a medium adequately removedfrom the same, whereas in adequate concentration of the element it grows and reproduces normally [16]. Different classifications are found in literature wherein Fe, Mn, Cu, Zn are classified as micro, trace and essential elements. All these chemical constituents play a vital and specific roles in all the living systems. Though the elemental constituents or nutrients are required in small quantities, they are essentially required and their absence or deficit can lead to disorders. The specific functions and roles played by them are listed in the table 1 [1].

These elements thus, play a very important role in all the biological processes in all the living systems, sourced from plants majorly which in turn reaches all the dependent living bodies as food. Major credit or importance has been given to the organics in the Ayurvedic preparations (extracts, tinctures, etc), as their efficacy has been directly related to these organics but these elemental constituents can as well have some specific role being bound to the organics (maybe as chelate or complex).

Elements	*Role and Functions In humans*
Na K	Essential electrolyte for maintaining normal fluid balance in cells. A delicate balance of K & Mg is reported to prevent an increase in blood pressure & maintain normal cardiac rhythm.
Ca	Involved in muscle contraction and relaxation, blood clotting, proper nerve function and body immune defenses.
Fe	Functioning of haemoglobin and RBC production
Zn	Functioning of enzymes, DNA synthesis, supporting healthy immune system, liver function
Cu	Functioning of nervous system,Essential for various biochemical processes, involved in functioning of nervous system and maintains the balance of other useful trace elements.
Mn	As antioxidant and production of energy
Cr	Required for normal carbohydrate metabolism & a critical cofactor for insulin action

Table 1 Role of minerals in humans

Before their activity is considered, their consntrations needs to be determined. With this view, the study has been taken up and the ash prepared from Manjistha root has been used as the enriched material to determine their concentration using the basic absorption and emission analytical techniques and their comparison with the sophisticated ones. The ash and the root powder have been characterized by analytical techniques to determine the constitution of the root powder and ash. The inquisitiveness to know if the ash obtained is not white, does it still have any residual carbon remaining in it and using this ash as a pre-concentration step to enrich the elemental constituents and determine them using the basic analytical techniques.

In order to use simpler analytical tools for determining elemental composition of plant material, we have used a step of ashing, followed by determination.

2b.2 Preparation of ash and optimization of parameters

To heal wounds, to cure diseases, over ages man used them and gained the knowledge and knowhow of using the medicinal plants. Various applications have been documented by our ancestors and their knowledge has been carried forward in the form of Ayurveda which scripts down the pros and cons of plants. *Rubia cordifolia* L. is one such medicinal plant to have various beneficial effects and its ethanol root extract has been known to reduce blood sugar in alloxan diabetic animals, and to prevent cold stress-induced gastric ulcers [17]. It contains a number of chemical constituents such as rubytheric acid, number of anthraquinones (alizarin, purpurin, munjistin, rubiadin), naphthaquinones, bicyclic hexapeptides, gallic acid and tannins, etc [17]. A huge amount of research has been done for the studies relating contribution of organics in the medicinal plants but the role of inorganics has not been explored much. Before addressing the role of inorganics in medicinal properties, their determination is necessary, as little has been known about the inorganic elemental composition of such plants, as also for *Rubia cordifolia* L. Techniques such as PIXE, XRF, ICP – OES have been used to determine the composition in the plants either by directly subjecting the plant material or by digesting the plant material. We have evaluated the elemental composition by the conventional analytical techniques such as Flame emission spectrometry and Atomic absorption spectrometry (AAS) and compared the obtained results with the sophisticated analytical technique- inductively coupled plasma – optical emission spectrometry (ICP – OES). To determine the elemental composition by the conventional techniques, ashing served as the enrichment step to enhance the concentration and make their determination in the sample easy and without interference as the organic framework is removed.

It can be questioned as to why perform ashing when directly the plant samples can be subjected to sophisticated analytical techniques. Conventional analytical techniques can be useful but the limitation lies in the complex plant matrix which makes the determination of elements difficult and not as efficient and the results reliable as exhibited by the advanced techniques. Further, results will also vary because of uneven distribution of the elements within the parts of plant body. To overcome this issue and make the basic techniques applicable, removal of the organics from the plant matrix is achieved by “ashing”, as it serves as a pre-

concentration step and enriches the ash with the elements to be determined. In absence of sophisticated analytical techniques, this cost effective and easily accessible technique can be used.

For characterization of plant material, several tests are performed like ash value, loss on drying, alcohol extracts, ash extracts etc. This work also describes using such tests of plant material characterization and comparing them with instrumental methods and see if the former can be replaced with later.

2b.2.1 Materials and instrumentation

Manjistha plant roots were procured from Manikarnika Aushadhalya, Pune. They were washed extensively with tap water and then with distilled water to ensure the removal of soil and dust particles. After initial sun drying, the material was dried at 80 °C for 24 hours in oven. A sample of the material was sent for Authentication and remaining was ground to powder in domestic mixer grinder. This powder was sieved in 0.425 mm mesh to obtain fine powder and this was labeled as "**M1**". The roots have been authenticated from the Botany Department of Maharaja Sayajirao University of Baroda, Vadodara and the herbarium of roots and root powder have been preserved as herbarium with reference number NK 03. Manjistha root powder was procured from a shop named "Saraiya" in local market of Vadodara, Gujarat and used directly as purchased and it was labeled as "**M2**".All the chemicals and solvents used for the preparation of samples for analysis are of analytical grade and were used as purchased. The ground root powder was sieved using Jayant make sieves, ASTM nos 40 and 80. **M1** was sieved through 0.425 mm mesh (ASTM no 40) and this was used in all studies. The other sieve used was of 0.180 mm (ASTM no 80), to study the effect of particle size of ash.

A BLS make muffle furnace with maximum temperature of 1000 °C was used to prepare ash. ^{1}H NMR spectra were recorded using $CDCl_3$ as a solvent on a Bruker AR X 400 Avance instrument at 400 MHz. The Infrared spectra were recorded using KBr pellets in the range of 400 – 4000 cm^{-1}on Bruker alpha FTIR system. TGA of the samples were performed using SII TG/DTA6300 EXSTAR instrument. M1 sample (1.673 mg) was heated up to 800 °C at a heating rate of 10 °C min^{-1}in platinum pan under inert N_2 atmosphere. The thermogram obtained was a plot of weight loss as a function of temperature. Systronics flame photometer equipped with Systronics FPM

125 digital meter, having sodium and potassium filters, was used for the analysis of these metal ions. ICP-OES analysis was carried out using Thermo Scientific instrument (Thermo Fisher Scientific-6300 duo) with plasma power of 1150 W, concentric nebulizer and a CCD detector. Nebulizer and auxiliary gas flow of 1.0 mL min^{-1} and 0.5 mL min^{-1} respectively were used. XRF analysis was carried out using EDX – 800, Shimadzu (Japan) equipped with rhodium target having 5 to 50 KV voltages and 1 to 1000 µA current ranges (applied 50 KV and 1000 µA) to analyze the root powder and its ash. An Airix STR 500 Micro Raman system was used to record the spectrum of the root powder and its ash. Excitation at 532 nm was provided by the laser (power 50 mW) for an exposure time of 3 minutes. Atomic absorption spectrophotometric determination was performed on a Perkin Elmer, AAnalyst 200 model for Cu and Zn determination

2b.2.2 Method

a. Preparation of ash (Total ash)

A fused silica crucible was pre-conditioned by heating at 100 °C for 1 hour in order to remove any contaminants, allowed to cool for 30 minutes in a desiccator. It was weighed and the procedure was repeated till constant weight was achieved (to the nearest 0.1 mg). 2.0 g root powder of *Rubia cordifolia* L. was placed in the pre-heated, pre-weighed, crucible and heated in muffle furnace at 550 °C for 3 hours, allowed to cool in desiccator and weighed. The difference in weight was computed as the weight of ash and further computed in terms of percentage (%). The general scheme for the ashing procedure is depicted in the figure 2.1.

