STANDARDIZATION OF AYURVEDIC DRUGS:
CHARACTERIZATION OF DASAMOOLARISTA

BY

TENNAKOON MUDIYANSELAGE SAMANTHA GOME TENNAKOON

M.Phil 2002
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## ABBREVIATIONS

<table>
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<th>Abbreviation</th>
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<td>5-HMF</td>
<td>5-Hydroxymethylfurfural</td>
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<td>AA</td>
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<tr>
<td>ABA</td>
<td>Abeyarista</td>
</tr>
<tr>
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<td>Asokarista</td>
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<td>AT-225</td>
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<td>Draksharista</td>
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<td>Food Drug and Cosmetic Act</td>
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<td>FP</td>
<td>Fingerprint</td>
</tr>
<tr>
<td>GLC-FP</td>
<td>Gas Liquid Chromatography- Fingerprint</td>
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<td>High performance chromatography- Fingerprint</td>
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CHARACTERIZATION OF DASAMOOLARISTA

TENNAKOON MUDIYANSELAGE SAMANTHA GOME TENNAKOON

ABSTRACT

Ayurveda plays a significant role in the health care system in Sri Lanka. Within the social context in which Ayurveda is practised in the present day, the standardization and quality assurance of Ayurvedic drugs is urgent and imperative.

Dasamoolarista (DMA) is a complex drug containing over 60 ingredients. Tests for identity in complex herbal drugs such as DMA can be devised through chromatographic methods to identify specific marker compounds which can be correlated with specific plant ingredients and representative compounds for groups of plant ingredients. A strategy for identifying such marker compounds by comparing the thin layer chromatograms of drugs (Arista) having closely related formulae, was developed. Thin layer chromatographic systems to detected the following plant ingredients through the marker compounds and representative compounds shown in parenthesis were developed; Aegle marmelos Correa. (Umbelliferone); Plumbago indica L. (Isoshinanolone and Epiisohinanolone); Saussurea lappa C.B. Clarke. (Dehydrocostus lactone and Dihydrodehydrocostus lactone); Glycyrrhiza glabra L., Pterocarpus marsupium Roxb. , and Myroxylon balsamum (L.) Harms. (Isoliquiritigenin); Rubia cordifolia L. (Alizarin and Purpurin); Eugenia caryophyllata Thumb, and Cinnamomum verum Presl (Eugenol);
Woodfordia fruticosa (Linn.) Kurz., Vitis vinifera L. and bees honey (Quercetin and Kaempferol).

It is proposed that tests for strength(potency) of drugs such as DMA of unknown pharmacological action, can consist of quantitative measurements of compounds of high biological activity found in the drug and specific marker compounds irrespective of their biological activity. Analytical methods based on TLC densitometry, HPLC and GLC were used to quantify gallic acid, isoliquiritigenin, umbelliferone, dehydrocostus lactone, dihydrodehydrocostus lactone, 5-hydroxymethylfurfural, isoshinanolone and epiisoshinanolone in DMA. The analytical methods were shown to be precise and accurate. These methods were then used to study the variability in composition of different commercial brands of DMA, and of different manufacturing batches of DMA of the same brand. Inter batch and inter brand variability was high, indicating a significant variation in the quality of crude drugs used in the manufacture of the drug. Changes in the level of dehydrocostus lactone, gallic acid and 5-hydroxymethylfurfural during the different stages of manufacture were monitored. It was concluded that levels of gallic acid and 5-hydroxymethylfurfural were more suitable as parameters for process control than those of dehydrocostus lactone.

Finally, eight chromatographic fingerprints covering a wide range of compounds were developed which could be used for routine quality control, and would provide an overall measure of identity and potency.
1.0 Introduction

1.1 Ayurveda and traditional medicine

The axioms and practices of traditional medical systems vary with the socioreligious and sociocultural structure of indigenous societies of the world.\textsuperscript{1} The World Health Organization (WHO) has given a detailed and comprehensive definition of traditional medicine. As stated by the WHO "Traditional medicine is the totality of all knowledge and practices, whether explicable or not, used in diagnosing, preventing or eliminating a physical, mental or social disequilibrium, which rely exclusively on past experience and observation handed down from generation to generation".\textsuperscript{2} Ayurveda which evolved in India is one of the oldest systems of traditional medicine in the world and has a history of over 3000 years.\textsuperscript{3,4} Ayurveda means "Science of life" or true knowledge of human life. Ayurveda is different from other forms of traditional medicine such as folk medicine due to its theoretical and systematic clinical methodology.\textsuperscript{5} The Ayurvedic system of medicine is described in traditional texts known as samhititas. The samhititas were written originally in Sanskrit. Some of them are now available in translation in several languages including English. Some of the better known samhititas\textsuperscript{5,6,7} are Charaka samhita (describes mainly general medicine), Susruhta samhita (describes mainly surgery) and Kasyapa samhita (describes mainly gynaecology and pediatrics).

1.2 Basic concepts of Ayurvedic medicine\textsuperscript{7,8}

The Ayurvedic system of medicine is founded on the concepts of Tridosa, Sapta Dhatu, Mala, Srota and Agni.
1.2.1 **Tridosha concept**

The *Tridosha* concept is extremely important in the Ayurvedic system of medicine. According to the Charaka samhita, the fundamental components of the human body are *Dosha, Dhatu* and *Mala*. The causes of diseases are disequilibria of the *Dosha* and *Dhatus*. *Dhatus* refer to human tissues which are regulated by *Tridosha*. *Tridosha* is composed of three units named as *Vata, Pitta* and *Kapha*. The *Tridosha*, like everything else in the universe is constituted of the five universal elements known as *Panchamahabutha*. The *Tridosha* and their constituent *Panchamahabutha* are given below:

<table>
<thead>
<tr>
<th>Dosha</th>
<th>Constituent of Panchamahabutha</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vata</td>
<td>Vayu (wind), Akasa (space)</td>
</tr>
<tr>
<td>Pitta</td>
<td>Apa (fire), Teja (water)</td>
</tr>
<tr>
<td>Kapha</td>
<td>Apa (fire), Prithivi (earth)</td>
</tr>
</tbody>
</table>

According to the Ayurvedic texts *Vata Dhosa* is described as a motivator of the body. It maintains all movements of the body such as transmission, respiration, circulation, and excretion etc. *Pitta* is the major factor for regulating thermo genesis, vision, intelligence and courage. The third unit *Kapha* regulates the immunity and resistance against disease. The other role of *Kapha* is to prevent excessive activity of the *Pitta* and *Vata* function in the body.
1.2.2 Saptha dhatu concept

The Sapta dhatu concept relates to the seven histological formations of the body. These are described in the Ayurvedic texts and are given below with the accepted modern equivalent.

1. Sukra dhatu - reproductive tissue
2. Majja dhatu - bone marrow and nervous tissue
3. Asthi dhatu - bone tissue
4. Meda dhatu - fatty tissue
5. Mansa dhatu - muscle tissue
6. Rakta dhatu - blood tissue
7. Rasa dhatu - plasma tissue

1.2.3 Mala Concept

Waste products and excretory products of the body are described as Mala. There are two types of Mala in the body, namely:

1. Ahara Mala - arising from undigested food
2. Dhatu Mala - arising from metabolic activity

1.2.4 Srota concept

Srota is defined as channels or pores in the body either visible or invisible. There are thirteen Srotas described in the Charaka samhita as listed below:

1. Pranavaha Srota - the channels carrying the breath
2. Annavaha Srota - the channels transporting solid and liquids
3. Udakavaha Srota - the channels transporting water
4. Rasavaha Srota - the channels carrying plasma and lymph
5. Rakthavaha Srota - the channels carrying blood
6. Medovaha Srota - the channels supplying the various adipose tissues
7. Majjavaha Srota - the channels supplying the bone marrow and nerves including the brain
8. Sukravaha Srota - the channels carrying the sperm and ova and their nutrient.
9. Purisavaha Srota - the channels carrying faeces
10. Mutravaha Srota - the channels carrying urine
11. Svedavalu Srota - the channels carrying perspiration.
12. Asthivaha Srota - the channels carrying osseous tissue
13. Mamsavaha Srota - the channels carrying component of muscle tissue

1.2.5 Agni concept

Agni is the factor responsible for the digestion of food and the regulation of cellular metabolism. Agni is described to be of four types.
1. Tiksan Agni - strong digestion
2. Manda Agni - weak digestion
3. Visam Agni - irregular digestion
4. Sam Agni - balanced digestion

1.3 Pharmacology in Ayurveda

In Ayurveda, drug action is characterized by the site of action and the nature of action. By site of action is meant the Dosha on which it acts. By nature of action is meant whether it increases, decreases or maintains the relevant Dosha. It is stated in
the Charaka samhita that the physician should have adequate knowledge about any
drug that is needed including, its preparation and usage.9

1.4 Ayurvedic pharmaceutical preparations 10-18

Ayurvedic pharmaceutical preparations are described in Ayurvedic texts such as
Bhaishajyaratnavali, Saranghadhara samhita, Yogaratnakara and Charaka samhita.
Some of the important Ayurvedic dosage forms and preparations are given below:

1. Kasaya

Five types of Kasaya are described in the Ayurveda pharmacopoeia, as given below.
1. Swarasa Kasaya - Expressed juice of fresh plants materials
2. Kalka Kasaya - Paste of dried or fresh plants materials
3. Kwatha Kasaya - Decoction
4. Hima Kasaya - Cold infusion
5. Phanta Kasaya - Infusion

2. Asava and Arista

These are fermented plant extracts. An asava is obtained by fermenting a cold extract
of plants while an arista is obtained by fermenting a decoction.

3. Choorna

The fine dry powder of a single substance or mixture of substances of plant or mineral
origin.

4. Ghritha

Ghirtha are extracts of various materials prepared by heating the material with ghee.

5. Leha (Avleha)

The paste obtained by heating powdered ingredients, sugar and kasaya of crude drugs
6. Thaila
Thaila are prepared by heating the expressed juice of plant materials with oil until all the water is evaporated.

7. Gutika (Vatika)
Sugar, Gugul (Resin of Commiphora mukul (Hook. ex Stocks) Engl.) are heated till molten and mixed with powdered ingredients and formed into pills when cold.

8. Bhasma
The Bhasma is obtained by calcining with other ingredients.

9. Rasa ousadha
Rasa Ousadha are obtained by “Kahajjali” combined with other ingredients. Kahajjali is made by grinding mercury and sulfur.

1.5 Ayurvedic Pharmacopoeias
Most drugs used in Ayurveda are based on plants and pharmacognosy plays an important part in Ayurveda. Much of the pharmacognostic knowledge in Ayurveda is contained in the texts known as “niganthus” the first of which was written around 700 AD. The Danvanthari niganthu which includes about 370 plants is the first medicinal glossary in Ayurveda. The subject matter of the niganthus cover identification of plants, synonyms, pharmacological properties, toxicity and medicinal uses and different systems of weights and measures. The different niganthus describe dosages and methods of preparation of drugs. The niganthus have been written in the regional languages of India. The Rajaniganthu and Madanapala niganthu were considered as master works for medicinal plants. The niganthus of different periods show the advancement of knowledge on medicinal plants.
The Dhantavari niganthu (1300 AD) describes about 490 plants, whereas the Madanapala niganthu (1431 AD) is composed of 820 plants and the Bhan nighanthu ratnakara (1861 AD) includes over 1600 plants. In addition to the niganthus much information on medicinal plants is found in the Charaka and Susruhta samhitas.

1.5.1 Comparison between niganthus and modern pharmacopoeias

The niganthus and other Ayurvedic texts do not describe plants using modern scientific terminology. There are conflicting descriptions of plants given in the different niganthus. For example, the plant *Syzygium cumini* (L.) Skeels. is described as having several different types. The different niganthus and related texts do not agree with each other on the number of types and the names given to them. The Niganthuratnakara describes the five types as jambu, mahajambu, ksudrajambu, kakajambu and bhumijambu. Bhavaprakasa describes also five types as jambu, mahajambu, ksudrajambu, rajajambu and jalajambu. The Rajaniganthu describes only three types as jambu, mahajambu and kakajambu. The Dravayagunavijana describes only three types as rajajambu, ksudrajambu and bhumijambu. Madanadi niganthu describes two types as jambu, and kakajambu. Abhidhanamanjari describes two types as jambu and shrasvaphala. Dhavanthariniganthu describes two types as jambu and kakajambu.

Krisnamurthi has discussed the problems associated with the correlation of traditional knowledge of plants with modern botany. Ayurvedic texts describe the morphology of plants in terms, which are not very much related to modern botany. Different Ayurvedic texts especially the niganthu give descriptions for a particular plant in different ways. One vernacular name is used for more than one single plant,
e.g., “Sanichapusphi” for *Evoluolus alsinoides* L. and *Ciltoria ternatea* L. The converse also happens, i.e., a single plant may be given more than one Sanskrit name, e.g., Bhunimba, Kalamegha, or Cirata for *Andrographis paniculata* Nees. Also some plant names in regional languages are not related to Ayurvedic texts. Under these circumstances it is necessary to correlate the traditional vernacular name with modern scientific nomenclature. In recent times, plant anatomy, palynology, embryology, genetics and chemotaxonomy have contributed to the systematic identification of the plants rather than morphological characters. There are only a few pharmacopoeias and related texts which carry monographs on medicinal plants. Among these are the British pharmacopoeia, the British Herbal Pharmacopoeia, the European Pharmacopoeia, the United State Pharmacopoeia, the National Formularies, the Indian Herbal Pharmacopoeia and the Indian Materia Medica. Many of these pharmacopoeias and related texts are of rather recent origin.

Problems relating to existing pharmacopoeias have been commented on by Penso.²⁶ There are often inconsistencies within the same pharmacopoeia with regard to the parameters used to evaluate the quality of different plants. The test methods are not standardised in the different pharmacopoeias. Not surprisingly different pharmacopoeias sometimes describe in the monographs e.g., for *Aloe* one pharmacopoeia states that the total ignition residue must not exceed 2 % while another mentions 5%. Parts used for therapeutic applications are often different and very different dosages are described in various monographs, e.g., one monograph recommends 8 g for *Mallotus philippinensis* (Lam.) Muell.Arg. and 15 g are indicated in another one. The therapeutic effects attributes to the same medicinal plants are
different in pharmacopoeias, e.g., *Euphorbia pilulifera* C. is described sometimes as a diuretic, sometimes as an antiasthmatic and sometimes as a cardiotonic or as an antigonorrhoeic.

1.6 Traditional medicine (TM) in the world today

In most developing countries about 80% of the population are in rural areas. Since traditional health care systems do not depend heavily on sophisticated technology and the drugs used for common ailments are abundantly available in the country, delivery of health care with TM is easier and cheaper than with modern medicine in these countries. Many developing countries are spending more than 30% of their health budget on modern medicine where the drugs used are manufactured by multinational companies and most of them are imported. As a result, governments in developing countries are faced with much economic pressure and are beginning to support and develop the long-standing traditional systems.

In the past two decades, TM systems around the world have become popular in developed countries as well. Due to the increasing age of the population the incident of chronic diseases have increased and the results of the orthodox medical treatment in these cases are often unsatisfactory. In the developed countries people are disillusioned with their artificial life style and are yearning for a more ‘natural’ way of life. Getting away from synthetic medicine to herbal medicine is an important aspect of this change. The fact that most TM systems use plant based drugs, has contributed to the increasing popularity of TM system. Traditional medicine is considered by the people who use it to be better than modern medicine as the medicines used are not
synthetic, and generally provide long term remedies in cases where modern medicine fails.\textsuperscript{30}

1.6.1 Barriers to Ayurvedic (AV) drugs
The spread of AV drugs in the developed countries is difficult due to competition from native herbalists, lack of qualified Ayurvedic practitioners and inadequate clinical and toxicological evidence for safety and efficacy. The other reason is the strict legislation in these countries. The evaluation of clinical effect and toxicity of drugs is costly in terms of time and money. Most of the Asian countries where the AV system is existing have insufficient resources for clinical and toxicological assays. In the Ayurvedic system of medicine where the synergistic effect of drugs is emphasized only a few single plant treatments were used. Due to the complexity of AV drugs clinical and toxicity evaluation is rather difficult. Often Ayurvedic practitioners prescribe drugs containing 5 or more ingredients. The Food Drug Administration (FDA) in USA and other agencies controlling the import and usage of drugs in advanced countries require well characterized products generally containing a single active ingredient. However, a few pharmaceutical companies in India are successfully expediting the evaluation of Ayurvedic drugs.\textsuperscript{31} For example, LIV 52 a herbo-mineral drug by Himalayan drug company in India is used in many countries as an Ayurvedic drug for treatment of hepatitis and liver disorders. A number of phytopharmaceutical companies, aware of the rapidly changing way of life in modern society, have introduced a more convenient form of drugs for instant relief-viz. herbal Over-The-Counter (OTC) products. OTC drugs are well established and making use of this trend some unscrupulous phytopharmaceutical companies in the Asian countries are gradually invading OTC and the herbal drug market introducing illegal and poisonous
substances for quick profit making. For example, Chinese herbal medicines sold in the Singapore market were found to contain Chlorpheniramine and Dipyrone to increase the efficacy of the drugs. Chlorpheniramine has several side effects and Dipyrone is a banned drug in the world. Further mercury, lead and arsenic toxic metals have been found as impurities in many herbal drugs. This trend is certain to adversely effect the popularity of TM in the world. 32,33

1.7 The status of Ayurveda in Sri Lanka

The Ayurvedic system of medicine has been in existence since 240 B.C. in Sri Lanka. 34 Over 80% of the population in Sri Lanka are spread out in rural areas where the people use the Ayurveda system of medicine and Ayurveda practitioners are highly regarded. Successive governments have understood the importance of Ayurveda in the country. Since 1961 the practice of the Ayurvedic system of medicine in Sri Lanka has being governed under the Ayurveda Act no 31.35 In 1980 the ministry of indigenous medicine was established and it became the governing body for Ayurveda and other traditional systems of medicine. A research institute, the Bandaranayake Memorial Ayurvedic Research Institute (BMARI) was established at Nawinna in 1960 to carry out research related to Ayurveda. Two Ayurvedic educational institutes, the Gampaha Ayurvedic College and the institute of indigenous medicine were upgraded in 1995 to University status.