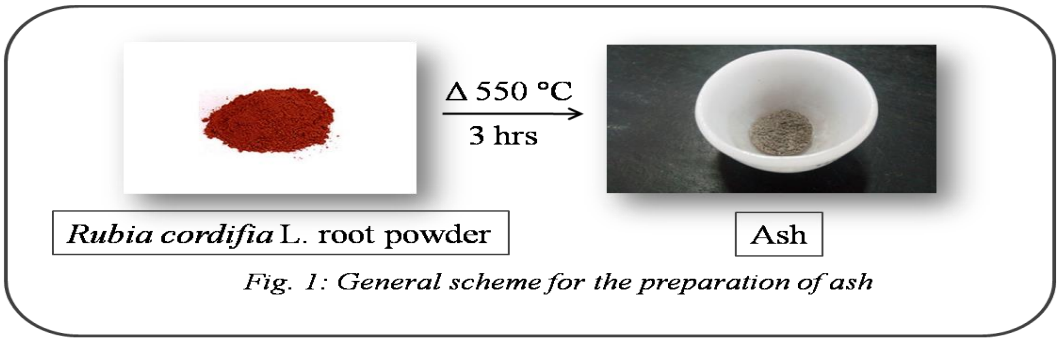


Fig. 1: General scheme for the preparation of ash

b. Loss on drying (LOD) (or moisture content)

LOD of the plant sample was determined by drying 5.0 g root powder in an already pre-conditioned pre-weighed porcelain evaporating dish by heating at 100 °C so as to remove burnable contaminants or volatiles and achieve constancy in weight

of the dish. The evaporating dish was then placed in a conventional heating oven at 105 °C for 5 hours, as the method in Pharmacopoeia. The loss in weight of the sample was determined as loss on drying (or moisture content) or loss of moisture by drying.

c. Extract ash

In a stoppered conical flask, 2.0 g root powder was weighed and 10 mL of solvent (CH_3OH and absolute alcohol) was added, kept at room temperature for extraction with occasional stirring for 24 hours. The extract was filtered through Whatmann® filter paper no.1 into a pre-weighed and conditioned crucible (as described in section a). The filtrate was allowed to dry at room temperature and then the crucible was heated in a muffle furnace for 3 hours at 550 °C, to result in methanol and absolute alcohol extract ash **(MEA and AEA** respectively**)**. Also, the residue that remained after filtration of extract was dried and was separately subjected to heating in the muffle furnace at the same temperature conditions and weighed, to result in ash of residue after methanol extraction.

d. Alcohol soluble extractive

To 5.0 g of root powder, 100 mL of absolute alcohol was added and kept for extraction at room temperature for 24 hours with shaking for initial 6 hours and allowed to stand for 18 hours. Filtered using Whatmann® filter paper no. 1, 25 mL of the filtrate was evaporated to dryness at 105 °C in a petri dish. Alcohol soluble extractive was calculated as the percentage extractive with reference to the initial sample taken.

e. Acid insoluble ash

In a crucible containing total ash, 25 mL of dilute HCl was added. After stirring the solution, it was filtered on an ashless filter paper (Whatmann filter paper no.41) and washed with hot water until neutralized. The filter paper containing the insoluble matter was transferred to a crucible and ignited to constant weight to yield acid insoluble ash content which was calculated with reference to the initial ash sample weighed.

f. Water soluble ash

Water soluble ash was determined by boiling the total ash with 25 mL distilled water, cooled, washed with water and filtered using ash less filter paper no. 41. The insoluble matter on the filter paper was ignited for 15 minutes at temperature 400 °C

(not exceeding 450 °C). The water soluble ash was calculated by subtracting the weight of insoluble matter from the weight of total ash, the difference in weight represents the water soluble ash. Its percentage was calculated with reference to the initial root sample.

All these physic-chemical properties of Manjistha root sample as determined by the pharmacopoeia method (2b.1.3) have been tabulated in the table for both the samples M1 and M2.

g. Sulphated ash

Sulphated ash was determined by moistening 2.0 g root powder sample with 1 mL concentrated H_2SO_4, heating it till all white fumes vanish in a fume hood and then heating it in muffle furnace at 550 °C for 3 hours. Weight of the ash obtained was converted to ash value reported in %.

h. Walkley black titration

To the black coarse ash (TA-S-III series) sample 10 mL of 0.167 M $K_2Cr_2O_7$ solution was added. To this 20 mL H_2SO_4 was added and the solution was swirled. It was allowed to stand for 30 minutes, avoiding excessive swirling. Then, 200 mL distilled water was added, further 10 mL 85% H_3PO_4 and 0.2 g NaF (0.199 g-amount weighed) were added. 10 drops ferroin indicator just before titration was added and then titrated with 0.5 M Fe^{2+} solution to achieve burgundy end point. The colour of the solution at the beginning is yellow-orange to dark green, depending on the amount of unreacted $Cr_2O_7^{2-}$ remaining, which shifts to a turbid grey before the endpoint and then changes sharply to a wine red colour at the endpoint. Similar titration was carried out with TA series ash sample, but no colour change was observed even after 50 mL FAS solution was consumed. Similarly, a blank titration without the sample was performed keeping all the conditions same [18].

2b.2.3. Results and discussion

The work was initiated by the identification of the plant material by determining and comparing its physico-chemical properties with the reported values. For this the physico-chemical properties namely total ash content (TA), loss on drying (LOD), acid soluble ash, acid insoluble ash, and alcohol soluble extractive were determined (table 1). For the determination of total ash, the procedure described in the pharmacopoeia [2] was followed which described heating the root powder **(M1 in our**

case) at temperature not exceeding 600 °C. In this way, if carbon free ash cannot be obtained. Exhaust the charred mass with hot water, on an ashless filter paper collect the residue, incinerate both of them (the residue and filter paper), add the filtrate, evaporate to dryness, and ignite at a temperature not exceeding 600 °C [2]. Even on performing this step, the colour of the ash remained same and there was no change observed.

Physico-chemical Properties	**Literature value**	**Observed values (%)**	
		M1	**M2**
1. Total ash	< 12.0%	8.97	7.28
2. Loss on drying (LOD)	< 5.0%	4.29	4.97
3. Acid insoluble ash	0.5 %	0.48	0.61
4. Water soluble ash	2.93	2.65	3.19
5. Alcohol soluble extractive	Not less than 3.0	5.74	6.10
6. Sulphated ash	NA	3.79	4.34
7. Hot water extractable matter [17]	15.55	10.69	9.82

Table 2 Physico-chemical properties of Rubia cordifolia L. M1 and M2, comparison with literature values (NA = Not Available)

During this experimental procedure, it was observed that even for long heating hours, the ash obtained was crème coloured, that sometimes contained black particles and was not uniform and when the same procedure was applied on sample **M2**, a white coloured ash was observed. Also, the colour and texture of ash along with their % ash values varied during the repeated experiment for the same sourced sample (M1). This led us to optimize the ashing procedure and to know what exactly constituted the ash. Also, this had led us to investigate the elemental composition in the plant material as discussed in section 2b.3. The ash produced during the experiments was thus scrutinized on the basis of appearance, texture, uniformity and of course % ash values and variance in % ash content. In order to study the effect of change on ash and its % ash values and to optimize the method parameters such as effect of heating time at same temperature, effect of heating temperature, change in the geometry of vessel, effect of particle size of sample for ashing have been studied.

i. Effect of heating time (or duration):

The effect of change in heating time (or duration) at the same temperature was studied to observe changes in the nature of ash. Fulfilling the criteria of Ayurvedic pharmacopoeia [2], the temperature chosen was 550 °C, not exceeding 600 °C. Sample **M1** was heated at 550 °C and the effect of heating time was varied from 3, 6 and 9 hours. The ash content (% ash) determined have been shown in table 2 along with the photographs of the resulting ash. By varying the heating time of the sample for ashing, it was observed that ash content for the same sample varied, though in all the cases it was below 10.0% (as reported in the pharmacopoeia), ash TA-P-3 exhibited the highest value. This indicated that heating the sample for duration of 3 hours 550 °C temperature was sufficient to produce ash with consistent % ash value.

Sample	Time	Temp.	% Ash	%RSD	Image
TA-P-1	3 hrs	**550 °C**	9.08±0.09	1.01	
TA-P-2	6 hrs		9.21±0.13	1.44	
TA-P-3	9 hrs		9.82±0.14	1.39	

Table 3 Effect of heating time for ashing M1 root sample, n=3

The same set of parameters were applied to sample **M2** (locally procured Manjistha root powder), to determine ash content, appearance and also, if the sample procured from the market had the same origin as **M1** (table 3). But it was observed that the root powder **M2** had different origin as the ash values and other physic-chemical parameters were different from that of **M1** but fell within the range/ limits for that of *Rubia cordifolia* L.

Sample	Time	Temp.	% Ash	%RSD	Image
TA-L-1	3 hrs	550°C	7.20±0.13	1.77	
TA-L-2	6 hrs		7.33±0.19	2.65	

Table 4 Effect of heating time (duration) on M2 sample (n = 3)

ii. Effect of heating temperature:

In this study, keeping the heating duration parameter constant, the temperature was varied. The other temperature that was screened for the analysis was 750 °C, it was chosen as all the organic matter would decompose and also to determine if it fell under our scrutiny parameters.

Sample	Time	Temp.	% Ash	%RSD	Image
TA-P-1	3 hrs	550 °C	9.08±0.09	1.01	
TA-P-4		750 °C	9.33±0.26	2.87	

Table 5 Effect of heating temperature, n = 3

It was observed the ash produced on heating the sample at 750 °C, had shrunk and stuck to the base of the crucible, as seen in the photograph (table 4) and the variance observed for TA-P-4 was high compared to TA-P-1. The total ash content for M1 sample was higher at 750 °C compared to that at 550 °C. Therefore, the heating temperature of 550 °C was used in all the further experimental studies.

iii. Effect of sample particle size

This study was carried out with an aim to understand if there is any effect of sample particle size on the ash value and if all the parameters fell under acceptable ranges. The ground Manjistha root powder M1 was sieved through mesh 40 (0.425 mm, resulting in TA-S-I) and 80 (0.180 mm – TA-S-II) and the third sample prepared was coarse powder of M1 (resulting in TA-S-III), which was not sieved. From the data (table 5) generated from the ashing of all these three samples, it was observed

that there was change in the ash content, texture and appearance of all the samples. Sample TA-S-III exhibited the lowest ash content but black coloured (sooty) ash was observed indicating presence of carbon.

Ash Sample	Total ash content	% RSD	Ash Image
TA- S - I (0.425 mm)	9.09 ± 0.06	0.73	
TA- S – II (0.180 mm)	9.28 ± 0.16	1.75	
TA- S –III (coarsely powdered)	6.29 ± 0.34	5.35	

Table 6 Effect of sample particle size, n =3

Hence, for all the studies here onwards sample which was sieved through mesh 40 resulting in sample particle size of 0.425 mm was used.

iv. Effect of geometry of vessel:

This study (table 6) was conducted to observe the effect of shape of the test vessel on the ash texture and value. Therefore, porcelain evaporating dish was used. The sample parameters optimized so far were applied and the sample M1 was heated in the muffle furnace at 550 °C for 3 hours.

Sample	Ash value	% RSD	Ash image
TA-V-1	9.06 % ± 0.08	0.91	

TA-V-5	10.15 % ± 0.61	6.18	

Table 7 Effect of vessel geometry, n = 3

To our least expectation, this study indicated that when the sample was subjected for ashing in a wider mouth vessel, the ash produced was not uniform as it had a combined grey and crème ash, with higher ash content and the variance observed in TA-V-5 series was higher than 5.0 %. This could be attributed to the sample dispersed in a wide mouth vessel leading to inappropriate heating and exposure of the sample which could significantly influence the fusion characteristic of sample [19] that would have resulted in a non-uniform ash.

Further, to our curiosity when sample M1 was extracted with CH_3OH, mixture of absolute alcohol-water (25:75) and chloroform. After filtration, the residue (with filter paper no.41) was placed in an already weighed to constant weight crucible and subjected to heating in muffle furnace at the optimized heating conditions, we observed white coloured ash similar to that as observed with sample **M2.** This could be attributed to extraction of more complex moieties from plant in the extract and thus the residue would have lesser content of more complex moieties and hence more clean burning (table 7).

Ash from sample	Name	Solvent	Residue ash %	Ash Image
M1	TA-P-5	_	8.97	
M1	TA-So-1	CH_3OH	7.03	
M1	TA-So-2	25:75 – Abs. C_2H_5OH: H_2O	7.20	

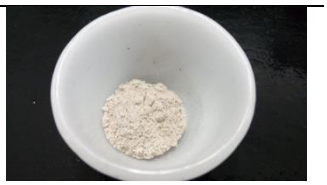
M1	TA-So-3	$CHCl_3$	6.87	
M2	TA-L-3	_	7.17	

Table 8 Total ash for M1 and M2 sample (total ash after extraction in different solvents)

Since most of the Ayurvedic preparations utilize water and alcohol as solvents. Thus, we have prepared ash from sample **M1** with different ratio of alcohol to water and the ash values of ash obtained from extract and the residue were recorded (table 8). And similar observation was recorded that on extraction, the residue achieved when subjected to heating by the optimized parameters resulted in white ash.

Sample name	Solvent Abs. C_2H_5OH: H_2O	% ash (extract)	Residue % ash	Image for residue ash
TA-AW-1	25:75	1.60	7.11	
TA-AW-2	50:50	1.18	7.39	
TA-AW-3	75:25	1.89	7.46	

Table 9 Ash values for extraction of M1 (extract & residue) in Absolute C_2H_5OH & H_2O in different ratios

To verify that there was no organic content present in TA, we prepared the ash by heating the sample M1 to form ash TA-P-5 by the optimized procedure. To confirm whether we have achieved carbon free ash which was not white in colour, characterization of ash using NMR, IR techniques was performed. Also, to know the chemical constitution of ash and affirm with our assumption that even for smaller

duration of heating hours can be sufficient to achieve ash which need not necessarily be white coloured but can be carbon free.

v. Analysis of ash and root powder:

The thermal analysis of the root powder was carried out as an attempt to compare the LOD and ash value amongst the physico-chemical parameters using conventional analysis with the thermal technique described in table 9.

Parameters	Temperature	Permissible limit (%)	% Loss M1 (TGA)	% Loss M1 Ashing	% Loss MPS Ashing	% Loss MLS (TGA)
Loss on drying (LOD)	105 °C	<5.0	4.12 %	4.29	6.16	6.66 %
Organic degradation	250°C	-	36.10 %	-	-	23.09 %
Total ash	550 °C	<10.0	91.03 % (8.96)	9.17	7.20 %	92.63 % (7.37)
	750 °C	-	96.32 %	9.03	NP	94.99 %

Table 10 Comparison of thermal and conventional ashing (NP= Not performed)

The thermal analysis performed for the root sample M1 indicated that at 105 °C, the temperature for determination of LOD, indicated weight loss was comparable with that achieved using the conventional method for LOD as seen in table 9.

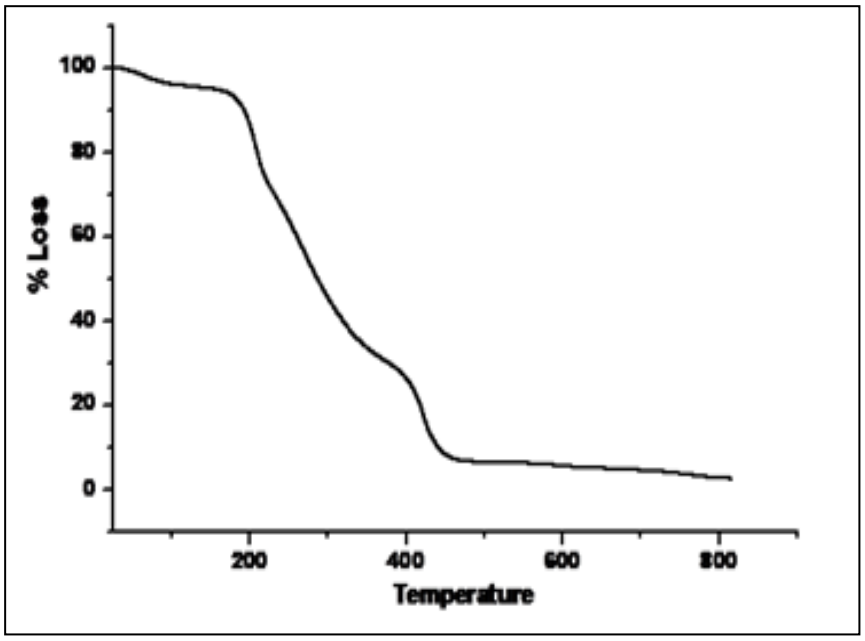


Figure 2 TGA of M1

On heating the sample further in TGA, the weight loss obtained at 550 °C comes out to be 8.96 % which compared well with our values of total ash for M1

obtained by the conventional ashing procedure. Thermogram for sample M1 is shown in figure 2. Similar is the case with sample M2, when subjected to ashing and that determined by TGA exhibited similar trend and the ash values, LOD values were comparable with each other.