More than 16000 Ayurvedic practitioners who are registered in the department of Ayurveda are practicing in the country.36 Some of them are traditional Ayurvedic practitioners and others are institutionally qualified practitioners. There are about 46
hospitals practicing Ayurvedic system of medicine and 230 outdoor patient dispensaries scattered all over the Island. There are 100 manufacturers of Ayurvedic medicine in the country of which about 10 could be considered as large scale manufacturers. The government has established a factory, the Ayurvedic drugs corporation, for manufacturing Ayurvedic medicine. All Ayurvedic drugs manufacturers have to be registered in the Ayurveda department and should renew their registration annually. Meanwhile the Ministry of indigenous medicine has published an Ayurveda pharmacopoeia in three volumes (volume one in 1976, volume two in 1982 and volume three in 1985) and an Unani pharmacopoeia (in 1998). In volume one of the Ayurveda pharmacopoeia around 700 recipes of Ayurvedic drugs, drug preparation methods and equipment are specified in detail. Generally it provides a guide for the manufacturing of Ayurvedic drugs. Volume two of the Ayurveda pharmacopoeia describes about 400 medicinal plants, 67 non-herbal materials, 37 animals and by products. It is also lists acceptable substitutes for certain raw materials, provides information on the purification and detoxification of raw materials and gives the modern equivalent of the traditional weight and measures. Volume three deals with properties and actions (Guna) of crude drugs (Dravya).

1.7.1 Health services and health problems in Sri Lanka

Sri Lankan public health services are completely free of charge. Since independence, successive governments have maintained a fairly high level of public health services. In 2000, total health expenditure was Rs 20,696 million and 6,266 western doctors, 1,352 assistant medical practitioners and 16,161 Ayurvedic physicians worked in the health service. The number of government hospitals (practising western medicine)
was increased up to the 578. In addition 389 central dispensaries (practising western medicine) and 40 private hospitals (practising western medicine) are existing in the country. At the end of the year 2000 the persons per doctor was 3197. The health service presently faces a shortage of para medical staff such as nurses, pharmacists and technicians. Changing life styles, demographic transition, poor environmental conditions and weakness in the existing health care delivery system are problems facing public health care system today. Nutritional deficiencies, poverty related diseases, malaria, mental health, heart diseases, physical disabilities, and diabetes are major issues. The newly emerged disease like AIDS and re-emerging diseases, hepatitis, dengue and Japanese encephalitis have become further burden to the health system. Awareness programs for newly emerged and re-emerging diseases and the campaign for the immunization of children against the epidemic diseases are operating throughout the country. Fake allopathic and Ayurvedic practitioners are a newly emerging situation in the country which adversely affect public health.\[36,41,42\]

All allopathic drugs used in state sector hospitals are imported by the State Pharmaceutical Corporation. Many imported drugs are evaluated for the manufacturer specifications by the National Quality Assurance laboratory before they are issued. The Cosmetic Devices and Drugs (CDD) Act No 27 of 1980 covers the manufacture, import, distribution, transport, sale and advertising of allopathic drugs, and cosmetics and medical devices. This act serves to minimize the improper uses of drugs.\[43\]
1.8 Cultivation of medicinal plants

A growing population, increasing popularity of herbal medicine, the commercialization of drug manufacturer and the diminishing of forest cover all contribute to a tremendous pressure on the existing sources of medicinal plants today. The global situation is that many plants such as *Rauwolfia serpentina* (L.) Benth.ex.Kurz. and *Dioscorea deltoidea* Watts. in India, *Ephedra sinica* Stapf.in China, *Artemisia maritima* Strand-Malurt. in Pakistan, *Hyocyamus muticus* L. in Egypt, *Cassia acutifolia* Del. in Ethiopia and *Catharathus roseus* (L.) G.Don. in West Indies are considered as endangered species. In Sri Lanka *Munronia pumilla* Wight., *Saraca asoca* (Roxb.) de Wild., *Strychnos nux-vomica* L. *Capparis moonii* Wight. are considered rare and some of them are nearly extinct. Herbal gatherers collect medicinal plants from wild sources in their country. Day to day there wild resources are being depleted from their natural habitat. Today plant based drug industries invest millions of dollars for gathering herbs in developing countries where biodiversity is the richest in the world. As a result, the world demand for medicinal plants is great and cultivation of medicinal plants has become important. The cultivation of medicinal plants has to take into account future demands of herbal based drug requirements in the world. The present knowledge of cultivation and breeding technology needs to be used to guide the development of cultivars to increase fertilizer responsiveness, and give high yields of active ingredients.

1.9 Need for standardization

All ancient civilizations had a group of persons who were responsible for public health. They prepared medicine and treated patients. They were highly regarded
members of their society, and their work was not influenced by commercial purposes. In Ayurveda, only the experienced Ayurvedic practitioners prepared drugs. Ayurvedic system of pharmacy concentrated more on quality assurance than quality control. In the absence of sophisticated technologies and equipment, they used to monitor the whole process of drug preparation by simple methods. Physical and organoleptic parameters were used to assure the quality of the drug and raw materials. Some times, novel methods such as observing the behaviour of a burning splinter placed in the mouth of the vessel, to determine the end of the fermentation process of arista and asava were used.\textsuperscript{49} At the time the Ayurvedic system of medicine developed there was no standardized scale for measuring amounts of substances. Hence simple, less accurate methods have been used in Ayurveda, e.g., Paddy seeds, Olinda (\textit{Abrus precatorius} L.) seeds, Madatiya (\textit{Adenanthera pavonia} L.) seeds and coins, were used for weighing the raw materials and drugs in ancient Sri Lanka.\textsuperscript{50} The Ayurvedic system of medicine had a well developed pharmacognostic knowledge of raw materials. The system gave priority to the quality of the raw materials to prepare quality medicine. Hence they attached importance to the maturity of the plant at harvesting time, part of the plant harvested, habitat of the plant (e.g., plants should not be collected from dirty place, ant hills, cemetery and flooded areas). Further, collecting time of the plant (e.g., Auspicious time is highly recommend), storage of the plants (e.g., shade dried, storing well in closed, fumigated storing places), the nature of the plants samples (e.g., healthy, non infected) are specified.\textsuperscript{14,51,52}

The Ayurvedic system of medicine describes the methods called “samaskara” by which medicines are prepared. The samaskara consists of two main activities,
“shodana” and “bhaishyajaya kalpana”. The main objective of the shodana processes is to minimize the side effects of the drugs. Various changes are brought about during the shodana process including elimination of impurities. The shodana is a set of physicochemical methods such as washing, boiling, trituration and separation.

The Ayurvedic pharmacist is aware of the plants and minerals, which need to be purified before using as medicine. eg; *Acorus calamus* L., *Aconitum napellus* Linn., *Plumbago indica* L., *Strychnos nux-vomica* L., *Croton tiglium* L., gum of guggul (*Commiphora mukul* (Hook ex Stock) Engl.), *Cannabis sativa* L. and shilajit. For example, *Plumbago indica* L. is purified by dipping in a saturated solution of Ca(OH)$_2$ over night. This extracts the toxic phenolic compounds. Tetrahydro cannabinol (THC) level of *Cannabis* is reduced by heating with ghee. Well defined parameters are not found in shodana processes to manipulate. Ayurvedic pharmacopoeias mentioned that different shodana processes for a single ingredient. Recent studies by the scientists at the National Botanical Research Institute at Lucknow in India, revealed that the level of toxic protein-abrin and toxic alkaloid-hypaphorine decreased when *Abrus precatorius* L. is processed with “Kanji” (Sour gruel).

The procedures by which the crude drugs are converted to the therapeutic dosage are called bhaishyajaya kalpana. The Ayurvedic system of medicine had a well defined concept of quality. The Charaka samhita describes the quality of an ideal drug as follows, “effective in small dose, fast acting, effective against multiple disorder, easily administered, easily digestible and metabolized, pleasant in taste, not producing
any disease as side effect, the drug should not have fatal side effect, smell-odour, and
taste should be pleasant and it should be given in an effective dosage".58

In a sophisticated urban society the demand for finished Ayurvedic products provides
a new opportunity for commercial manufacturer of drugs on a large scale. Today, the
Ayurvedic drugs preparation has shifted from the professional to the commercial
sphere. Competition, profit motive and lack of legally enforceable pharmacopoeial
standards have led to the market being flooded with low quality products.
Standardization and quality control are needed to ensure the quality, safety and
efficacy of the drugs in the market. According to Bonati,59 “Standardization of a
vegetable drug and of a preparation thereof is not just an analytical operation: it does
not end with the identification and assay of an active principle or of a marker.
Standardization signifies the body of information and control that are necessary to
guarantee constancy of composition hence the standardized quality of a
phytopharmaceuticals drug”.59

Safety is an important factor in the usage of herbal drugs. WHO's guidelines for the
assessment of herbal medicines emphasizes the necessity of safety evaluation by risk-
to-benefit ratio analysis for herbal drugs. WHO states that “although experience on
long term use without any evidence of risks may indicate harmlessness of a medicine,
it is not certain in some cases to what extent reliance can be placed solely upon long-
term usage to provide assurance of innocuity in the light of concern generated in
recent years over long term hazards of some herbal medicines”.60
1.9.1 Problems and methods in the standardization of Ayurvedic drugs

1. Raw materials

The quality of the raw materials used has a direct effect on the quality of the final drug. Many problems are encountered in the authentication of plants. For example, the same common Chinese name (Fang Ji) is given for the two plants *Stephania tetrandra* and *Aristolochia fangchi*, out of which the latter is known to be nephrotoxic and carcinogenic. Medicinal plants contain complex mixtures of constituents found in different parts of the plant in different composition. Environmental and genetic factors are mainly responsible for the variation in the levels of the constituents in a plant. It is essential to ensure the reproducible quality of starting material. Variations in the alkaloid content of *Adhatoda vasica* Nees. Illustrate these points. The total alkaloids varied from 2% in August to 0.4-0.6% in March. Further, the big leaf variety of *Adhatoda vasica* Nees had a significantly higher level of alkaloid than in the small leaf variety. The alkaloid content of *Datura metel* L. was shown to vary significantly with altitude. Thus, the restrictions placed on time and place of harvesting plants in the Ayurvedic texts may be significant for certain plants. The substitution of one plant for another is a common problem. The fact that different parts of the plant differ significantly in their chemistry from each other is too well known to require documentation. The substitution of one plant by another is more problematical, as Ayurveda permits substitution. However some of the permitted substituents do not appear to be chemically or phylogenetically connected. For example, *Curcuma longa* L. (Zingiberaceae), for *Berberis aristata* DC. (Berberidaceae) and *Gmelina arborea* Roxb. (Verbenaceae) for *Myrica nangi* Thumb. (Myricaceae).
Non permitted substitutes are adulterants and methods for identifying them need to be
developed. For example, *Cupressus macrocarpa* Hartw. ex Gord., and *Myroxylon*
balsamum (L.) Harms. are common adulterants for *Pterocarpus santalinus* L.f. and *Acacia catechu* Willd. respectively and can be identified by chromatography. Post-harvest conditions influence significantly the levels of the active ingredients in plants and production of mycotoxins. For example, Gupta et al have shown that the Bacoside—A content in the *Bacopa monneria* (L.) Wettst. herbage varied significantly with the method of drying.

The WHO recommends the following of specifications for starting materials for herbal preparations.

- The botanical name, with reference to authors.
- Details of the source of the plant (country or region of origin, and where applicable, method of cultivation, time of harvesting, collection procedures, possible pesticides used, etc).
- Whether the whole plant or only a part is used.
- A description of plant material based on visual and/or microscopical inspection.
- Suitable identification test including, where appropriate, identification tests for known active ingredient, or markers.
- The assay, where appropriate, of constituents of known therapeutic activity or markers.
- Suitable method for determination of possible pesticide contamination and the acceptable limits for such contamination.
- The results of tests for toxic metals and for likely contaminants, foreign materials
and adulterants.

• The results of tests for microbial contamination and aflatoxins.

Any treatment used to reduce fungal/microbial contamination or other infestation should be documented. Instructions on the conduct of such procedures should be available and should include details of the process, tests and limits for residue.

2. Processing\textsuperscript{12,13}

The ayurvedic system of medicine describes various processes for preparing drugs. The quality of the drugs is ensured by process control. All quality parameters used are based on organoleptic and simple physical methods, which do not involve the instruments. Several different physical methods are used for the extraction of raw materials. Hydro-distillation method is used to extract the volatile components from the raw materials. They use simple distillation apparatus for this purpose. The end of the distillation is indicated when dark fumes escape from the exhaust materials. The most common method of extraction is boiling with water. Extraction with cold water by percolation is also used. Hardness of the materials extracted determines the boiling time used to prepare a decoction. Soaking the raw materials overnight and squeezing it carries out maceration. Fat soluble materials are incorporated into drugs first by expressing the juice of fresh raw materials which have been crushed with water, and then heating the aqueous phase with fats or oils. These preparations are known as ghritha and thaila respectively. Residual water affects the quality of the product. The product is tested for residual water by burning in an open flame and noting the absence of sputtering. Ayurvedic pastes (leha) are prepared by mixing finely powdered raw materials with thick syrup of sugar blended with ghee. Fineness of
powdered materials and efficient mixing at the correct stages affect the quality on the final product. Ayurvedic powders (choorna) are made by blending finely powdered ingredients. Fineness of powdered material, homogeneity and moisture content determine the product quality. Metallic powders known as Bhasma are made by calcining a mixture of herbal and mineral materials in a crucible. Traditionally, different test methods are described to determine the final quality of Bhasma. eg: colour, particle size and density as determined by the ability to float on water, and the ability to enter the fine grooms in the skin of the thumb as determined by rubbing the powder between the thumb and the index finger. Traditional Ayurvedic drug manufacturing methodologies are described for cottage level manufacturers. Today, Ayurvedic drug manufacturing is commercialized. Traditional process control methods and the quality parameters are not adequate for large-scale industrial drug manufacture. There is a trend for large-scale manufacturers to modify the processing and quality control methods by using modern technology and science. Raw materials processing such as cutting, chopping, grinding and sieving are carried out by electrically operated machineries while steam jacketed vessels are introduced to accelerate the boiling process. Some manufacturers prepare water extracts by boiling raw materials with water under pressure for a short period. Thereafter the decoction is concentrated in wide mouth stainless steel pan. This method is more economical than the conventional boiling process.

Properly manufactured modern drugs must meet the standards set for identity, purity, quality, and strength (or potency). Standards for the above four tests are described by Banes as follows: “Standards for identity are qualitative criteria designed to ensure
that the proper drug substance has been incorporated as the active ingredient of the
drug. Standards of purity for modern drugs are expressed numerically as maximum
tolerances for designated impurities in the drug, or as the absence of an impurity.
Standards of quality is expressed as a permissible range of value for a measured
property of the drugs. Standards of strength or potency are expressed quantitatively as
the required level for the concentration of the active substance in the drug. The
Federal Food Drug and Cosmetic act (FDC) US describes the testing methods for the
above criteria. The identity of drugs is determined by chemical and physical methods
such as UV and IR spectroscopy and chromatography. The tests for purity depend on
the measurement of quantitives such as the residue on ignition, heavy metals content,
acidity, chloride and sulfate content. Quality is basically evaluated by physical
measurements such as melting range, optical rotation, specific gravity and quantitative
spectra. The strength of drugs is determine by the concentration or activity of the
active component which is determined by various techniques such as gravimetry,
titrimetry, spectrophotometry, colorimetry, spectroscopy and bioassays. 69

Chromatographic techniques are complementary to the non-selective and non-specific
analytical procedures such as total extractives in the total range of assay methods.
Marker compounds and “fingerprints” (FP) play important roles in the
Standardization of Ayurvedic medicine. The use of gas chromatographic fingerprints
for the qualitative and quantitative characterization of essential oils is well known. 70
A chromatographic fingerprint is representative of a group of compounds selected
from the large number present in the drug. A set of properly selected chromatographic
fingerprints can thus provide a “total picture” of a complex mixture, which is
characteristic and specific for that mixture. Marker compounds are those which are characteristic specific for a mixture of compounds. The observation of a previously noted compound in a mixture is then taken to signify the presence of all the compounds that should be present in the mixture. The availability of reasonably priced densitometry equipment in recent times has made thin layer chromatography a very powerful tool in the standardization of complex herbal products.

Efficacy is described as the property of a drug to achieve the desired response and as the primary determinant of the choice of a drug. Scientific validation of the efficacy of herbal drugs is difficult to establish due to many reasons. Most pharmacological studies are focused on a crude extract of a single plant and not a formulated product. The complexity of polyherbal drugs and lack of standard specifications for formulated herbal medicine which are used in clinical studies also present problems. Formulated herbal medicines containing plant extracts are not bioequivalent to the crude extracts of individual plant. Pharmacopoeially recommended dosage levels are not available in commercial Ayurvedic products. WHO's 'guideline for assessment for herbal medicine' has accepted the documentation of the traditional use of herbal preparations as a documentation of efficacy. However the current drive towards evidence based medicine makes clinical evaluation of standardized Ayurvedic drugs a necessity.

1.10 Arista

Arista are fermented products mainly prescribed during the convalescent phase of a disease. Thirty five recipes for different arista have been described in the Ayurvedic pharmacopoeia. Fermentation is traditionally carried out in clay or wooden vessels.
An arista is prepared by allowing a decoction of primary plant materials, mixed with sugar to ferment in the presence of secondary plant materials (Kalka) for 30 days in seasoned wooden or clay vessels. When a fresh clay fermentation vessel is used it has to be seasoned by boiling with *Woodfordia fruticosa* (L.) Kurz flowers, *Terminalia arjuna* Wight & Arn. bark and *Syzygium cumini* (L.) Skeels. bark with water. This treatment is supposed to close larger holes and cracks of the vessel. When a wooden vessel is used the vessel has to be washed with a hot water extract of the above mentioned plant materials. This is followed by fumigating the vessel by burning *Aquilaria malaccensis* Lam. Camphor, *Santalum album* L. *Nardostachys jatamansi* DC. Immediately after that the inside of the vessel is smeared with cow's ghee and dried. Then a paste of *Woodfordia fruticosa* (L.) Kurz. flowers are applied on the interior wall of the vessel and allowed to dry. A well-seasoned fermentation vessel is used for successive fermentations of the same arista, and not washed in between.

Today the majority of large-scale manufacturers do not carry out fermentations in traditionally prepared wooden or clay fermentation vessels and use stainless steel vessels instead. There are three main stages in the preparations of an arista: Preparation of the decoction of primary plants, fermentation of decoction after adding sugar and secondary plants and sedimentation.