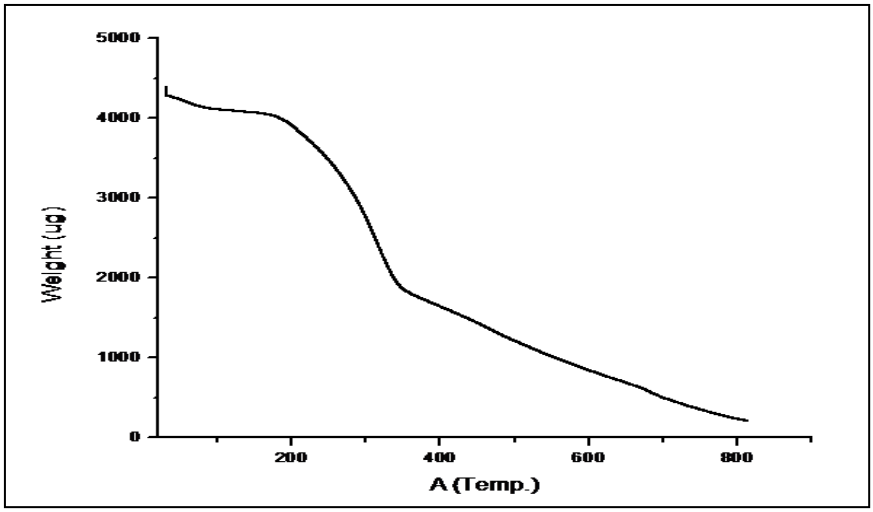


Figure 3 TGA of M2

Also, most of the organic portion of the sample is lost within 600 °C. This study helped us establish the fact that the thermal (TGA) analysis can be used to determine the LOD and total ash content of plant material.

To determine the functional groups in the samples, FT-IR analysis of both root sample and ash samples was performed. Also, a comparison could be performed to know if there is any change on performing the ashing procedure. All the ash samples and even M2 was characterized by IR spectroscopy.

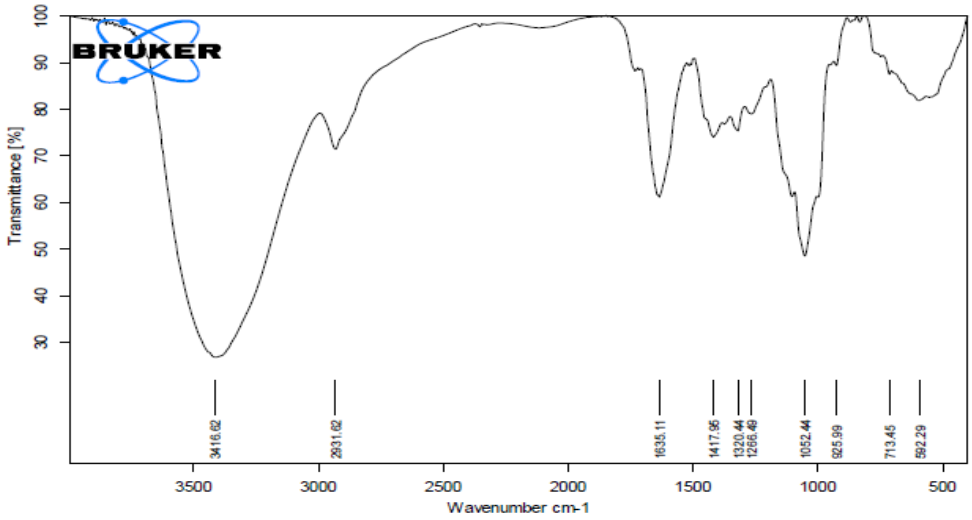


Figure 4 FT-IR analysis of M1

The spectrum of M1 (figure 4) exhibits a peak at 3416 cm^{-1} due to O-H stretch indicating presence of phenolics, flavanoids, anthraquinones (probable functional groups) as observed in figure 2. A small peak at 2931 cm^{-1} is due to CH_3 (alkane) stretch of alkaloids, flavonoids and polyphenols. At 1635 cm^{-1} NH_2 scissoring stretch due to amines indicating the presence of alkaloids, triterpenoids, flavanoids. A small absorption peak at 1417 cm^{-1} was observed due to CH_3-N (1440 – 1410 cm^{-1}) indicating presence of the probable phytochemicals alkaloids, triterpenes, flavanoids. 1266 cm^{-1} and 1320 cm^{-1} were due to >C=C< and CH_2 groups. This together indicated presence of a carboxylic group thus indicating probability of flavanoids, alkaloids. A peak at 1052 cm^{-1} was observed due to O-H stretch of alcohols and phenols and N-H stretch of amines indicating presence of probable phytochemicals coumarins, triterpenes, flavanoids, alkaloids, polyphenols. Similar absorption peaks were observed for sample **M2** (figure 5)**.**

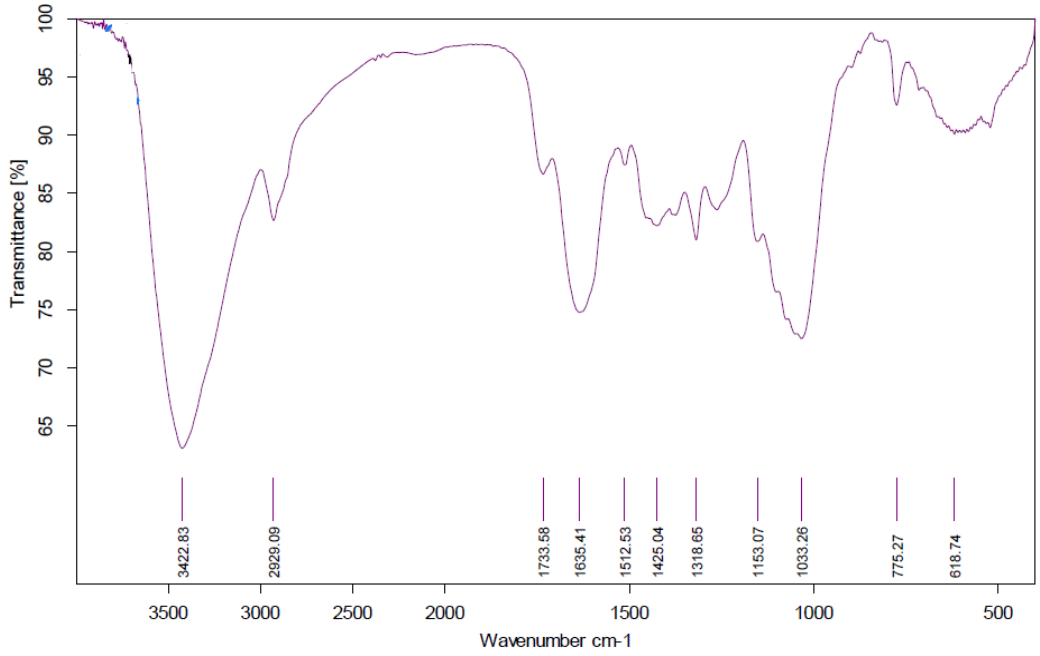


Figure 5 FT-IR analysis of M2

The FT-IR analysis of TA was carried out and its spectrum is exhibited in figure 6 and the peaks 1436 cm^{-1}, 878 $cm^{-1}$and 702 cm^{-1} confirm the presence of C-O stretch due to carbonate as observed in calcite. A weak peak at around 997 cm^{-1} corresponds to Si-O stretch. The limestone minerals are characterized by the following prominent peaks at 1437, 874, 712 cm^{-1} confirming presence of carbonate (figure 6).

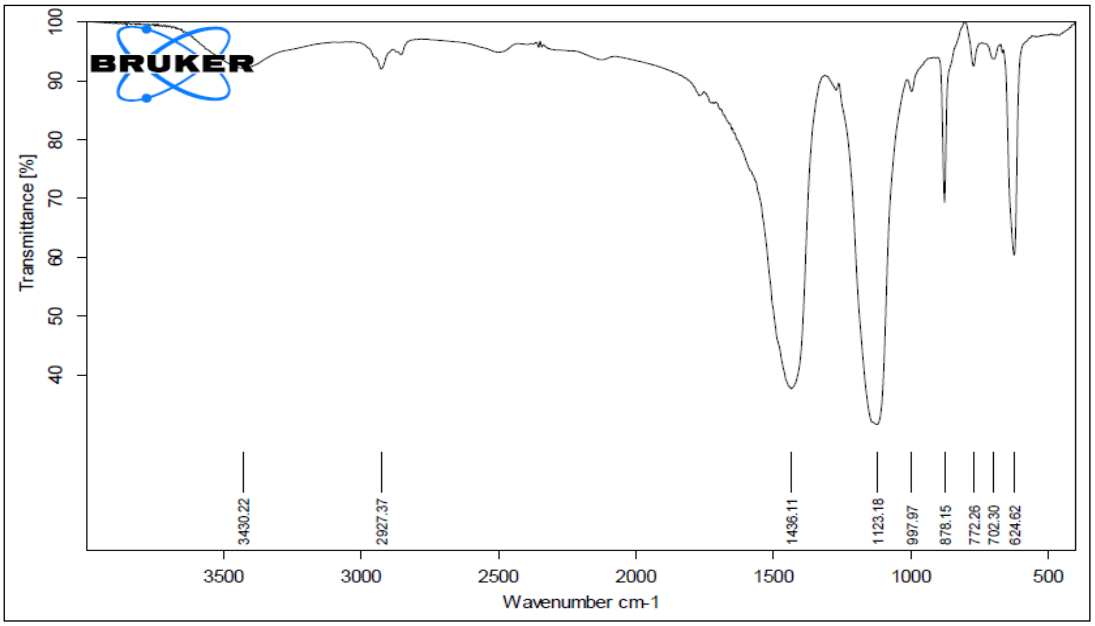


Figure 6 FT-IR of TA

The band positions of all the Raman- and infrared active modes found in the sample match the values of calcite reference bands. The minor shift in positions may be due to the effect of trace metals in the samples. It is observed from the infrared and Raman spectra of limestone that the asymmetric vibration gives rise to a very broad infrared absorption at 1437 cm^{-1} but which appears weak in Raman spectra. The symmetric stretching vibration, on the other hand, gives rise to a very strong Raman band at 1092 cm^{-1}, which is normally inactive in infrared. The frequency values for all the ash prepared have been tabulated in table 10 and 11.

Ash (Frequency in cm^{-1})			**Functional groups**
TA-1	TA-2	TA-3	
3430	3171	3171.81	-OH stretch
1436	1461	1461.97	Vibrational mode of carbonate
997	1034	1031.84	Kaolinite (Si-O stretching (1032 cm^{-1}))
878	871	866.33	C-O bending for calcite
702	708	708	Carbonate
624	687	686	
570	570	565	(560) αFe_2O_3

Table 11 FT-IR data for TA

Ash (Frequency in cm^{-1})		Functional groups	Ref
TA-L-1	TA-L-2		
3223	3067	-OH stretch	
1465	1418	Vibrational mode of carbonate	20
1011	1047	Kaolinite (Si-O stretching (1032 cm^{-1}))	20
864	874	C-O bending for calcite	21
738	708	Carbonate	20
547	464	(560) αFe_2O_3	20

Table 12 FT-IR tableTA-L-1 ash of M2 sample

Samples of root powder (M1) and ash (TA) were subjected to Raman analysis, a sharp peak was observed at 1070 cm^{-1} accompanied with a satellite peak at 1052 cm^{-1} for the ash sample whereas on analysis of the root powder, no signal was recorded as it degraded on application of laser and thus no peak was observed.

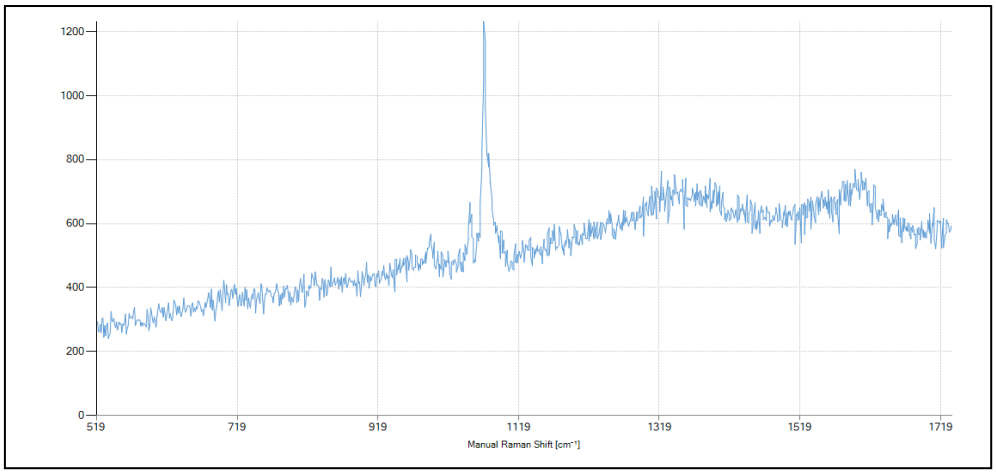


Figure 7 Raman spectrum for TA

The peak obtained at 1070 cm^{-1}, as seen in figure 7, confirms the presence of carbonate, the value matches with the literature value for calcite at 1092 cm^{-1} [22]. The slight shift in value is due to presence of trace metals in the ash sample [22, 23]. Further, the presence of carbonate is evident from the FT-IR analysis.

Thus, the determination of ash content and its comparative study with the root powder has exhibited that the heating time of 3 hours at 550 °C was sufficient enough to remove the organic framework and the residual carbon content remaining in the ash was mainly due to the presence of metal ions in the form of carbonate. To confirm our statement and thought process, NMR analysis of ash sample was carried out to determine that the presence of residual carbon.

The ash sample (TA-1) was treated with nitric acid and extracted with diethyl ether and chloroform. A clean NMR spectrum of the extract as shown in figure 2, with solvent signal at 7.280 ($CDCl_3$) and signals at δ = 1.2 ppm, 1.599 and 3.48 ppm which corresponds to —CH_3 (methyl),–CH (methine) and –CH_2 (methylene) groups of diethyl ether present in trace amounts in the NMR tube, indicated that there was no remarkable presence of any residual carbon (undecomposed carbon) because of ash, which is also indicated by IR analysis (figure 6).

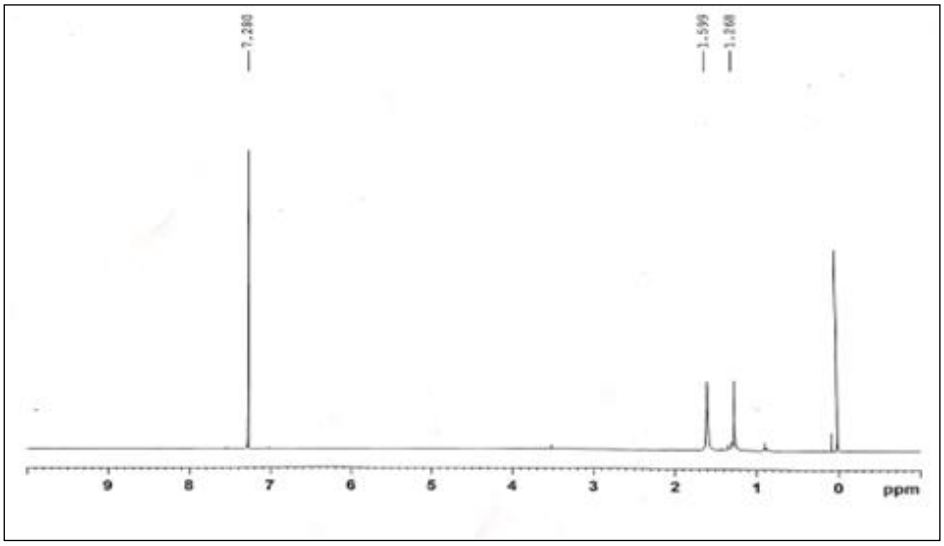


Figure 8 NMR of TA

Thus, it can be concluded that major amount of carbon present was in the form of carbonate in ash, which was qualitatively indicated by the rigorous effervescences obtained on addition of dilute acid to TA. Also, a sharp peak obtained in Raman analysis confirmed the same. Since the colour of ash obtained was not white, to ascertain absence of any organics, this study was undertaken. The study exhibited that in majority of the cases when the blackness in the ash disappeared and even when the ash was not white, the ash was carbon free.