### 1.10.1 Preparation of decoction

Water, weighing sixteen times the weight of the finely cut primary raw materials is added and boiled long enough to reduce the volume of water to 1/4\textsuperscript{th} the original volume. The actual sizes of raw materials that are ready to be used for the decoction are not specified in the Ayurveda pharmacopoeia. For our study, cut pieces where the maximum length of any dimension was not greater than 2.5 cm were used. The
boiling time varies from manufacturer to manufacturer. Kroes reported that some manufacturers carried out the boiling process for 5-7 days.\textsuperscript{74} Our reference arista was boiled for 16 to 24 h. Kroes has shown that such prolonged boiling periods reduces the \textit{in vitro} bio-activity of the extract. Shorter boiling times of decoction resulted in higher potencies of the drug.\textsuperscript{75} Further, Manike has shown that over 80\% of the extraction of biologically active low molecular weight compounds is complete in the first 24 h, of the root bark extract of \textit{Adathoda vasica} Nees.\textsuperscript{76}

1.10.2 Fermentation

After the decoction is prepared, it is mixed with sugar, bees honey, an aqueous extract of raisins and powdered plant materials (Secondary plant materials known as kalka) and allowed to ferment in the vessel. After leaving about 10 day the mouth of the fermentation vessel is closed and sealed. Traditionally the sealing was carried out by smearing clay and ghee.\textsuperscript{77} Today, the sealing is carried out mostly by means of a wooden lid. Kroes mentioned that gas production occurs within 2 days after the addition of kalka materials and sugar in Nimbarista. If properly agitated, evolution of gas is over in 5 to 7 days.\textsuperscript{78} About 1/3 of the space of the vessel is unfilled and accommodates the froth that arises during the fermentation process. Traditionally, end of the gas evolution phase was determined by bringing a burning splinter to the mouth of the vessel. If the vigorous fermentation is taking place, the flame is extinguished. After sealing, the vessel is left undisturbed in a dark place. The seal is broken after 30 days from the commencement of the fermentation stage.\textsuperscript{49,77} Kroes isolated \textit{Schizosaccharomyces pombe}, \textit{Zygosaccharomyces baillii} and \textit{Saccharomyces cerevisiae} from the sediments of Nimbarista and other aristas.\textsuperscript{79} Studies of Kroes and Manike, both pointed to the presence of an endogenous invertase in \textit{Woodfordia}
This has now been isolated and characterized by Weerasooriya et al. This invertase shows optimum activity at pH 4–5.\textsuperscript{82} The lowering of the pH by the release of gallic acid from gallotannins serves to provide optimum condition for invertase activity. Flowers of \textit{Woodfordia fruticosa} (L.) Kurz., \textit{Mesua ferrea} L. and \textit{Madhuca longifolia} (L.) J.F. Macbr. are commonly found kalka materials.\textsuperscript{55} Kroes mentioned that bio-transformations occur during the fermentation process of Nimbarista. \textit{Aspergillus niger} strains which were isolated from flowers of \textit{Woodfordia fruticosa} (L.) Kurz. were able to convert gallotannin into gallic acid.\textsuperscript{83} The alcohol levels of commercial Nimbarista are 6-10\% (v/v) and reducing sugar level 22.3-33.5 \% (w/w).\textsuperscript{84} Our studies have shown that some commercial samples of arista had incomplete inversion of sucrose to glucose and fructose. These contained 0.12-1.37 \% (w/w) of sucrose.\textsuperscript{85}

1.10.3 Sedimentation

At the end of fermentation period, the arista is filtered and allowed to stand for 2 to 14 days for sedimentation.\textsuperscript{86} Thereafter, the arista is stored in well closed bottles or clay pots. The medicinal value of the arista is considered to increase with age of the arista.\textsuperscript{77} Kroes studied the storage of Nimbarista at 25° C during 26 months. According to his studies, the \textit{in vitro} bioactivity (complement activity and chemiluminescence effect) did not change substantially during this period.\textsuperscript{87}

1.11 Dasamoolarista (DMA)

Dasamoolarista is a complex poly-herbal fermented Ayurvedic drug, which is one of the most popular Ayurvedic arista in Sri Lanka. The Ayurvedic pharmacopoeia
describes 64 plants for its recipe. The name dasamoola mean 10 roots and refers to the root of *Aegle marmelos* (L) Correa., *Stereospermum suaveolens* (Roxb.) DC., *Oroxylum indicum* Vent., *Gmelina arborea* Roxb., *Premna serratifolia* L., *Tribulus terristris* L., *Aerva lanata* Juss., *Alysicarpus vaginalis* DC., *Solanum xanthocarpum* Schard & Wendl., *Solanum melongena* L. Burm. f. which can be considered the most important ingredients of the drug. Some of the plant ingredients of DMA are controversial. The Ayurvedic pharmacopoeia lists substitutes for genuine plants in case where genuine plants are not available.

The objective of this research project was to develop methods to chemically characterize DMA, both qualitatively and quantitatively and to use these methods for the quality assurance of DMA. The following studies were carried out.

- Development of chromatographic identity tests for DMA through marker and representative compounds

- Isolation, identifications and structure elucidation of the markers and representative compounds.

- Development of methods for the quantitative determination of marker and representative compounds, and use of these methods to evaluate different brands of DMA available in the market.

- Study the fate of selected marker and representative compounds during the manufacturing process

- Develop chromatographic “fingerprints” (FP) for DMA.
2.0 Materials and Methods

2.1 Spectra
Proton magnetic resonance spectra were measured at 200.132 Hz and $^{13}$C NMR spectra were measured at 50.323 Hz on a Bruker AC-F 200 spectrophotometer. TMS was used as the internal standard. IR spectra were recorded on a JASCO 5300 FT-IR spectrophotometer. UV and Visible spectra were recorded on a JASCO V-560 UV-Visible spectrophotometer or Perkin-Elmer 551 double beam spectrophotometer or a Shimadzu 160 A UV-Visible spectrophotometer. Mass spectra were measured on a Hewlett-Packard 5989 A MS and 5890 series ii instruments in the EI mode. The GC column was 5 % diphenyl 95 % dimethylpolysiloxane, 30 m, 0.25 mm ID, film thickness 0.25 μm.

2.2 High performance liquid chromatography (HPLC)
HPLC studies were carried out on a Waters high performance liquid chromatograph equipped with a Waters model 616 quaternary gradient system, 55920 manual injector, and a 57002 Waters model 996 photodiode array (PDA).

2.3 Gas liquid chromatography (GC)
GC studies were carried out on a GL Sciences GC-380 gas chromatograph and Finnigan 9001 gas chromatograph. Detector is flame ionization detector (FID).

2.4 Densitometry
Densitometric studies were carried out on a Shimadzu CS 9000 and CS 9301 PC densitometer.
2.5 Melting points

Melting points were recorded on a Reichert Thermovar hot stage melting block apparatus. All melting points recorded are uncorrected.

2.6 Thin layer chromatography (TLC)

Pre coated (Merck) analytical, preparative and high performance silica plates with a fluorescence indicator were used. Preparative plates were also prepared in the laboratory using Merck silica gel 60 HF254 (15 μm). Layer thickness of analytical and preparative plates were 0.25 mm and 0.5 mm respectively. All analytical plates were pre washed by eluting once with methanol. They were air dried and activated at 110°C for 30 min prior to use.

2.6.1 Chromatographic reagents

i. Anisaldehyde sulphuric acid reagent

To a solution made up of 1 ml of anisaldehyde in 85 ml of methanol, added 2 ml of concentrated sulfuric acid in 10 ml of glacial acetic acid.

ii. Phosphomolybdic acid reagent

Phosphomolybdic acid (250 mg) was dissolved in 50 ml of absolute ethanol.

iii. Natural product/ Polyethylene glycol 4000 (NP/PEG) reagent

Diphenylboric acid-2-aminoethyl ester (Diphenylboric acid β-aminoethyl ester) (1 g) was dissolved in 100 ml of methanol. Polyethylene glycol 4000 (1 g) was dissolved in 100 ml of ethanol.

iv. Ethanolic potassium hydroxide reagent (Bornträger reagent)

Potassium hydroxide pellets (5 g) was dissolved in 100 ml of absolute ethanol.
v. **Ethanolic aluminum hydroxide reagent**

Aluminum chloride hexahydrate (1 g) was dissolved in 100 ml of absolute ethanol.

vi. **Ethanolic n-hexane reagent**

Mixed 50 ml of n-hexane with 50 ml of absolute ethanol.

vii. **Gibbs reagent**

2,6-dichloroquinonechloroimide (1 g) was dissolved in 100 ml of chloroform.

viii. **Folin-Ciocalteu reagent**

Sodium tungstate (10 g) and sodium molybdate (2.5 g) were dissolved in 70 ml of distilled water. Added 5 ml of 85 % phosphoric acid and then added (10 ml) of 36 % hydrochloric acid. The mixture was heated under reflux for 10 h and added 15 g of lithium sulfate, 5 ml distilled water and 1 drop of bromine. The resulting solution was refluxed for 20 min, cooled and made up to 300 ml with distilled water. TLC plates were exposed to ammonia vapour after spraying.

ix. **Zinc-Hydrochloric acid reagent**

Zinc dust was lightly sprinkled evenly on a TLC plate as a suspension in acetone, followed by spraying with 6 M hydrochloric acid.

x. **Lieberman-Burchard (LB) reagent**

Acetic anhydride (5 ml) and 5 ml of sulphuric acid were added carefully to 50 ml of absolute ethanol, while cooling in ice.

xi. **Hydrochloric acid reagent**

Hydrochloric acid (36 % w/w) was used.

xii. **50% Sulfuric acid reagent**

Added carefully 50 g of absolute ethanol to 50 g of sulfuric acid and mixed well.
2.7 Column chromatography

Dry and wet column chromatography were carried out using silica gel 0.04-0.063 mm (E.Merck) using 2.5 x 60 cm and 2 x 60 cm glass columns. In the isolation of flavonoids, silica gel washed with concentrated hydrochloric acid was used. Flash chromatography was carried out over silica gel HF254 (15 μm), at a pressure of one bar, using a glass column of 2.5 x 30 cm.

2.8 Plant materials and other ingredients

Plant materials were obtained from the herb stores at Link Natural Product (Pvt) Ltd. Plants were authenticated by comparison with herbarium specimens at the National Herbarium at Peradeniya, Sri Lanka. *Nardostachys jatamansi* DC. was identified by Dr. A.K.S Rawat of the National Botanical Research institute, at Lucknow, India. *Cryptolepis buchananii* R & S, *Saussurea lappa* C.B. Clarke. and *Inula racemosa* Hook. were identified by Dr. T.N. Srinivasan of the Regional Research Laboratory at Jammu, India. *Plumbago indica* L. samples were collected at Matale, Gampha, Kurunagala, Anuradhapure and Colombo districts in February 1997. Authentic bees honey sample was collected from Kekirawa in Anuradhapura district.

2.9 Dasamoolarista (DMA) samples

DMA samples were purchased from dealers in Colombo and Kandy. Samples prepared and supplied from the Link Natural Products (Pvt) Ltd factory at Dompe were used as reference DMA preparations.
2.12.2 Extract of the phenolic fraction of DMA

The diethyl ether extract of DMA (50 ml) prepared as described in 2.12.1 was made up to 50 ml in diethyl ether and partitioned with 0.5 M potassium hydroxide solution (50 ml x 3). The aqueous phase was acidified with 0.1 M hydrochloric acid (pH = 3-4) and partitioned with diethyl ether (50 ml x 3). The ether extract was dried with anhydrous sodium sulfate (10 g) and evaporated. The residue was dissolved in 1.00 ml of methanol. Two microlitres were applied on the TLC plate.

2.12.3 Aqueous extracts of plant ingredients of DMA

The dried powder (5 g) of the plant ingredient was refluxed with 100 ml of distilled water for 2 h. The extract was filtered thorough a G-4 sintered crucible.

2.12.4 Laboratory scale preparation of the decoction of DMA

Appropriate amounts of the plant ingredients (1 kg) according to the recipe, were heated in a stainless steel vessel with 16 l of water until the volume reduced to 4 l. The decoction was allowed to cool to room temperature, filtered through cotton wool and centrifuged.

2.13 Isolation of marker and representative compounds

2.13.1 Kaempferol (i) and Quercetin (ii)

The dried flower of *Woodfordia fruticosa* (L.) Kurz. (800 g) was extracted in a soxhlet apparatus with 1000 ml of methanol for 48 h, and the methanol was evaporated under reduced pressure to obtain 119 g of a sticky solid. Ten grams of the solid was heated under reflux with 250 ml of 2 M hydrochloric acid for 1 h. The
solution was extracted with ethyl acetate (250 ml x 4). The residue obtained by evaporation of the ethyl acetate (3.5 g) was chromatographed on a dry silica column, using a methylene chloride–ethyl acetate gradient elution. Fractions (125 x 30 ml) were collected and compounds detected on TLC using as the solvent system, toluene/diethyl ether (1:1), saturated with 10 % acetic acid ($R_f$ kaempferol = 0.33, $R_f$ quercetin = 0.55) and visualized by the natural product reagent. Kaempferol and quercetin eluted with methylene chloride/ethyl acetate (98: 2) and methylene chloride/ethyl acetate (95:5) respectively. The compounds were crystallized from n-hexane/acetone (8:1) (Kaempferol 90 mg, Quercetin 25 mg). Purity of the compounds were checked by TLC using several systems.

**Kaempferol (i)**

mp: 250 °C (276-278 °C lit.$^{92}$)

$\lambda_{MeOH max}^{\text{nm}}$: 252 (sh), 267, 294 (sh), 322 (sh), 365

$^1$H NMR (CD$_3$COCD$_3$): $\delta$ 6.2 (1H, $d$, $J$ = 2 Hz, H-6), 6.5 (1H, $d$, $J$ = 2 Hz, H-8), 7.0 (1H, $d$, $J$ = 8.5 Hz, H-3’ H-5’), 8.2 (1H, $d$, $J$ = 9 Hz, H-6’), 8.2 (1H, $d$, $J$ = 9 Hz, H-2’), 12.15 (1H, s, 5-OH).

$^{13}$C NMR (CD$_3$COCD$_3$): $\delta$ 146.98 (C-2), 136.60 (C-3), 176.55 (C-4), 162.26 (C-5), 99.13 (C-6), 164.96 (C-7), 94.45 (C-8), 157.73 (C-9), 104.10(C-10), 123.26 (C-1’), 130.42 (C-2’, C-6’), 116.29 (C-3’, C-5’), 160.14 (C-4’).

**Quercetin (ii)**

mp: 278-280 °C (313-314°C lit.$^{93}$)

$\lambda_{MeOH max}^{\text{nm}}$: 257, 269 (sh), 301(sh), 370

$^1$H NMR (CD$_3$COCD$_3$): $\delta$ 6.3 (1H, $d$, $J$ = 2 Hz, H-6), 6.51 (1H, $d$, $J$ = 2 Hz, H-8), 7.0 (1H, $d$, $J$ = 8.5 Hz, H-5’), 7.65 (1H, $dd$, $J$ = 2.1, 8.5 Hz, H-6’),
7.81 (1H, d, J = 2.1 Hz, H-2’), 12.15 (1H, s, 5-OH).

$^{13}$C NMR (CD$_3$COCD$_3$): δ 146.91 (C-2), 136.72 (C-3), 176.51 (C-4), 162.26 (C-5), 99.10 (C-6), 164.95 (C-7), 94.40 (C-8), 157.71 (C-9), 104.08 (C-10), 123.71 (C-1’), 115.69 (C-2’), 145.77 (C-3’), 148.29 (C-4’), 116.15 (C-5’), 121.41 (C-6’).

2.13.2 Isoliquiritigenin (iii)

The dried stem of Glycyrrhiza glabra L. (700 g) was extracted in a soxhlet apparatus with 1000 ml of methanol for 48 h, and the methanol was evaporated under reduced pressure to obtain 156 g of a sticky solid. Ten grams of the solid was heated under reflux with 250 ml of 2 M hydrochloric acid for 1 h. The solution was extracted with ethyl acetate (250 ml x 4). The residue obtained by evaporation of ethyl acetate (7.3 g) was chromatographed on a dry silica column, using a toluene/chloroform/acetone gradient elution. Isoliquiritigenin eluted with toluene/ chloroform/ acetone (70:70:8).

It was recrystallized from n-hexane/acetone (130 mg). Purity of the compound was checked by TLC using several systems.

Isoliquiritigenin (iii)

mp: 198 °C (200 °C lit.94)

$\lambda_{\text{MeOH}}^{\text{max}}$ nm : 241.5, 303 (sh), 371.

$\nu_{\text{KBr}}^{\text{max}}$ cm$^{-1}$: 1640 (C=O), 3350 (3xOH), 1613, 992 (-C=C-).

$^1$H NMR (CD$_3$OD): δ 6.28 (1H, d, J = 2.4 Hz, H-3’), 6.40 (1H, dd, J = 2.4, Hz, 9Hz, H-5’), 6.85 (2H, d, J = 8 Hz, H-5, H-3), 7.56 (2H, d, J = 8.6 Hz, H-2, H-6),

7.57 (1H, d, J = 15.4 Hz, H$_a$) 7.78(1H, d, J = 15.4 Hz, H$_b$), 7.95 (1H, d, J = 9 Hz, H6’).
$^{13}$C NMR (CD$_3$OD): $\delta$ 127.59 (C-1), 131.6 (C-2), 116.7 (C-3), 161.2 (C-4), 116.7 (C-5), 131.6 (C-6), 109 (C-5'), 166 (C-2'), 103.6 (C-3'), 167.2 (C-4'), 114.5 (C-1'), 133 (C-6'), 145.5 (C-\(\beta\)), 118 (C-\(\alpha\)), 193 (C=O)

GCMS, $m/z$ (rel.int.): 256 [M]$^+$ (100), 163 (38), 137 (98), 125 (46).

2.13.3 Plumbagin (iv) and a 4:1 mixture of Isoshinanolone (v) and Epiisoshinanolone (vi) (ISMIX)

The dried root of *Plumbago indica* L. (1 kg) was extracted in a soxhlet apparatus with 5 l n-hexane for 72 h, and the solvent was evaporated under reduced pressure to obtain 35 g of sticky solid. Thirty grams of the solid was chromatographed on a silica column using a n-hexane/chloroform gradient elution. Plumbagin eluted with n-hexane and was recrystallized from n-hexane/ethyl acetate (8:2) to obtain 1.5 g of plumbagin as dark orange needles. Purity of the compound was checked by TLC. ISMIX was obtained as a dark red semi solid after concentration of the n-hexane/ethyl acetate (1:1) fractions. The dark red semi solid (15 g) was dissolved in 250 ml of hexane and partitioned with 1 M potassium hydroxide solution (250 ml x 4). The aqueous layer was acidified with concentrated hydrochloric acid and extracted with diethyl ether (250 ml x 4). The ether phase was evaporated off and the residue obtained was subjected twice to prep. TLC. The first prep. TLC was carried out using n-hexane/ethyl acetate (70:30) as the solvent. The second prep. TLC was carried out using toluene/chloroform (1:1) as the solvent. ISMIX was identified as a yellow fluorescent band ($R_f = 0.5$, in first prep. TLC and $R_f = 0.35$, in second prep. TLC). It was extracted from the silica with chloroform to obtained a pale yellow semi solid (200 mg) which was shown to be a 4:1 mixture of isoshinanolone and
epiisoshinanolone purity of the isolate was determined by TLC using several systems and HPLC.

**Plumbagin (iv)**

mp: 73.6 °C (76 °C lit95)

$\lambda_{\text{MeOH}}^{\text{max}}$ nm: 210, 245 (sh), 267, 418.

$^1\text{H NMR (CDCl}_3\text{)}$: $\delta$ 2.15 (1H, d, $J = 1.6$ Hz, 2-CH$_3$), 6.79 (1H, d, $J = 1.6$ Hz, H-3), 7.2-7.65 (3H, m, H-6, H-7, H-8), 11.85 (1H, s, 5-OH).

$^{13}\text{C NMR (CDCl}_3\text{)}$: $\delta$ 184.73 (C-1), 149.56 (C-2), 135.39 (C-3), 161.10 (C-5), 124.11 (C-6), 136.06 (C-7), 119.23 (C-8), 131.98 (C-9), 115.05 (C-10), 190.21 (C-4), 16.50 (2-CH$_3$).

**Mixture of Isoshinanolone (v) and Epiisoshinanolone (vi)**

$\lambda_{\text{MeOH}}^{\text{max}}$ nm : 215, 259, 267, 332.5.

$\nu_{\text{film cm}^{-1}}^{\text{max}}$: 3441, 2930, 1631, 1524, 1452.

$^1\text{H NMR (CDCl}_3\text{)}$: $\delta$ 1.17 (3H, d, $J = 6$ Hz, 2-CH$_3$), 2.17-2.91, (4 H, m, H-2, H-3, 1-OH), 4.47 (br, d, ca.1/5 H, $J = 8$ Hz, H-1), 4.72(1H, d, $J = 2.5$ Hz, H-1), 6.89-7.50 (3H, m, H-6, H-7, H-8), 12.38 and 12.35 (1H, s, 5-OH).
\[ ^{13}\text{C NMR (CDCl}_3): \]

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GCMS, m/z (rel.int.): 192 [M]+ (100), 177 (17), 150 (50), 122 (42), 121 (86), 84 (20) 93 (16), 65 (26).

**Isoshinanolone (v)**

\[ \lambda_{\text{MeOH}} \text{ nm: 215, 259, 332.5} \]

\[ ^{1}\text{H NMR (CDCl}_3): \delta \text{1.17 (3H, d, } J = 6\text{Hz, 2-CH}_3), 2.0-2.98, (4 \text{H, m, H-2, H-3, 1-OH), 4.72 (1/2 H, } d, J = 2.5 \text{Hz, H-1), 6.69-7.60 (3 \text{H, m, H-6, H-7, H-8), 12.35 (1H, s, 5-OH).} \]

\[ ^{13}\text{C NMR (CDCl}_3): \delta \text{71.00 (C-1), 34.38 (C-2), 40.67 (C-3), 204.75 (C-4), 162.62 (C-5), 118.62 (C-6), 136.91 (C-7), 118.11 (C-8), 144.97 (C-9), 114.88 (C-10), 16.14 (2-CH}_3). \]

2.13.4 Acetylation of ISMIX \(^{96}\)

Added 2 ml of acetic anhydride and 2 ml of pyridine to 50 mg of ISMIX in 10 ml of chloroform. The reaction mixture was kept at room temperature for 12 h. Products
were checked on TLC using the following solvent systems: toluene/ethyl acetate (9:7), n-hexane/ diethyl ether: (9:1) / (8:2) / (7.5:2.5) / (6:4).

2.13.5 5-Hydroxymethylfurfural (5-HMF) (vii)

DMA (2 l) was dealcoholized and partitioned with dichloromethane (2 l x 7). The dichloromethane was evaporated under reduced pressure to obtain an oily mass (2.16 g). It was subjected to flash chromatography on a dry silica column, using n-hexane followed by n-hexane/methylene chloride (80:20). Impure 5-HMF (339 mg) eluted with n-hexane/methylene chloride (80:20). It was rechromatographed on a silica column using a n-hexane/methylene chloride gradient elution. Partially purified 5-HMF eluted (180 mg) with n-hexane/ethyl acetate (70:30), fraction 15-25. Further purification was achieved by prep.TLC (silica gel HF254, 0.25 mm), using toluene /diethyl ether (50:50) saturated with 10% glacial acetic acid as the solvent. The 5-HMF band observed as a fluorescence quenching band ($R_f = 0.78$) under UV 254 nm was scraped and eluted with, dichloromethane. Evaporation of the solvent gave 5-HMF as pale yellow oil (125 mg). The purity was checked by TLC using several systems.

5-Hydroxymethylfurfural (5-HMF) (vii)

$\lambda_{MeOH}^{\text{max}}$ nm: 230(sh), 278

$\nu \text{film}^{\text{max}}$ cm$^{-1}$: 1668, 1521, 1024, 2851, 2926, 3396, 810, 779

1H NMR (CDCl3): $\delta$ 4.7 (2H, s, 5-CH$_2$OH), 6.53(1H, d, $J = 3.5$ Hz, H-4), 7.25 (1H, d, $J = 3.5$ Hz, H-3), 9.2(1H, s, 2-CHO).

13C NMR (CDCl3): $\delta$ 161.11 (C-2), 123.57 (C-3), 109.64 (C-4), 151.88 (C-5), 57.01 (5-CH$_2$OH), 177.70 (2-C=O).
GCMS, \( m/z \) (rel. int.): 126 \([M]^+\) (27), 95 (100), 39 (42), 38 (25), 29 (19).

2.13.6 Umbelliferone (viii)

The dried root of *Aegle marmelos* Correa. (500 g) was extracted in a Soxhlet apparatus with 2 l of methanol for 48 h and the methanol was evaporated under reduced pressure to obtain 32 g of a sticky mass. Five grams of the material was flash chromatographed on dry silica, using chloroform followed by chloroform/acetone (80:10). Impure umbelliferone (400 mg) was eluted in the chloroform/acetone (80:10) fractions (2-20) and further purification was carried out by prep. TLC (silica gel HF254 15 μm) using chloroform/acetone/toluene (80:10:20) as the solvent. The blue florescent band (\( R_f = 0.5 \), UV 366 nm) was scraped off and extracted with ethyl acetate. Umbelliferone (52.5 mg) was recrystallized in methanol/chloroform. Purity was checked by TLC using several systems.

**Umbelliferone (viii)**

mp: 225 °C (225-228°C lit. 97)

\[ \lambda_{\text{MeOH}} \text{nm: 324, 252} \]

\( ^1H \text{NMR (CD}_3\text{COCD}_3): \delta \]

6.15 (1H, \( d, J = 9.5 \) Hz, H-3), 6.7 (1H, \( d, J = 2 \) Hz, H-8),

6.8 (1H, \( dd, J = 3 \) Hz, 9 Hz, H-6), 7.5 (1H, \( d, J = 9 \) Hz, H-5), 7.95 (1H, \( d, J = 9 \) Hz, 4 H), 9.5 (1H, s, \( br, 7-\text{OH} \)).

\( ^{13}C \text{NMR (CD}_3\text{COCD}_3): \delta \]

161.03 (C-2), 112.90 (C-3), 144.66 (C-4), 130.42 (C-5), 113.7 (C-6), 161.91 (C-7), 103.23 (C-8), 156.9 (C-9), 112.90 (C-10).

2.13.7 Gallic acid (ix)

DMA (2 l) was dealcoholized and acidified with 6 M hydrochloric acid (pH = 1) and extracted with ethyl acetate (2 l x 3). The ethyl acetate extract was evaporated under
reduced pressure to obtain 6.22 g of an oily mass. The mass was dissolved in 100 ml of ethyl acetate and extracted into with 10 % sodium hydrogen carbonate solution (100 ml x 10). The aqueous layer was acidified with conc. sulfuric acid (pH = 1) and partitioned with ethyl acetate (200 ml x 4) to obtain 2.58 g of a sticky mass on evaporation. This was flash chromatographed on a dry silica column using ethyl acetate as the solvent. Fractions 5-10 were concentrated to obtain crude gallic acid (1.29 g). Further purification was carried out by prep. TLC. Toluene/ethyl acetate (40:60) saturated with 100 ml of 10% glacial acetic acid was used as the solvent system. Gallic acid band was detected as a brownish black colored zone on prep. TLC after treatment of a small strip with 10 % aqueous FeCl₃ solution. The band was scraped off (Rf = 0.32) and extracted with ethyl acetate (100 ml x 3). Gallic acid was recrystallized from methanol to obtain 55 mg as a white amorphous powder. Purity was checked by TLC using several systems.

**Gallic acid (ix)**

mp: 235 °C (240 °C lit.⁹⁸)

$$\lambda_{\text{MeOH}}^{\text{max}}$$ nm: 224, 271

$^1$H NMR (CD₃COCD₃): $\delta$ 7.1 (2 H, s, H-6, H-2)

$^{13}$C NMR (CD₃COCD₃): $\delta$ 121.67 (C-1), 110.09 (C-2, C-6), 145.94 (C-3, C-5), 138.69 (C-4), 168 .11 (C-7).
2.13.8 Isolation of dehydrocostus lactone (x) and dihydrodehydrocostus lactone (xi)

The dried rhizome of *Saussurea lappa* C.B. Clarke. (500 g) was extracted in a Soxhlet apparatus with 2 l of methanol for 72 h, and the methanol was evaporated under reduced pressure to obtain 107 g of a sticky solid. Eight grams of the solid was subject to flash chromatography, using n-hexane as the solvent to obtain 700 mg of the crude product. This was rechromatographed on silver nitrate impregnated silica (100 ml of 1.5 % methanolic silver nitrate for 50 g of silica 0.04-0.063 mm, 2 x 60 cm), using a n-hexane/aceto
tone gradient elution. The product which eluted with n-hexane/aceto

tone (95:5) fractions 6-15 as a pure pale yellow fragrant oil was either dehydrocostus lactone or a mixture of dehydrocostus lactone and dihydrodehydrocostus lactone. Certain sample of *Saussurea lappa* C.B. Clarke yielded pure dehydrocostus lactone instead of the mixture. Purity of the product was checked by TLC using several systems.

Dehydrocostus lactone (x)

\[
\lambda_{\text{MeOH}} \text{ nm: } 215 \\
\text{max}
\]

\[
\nu_{\text{RIR}} \text{ cm}^{-1}: 1766, 1642, 1440, 1404, 1305, 1257, 1147, 999, 893, 814, 652, 503. \\
\text{max}
\]

\[
^1\text{H NMR (CDCl}_3): \delta 1.30-3.00 (11 \text{ H, } m, \text{ H-1, H-2, H-3, H-5, H-7, H-8, H-9}), \\
3.92 (1\text{H, } dd, J = 9 \text{ Hz, } J = 9 \text{ Hz, } \text{H-6}), 4.80 (1\text{H, } s, \text{ H-15}), 4.9 (1\text{H, } s, \text{ H-15}), \\
5.05 (1\text{H, } d, J = 2 \text{ Hz, } \text{H-14}), 5.28 (1\text{H, } d, J = 2 \text{ Hz, } \text{H-14}), 5.5 (1\text{H, } d, J = 3 \text{ Hz, } \text{H-13}), 6.2 (1\text{H, } d, J = 3.5 \text{ Hz, } \text{H-13}).
\]
51.92 (C-5), 85.20 (C-6), 45.02 (C-7), 30.84 (C-8), 36.19 (C-9), 149.14 (C-10), 139.64 (C-11), 170.22 (C-12), 120.14 (C-13), 109.47 (C-14), 112.52 (C-15).

GCMS, m/z (rel. int.): 230 [M]+ (1.4), 220(41), 184(49), 119(43), 105(85), 91(100), 79(97), 67(75), 55(97).

2.13.9 Reduction of dehydrocostus lactone (x) to dihydrodehydrocostus lactone (xi) 99

To a solution of 1.22 g of dehydrocostus lactone in 60 ml of dried methanol (dried over molecular sieve-4 A) was added 1 g sodium borohydrate and the reaction mixture was kept at room temperature for 48 h. The mixture was poured into 100 ml of water, acidified with 2 M hydrochloric acid and extracted with methylene chloride (100 ml x 4). Methylene chloride was evaporated under reduced pressure to obtain 1.19 g of a white solid which was chromatographed on dry silica, using a n-hexane/methylene chloride gradient elution. Dihydrodehydrocostus lactone (0.5 g) eluted with n-hexane/methylene chloride (70:30). Purity was checked on TLC (n-hexane/diethylamine, 99.5:0.5). The pure compound was characterized as dihydrodehydrocostus lactone by 1H NMR, 13C NMR, DEPT, HOMOCOSY, HETEROCOSY and GCMS experiments.

Dihydrodehydrocostus lactone (xi)

\[ \lambda_{MeOH} \text{ nm: } 206 \]

\[ \nu_{\text{film}} \text{ cm}^{-1}: 1759, 1660, 1439, 1383, 1228, 1138, 1055, 966, 953, 895, 814, 708, 498, 440. \]

1H NMR (CDCl3): \( \delta \) 1.50-3.00 (11H, m, H-1, H-2, H-3, H-5, H-7, H-8, H-9), 1.22 (3H, d, \( J = 7 \) Hz, 13-CH3), 3.92 (1H, t, \( J = 9 \)Hz, H-6), 4.78 (1H, s, H-15),
4.81 (1H, s, H-15), 5.08 (1H, d, J = 2 Hz, H-14), 5.20 (1H, d, J = 1.7 Hz, H-14).

\[^{13}\text{C NMR (CDCl}_3\]): \delta 49.71 \text{(C-1)}, 30.06 \text{(C-2)}, 32.38 \text{(C-3)}, 149.84 \text{(C-4)},

51.82 \text{(C-5)}, 85.18 \text{(C-6)}, 46.92 \text{(C-7)}, 30.06 \text{(C-8)}, 37.54 \text{(C-9)}, 151.64 \text{(C-10)},

41.92 \text{(C-11)}, 178.8 \text{(C-12)}, 13.11 \text{(C-13)} 108.98 \text{(C-14)}, 111.69 \text{(C-15)}.

GCMS, m/z (rel.int.): 232 [M] (21), 158 (100), 152 (87), 159 (53), 105 (36), 106 (31), 91 (61), 79 (59), 77 (37), 55 (44), 53 (30), 55 (44).

2.13.10 Alizarin (xii) and Purpurin (xiii)

The dried stem (25 g) of \textit{Rubia cordifolia} L. was extracted with in a Soxhlet apparatus
with 500 ml of n-hexane for 5 h and the n-hexane was evaporated under reduced
pressure to obtain 171.5 g of a dark red sticky solid. Trituration of the solid with n-
hexane and acetone (1:1), converted it to a dark red powder. Forty seven milligrams
of powder was flash chromatographed on a dry silica column, using toluene. Alizarin
eluted with toluene fractions 25-80. Alizarin (30 mg) was recrystallized from acetone.

Purity was checked by TLC. A dark purple substance was stratified at the top of the
column. This layer was extruded from the column, by carefully pressurizing with
nitrogen, the column outlet. The silica was washed with excess toluene and eluted
with acetone (250 ml x 3) to obtain purpurin (6 mg). Purity was checked by TLC
using several systems.

\textbf{Alizarin (xii)}

\begin{align*}
\text{mp: } & 265-270 \degree C (288-289 \degree C \text{ lit.}^{100}) \\
\lambda^{\text{MeOH}} \text{ nm: } & 247, 263, 275, 335 \text{ (sh), 434} \\
\end{align*}

\[^{1}H \text{ NMR ((CD}_3)_2\text{SO): } \delta 7.16 \text{(1H, d, J = 8 Hz, H-3)}, 7.55\text{(1H, d, J = 8 Hz, H-4)}, 7.8 \text{(2}

H, m, H-6, H-7), 8.07\text{(2H, m, H-5, H-8), 12.53\text{(1H, s, OH), 12.85 \text{(1H, s, OH).}}

\text{H}, s, H-15, s, H-15, d, J = 2 Hz, H-14, d, J = 1.7 Hz, H-14).
$^{13}$CNMR($CD_3$)$_2$SO): $\delta$ 150.74 (C-1), 152.69 (C-2), 121.07 (C-3), 120.75 (C-4), 126.62 (C-5), 135.01 (C-6), 133.94 (C-7), 126.37 (C-8), 188.65 (C-9), 180.38 (C-10), 132.69 (C-11), 133.43 (C-12), 116.11 (C-13), 123.68 (C-14).

**Purpurin (xiii)**

mp: 260-261°C (262-263°C lit.$^{101}$)

$\lambda^{MeOH}$ nm: 220, 264, 282sh, 515.

$\lambda_{max}$
2.14 Quantitative determination of marker and representative compounds

2.14.1 TLC-densitometric quantification

Following general settings were used.