Carbon for the ash samples which had black particles (TA-S-III) and TA-I ash samples were tested by Walkley black titration and the amount of carbon remaining was computed using the following equation A1:

Equation A1: % easily oxidisable organic C:

$$\%\ \mathrm{C} = (B - S) \times \frac{\text{M of ferrous solution } \times 12 \times 100}{\text{amount of sample } \times 4000}$$

where:

B = Burette reading of FAS solution in blank

S = Burette reading of FAS solution in sample containing the plant ash [18]

Reading of titration for sample:

Weight of sample from which ash was prepared	Weight of ash	Burette reading for ash sample (TA-S-III)	Burette reading for blank (without ash sample)
2.0053	0.1321 g	18.0 mL	19.8 mL
2.0031	0.1386 g	18.2 mL	19.6 mL
2.0022	0.1275 g	17.8 mL	19.7 mL
Average		18.0 mL	19.7 mL

$$\%C = \frac{(19.8 - 18.0) \times 0.5\ \text{M} \times 12 \times 100}{0.1321 \times 4000} \quad \textbf{=2.0439\%}$$

The ash prepared from coarsely powdered root material was black in colour (TA-S-III series) and the ash prepared from sieved root powder (TA-I series) was not white in colour. Walkley black titration helped us determine that the TA-S-III series ash had 2.0439 % carbon content present in it while the TA-I series ash though not in white colour had not carbon content present in it. And thus, the sieved powder when subjected to heating at 550 C for 3 hours was sufficient to achieve ash free from organic carbon.

XRD analysis:

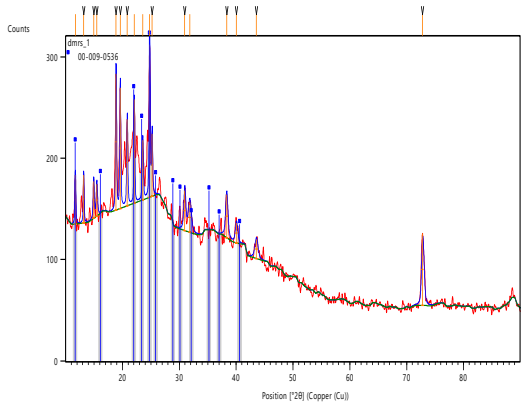


Figure 9 XRD diffractogram for M1

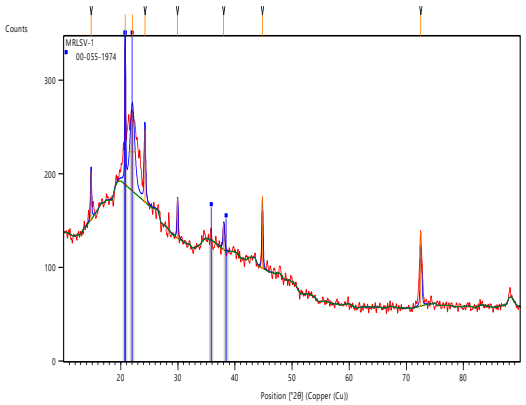


Figure 10 XRD diffractogram for M2

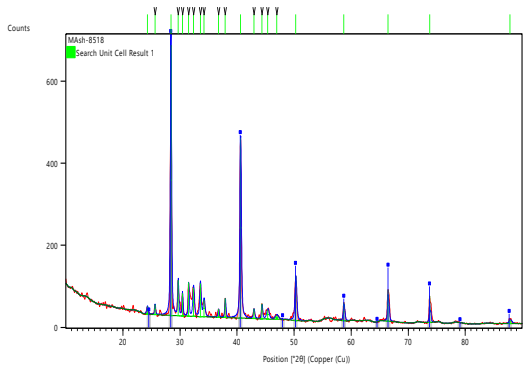


Figure 11 XRD diffractogram for TA-1

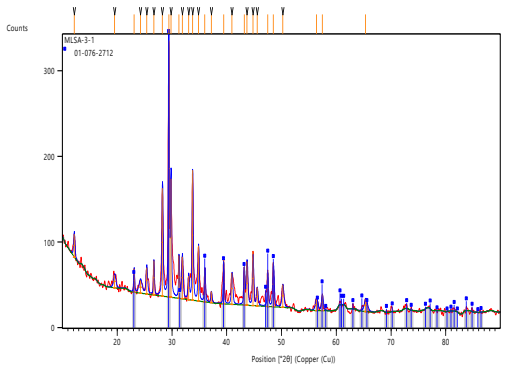


Figure 12 XRD diffractogram for TA-L-1

XRD analysis of M1 and M2 (figure 9 and 10) exhibits amorphous multiphase system due to presence of mixture of organic compounds. This had disappeared in the case of their respective ash samples. Though not a single phase was confirmed from the diffractogram but still the organic framework removed due to ashing was absent. Also, the library search correlated the diffractogram of TA-1 (figure 6) and TA-L-1 (figure 7) with KCl and $CaCO_3$ respectively. This can be further confirmed by comparison with standard $CaCO_3$. Since they are present in not just the form of carbonates but also in the form of elemental oxides and silicates. Thus, a mixture of these elemental carbonates, oxides and silicates in the ash were identified in the data acquired from XRD analysis.

We performed acid hydrolysis of *Rubia cordifolia* L. root powder, for which 2.0050 g root powder was refluxed for half an hour with 2M 50 mL H_2SO_4. On cooling, the solution was filtered and after the solution was allowed to stand for some time, crystals were observed in the filterate. The crystals were separated and allowed to dry after which its melting point was measured. Melting point of the compound was in the range of 120 – 125 °C (124 °C). The material charred afterwards. The crystals were soluble in water.

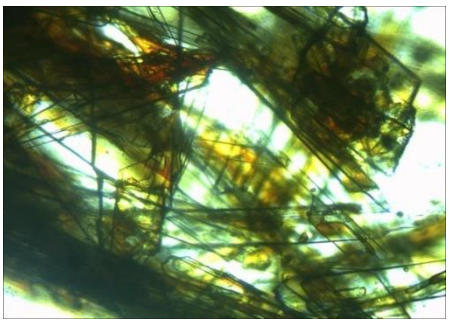


Figure 13 Crystals obtained after acid hydrolysis

Since anthraquinones are present largely as glycosides, we attempted to hydrolyse and isolate and identify the sugar part bound to anthraquinone. The crystals obtained from osazone test observed under the microscope confirm the presence of sugar (disaccharide). The filtrate was partitioned with $CHCl_3$ layer and was subjected to GC-MS analysis and following anthraquinones were found to be present.

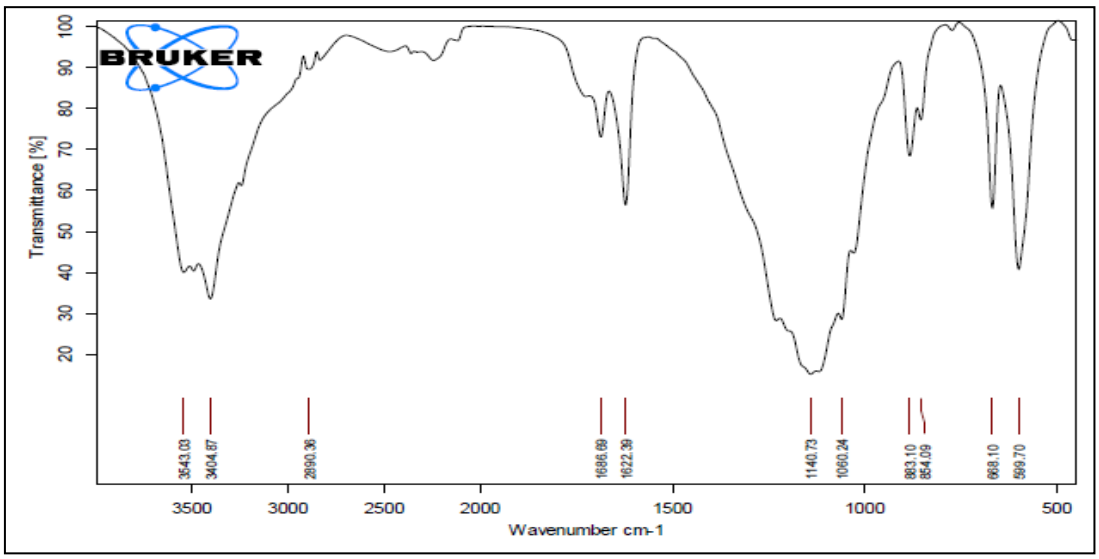


Figure 14 FT-IR spectrum of crystals obtained

Characterization of these crystals was performed by FT-IR analysis. Peak in the region of 3400 cm^{-1} indicated presence of –OH group. Peak at 1686 cm^{-1} exhibited the presence of –C=O group. GC-MS analysis of the chloroform layer exhibited that it contains anthraquinones.