<table>
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<tr>
<th>TLC-densitometer settings</th>
<th>Reflection</th>
<th>Fluorescence</th>
<th>Spectral scanning</th>
</tr>
</thead>
<tbody>
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<td>Reflection</td>
<td>Fluorescence</td>
<td>Reflection</td>
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<tr>
<td>Scan mode</td>
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<td>0.04</td>
<td>0.10</td>
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<td>Swing width</td>
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<td>Not applicable</td>
</tr>
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<td>1</td>
</tr>
<tr>
<td>Lamp</td>
<td>Deuterium and Xenon</td>
<td>Deuterium and Tungsten</td>
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</tr>
<tr>
<td>Linearizer</td>
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<td>Off</td>
<td>Off</td>
</tr>
</tbody>
</table>

Table-1: TLC Densitometer settings

Beam size was 0.4 mm x 0.4 mm for isoliquiritigenin, gallic acid, costuslactones and 5-HMF and 4 mm x 10 mm for umbelliferone and 1 mm x 5 mm for ISMIX.
2.14.1.1 Isoliquiritigenin (iii) by TLC-VIS method

i. Standard curve

Standard solutions of isoliquiritigenin in methanol containing 160, 240, 320, and 480 \( \mu g/ml \) were prepared by dilution of a stock solution containing 1600 \( \mu g/ml \). Two microlitres of each solution (6 replicates) were applied using a Hamilton micro syringe to a TLC (10 cm x 20 cm) which had been pre washed with methanol. The plate was developed in a pre saturated chamber using toluene/ethyl formate (5:5) as solvent (solvent front 8.0 cm). The plate was dried by hot air and dipped in 1% methanolic diphenylboric acid -\( \beta \)-ethyl amino ester reagent and heated at 100 °C for 2 min. Isoliquiritigenin is visualized as a bright yellow spot at \( R_f = 0.67 \). Densitometric scanning was carried out at 435 nm after leaving the plate for 15 min at room temperature to stabilize the colour. The standard curve is given in fig-1 (\( Y = 21414 X + 844, R^2 = 0.9999 \)).

ii. Preparation of Isoliquiritigenin extract from DMA

DMA (50.0 ml) was dealcoholized and extracted with diethyl ether (50 ml x 4). The combined ether extracts were dried over anhydrous sodium sulfate and concentrated under reduced pressure. The concentrated ether extract was transferred to a graduated separating funnel, the volume adjusted to 50 ml by the addition of diethyl ether, and extracted with 0.5 M aqueous potassium hydroxide (50 ml x 4). The combined aqueous extract was acidified with dilute hydrochloric acid to pH 3-4 and extracted with diethyl ether (200 ml x 3). The combined ether extracts were dried over anhydrous sodium sulfate and filtered through Whatman No: 1 filter paper and
evaporated under reduced pressure. The resultant solid was dissolved in 1.00 ml of methanol and 2 µl of this solution was applied on the TLC plate. The plates were developed and scanned as described for the standard curve above.

Fig-1 TLC-VIS densitometric standard curve for isoliquiritigenin
2.14.1.2 Gallic acid (ix) by TLC–UV method

i. Standard curve

Standard solutions of gallic acid in methanol containing 100, 200, 400, 600, 800, 1000, 2000 and 4000 μg/ml were prepared by dilution of a stock solution containing 5000 μg/ml. One microlitre of each solution (6 replicates) was applied using a Hamilton microsyringe to a TLC (10 x 20 cm) which had been pre washed with methanol. The plate was developed in a pre saturated chamber using toluene/ethyl acetate (4:6) saturated with 10% glacial acetic acid as solvent system (solvent front 8.0 cm). The plate was dried by hot air. Gallic acid was observed under UV 254 nm as a black fluorescence quenching spot at \( R_f = 0.40 \). The spots were scanned at 270 nm, the absorbance maximum of gallic acid as determined by spectral scanning. Standard curve is given in Fig–2 \( (Y = 62061X +5608, R^2 = 0.9885) \).

ii. Preparation of gallic acid extract from DMA

One millilitre of DMA was accurately pipetted out and added into a separating funnel. Thereafter it was diluted with 25 ml of distilled water and acidified with 6 M hydrochloric acid (pH = 1). The acidified extract was partitioned with ethyl acetate (25 ml x 5). The combined ethyl acetate extract was dried over anhydrous sodium sulfate and evaporated under reduced pressure. The residue was dissolved in 1.0 ml of methanol and 1 μl sample was applied on TLC. The plates were developed and scanned as described for the standard curve above.
Fig-2 TLC-UV densitometric standard curve for gallic acid
2.14.1.3 Gallic acid (ix) by TLC-FD method

i. Standard curve

Standard solutions of gallic acid in methanol containing 2.5, 5, 10, and 20 µg/ml were prepared by dilution of a stock solution containing 50 µg/ml. One microlitre of each solution (6 replicates) were applied using a Hamilton microsyringe to a TLC (10 x 20 cm) which had been pre washed with methanol. The plate was developed in a pre saturated chamber using toluene/ethyl acetate (4:6) saturated with 10% glacial acetic acid as solvent system (solvent front 8.0 cm). The plate was dried by hot air and dipped in 1% methanolic diphenylboric acid -β-ethyl amino ester reagent and heated at 100 °C for 2 min, after which the plate was dipped in 1 % ethanolic polyethylene glycol 4000 solution and kept in a desiccator for 30 min. The gallic acid was observed under UV 366 nm as a blue spot at \( R_f = 0.40 \). Densitometric scanning was carried out with excitation at 315 nm and emission was measured at 330-400 nm. The standard curve is given Fig-3 (\( Y = 1182397 X -3682, R^2 = 0.9996 \)).

ii. Preparation of gallic acid extract from DMA

The preparation was carried out as described in section 2.14.1.2. However, the residue from the ethyl acetate extract was dissolved in 10.00 ml of methanol instead of 1.00 ml. One microlitre was applied on TLC. The plates were developed and scanned as described in section for the standard curve above.
Fig-3 TLC-FD densitometric standard curve for gallic acid
2.14.1.4 5-Hydroxymethylfurfural (vii) (5-HMF) by TLC-UV method

i. Standard curve

Standard solutions of 5-HMF in methanol containing 125, 250, 500 and 1000 µg/ml were prepared by dilution of a stock solution containing 10000 µg/ml. One millilitre of each standard solution was transferred to a 5 ml sample vial and the solvent were evaporated by using a stream of nitrogen. The solvent free solid was dissolved in 1 ml of internal standard solution. 4-Hydroxybenzaldehyde (4 g/1000 ml in methanol) was used as an internal standard. One microlitre of each solution (6 replicates) was applied using a Hamilton microsyringe to a TLC (10 x 20 cm) which had been pre-washed with methanol. The plate was developed in a pre-saturated chamber using toluene/ethyl acetate 1:1 saturated with 10 % glacial acetic acid as solvent system (solvent front 8.0 cm). The plate was dried in a stream of hot air, 5-HMF was observed under UV 254 nm as a black spot at Rf = 0.64. Densitometric scanning was carried out at 283 nm. The standard curve is given fig-4 (Y = 0.268487 X + 0.036922, R² = 0.9956).

ii. Preparation of 5-HMF extract from DMA

DMA (2 ml) was quantitatively transferred to a 50 ml separating funnel and diluted to 25 ml by the addition of distilled water. Adjusted the pH to 7 by adding 0.1M sodium hydroxide solution. The solution was extracted with ethyl acetate (25 ml x 5). The combined ethyl acetate extract was evaporated under reduced pressure. The residue was dissolved in 1 ml of methanolic 4- hydroxybenzaldehyde solution and 1µl was applied on the TLC. The plates were developed and scanned as described for the standard curve above.
Fig-4 TLC-UV Densitometric standrad curve for 5-HMF
2.14.1.5 Costuslactones by TLC-VIS method

i. Standard curve

Standard solutions of dehydrocostus lactone in chloroform containing 125, 250, 500, 1000, 2000 and 4000 µg/ml were prepared by dilution of a stock solution containing 8000 µg/ml. One microlitre of each solution (6 replicates) was applied using a Hamilton microsyringe to a TLC (10 x 20 cm) which had been pre-washed with methanol. The plate was developed in a pre-saturated chamber using toluene/diethylamine (99:1) as solvent system (solvent front 8.0 cm). The plate was dried in a stream of hot air and dipped in Liebermann-Burchard reagent and heated at 80 °C for 2 min. Dehydrocostus lactone was visualized as a pink violet spot at Rf = 0.68. Densitometric scanning was carried out at 535 nm after leaving the plate for 20 min at room temperature to stabilize the colour. The standard curve is given fig-5 (Y = 31805 X + 5684, R² = 0.9946). This standard curve is used to determine total costuslactones expressed as dehydrocostus lactone (See section 3.2.4).

ii. Preparation of total lactones extract from DMA

DMA sample for determination of DMA (50 ml) was extracted with chloroform (50 ml x 3). The combined chloroform extract was dried over anhydrous sodium sulfate and concentrated under reduced pressure. The resultant solid was dissolved with 0.5 ml of chloroform and 10 µl was applied on the TLC. The plates were developed and scanned as described for the standard curve above.
Fig-5 TLC-VIS densitometric standard curve for dehydrocostus lactone
2.14.1.6 Umbelliferone (viii) by Thin layer chromatography-Fluorescence densitometry (TLC-FD) method

i. Standard curve

Standard solutions of umbelliferone in methanol containing 1.25, 2.5, 5, 10, 20, 40, and 80, μg/ml were prepared by dilution of a stock solution containing 1000 μg/ml. One microlitre of each solution (6 replicates) was applied on the HPTLC. The plate was developed in a pre-saturated chamber using toluene/ethyl methyl ketone (9:1). The developed TLC plate was dried in a stream of hot air. Umbelliferone is visualized at \( R_f = 0.4 \) as a blue fluorescent zone under excitation at 366 nm. Densitometric scanning was carried out with excitation at 340 nm and the emission was measured at 400-500 nm. Standard curve is given in fig-6 (\( Y = 126887X + 196, R^2 = 0.9945 \)).

ii. Preparation of Umbelliferone extract from DMA

DMA (50 ml) was dealcoholized and extracted with chloroform (50 ml x 3). Combined chloroform extract was dried over anhydrous sodium sulfate (10 g) and concentrated under reduced pressure. The resultant solid was dissolved in 2 ml of methanol and 1μl applied on the HPTLC. The plates were developed and scanned as described for the standard curve.
Fig-6 TLC-FD densitometric standard curve for umbelliferone
2.14.1.7 Mixture of Isoshinanolone (v) and Epiisoshinanolone (vi) (ISMIX) by TLC-FD method

i. Standard curve

Standard solutions of isoshinanolone in methanol containing 5, 10, 15, 20, and 40 µg/ml were prepared by dilution of a stock solution containing 1000 µg/ml. One microlitre each solution (6 replicates) were applied on the TLC. The plate was developed in a pre saturated chamber using toluene/ethyl acetate (8:2) as solvent (solvent front 8.0 cm). The plate was dried in a stream of hot air and dipped in 1% ethanolic aluminum chloride hexahydrate reagent and heated at 100 °C for 2 min and immediately immersed in n-hexane reagent. Isoshinanolone is visualized at $R_f = 0.62$ as a blue fluorescent spot with excitation at 366 nm. The plate was kept in dark for 30 min to stabilize the fluorescent and densitometric scanning was carried out at 384 nm excitation wave length and emission was measured at 400-500 nm. Standard curve is given in Fig-7 ($Y = 220227X + 176$, $R^2 = 0.9936$). This standard curve is used to determine the mixture of isoshinanolone and epiisoshinanolone expressed as isoshinanolone. (See discussion on section 3.2.3)

ii. Preparation of ISMIX extract from DMA

DMA (50 ml) was dealcoholized and extracted with methylene chloride (50 ml x 4). The combined methylene chloride extract was dried over anhydrous sodium sulfate and concentrated under reduced pressure. The resultant solid was dissolved in 1 ml of methanol and 1µl was applied on the TLC plate. The plates were developed and scanned as described for the standard curve above.
Fig-7 TLC-FD densitometric standard curve for Isoshinanolone
### 2.14.2 HPLC quantification.

The following settings were used.

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<th>Parameter</th>
<th>Analyte</th>
<th>Gallic acid</th>
<th>5-HMF</th>
<th>Ethyl acetate extract of DMA</th>
<th>ISMIX</th>
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<td>MeOH : Water (50:50 v/v)</td>
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Table-2: HPLC settings
2.14.2.1 Gallic acid (ix) in DMA and its decoction

i. Standard curves

Standard solution of gallic acid in methanol containing 10, 50, 100, 200, 500, and 1000 µg/ml were prepared by dilution of a stock solution containing 5000 µg/ml. Five microlitres of each solution (3 replicates) were injected using a Hamilton micro syringe to HPLC injector (R for gallic acid = 3.3 min). The standard curve is given in Fig. 8. (Y = 6332155 X - 68081, R² = 0.9986)

ii. Sample preparation

DMA (10 ml) and decoction (10 ml) were filtered through Millipore HIV 0.22 µl filter separately. Five microlitre of each samples were injected into HPLC. Gallic acid level of five different batches of five brands of DMA were determined. In the case of the decoction, samples were collected at regular intervals during the boiling process and their gallic acid levels were determined.
Fig- 8 HPLC standard curve for gallic acid
2.14.2.2 5-HMF (vii) in DMA and its decoction

i. Standard curve

Standard solutions of 5-HMF in methanol containing 0.1, 1.0, 10, 20, 50, 100, 500 and 1000 µg/ml were prepared by dilution of a stock solution containing 5000 µg/ml. Five microlitres each solution (3 replicates) were injected using a Hamilton micro syringe to HPLC injector. (R<sub>t</sub> for 5-HMF = 5.2 min) The standard curve is given in fig-9 (Y = 6313928 X - 27851, R<sup>2</sup> = 0.9986).

ii. Sample preparation

5-HMF level of decoction of DMA and five different batches of five brands of DMA were calculated utilizing the same chromatograms used for the determination of gallic acid which were obtained previously in experiment (2.14.2.1). (R<sub>t</sub> for 5-HMF is 13.8 min).
Fig -9 HPLC standard curve for 5-HMF
2.14.2.3 Preparation of *Plumbago indica* L. root extract for determining the ratio of Isoshinanolone (v) and Epiisoshinanolone (vi)

*Plumbago indica* L. fresh root was dried at 40° C in a plant drier. Dried plant powder (500 mg) was refluxed with methanol for 30 min. The filtered extract was evaporated under reduced pressure. The residue was dissolved in 5.00 ml methanol and filtered through Millipore HIV 0.22 μm filter and 5 μl was injected to the HPLC.
2.14.3 GLC quantification

Following settings were used.

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*TC-1 : 100% dimethylpolysiloxane (30 m, 25 mm, film thickness 0.25μm)

**AT 225 : 50% Cyanopropylmethyl,50% phenylmethylpolysiloxane(30 m, 25 mm, film thickness 0.25μm)

N/A : not applicable    DHC : Dehydrocostus lactone    DDHC : Dihydrodehydrocostus lactone

Table - 3 : GLC settings

67
2.14.3.1 Dehydrocostus lactone (x) (DHC) and dihydrodehydrocostus lactone (xi) (DDHC) by GLC

i. Standard curve for dehydrocostus lactone (DHC)

Standard solutions of dehydrocostus lactone in chloroform containing 50, 100, 200, 400 and 800 µg/ml were prepared by dilution of a stock solution containing 4000 µg/ml. One millilitre of each standard solution was transferred to a 5 ml sample vial and the solvent was evaporated by using a stream of nitrogen. The solvent freed solid was dissolved in 1 ml of internal standard solution. Anthracene (48 g/100 ml in chloroform) was used as an internal standard. One microlitre of each solution (6 replicates) was applied using a Hamilton microsyringe to the GC (Rt for anthracene is 5.6 min and Rt for dehydrocostus lactone is 8.4 min). The standard curve is given in Fig-10 (Y = 1.037 X - 0.00032, R² = 0.9999).

ii. Standard curve for dihydrodehydrocostus lactone (DDHC)

Standard solutions of dihydrodehydrocostus lactone in chloroform containing 50, 100, 200, 400 and 800, µg/ml were prepared by dilution of a stock solution containing 4000 µg/ml. One millilitre of each standard solution was transferred to 5 ml sample vial and the solvent was evaporated by using stream of nitrogen.

The solvent freed solid was dissolved in 1 ml of internal standard solution. Anthracene (48 g/100 ml in chloroform) used as an internal standard. (Rt for dihydrodehydrocostus lactone is 7.6 min) One microlitre of each solution (3replicates) was injected to GC using a Hamilton microsyringe. The standard curve is given Fig-11 (Y = 1.06575 X + 0.001044, R² = 0.9998).
Fig -10  GLC standard curve for dehydrocostus lactone
Fig-11 GLC standard curve for dihydrodehydrocostus lactone
2.14.3.2 Monitoring of DHC and DDHC contents during industrial scale manufacture of DMA (at Link Natural Products)

2.14.3.2.1 Determination of DHC content in decoction.

Three vessels used in the preparations of decoction and labeled as R1, R2, and R3. Samples (100 ml) were taken every 2 h from the stoves during the boiling process. The centrifuged sample (50 ml) was extracted into methylene chloride (50 ml x 3). The combined methylene chloride extracts were dried over anhydrous sodium sulfate and filtered and concentrated under vacuum. The concentrated extract was subjected to a pre-clean up procedure as follows; Five grams of silica gel was packed in small glass column (12 x 1 cm) and the sample was introduced to the top of the column and covered with a sand layer using pre-washed sand (2 mm layer thickness). Thereafter, the column was eluted with 250 ml of methylene chloride. The methylene chloride was evaporated under reduced pressure. The resultant solid was dissolved in 1 ml of internal standard solutions and 1 µl (3 replicates) was injected into GC.

2.14.3.2.2 Determination of DHC content in fermenting decoction

The fermentation vessel contained three external channels, top, middle and bottom with outlet valves which facilitated sampling from different levels of the fermenting decoction. Fifty millilitres of samples were taken from top, middle and bottom of the vessel and bulked. One hundred millilitres of bulk sample were used for determination of lactones. Samples were taken daily in the first ten days. Thereafter samples were taken every two days in same manner. Samples (100 ml) were prepared for GC as described in section 2.14.3.2.1.
2.14.3.2.3 Determination of DHC content in fermented decoction (DMA) samples preparation for GLC analysis

DMA (100 ml) were extracted with methylene chloride (100 ml x 4). The combined methylene chloride extract was dried over anhydrous sodium sulfate (10 g) and evaporated under reduced pressure. Samples were prepared for GC as described in section 2.14.3.2.1.

2.14.3.2.4 Preparation of a mixture of DHC and DDHC extract from Saussurea lappa C. B. Clarke. rhizomes

Powdered Saussurea lappa C.B. Clarke. (50 mg) was refluxed at 60 °C with 50 ml of 50 % aqueous methanol for 30 min, allowed to cool and filtered. A second extraction was carried out with 50 ml of methanol. The two extracts were combined and partitioned with methylene chloride (100 ml x 4). The methylene chloride extracts were dried over anhydrous sodium sulfate and evaporated under reduced pressure. The residue was dissolved with 5.00 ml of internal standard solution. One microlitre was injected (3 replicates) to GC.

2.15 Preparation of extracts for chromatographic analysis of plant with problems of identity

2.15.1 Methanol extract of Acacia catechu Willd., and Myroxylon balsamum (L.) Harms. heartwoods

Coarsely powdered (1 g) Acacia catechu Willd., and Myroxylon balsamum (L.) Harms. heartwoods were extracted separately into 25 ml of methanol by heating under reflux for 30 min. The extract was evaporated under vacuum, and the residue
dissolved in 1 ml of methanol and 2 μl was applied on TLC. The plates were
developed in a pre saturated chamber using three different solvents, toluene/ethyl acetate (9.8:0.2), chloroform/toluene/ethyl formate/formic acid (5:3:2:1) and toluene/ethyl formate (5:5).

2.15.2 Hydrodistillation of Nardostachys jatamansi DC. rhizome
Coarsely powdered (100 g) Nardostachys jatamansi DC. rhizome was hydrodistilled using (1.2 l of water) a Clevenger apparatus. Distillation was carried out for 16 h to obtained 1.2 ml of light bluish oil. The oil (1.00 ml) was dissolved with 5 ml of n-hexane and 0.2 μl was injected into GC.

2.15.3 Chloroform extract of Saussurea lappa C. B. Clarke., and Inula racemosa Hook.f. rhizomes
Finely powdered (1g) Saussurea lappa C.B. Clarke., and Inula racemosa Hook.f. rhizomes were extracted into 25 ml of chloroform by heating under reflux for 30 min. The extract was evaporated under vacuum, and the residue dissolved in 1 ml of chloroform and 2 μl was applied on TLC. The plate was developed in a pre saturated chamber using, n-hexane/diethyl ether (9:1).

2.15.4 Chloroform extract of Nardostachys jatamansi DC. and Flickingeria macraei (Lindely) Seidenf. rhizomes
Finely powdered (1 g) Nardostachys jatamansi DC and Flickingeria macraei (Lindely) Seidenf rhizomes were separately extracted in to 25 ml of chloroform by heating under reflux for 30 min. The concentrated extract was dissolved in 1 ml of
chloroform and 2 µl was applied on TLC. The plate was developed in a pre saturated chamber using, n-hexane/diethyl ether (9:1).

2.15.5 Methanol extract of *Hemidesmus indicus* R. Br. and *Cryptolepis buchananii* Rome & Shults. roots

Coarsely powdered (1g) *Hemidesmus indicus* R. Br. and *Cryptolepis buchananii* Rome & Shults. roots were extracted separately in to 25 ml of methanol by heating under reflux for 30 min. The extract was evaporated under vacuum, and the residue dissolved in 1 ml of methanol and 2 µl was applied on TLC. The plate was developed in a pre saturated chamber using, n-hexane/diethyl ether (9:1).

2.15.6 Diethyl ether extract of bees honey

Five samples of bees honey were collected from the local market and the standard sample was extracted from honey comb which was collected from Kekirawa. Two of the five were imported samples. The sources of rest of the samples are not known. Bees honey (25 g) was diluted upto 50 ml with water and extracted (50 ml x 3) with diethyl ether. The residue from evaporation of the extract was dissolved in 1 ml of acetone and 5 µl was applied on TLC. The plate was developed in a pre saturated chamber using, toluene/ethyl formate (9:1).
2.16 Development of fingerprints (FP)

2.16.1 GLC fingerprints (GLC-FP)

Dasamoolarista (100 ml) was extracted with chloroform (100 ml x 3). The extract was evaporated under reduced pressure and the residue was dissolved in 1 ml of chloroform and 0.2 μl was injected. Some of the GLC profiles used for quantifications were also used as fingerprints. These are described in discussions in section 3.5.

2.16.2 HPLC fingerprints (HPLC-FP)

Dasamoolarista (100 ml) was extracted with ethyl acetate (100 ml x 3). The extract was evaporated under reduced pressure and the residue was dissolved in 1 ml of the methanol. The extract was filtered through 0.22μm microfilter and 5μl were injected to the HPLC.

2.16.3 TLC fingerprints (TLC-FP)

Some of the densitograms used for quantification were also used as fingerprints. These are described in results and discussions in section 3.5.
3.0 Results and Discussion

3.1 Development of identity tests

Identity tests are used to confirm the presence or absence of specific compounds in the final product. The most useful identity tests are those for compounds which are unique to a single plant or a group of plants used in the preparation of the drug. The test for the compound then becomes a test for a plant or representative compounds for a group of plants. Such compounds are marker compounds for a plant or representative compounds group of plants. In the case of single plant there are important in determining whether the correct ingredients have been used in the preparation of a drug. It is difficult to clarify those plants in cases where a compound indicates a group of plants.

Identity tests were developed for 11 plants. These tests were based on TLC or GLC. Given the complexity of the drug DMA, meaningful chromatography is possible only after fractionation into groups of compounds based on physicochemical properties. DMA was extracted with the following solvents; n-hexane, methylene chloride, chloroform, diethyl ether and ethyl acetate. These extracts were subjected to GLC, HPLC and to extensive TLC on silica, using a variety of solvent systems and a variety of spray reagents. Prominent spots on the TLC and relevant peaks in the GLC chromatograms representing marker compounds or representative compounds were then correlated with specific plants or groups of plants.
Comparison of the chromatograms of DMA extracts with those of other arista having closely related recipes enabled us to quickly “home in” on the most likely plants for correlation. The method is described in detail in sections 3.1.1.1 and 3.1.1.2. Correlations were confirmed by isolating the marker compounds and representative compounds from the plants and correlating them with corresponding compounds on the chromatograms of the DMA extracts by conventional methods such as spiking and comparison of \( R_f \) values and retention times. More sophisticated combined chromatographic spectroscopic techniques such as LC-MS, LC-NMR, LC-UV-NMR, GC-MS and LC-UV/VIS are now available for the rapid detection of compounds in complex matrices. HPLC –UV/VIS was used to further confirm the correlation of plants with marker compounds and representative compounds containing chromophores. An UV-VIS library of pure compounds was first created using the Millenium chromatographic manager package. The spots of interest on the TLC were scraped off, and the compound taken up in methanol and injected into the HPLC. The UV-VIS spectrum of the compound from the chromatogram was matched with the corresponding spectrum in the library.

3.1.1 Identity tests based on TLC

3.1.1.1 Identity tests for *Plumbago indica* L.

The n-hexane and chloroform extracts of DMA and its decoction gave a bright yellow fluorescent zone under excitation at 366 nm, at \( R_f = 0.63 \) when chromatographed on silica using toluene/diethyl ether (1:1, saturated with 10 % acetic acid) as the solvent system. (TLC-2, *photographs of thin layer chromatograms are given in appendix i*). This zone exhibited weak quenching of background fluorescent when excited at 254 nm.
With anisaldehyde sulfuric acid reagent this appeared as a blue zone (TLC-1). The bright yellow fluorescent zone was correlated with *Plumbago indica* L. in the following manner. At the outset chloroform extracts of 12 arista with recipes related to DMA namely *Danthyarista* (DA), *Amurtharista* (AMA), *Nimbarista* (NBA), *Asokarista* (AKA), *Kadirarista* (KA), *Musthakarista* (MA), *Aswagandharista* (ASA), *Sarawastharista* (SA), *Darksharista* (DRA) *Arjunarista* (AA), *Abeyarista* (ABA), *Balarista* (BA) were chromatographed under identical conditions (appendix-iii). Three of them DA, MA and ASA contained a yellow zone corresponding the one observed in DMA. Of the three arista, MA had the simplest recipe, containing 9 plants. Of these 9 plants *Cyprus rotundus* L., *Woodfordia fruticosa* (L.) Kurz., *Carum corpticum* Bentha Hook., *Zingiber officinale* Rosc., *Piper nigrum* L., *Eugenia caryophyllata* Thumb., *Trigonella foenum-graceum* L., *Plumbago indica* L., *Cuminum cymium* L., only 4 plants, *Cyprus rotundus* L., *Woodfordia fruticosa* (L.) Kurz., *Eugenia caryophyllata* Thumb., *Plumbago indica* L., are found in DMA and therefore the yellow spot could arise from one of these. Of these four plants two plants (*Woodfordia fruticosa* (L.) Kurz., and *Cyprus rotundus* L.) are found in AKA and one plant (*Eugenia caryophyllata* Thumb.) is found in KA, neither of which exhibited a corresponding yellow zone in their TLC. The remaining plant was *Plumbago indica* L. The chloroform extract of *Plumbago indica* L. showed a yellow zone corresponding to the one in the TLC of the chloroform extract of DMA. The identification was confirmed by noting the absence of the yellow zone in a decoction prepared as for DMA but leaving out *Plumbago indica* L., and comparing it with a decoction of DMA. The compound corresponding to the yellow band was isolated from *Plumbago indica* L. by column chromatography (section 2.13.3) and was shown
to be a mixture of two isomers, isoshinanolone (cis-isomer) and epiisoshinanolone (trans-isomer) by NMR spectroscopy. They and can thus be considered as specific marker compounds for *Plumbago indica* L. The presence of isoshinanolone (v) and epiisoshinanolone (vi) in *Plumbago indica* L. have not been reported earlier, although they have been reported from other *Plumbago* species.

### 3.1.1.2 Identity tests for *Aegle marmelos* Correa.

The chloroform extract of DMA and its decoction revealed a prominent blue fluorescent zone under excitation at 366 nm at \( R_f = 0.59 \) when chromatographed on silica using toluene/ethyl methyl ketone (9:1) as the solvent system (TLC-4) and this zone exhibited blue fluorescence against the of background green fluorescence when excited at 254 nm (TLC-3). Out of the 12 related arista, this blue fluorescent zone was found only in DA and AMA. The decoction of AMA was used as the basis for further analysis owing to it being less complex than DA. Decoction of DA contains the ten main roots of DMA and *Plumbago indica* L. and *Baliospermum montanum* (Willd.) Muell-Arg. The decoction of AMA also contains these ten roots and *Tinospora*
cordifolia (Willd.), Hook. f. & Thom. Therefore it can be concluded that the corresponding zone could be from one of the ten roots which are common in both arista. According to Ayurvedic principles these ten plants are divided into two groups named “Mahapasmul” (*Aegle marmelos* Correa., *Sterospermum suaveloens* DC., *Oroxyllum indicum* L., *Gmelina arborea* Roxb., *Premna serratifolia* L., and “Sulupasmul” (*Tribulus terrestris* L., *Aerva lanata* Juss., *Alysicarpus vaginalis* DC., *Solanum xanthocarpum* Schard & Wendl., *Solanum melongena* L.). These two groups of plants were extracted with chloroform and analysed by TLC as described above. Only the extract from the “Mahapasmul” group gave a blue fluorescent zone corresponding to that observed for DMA. The five plants in “Mahapasmul” were divided into three groups on the basis of plant families, namely Verbenaceae, Bignoniaceae and Rutaceae. It was found that the blue fluorescent zone was given only by the Rutaceae family, which contained only one plant, *Aegle marmelos* Correa. The compound corresponding to the blue fluorescent zone was isolated and was found to contain one compound, identified as umbelliferone (viii). The recipe for DMA contains several other plants which contain umbelliferone, namely *Feronia limonia* (L.) Swingle. (Rutaceae) *Anethum graveolens* L. (Astraceae). However, the umbelliferone content is low in these plants, and the contribution of these plants towards the umbelliferone content in DMA was shown to be below the detectable level under these experimental conditions. Therefore, umbelliferone can be considered as a suitable marker compound for *Aegle marmelos* Correa.
3.1.1.3 Identity tests for *Saussurea lappa* C. B. Clarke.

The chloroform extract of DMA revealed a bluish purple visible zone at $R_f = 0.84$ when chromatographed on silica using n-hexane/diethylamine (99:1) as the solvent.

![Diagram of Umbelliferone](image)

Umbelliferone

![Diagram of Dehydrocostus lactone](image)

Dehydrocostus lactone

![Diagram of Dihydrodehydrocostus lactone](image)

Dihydrodehydrocostus lactone
system and treatment thereafter with anisaldehyde sulphuric acid reagent (TLC-6). Using procedures similar to that described in section 3.1.1.1 and 3.1.1.2, this was identified as a specific marker for Saussurea lappa C.B. Clarke. in DMA. The compound (s) corresponding to the blue zone was isolated by column chromatography (section 2.13.8 and 2.13.9) and was shown to be a mixture of dehydrocostus lactone (x) and dihydrodehydrocostus lactone (xi). 109-114

3.1.1.4 Identity test for Woodfordia fruticosa (L.) Kurz.

The ethyl acetate extract of DMA gave a bright orange fluorescent zones at Rf = 0.19 excitation at 366 nm when chromatographed on silica using toluene/ethyl formate/formic acid (5:4:1) as the solvent system and treatment thereafter with NP/PEG reagent (TLC-7). Using procedure similar to that described in section 3.1.1.1 and 3.1.1.2, this was identified as a specific marker for Woodfordia fruticosa (L.) Kurz. DMA, even though its identity has not been established as yet. The diethyl ether
extract of DMA gave a prominent yellow bright orange zone and a greenish fluorescent zones under excitation at 366 nm at $R_f = 0.47$ and 0.61 respectively, when chromatographed on silica using toluene/ethyl formate (5:5) as the solvent system (TLC-8). The compounds corresponding to the greenish yellow zone and a bright fluorescent zones were isolated by column chromatography (section 2.13.1) and were shown to be kaempferol (i) and quercetin (ii) respectively. Although these are very common flavonoids, they are both found in high concentrations only in *Woodfordia fruticosa* (L.) Kurz. and bees honey. Quercetin is found in high concentration in raisins. Thus arista which do not contain *Woodfordia fruticosa* (L.) Kurz, bees honey or raisin does not display this band (TLC-9).

![Quercetin](image)

**Quercetin**

### 3.1.1.5 Identity test for *Pterocarpus marsupium* Roxb.

The ethyl acetate extract of DMA and its decoction gave a greenish yellow fluorescent zone at $R_f = 0.12$ under excitation at 366 nm when chromatographed on silica using chloroform/toluene/ethyl formate (5:4:1) as the solvent system, and
treatment thereafter with NP/PEG reagent (TLC-11). When sprayed with 10 % aqueous ferric chloride reagent instead NP/PEG this appeared as blackish brown zone (TLC-10). Using procedures similar to that described in section 3.1.1.1 and 3.1.1.2, this was identified as a specific marker for *Pterocarpus marsupium* Roxb. in DMA. Its identity has not been established as yet.

### 3.1.1.6 Identity test for *Rubia cordifolia* L.

The chloroform extract of DMA revealed purplish pink and bright pink visible zones at R$_f$ = 0.73 and 0.56 respectively when chromatographed on silica using toluene/ethyl methyl ketone/formic acid (8:1.9:0.1) as the solvent system (TLC-12). Using procedures similar to that described in section 3.1.1.1 and 3.1.1.2, these were identified as specific markers for *Rubia cordifolia* L. in DMA. The compounds corresponding to the purplish pink and bright pink zones were isolated by column chromatography (section 2.13.10) and were shown to be alizarin (xii) and purpurin (xiii), respectively.$^{100, 101, 123-126}$

![Diagram](attachment:image.png)
3.1.1.7 Identity test for *Woodfordia fruiticosa* (L) Kurz and *Glycyrrhiza glabra* L.
The diethyl ether extract of DMA and its decoction gave a greenish yellow fluorescent zone at $R_f = 0.49$ under excitation at 366 nm, when chromatographed on silica using toluene/ethyl formate (5:1) (TLC-13). Using procedure similar to that described in section 3.1.1.1 and 3.1.1.2, this was identified as representative "compounds" for the two plant ingredients or either *Woodfordia fruiticosa* (L.) Kurz and *Glycyrrhiza glabra* L. Its identity has not been established as yet.

3.1.1.8 Identity test for *Eugenia caryophyllata* Thumb. and *Cinnamomum verum* Presl.
The chloroform extract of DMA revealed a dark brown zones at $R_f = 0.47$ when chromatographed on silica using n-hexane/diethylamine (8: 2) as the solvent system, and treatment thereafter with anisaldehyde sulfuric acid (TLC-16). Using procedure similar to that described in section 3.1.1.1 and 3.1.1.2, this was identified as a representative compound for the two plant ingredients *Eugenia caryophyllata* Thumb. and *Cinnamomum verum* Presl. in DMA. The compound responsible dark brown zone was identified as eugenol. This is not specific for one plant.

3.1.1.9 Identity tests for *Pterocarpus marsupium* Roxb., *Glycyrrhiza glabra* L. and *Myroxylon balsamum* (L.) Harms.
The diethyl ether extract of DMA revealed bright yellow and pink visible zones at $R_f = 0.67$ and $R_f = 0.45$ respectively when chromatographed on silica using toluene/ethyl formate (5:5) as the solvent system and treatment thereafter with
NP/PEG reagent (TLC-14). Using procedures similar to that described in section 3.1.1.1 and 3.1.1.2, these were identified as representative “compounds” in three plant ingredients *Pterocarpus marsupium* Roxb., *Glycyrrhiza glabra* L. and *Myroxylon balsamum* (L.) Harms. in DMA. The compound responsible for the bright yellow zone was isolated by column chromatography (section 2.13.2) and was shown to be isoliquiritigenin (iii).\(^{94,120,127,128,129}\) The identity of compound responsible for the pink zone has not been established as yet.

### 3.1.2 Identity test based on GLC

GLC was used to establish identity tests for some of the secondary plant materials which contain essential oils. It was possible to separate and identify peaks for eugenol as a representative compound for *Eugenia caryophyllata* Thumb. and *Cinnamomum verum* Presl. leaf and cinnamaldehyde as a marker for cinnamon bark. Although
cinnamaldehyde is difficult to observe in TLC with anisaldehyde sulfuric acid reagent, it can be observed very easily in GLC.