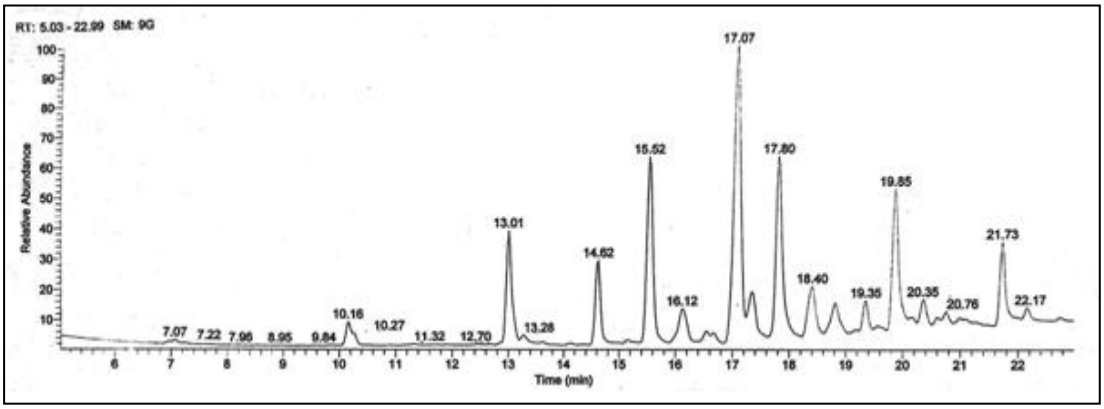


Figure 15 GC-MS of chloroform extract (obtained as a result of partition with aqueous filtrate)

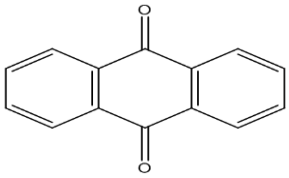
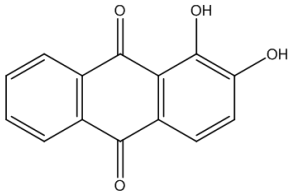
Anthraquinone	Structure	Retention time	Molecular weight
9,10-anthracenedione		13.01	208
1,2-dihydroxyanthracene-9,10-dione (Alizarin)		17.80	240

Table 13 GC-MS data for anthraquinones present in Manjistha

2b.3. Determination of elements in *Rubia cordifolia* L.

2b.3.1 Sample preparation

Individual stock solutions of 100 µg mL^{-1} of NaCl, KCl and $CaCl_2$ were prepared using deionized water. The working standards of the metal ions were prepared in the concentration range of 0.5 µg mL^{-1} to 10 µg mL^{-1} for flame photometric analysis. Similarly, 100 µg mL^{-1} stock solutions of Cu and Zn were prepared using Cu $(OAc)_2$ and $ZnCl_2$ salt respectively. Appropriate dilution of stock solutions resulted in working standards in the range of 0.5 µg mL^{-1} to 5.0µg mL^{-1} which were used for AAS analysis.

The ash prepared with optimized parameters was used for sample preparation for analysis by flame photometric and AAS analyses to determine elemental constituents. These were extracted in dilute acid from M1 (root powder sample) and TA (ash prepared from the root powder) sample. In a crucible, samples were boiled with 2M 25 mL HCl for 5 mins covered with a watch glass. The crucible was allowed to cool, the extracted solution was filtered and the contents over the watch glass were rinsed with 5 mL distilled water. The contents were transferred to a 100 mL volumetric flask and the volume was made up to 100 mL. From this, 2 mL of the solution was taken and further diluted to 100 mL and from this 25 mL of solution was used for the flame analysis.

For ICP-OES analysis, root powder (M1) and ash (TA), were separately treated with 25 mL of 2M HNO_3 each for 5 minutes, cooled to room temperature and

filtered into 100 mL volumetric flask. The filtrates were then diluted to the volume with milli-Q water and a 10 mL aliquot of this solution was used for analysis. Foe AAS analysis, the samples M1 and TA were boiled gently with 2M 25 mL HCl for 5 minutes then filtered and made upto 100 mL using distilled water. From this 25 mL of solution were used directly for the analysis.

For XRF analysis, the root sample and the ash sample were directly subjected without any sample pretreatment to them.

2b.3.2 Results and discussion

We began our experimental work with XRF analysis to determine the distribution of elemental constituents in the root and ash samples.

Root sample M1, M2 and ash samples TA, MEA,MRA and AEA were analysed. To our curiosity, samples M1 and M2 exhibited variation in the distribution of elements as they both have different origin. Thus, this study can be useful to determine if the root samples have different origin and also if there is any adulteration in the root sample, which are sold locally in the Indian market.

Along with these samples, ash of methanolic extract and its residue obtained over filtration was subjected to ashing in similar manner. For medicinal applications, herbal extracts are prepared in solvents of varying polarities; types of organics extracted are different. We assume that this should also affect the composition of inorganics that get extracted along. To verify this argument, all the data for the samples were compared and the results are listed in table no 14. Determination of elements by XRF in the powdered plant material may not show presence of some elements since they may be located inside the bulk of the material. Therefore, ash prepared from the roots was used to determine the elements present.

Elements	M1	M2	TA	MEA	MRA	AEA
			%			
K	47.563	34.848	45.295	47.275	45.494	42.714
Ca	43.489	54.974	29.881	-	39.999	2.382
Fe	4.521	3.165	1.975	0.304	4.406	0.373
Si	1.924	1.501	1.255	0.694	2.291	11.734
S	1.711	1.788	1.057	0.714	1.128	0.868
Sr	0.515	0.360	0.883	-	1.307	-
Zn	0.278	-	0.164	-	0.215	0.048
Ti	-	0.867	0.275	-	0.459	-
Na	-	-	7.753	12.688	-	10.170
Cl	-	-	8.567	37.329	-	31.020
Mg	-	-	1.363	-	2.908	-
P	-	-	1.168	0.509	1.305	0.300
Rb	-	-	0.142	-	0.184	0.083
Br	-	-	0.105	0.362	0.131	0.181
Cu	-	-	0.079	-	0.113	0.051
Zr	-	-	0.038	-	0.059	-
Ni	-	-	-	0.123	-	0.078
Mn	-	2.498	-	-	-	-

Table 14 XRF data for all samples

To our inquisitiveness, in both the samples M1 and M2, four macro elements (K, Ca, S, Si and (Ti in case of M2) and microelement (Fe and Zn in case of M1 and Mn in case of M2) and one non-essential element (Sr) were determined from spectra (figure 16 to 21).

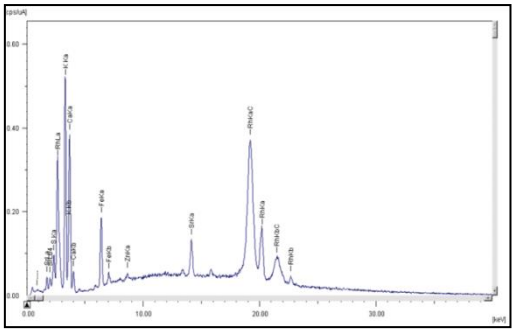


Figure 16 XRF of M1

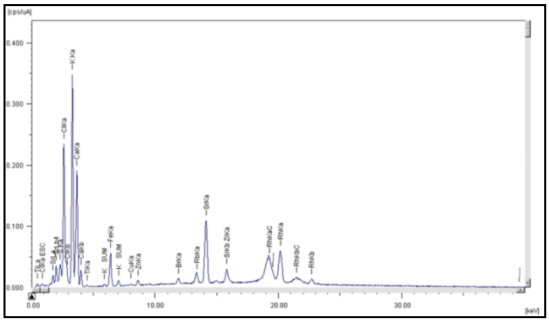


Figure 17 XRF of TA

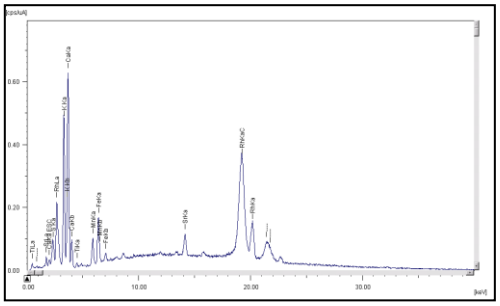


Figure 18 XRF of M2

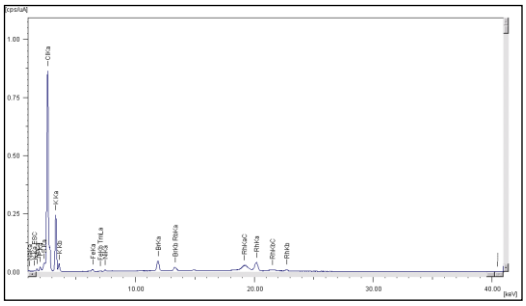


Figure 19 XRF of MEA

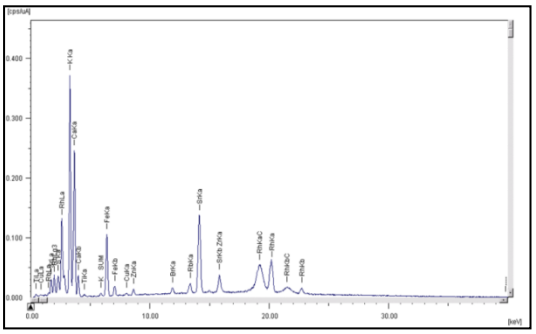


Figure 20 XRF of RA

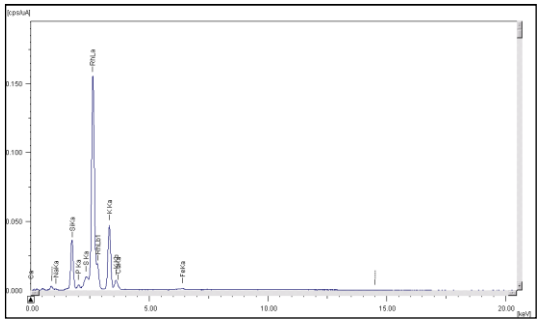
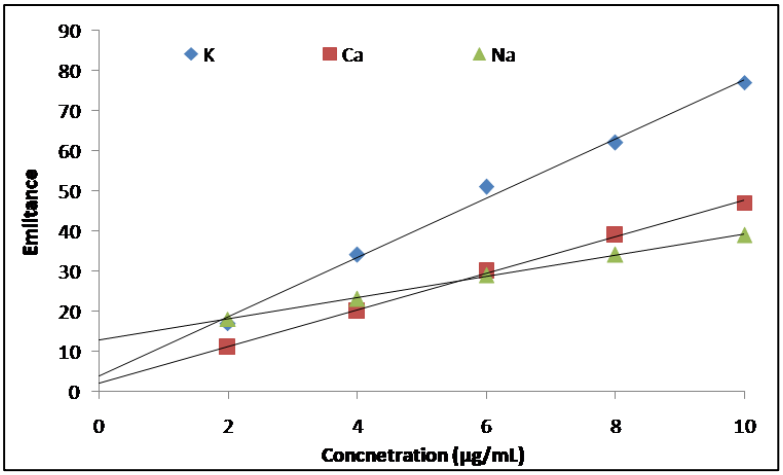


Figure 21 XRF of AEA

The data showed that many trace elements which are not detected by XRF in root powder are detected in ash of the same material. Also, plant material from different places shows different trace elements composition. Hence this can be considered as a useful tool to validate source of plant material. Composition of inorganics in ash prepared from different extracts is different, validating our proposed statement above.

Quantitative estimation of elements sodium, potassium and calcium was carried out using flame photometric technique.

The calibration curve was plotted for the analysis of Na, K and Ca metal ion in the concentration range of 2 to 10 µg mL^{-1} and the metal ions dissolved from ash samples were quantified using these calibration curves, Table 15 shows the statistical data:



Elements	R	y= mx±c	Measured value (in mg g^{-1})					
			Total ash (TA)			M1		
			%RSD	mg g^{-1}	In %	%RSD	mg g^{-1}	In %
Na	0.9989	y= 2.65 x + 12.7	4.65	7.26 ± 0.74	7.40	2.03	1.45 ± 0.37	0.56
K	0.9979	y= 2.95 x + 2.4	2.70	17.7 ±0.57	19.85	4.52	6.99 ± 0.33	1.39
Ca	0.9986	y= 4.55 x + 2.1	3.43	12.24 ± 0.42	7.10	3.45	5.34 ± 0.19	1.07

Table 15 flame photometric determination of M1 and TA

ICP-OES analysis of M1 and its ash (TA) were carried out to estimate and quantify the elements present. It was observed that K was the metal ion present in the highest concentration in both the samples. The other elements estimated were Na, Ca, Cu, Mg, Zn and P. Multi element standards were run in the range of 0.5 to 10 µg ml^{-1} for preparing the calibration curve.

Elements	R	Ash	Root
K	0.9992	20.48%	1.705%
Na	0.9975	9.38%	-
Fe	0.9996	0.27%	0.058%
Si	0.9993	1.38%	0.0909%
Zn	0.9989	0.0307%	0.0106%
Mg	0.9991	2.66%	0.276%
P	0.9969	2.43%	0.224%
Ti	0.9981	0.0127%	0.00067%
Ca	0.9998	9.179%	0.959%
Sr		0.149%	0.0157%
Cu		0.0047%	0.0017%

Table 16 ICP-OES analysis of M1 and TA

Thus, it can be said that in direct determination of elements in the root powder the concentration of most of the elements observed was lower due to the overall matrix effect. To exactly determine the concentration of elements in it, the organic framework needs to be removed. Converting the plant material into ash therefore serves as a preconcentration step. It was observed that macro elements Na, K and Ca were present in highest concentration in root powder of Manjistha with concentration of 9.38%, 20.48% and 9.17 %. And these could be determined by using flame photometry when the root powder was pre-concentrated in the form of its ash.

Elements	R	y = mx ± c	Measured value (µg/g) in M1	In % M1	Measured value (µg/g) in TA	In % TA
Cu	0.9986	y= 0.147x – 0.006	3.6	0.00036	265.3	0.28
Zn	0.9969	y= 0.222x + 0.200	15.09	0.0015	396.2	0.42

Table 17 AAS analysis for Cu and Zn determination

AAS analysis of both M1 and TA was performed for the determination of Cu and Zn. Both Cu and Zn are present in trace amounts and their determination directly in the root powder led to lower values compared to their determination in the ash.

Conclusion

All the studies conducted gave rise to the following optimized parameters of performing the dry ashing by sieving the sample by mesh 40 (0.425 mm) at 550 °C for 3 hours in a fused silica crucible for preparing ash which was further characterized and determination of chemicals that constituted both the root powder and ash sample was performed. The studies and their analysis exhibited that the ash was mainly composed of carbon in the form of carbonate. Also, we were able to separate sugars by acid hydrolysis of Manjistha which was found to be a disaccharide. Anthraquinones were found to be present in the chloroform layer which was extracted with the aqueous filtrate. This confirmed that anthraquionones were present as glycoside in Manjistha.

Roots of *Rubia cordiolia* L. were found to be rich in macro elements K, Na and Ca. The determination of the elements through basic analytical techniques using ash as a precocnetration step are comparable with that of ICP analysis. Cl, Fe, Zn, Cu, Sr, P and Mg were the elements found to be present in trace amounts in the roots of *Rubia cordifolia* L.

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