3.1.2.1 Tests for compounds other than marker and representative compounds in DMA

3.1.2.1.1 Gallic acid (ix)

Ethyl acetate extract of DMA and its decoction revealed a prominent blackish blue zone at $R_f = 0.48$, when chromatographed on silica using chloroform: ethyl formate/formic acid (5:4:1) as the solvent system and treatment with 10% ferric chloride reagent (TLC-17). This zone also strongly quenched background fluorescence when excited at 254 nm and exhibited as a blue fluorescence zone with excitation at 366 nm and 254 nm after treatment with NP/PEG reagent (TLC-18, TLC-19, and TLC-20). The compound was isolated from DMA by column chromatography (section 2.13.7) and was shown to be gallic acid (ix). Large quantities of gallic acid are extracted into DMA from *Woodfordia fruticosa* (L.) Kurz. flowers.

\[
\begin{align*}
\text{COOH} & \\
\text{HO} & \\
\text{OH} & \\
\text{OH} & \\
\text{OH} & \\
\text{HO} & \\
\end{align*}
\]

(ix)

Gallic acid
3.1.2.1.2 5-Hydroxymethylfurfural (5HMF) (vii)

Chloroform extract of DMA and its decoction gave a prominent dark bluish visible zone at \( R_f = 0.43 \) chromatographed on silica using toluene/ethyl acetate (7:3) as the solvent system and treatment thereafter with anisaldehyde sulfuric acid reagent (TLC-21). This zone exhibited strong quenching of background fluorescent when excited at 254 nm (TLC-22). The compound responsible was isolated from DMA and identified as 5-hydroxymethylfurfural.\(^{132}\) All other arista also showed the presence of considerable amount of 5-HMF and it is possible that the compound is formed during the boiling process from plant carbohydrates. 5-HMF is found in DMA, its decoction, raisin, bees honey and raw sugar.

\[
\text{HO} \quad \text{vii} \quad \text{HO} \\
\]

5-Hydroxymethyl furfural

3.2 Quantitative analysis

Although pharmacological information on DMA is not available, all the marker compounds and representative compounds identified for plants in the previous section have been reported to have various biological effects. Therefore developing methods for the quantification of these compounds in the drug is important from the viewpoint of standardization and quality control. We were also interested in determining whether the concentration of these compounds in the final product could be correlated with the initial amount of the plants used and the processing methods used in the preparation of the drug. Thus, methods were developed for the quantification of gallic
acid, 5-hydroxymethylfurfural, umbelliferone, isoliquiritigenin, the mixture of isoshinanolone and epiisoshinanolone and the mixture of dehydrocostus lactone and dihyrodehydrocostus lactone in DMA.

3.2.1 Quantitative determination of gallic acid (ix)

Gallic acid is one of the common low molecular weight phenolic acids found in plants. A wide range of biological activities for gallic acid have been reported including phagocytic activity of polymorphonuclear leucocytes, antimutagenic and anticarcinogenic activities, inhibition of thiopurine methyltransferase activity, blocking of anion channels, antiinflammatory and hepatoprotective activity.\textsuperscript{133,134,135} An LD\textsubscript{50} value of 5000 mg/kg of body weight (subcutaneously) for gallic acid in rats has been reported.\textsuperscript{136}

It can be visualized on TLC by treatment with 10 \% ferric chloride reagent (Brownish black) or NP/PEG reagent (purple fluorescence under 366 nm). \textit{Woodfordia fruticosa} (L.) Kurz is a major source of gallic acid in DMA. DA and NA which do not use \textit{Woodfordia fruticosa} (L.) Kurz in their products contain relatively smaller amount of gallic acid (TLC-23). It must be noted however, that some arista which do not use \textit{Woodfordia fruticosa} (L.) Kurz in their products may contain large amounts of gallic acid, due to other ingredients such as \textit{Terminalia bellerica} (Gaertn) Roxb., \textit{Terminalia chebula} Retz. and \textit{Azadirachta indica} A.Juss. (TLC-23). The determination of gallic acid in various fermented products including arista, have been reported using both TLC and HPLC methods.\textsuperscript{137-140} We determined gallic acid in DMA by TLC-FD, TLC-UV and HPLC-UV and observed a good agreement amongst the values obtained by the three methods. Ethyl acetate was used to extract of gallic acid from DMA for TLC
estimation. The TLC-FD method depends on fluorescence of the compound of interest. Although gallic acid does not fluorescence by itself. When treated with NP/PEG reagent, it gives an intense purple fluorescence zone under UV 366 nm. Gallic acid -Natural product reagent (NP/PEG reagent) complex was treated with the hydrophilic solution PEG 4000. This stabilizes the complex by reducing the molecular rotation and preventing contact with atmospheric air. The NP/PEG reagent treated TLC plate was stored in a dark room for one hour to stabilize the fluorescence.

Gallic acid was quantified by HPLC using direct sample injection. In the reported mobile phase of acetonitrile and methanol (RP C₁₈ column) the peaks for gallic acid and 5-HMF overlapped. These could be separated using methanol and 0.1 % acetic acid (10:90 v/v) as the mobile phase. Gallic acid and 5-HMF were identified by UV spectra. The gallic acid content of different brands of DMA were determined by HPLC. The content of 5-HMF in these different brands of DMA was also successfully quantified using the same chromatogram. Gallic acid content of five different batches of five different brands of DMA were determined. The results are given in Table-4.
<table>
<thead>
<tr>
<th>Label assigned to brand</th>
<th></th>
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</thead>
<tbody>
<tr>
<td></td>
<td>TLC-FD method</td>
<td>TLC-UV method</td>
<td>HPLC method</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
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<td>3</td>
<td>4</td>
</tr>
<tr>
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<td>1.680</td>
<td>0.990</td>
<td>1.290</td>
<td>1.060</td>
<td>1.168</td>
<td>1.688</td>
<td>0.992</td>
<td>1.290</td>
<td>1.085</td>
<td>1.138</td>
<td>1.705</td>
<td>1.100</td>
</tr>
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<td>B</td>
<td>0.660</td>
<td>0.642</td>
<td>0.665</td>
<td>0.670</td>
<td>0.760</td>
<td>0.658</td>
<td>0.655</td>
<td>0.668</td>
<td>0.660</td>
<td>0.755</td>
<td>0.663</td>
<td>0.650</td>
<td>0.680</td>
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<td>0.998</td>
<td>1.180</td>
<td>1.655</td>
<td>1.120</td>
<td>0.990</td>
<td>1.000</td>
<td>1.080</td>
<td>1.643</td>
<td>1.080</td>
<td>1.021</td>
<td>1.120</td>
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<td>D</td>
<td>1.060</td>
<td>0.482</td>
<td>0.695</td>
<td>0.860</td>
<td>0.845</td>
<td>1.072</td>
<td>0.478</td>
<td>0.690</td>
<td>0.870</td>
<td>0.840</td>
<td>1.080</td>
<td>0.480</td>
<td>0.700</td>
</tr>
<tr>
<td>E</td>
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<td>0.480</td>
<td>1.090</td>
<td>1.070</td>
<td>0.731</td>
<td>0.867</td>
<td>0.450</td>
<td>1.000</td>
<td>1.068</td>
<td>0.730</td>
<td>0.865</td>
<td>0.470</td>
<td>1.057</td>
</tr>
</tbody>
</table>

Table-4 Gallic acid content of reference samples and commercial samples of DMA by TLC-UV, TLC-FD and HPLC methods (Reference sample = A)
Gallic acid content in different brands of DMA was determined by three different methods. A statistical analysis of the results using EXCEL 97 package showed that there was no significant difference between the three methods ($P < 0.05$). The accuracy of the HPLC, TLC-FD, and TLC-UV methods were established by adding known amount of standard and calculating recoveries. The percentage recoveries were 99.5-102 % for HPLC, 99-101 % for TLC-FD, and 98.2-99 % for TLC-UV. The precision determined by 4 replicates of the analytical methods, were 1.0 % CV for HPLC, 1.8 % for TLC-FD, and 2 % for TLC-UV. Thus, all three methods are precise and accurate. The results also show clearly that there is a wide variation in the gallic acid content among the different brands, and within different batches of the same brand. The causes for these variations need to be studied and process control measure set up to obtain a standardized product.

3.2.2 Quantitative determination of 5-HMF (vii)

5-HMF was isolated from DMA and identified by its NMR spectra. Various toxic effects of 5-HMF on animals have been reported. It was also identified as an aldose reductase inhibitor found in Kampo medicine. 5-HMF arises during the processing of many food stuffs, from the breakdown of carbohydrates. It has been shown that the 5-HMF can arise from the acid catalyzed decomposition of dextrose and laevulose. Dextrose and Laevulose containing injections have a limit test for 5-HMF to control the extent of decomposition.

Quantitative determination and control of 5-HMF in DMA is important. Both colorimetry and HPLC have been widely used for quantitative determination of
5-HMF.\textsuperscript{145-151} 5-HMF standard was synthesised from D-fructose.\textsuperscript{152} Although 5-HMF produce a dark blue colour with anisaldehyde sulfuric acid reagent, the colour is not stable. Therefore direct UV scanning at the absorption maximum of 5-HMF (283nm) was used for densitometric quantification. 4-hydroxybenzaldehyde was used as the internal standard. HPLC–UV quantification was carried out using the same chromatogram used for the determination of gallic acid described above in section 3.2.1. The HPLC method is much more sensitive (detection limit 0.5 ng) than the TLC method. 5-HMF content of five different batches of five different brands of DMA were determined.

<table>
<thead>
<tr>
<th>Label assigned to brand</th>
<th>5-HMF level (mg/ml) by TLC-UV</th>
<th>5-HMF content (mg/ml) by HPLC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Number assigned to batch</td>
<td>Number assigned to batch</td>
</tr>
<tr>
<td>A</td>
<td>1 0.081 0.131 0.043 0.097 0.084</td>
<td>1 0.081 0.132 0.043 0.098 0.084</td>
</tr>
<tr>
<td>B</td>
<td>2 0.040 0.072 0.057 0.076 0.165</td>
<td>2 0.050 0.073 0.059 0.077 0.166</td>
</tr>
<tr>
<td>C</td>
<td>3 0.049 0.029 0.049 0.050 0.082</td>
<td>3 0.049 0.028 0.050 0.048 0.080</td>
</tr>
<tr>
<td>D</td>
<td>4 0.455 0.176 0.217 0.198 0.095</td>
<td>4 0.455 0.177 0.216 0.200 0.095</td>
</tr>
<tr>
<td>E</td>
<td>5 0.283 0.232 0.270 0.229 0.181</td>
<td>5 0.284 0.230 0.268 0.231 0.182</td>
</tr>
</tbody>
</table>

Table: 5 5-HMF content of reference samples and commercial samples of DMA determined by TLC-UV and HPLC methods (Reference sample = E)

A t-test of these data shows no significant difference ($P < 0.05$) in the results obtained by the two methods. The accuracy of the TLC and HPLC methods were established by adding known amount of standard and calculating recoveries. The percentage
recoveries were 98-99.5 % for the TLC-UV and 99.7-102 % for the HPLC. The precision as determined 4 replications of the analytical methods, were 1.75 % CV for the TLC-UV and 1.2 % for the HPLC. In conclusion, the TLC-UV method is precise, accurate, simple, rapid and can be used routinely instead of HPLC. We have observed that the 5-HMF content changes during the different stages of manufacture such as, boiling, fermentation and storage. It is therefore useful as a parameter to quantify for process control.

3.2.3 Quantitative determination of Isoshinanolone (v) and Episoshinanololone (vi)

Isoshinanolone and mixtures of isoshinanolone and episoshinanolone have been reported from several Plumbago species (Plumbago zeylanica L., Plumbago indica L., Plumbago scadens). Of the two Plumbago species found in Sri Lanka, Plumbago zeylanica L. and Plumbago indica L., the chemistry of Plumbago zeylanica L. has been studied, and the presence of isoshinanolone in it has been reported. DMA contains Plumbago indica L. as a major ingredient. Our investigations revealed that it contains isoshinanolone and it trans-isomer. Mixtures of isoshinanolone and episoshinanolone (ISMIX) have also been reported from Plumbago scadens, Diospyros species, Ceratostigma minus and Dioncophyllum thollani. Apart from weak antiplasmodial activity, no pharmacological effect has been reported from isoshinanolone.

Isoshinanolone and episoshinanolone are useful as specific marker compounds for Plumbago indica L. in DMA. They move at the same rate on TLC in different mobile
phases and could not be separated even after acetylation or benzylation. As it was necessary to observe the absorbance emission characteristic of two isomers before developing a TLC-FD method for ISMIX, they were separated by HPLC, on a C18 column using methanol/water (50: 50 v/v). The LC-UV absorption spectra of the two isomers were identical (Fig-12).

Fig-12  HPLC chromatogram and LC-UV spectra of mixture of isoshinanolone and epiisoshinanolone

Peak-1 : Epiisoshinanolone  Peak-2 : Isoshinanolone

It was assumed that their emission characteristics would be the same. However, this needs to be confirmed by spectrofluorometry. The compounds are quantified on TLC by measuring their fluorescence at 400 nm to 450 nm, when excited at 384 nm. In order to increase the sensitivity of the method, different fluorescence enhancing
substances were sprayed. Of the substances tested, fluorocene, ethanolic potassium hydroxide, polyethylene glycol 4000, n-hexane, liquid paraffin and 1 % ethanolic aluminum hydroxide, 1 % ethanolic aluminum hydroxide proved to be the most effective. The fluorescence was stabilized by a final spraying of the plate with 50 % ethanolic paraffin solution. The change in fluorescence emission with time for different loading of ISMIX on TLC was studied (Fig-13). Emission was stable after 30 minutes. Another fluorescent compound present in DMA, umbelliferone, runs very close to the compounds of interest. An adequate $\Delta r_f$ value of 0.1 can be obtained with toluene/ethyl acetate (8:2) as the solvent. The preparation of the standard curve for the TLC-FD method is described in section 2.14.1.7.

Concentrations of ISMIX solutions:

- 0.03 mg/ml
- 0.02 mg/ml
- 0.01 mg/ml
- 0.005 mg/ml

![Figure 13: Stability of fluorescent intensity of ISMIX after treatment with ethanolic aluminium hydroxide and ethanolic paraffin reagent](image.png)
The accuracy of the TLC method was established by adding known amount of standard and calculating recoveries. The percentage recoveries were 99.2-100.8 %. The precision as determined by 4 replicates of the analytical method, was 1.52 % CV. In conclusion, the TLC-FD method is precise, accurate and simple. ISMIX content of five different batch samples of five different brands of DMA were determined. The results are given in Table-6.

<table>
<thead>
<tr>
<th>Label assigned to brand</th>
<th>Label</th>
<th>Number assigned to batch</th>
<th>ISMIX content (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>A</td>
<td>0.0151</td>
<td>0.0092</td>
<td>0.0446</td>
</tr>
<tr>
<td>B</td>
<td>ND</td>
<td>ND</td>
<td>0.0071</td>
</tr>
<tr>
<td>C</td>
<td>0.0068</td>
<td>0.0079</td>
<td>0.0050</td>
</tr>
<tr>
<td>D</td>
<td>ND</td>
<td>0.0044</td>
<td>0.0080</td>
</tr>
<tr>
<td>E</td>
<td>0.0060</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

Table-6: ISMIX content of reference samples and commercial samples of DMA determined by TLC-FD method (Reference sample = A)

There is a very wide variation in the amount of ISMIX found in these samples. Some batches of some brands did not contain detectable amounts (detection limit 1ng) of these compounds while a few batches contained much higher than the general range (TLC-25). These variations probably reflect the variation in the quality of the crude drug used in the manufacturing process.
Plumbago indica L. is one of more expensive raw material in local drug market. The price is around 600-500 Rs per kg. In the local market, an inferior quality Plumbago indica L. called “indian ratnitul” is sold at a low price of 50-75 Rs per kg. The level of ISMIX in “Indian Ratnitul” is very low. Some samples of Plumbago indica L. roots are adulterated with aerial part of the plant, which have a lower concentration of ISMIX. Plumbago indica L. root is washed with aqueous Ca(OH)\textsubscript{2} solution prior to incorporation in the drug. This washing results in the loss of some of the phenolics in the plant material including ISMIX. If the washing is inadequate high levels of ISMIX may be present in the final product. It is interesting to note that the major phenolic compound in Plumbago indica L. plumbagin, is not detected in DMA. The dissolution of plumbagin into the Ca(OH)\textsubscript{2} solution can be demonstrated easily by acidification of the solution and extraction of plumbagin from it with chloroform. Plumbagin is also steam volatile and what ever remains in the Plumbago indica L. roots after purification is lost during the long boiling process in preparing the decoction. Plumbagin is a low melting (78 °C) phenolic compound which sublimes at 90 °C.\textsuperscript{160} It has to be borne in mind however, that minute quantities of plumbagin may be present in DMA.

![Diagram of Plumbagin](image)

(iv) Plumbagin
Plumbagin shows several biological activities such as antifertility, antifungal, antibacterial, anticonagulant, anticancer and antimalarial activity.\textsuperscript{159} Studies of biological activity has shown that plumbagin stimulated the isolated rabbit uterus at a concentration of 0.000001 % and at a concentration of 0.00001 % it inhibited this action.\textsuperscript{158} Recently it was reported that plumbagin shown a positive inotropic response on guniea pig papillary muscles at concentration $10^{-7}$ M to $3 \times 10^{-6}$ M.\textsuperscript{161} The fact that ISMIX is present in the final product indicates that its calcium salt may be less soluble in water than that of plumbagin, and that it is also less steam volatile, despite its lower polarity (TLC-24).

The level of ISMIX of plants growing two different districts Kurunagala and Gampha were studied (Table-7 and Fig-14) and found to be significantly different. Thus variation in the final product can also arise due to the locality from which the plants were harvested.

<table>
<thead>
<tr>
<th>Location of samples</th>
<th>% (w/w) ISMIX or Isoshinanolone</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sample-1</td>
</tr>
<tr>
<td>Kurunagala</td>
<td>0.0059± 0.00012</td>
</tr>
<tr>
<td>Gampha</td>
<td>0.0034± 0.00015</td>
</tr>
</tbody>
</table>

Table-7 ISMIX content of Plumbago indica L. root from two locations

It was also found that the ratio of isoshinanolone to epiisoshinanolone varied in the plants growing in different parts of the country. The ratio was determined by HPLC (Fig-12) and $^1$H NMR (Fig 45 & 46).
Fig-14 TLC-FD densitometric fingerprint for methanol extract of *Plumbago indica* L. root

Peak-1: isoshinanolone and the mixture of isoshinanolone and epiisoshinanolone

$^1$H NMR spectra of the mixture shows doublet at $\delta$ 4.72 ($J = 2.5$ Hz) for H-1 of Isoshinanolone and small doublet at $\delta$ 4.47 ($J = 8$ Hz) appears for H-1 of epiisoshinanolone. Intensity of the doublet at $\delta$ 4.47 is 25% of doublet at $\delta$ 4.72. This indicated that mixture containing 4:1 ratio of isoshinanolone and epiisoshinanolone. Therefore smaller signal of $^{13}$C NMR spectrum of the mixture can be assigned unambiguously to carbon atoms of epiisoshinanolone. There was good agreement between the results of the two methods (Table 8).

<table>
<thead>
<tr>
<th>Location</th>
<th>Ratio of Isoshinanolone/Epiisoshinanolone</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>1HNMR</td>
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<tr>
<td>Matale</td>
<td>4:1</td>
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<tr>
<td>Anuradhapure</td>
<td>4:1</td>
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<tr>
<td>Gampha</td>
<td>1:0</td>
</tr>
<tr>
<td>Kurunagala</td>
<td>1:0</td>
</tr>
<tr>
<td>Colombo</td>
<td>1:0</td>
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</tbody>
</table>

Table-8 Ratio of isoshinanolone and epiisoshinanolone in *Plumbago indica* L. root determined by HPLC and $^1$H NMR.
3.2.4 Quantitative determination of dehydrocostus lactone (x) and
dihydrodehydrocostus lactone (xi)

*Saussurea lappa* C. B. Clarke. root is rich source of the guaianolide type lactones
dehydrocostus lactone, dihydrodehydrocostus lactone and germacranolide type
lactones costunolide. The vasoactive and neuroleptic properties of pure
dehydrocostus lactone has been reported. Dehydrocostus lactone (DHC) and
dihydrodehydrocostus lactone (DDHC) can be observed as purplish blue spots on TLC when sprayed with anisaldehyde sulfuric acid reagent. However the colour fades rapidly. The pink colour displayed by spraying

![Graph](image_url)

Figure-15 Stability of colour complex of the mixture of DHC and DDHC with LB
spray reagents on TLC.

with Liebermann-Burchard reagent (LB reagent) was more stable (Fig-15), and more
suitable for densitometry.
Cinnamaldehyde was an interfering substance with most solvent systems. It could be separated from the DHC and DDHC spots by using of n-hexane/diethylamine (8:2) as the mobile phase. Changing the ratio to 99.5:0.5 resulted in the separation of the two lactones as well (Fig-16 and Fig-17). DHC standard for chromatography was isolated from *Saussurea lappa* C. B. Clarke. The compound was separated from other lactone including DDHC, by the use of argentative silica. resulting in the formation of π complexes between the unsaturated lactones and Ag⁺ ions, helps in the chromatographic resolution of many closely related lactones.¹⁶⁴ DDHC standard for chromatography was synthesised from DHC by borohydrate reduction. The identity and purity of the standard compounds were established by chromatography and ¹H NMR, ¹³C NMR (Fig-57, 58, 59, 60, 61, 62, 63) and GC/MS. TLC densitometry was carried for the mixture of DHC and DDHC (DDMIX). The standard curve was drawn using pure DHC as the UV-VIS densitometric scan of DHC and DDHC, showed them to have identical spectra (Fig-18). It is easier to detect DHC and DDHC separately using GC than TLC. The chloroform extract of DMA can be cleaned up by passing it through a short silica column, prior to injecting directly to the GC. Anthracene was found to be a suitable internal standard.
Fig-16 TLC- densitogram of chloroform extract of reference DMA - solvent system: hexane: diethylamine (80:20)

1. Eugenol
2. Mixture of dehydrocostus lactone and dihydrodehydrocostus lactone
3. Cinnamaldehyde

Fig-17 TLC- densitogram of chloroform extract of reference DMA - solvent system: hexane: diethylamine (99.5:0.5)

1. Eugenol
2. Dehydrocostus lactone
3. Dihydrodehydrocostus lactone
4. Cinnamaldehyde
The DHC and DDHC content of 5 different batches each of 5 different brands of DMA were determined by GC. The DDMIX content of the same sample were measured by TLC -VIS densitometry and compared with the values obtained by GC (Table-9)
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</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DHC(μg/mL)</td>
<td>DDHC(μg/mL)</td>
<td>Total lactone(DHC+DDHC) (μg/mL)</td>
<td>Total lactone(DHC+DDHC) (μg/mL)</td>
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<td>5</td>
<td>1</td>
<td>2</td>
<td>3</td>
</tr>
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<td>0.61</td>
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<td>ND</td>
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<td>0.31</td>
<td>0.92</td>
<td>0.47</td>
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<td>1.16</td>
</tr>
<tr>
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<td>ND</td>
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<td>7.5</td>
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<td>0.94</td>
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<td>0.92</td>
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<td>0.8</td>
<td>8.53</td>
<td>2.61</td>
<td>8.42</td>
</tr>
<tr>
<td>E</td>
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<td>0.17</td>
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<td>3.81</td>
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<td>3.12</td>
<td>2.47</td>
<td>4.12</td>
<td>4.23</td>
<td>1.12</td>
</tr>
</tbody>
</table>

ND : Not detected      (Reference sample = A)

Table: 9  Costuslactones content of reference sample and commercial samples of DMA determined by GLC and TLC-VIS methods

105
The accuracy of the GLC and TLC methods were established by adding known amount of standard and calculating recoveries. The percentage recoveries were 99-102.5 % for the GLC method and 98.5-99 % for TLC method. The precision determined 4 replicates of the analytical methods, were 1.0 % CV for GLC method and 1.82 % for the TLC method. In conclusion, the GLC method and TLC-VIS methods are both precise, accurate and simple. There was good agreement between the results obtained by the two methods. These two methods shows no significant differences between the results (P < 0.05). There is a very wide variation in the amount of DDMIX found in these samples. These variations probably reflect the variation in the quality of the crude drug used in the manufacturing process. *Saussurea lappa* C. B. Clarke. is commonly adulterated with inferior quality *Inula racemosa* Hook. f. *Inula racemosa* Hook. f. is rich in eudesmanolide sesquiterpene lactones and *Saussurea lappa* C.B. Clarke. contained mainly guaianolide sesquiterpene lactones. We observed that the chloroform extract of *Saussurea lappa* C. B. Clarke. gave a purple zone at $R_f = 0.59$ and dark green zone at $R_f = 0.52$ when chromatographed on silica using n-hexane/ethyl acetate (7:3) as the solvent system and treatment thereafter with 5 % ethanolic sulphuric acid reagent. *Inula racemosa* Hook. f. showed a brown spot on TLC at $R_f = 0.95$ under same conditions (TLC-31). This was in agreement with a method reported for distinguishing the two plants by TLC. However, a much clearer differentiation of the TLCs can be obtained by spraying with anisaldehyde sulfuric acid reagent rather than sulfuric acid reagent (TLC-32). The DHC and DDHC content in market samples of the rhizomes of *Saussurea lappa* C. B. Clarke were measured by GC. Sample preparation was carried out as follows; The extraction of the lactones could not be achieved by use of diethyl
ether, methylene chloride, chloroform, ethyl acetate, methanol and acetonitrile as the solvents. Therefore a double extraction procedure was developed: extraction first with 50 % aqueous methanol followed by methanol resulted in total extraction. The extract was analysed by GC (Fig-19) and NMR (Fig-64, 65). It was observed that some samples contained only DHC, while others contained both DHC and DDHC. However, the DHC content (1.4 ± 0.03 %) is higher than the DDHC content (0.12 ± 0.02 %) in the rhizome, when both are present.

Fig -19  GLC chromatogram of methylene chloride extract of *Saussurea lappa* C.B. Clarke rhizome

IS : internal standard  
Peak-1 : dihydrodehydrocostus lactone  
Peak-2 : dehydrocostus lactone
3.2.5 Quantitative determination of umbelliferone (viii)

Umbelliferone is easily recognized on TLC due to its characteristic blue fluorescence. A range of biological activities have been reported for it, including antitumor and immunomodulatory activity. In addition, umbelliferone is used as an UV protector in some sun cream formulations. HPLC methods for the quantitative determination of umbelliferone in human serum, plasma, bovine liver, and extract of Oak wood and in matured distilled alcoholic beverages have been reported. TLC-FD has been used to determine umbelliferone content in “Navaratne Kalkaya”, an Ayurvedic drug, and in human urine.

Umbelliferone in DMA arises mainly from the root of Aegle marmelos Correa. Quantification of umbelliferone is of value because Aegle marmelos Correa is one of the roots out of the ten roots which gives the drug DMA its name. Development of a mobile phase for the estimation of umbelliferone in DMA by TLC-FD was difficult due to the presence of background interfering substances, especially the flavonoids. It was necessary to use HPTLC plates instead of conventional TLC plates. This reduced diffusion of components and achieved a better separation than conventional TLC plates. Toluene/methyl ethyl ketone (9:1) proved to be the best solvent out of a number of toluene-based solvents such as toluene/ethyl acetate (7:3) and toluene/diethyl ether (1:1). The umbelliferone content of five different batch samples of three different brands of DMA were determined. The accuracy of the method was established by adding known amounts of standard and calculating recoveries. The percentage recoveries were 99.2-104%. The precision determined by 4 replicates of the analytical method, was 1.34% CV. Limit of detection is 1 ng. In conclusion, the
TLC-FD method is precise, accurate, simple, rapid and sensitive. The results are given in Table-10.

<table>
<thead>
<tr>
<th>Number assigned to batch</th>
<th>Umbelliferone content (µg/ml)</th>
<th>Label assigned to brand</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A</td>
<td>B</td>
</tr>
<tr>
<td>1</td>
<td>1.1</td>
<td>1.02</td>
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<tr>
<td>2</td>
<td>0.71</td>
<td>0.77</td>
</tr>
<tr>
<td>3</td>
<td>0.67</td>
<td>0.94</td>
</tr>
<tr>
<td>4</td>
<td>1.51</td>
<td>0.74</td>
</tr>
<tr>
<td>5</td>
<td>1.68</td>
<td>1.18</td>
</tr>
</tbody>
</table>

Table-10 Umbelliferone content of reference sample and commercial samples of DMA determined by TLC-FD method (Reference sample = A)

There is a variation in the amount of umbelliferone found in these samples. These variations probably reflect the variation in the quality of the crude drug used in the manufacturing process. Often, the roots are adulterated with the stem, which contains a much lower umbelliferone content. Umbelliferone contents of 0.1 % for the root and 0.02 % for the stem bark have been reported.\(^{174}\) Thus adulterant can be responsible for the variation of umbelliferone content observed. Although there are other plants of DMA that has umbelliferone, extraction studies show that these make a negligible contribution to the umbelliferone content in the final product.
3.2.6 Quantitative determination of Isoliquiritigenin (iii)

Isoliquiritigenin is one of the biologically active compounds found in DMA. Aldol reductase activity, monoamine inhibitory effect, strong inhibition of adenosine 3’5’-cyclic monophosphoterase, inhibition of platelet aggregation, inhibitory effect on the activation of hyaluronidase, inhibitory effect on the tube formation from vascular endothelial cells, and the antioxidant and vasorelaxant effect of isoliquiritigenin have been reported.129,175-185

Three plants, *Glycyrrhiza glabra* L., *Pterocarpus marsupium* Roxb. and *Myroxylon balsamum* (L.) Harms. are mainly responsible for the isoliquiritigenin in DMA. HPLC has been used for the determination of isoliquiritigenin content in *Glycyrrhiza* species.186,187 Diethyl ether was chosen over the more commonly used ethyl acetate as the solvent for extracting isoliquiritigenin from DMA, as it was more selective. Some non-polar compounds and some oxygenated compounds such as terpenes and carotenoids were seen on TLC as interfering substances. The interfering non phenolic components of the ether extract of DMA were removed by solvent extraction as described earlier (section 2.14.1.1). Mixtures of toluene with diethyl ether or ethyl acetate, with or without saturation with 10 % acetic acid were not capable of fully resolving isoliquiritigenin from kaempferol. However, a good separation was achieved by toluene/ethyl formate (5:5) with $R_f$ value of 0.58 and 0.67 (TLC = 14) for kaempferol and isoliquiritigenin respectively. Isoliquiritigenin can be distinguished from kaempferol on TLC by its colour on being sprayed with NP/PEG reagent. Isoliquiritigenin is a dark yellow while kaempferol is a light greenish yellow. Furthermore, isoliquiritigenin displays only a dark zone (TLC-15) under 366 nm
(after spraying with NP/PEG), whereas kaempferol like most other flavonols gives a strong greenish yellow fluorescence. Isoliquiritigenin content in DMA was measured by TLC-VIS densitometry (section 2.14.1). The stability of colour complex was studied by TLC densitometry and it was observed that the absorbance stabilized after 10 mins. The accuracy of the TLC method was established by adding known amount of standard and calculating recoveries. The percentage recoveries were 89-95 %. The precision determined 4 replicates of the analytical method was 1.96 % CV. In conclusion, the TLC-VIS method is precise, accurate, simple and rapid. Isoliquiritigenin content of five different batch samples of five different brands of DMA are given below:

<table>
<thead>
<tr>
<th>Number assigned to batch</th>
<th>Isoliquiritigenin content (mg/ml))</th>
<th>Label assigned to brand</th>
</tr>
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<td></td>
<td>A</td>
<td>B</td>
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<tr>
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<td>0.0042</td>
<td>0.0041</td>
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<td>0.0046</td>
</tr>
<tr>
<td>5</td>
<td>0.0035</td>
<td>0.0032</td>
</tr>
</tbody>
</table>

Table-11 Isoliquiritigenin content of reference samples and commercial samples of DMA by TLC-VIS method.(reference sample = B)

There is a variation in the amount of isoliquiritigenin found in these samples. These variations probably reflect the variation in the quality of the crude drug used in the manufacturing process. Many factors, which can cause this variation, can be
identified. Isoliquiritigenin is found in three of the plants used in the preparation of DMA, names of plants, *Pterocarpus marsupium* Roxb., *Myroxylon balsamum* (L.) Harms. and *Glycyrrhiza glabra* L. *Glycyrrhiza glabra* L. found in the market often contains material that has been extracted for various active compounds. This low grade *Glycyrrhiza glabra* L. with a low isoliquiritigenin content is commonly available in the market and is used by manufacturers. Sometimes, the sapwood of *Pterocarpus marsupium* Roxb. which contain very little Isoliquiritigenin is substituted for the heartwood. Often, *Myroxylon balsamum* (L.) Harms. which contains only a low amount of isoliquiritigenin is substituted for *Acacia catechu* Willd. Although these plants are in the same family, they can be clearly differentiated by TLC (TLC-26, 27, 28, 29, 30).

### 3.3 Parameters for process control

In order to determine whether the methods developed for the quantification of compounds in the final product could be used for the purpose of process control, the changes in the concentration of selected compounds during the two major processing steps, boiling and fermentation were measured.

#### 3.3.1 Boiling process

Three vessels labeled R1, R2 and R3 in which portions of the same batch of DMA produced at Link Natural Products were being boiled were studied. The variations in the pH and the temperature during the boiling period are shown in Fig-21. It is to be noted that boiling is continued until the desired change in volume is achieved. As the rate of heat supply to the vessels are not all the same, the boiling period varies from
vessel to vessel. The lowering of temperature around the 15th hour in R1 and R2 was caused by a fluctuation in the gas supply. The variation in gallic acid and 5-HMF was monitored by HPLC (Fig-20) and is shown in Fig–22 and 23.

![HPLC chromatogram](image)

**Fig -20** HPLC chromatogram (direct injection) for gallic acid and 5-HMF in reference DMA

Peak-1: Gallic acid
Peak-2: 5-HMF
Fig-21 Variation of pH value and temperature of decoction of reference DMA during boiling (R1, R2, R3 are selected vessels)

Fig-22 Variation of gallic acid content of decoction of reference DMA during boiling

Fig-23 Variation of 5-HMF content of decoction of reference DMA during boiling
The gallic acid content rises gradually due to the hydrolysis of gallates, while the 5-HMF content rises gradually due to the conversion of carbohydrates in the acid medium. However it is difficult to explain the sudden rise in the concentration of gallic acid and 5-HMF in R1 and R2. The rise in gallic acid content is also not reflected in the pH. The variation in DHC level is shown in Fig-24, 25. It is noted that the concentration achieved in the first 7 hours remain more or less constant during the entire boiling process. Analysis of the marc shows that it contains a large amount of unextracted DHC. Thus, DHC which steam distills out of the vessel during the boiling is replaced by extractive from the marc.
Fig- 25 GLC profile of methylene chloride extract of
decocction of reference DMA after 12 hours of boiling

IS- Internal standard
Peak-1 : Dehydrocostus lactone

Therefore the final level of DHC in the finished product does not reflect the amount
ofSaussurea lappa C. B. Clarke. used to manufacture the drug. The DHC level does
not show a significant change during the fermentation process (Fig-26, 27).

Fig-26 Variation of dehydrocostus lactone content in fermenting decoction
We conclude that DHC is more useful as a marker for identity than a quantitative measure of drug quality.

3.4 Adulterants and substituents

Establishing the identity and quality of the crude drugs used in the preparation of DMA is an important aspect of its quality assurance. Adulteration and substitution are two common problems that have to be dealt with. Adulterants are spurious materials mixed with the genuine materials, while substituents are materials which totally replace the genuine material. In Ayurveda, the pharmacopoeia mentions officially acceptable substitutents for some plant materials. There are also unofficial substitutes.
commonly in use. Table-10 lists the official substitutes, unofficial substitutes and adulterants for some of the plants used in DMA. ²⁰,६५,१८८

<table>
<thead>
<tr>
<th>Plant</th>
<th>Official Substitute</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saussurea lappa C.B.Clarke (rhizome)</td>
<td>Inula racemosa Hook. f.</td>
</tr>
<tr>
<td>Plumbago indica L.</td>
<td>Plumbago zeylanica L./Piper chaba Hunter.</td>
</tr>
<tr>
<td>Piper longum L.</td>
<td>Piper nigrum L.</td>
</tr>
<tr>
<td>Glycyrrhiza glabra L.</td>
<td>Abrus precatorius L./Woodfordia fruticosa (L.) Kurz.</td>
</tr>
<tr>
<td>Desmodium gangeticum (L.) D. C.</td>
<td>Alysicarpus vaginalis (L.) DC.</td>
</tr>
<tr>
<td>Uvaria picta Desv. Ex. D. C</td>
<td>Aerva lanata (L.) Juss. Ex Schultes</td>
</tr>
<tr>
<td>Fritilaria roylei Hook. F</td>
<td>Withania sominifera Dunal/ Asparagus racemosus Willd.</td>
</tr>
<tr>
<td>Lilium polyphyllum D. Don.</td>
<td>Asparagus racemosus Willd./ Ipomea mauritiana Jacq.</td>
</tr>
<tr>
<td>Habenaria edgeworthii Hook. f. ex Collett.</td>
<td>Sida cordifolia L.</td>
</tr>
<tr>
<td>Polygonatum verticillatum Allioni</td>
<td>Withania sominifera Dunal / Asparagus racemosus Willd./ Hemidesmus indicus R. Br.</td>
</tr>
<tr>
<td>Polygonatum cirricfolium Royle</td>
<td>Withania sominifera Dunal / Asparagus racemosus Willd./ Hemidesmus indicus R. Br.</td>
</tr>
<tr>
<td>Callicarpa macrophylla Vahl</td>
<td>Myristica horsfieldii Blume</td>
</tr>
<tr>
<td>Prunus cerasoides D. Don.</td>
<td>Calophyllum cuneifolium Thw.</td>
</tr>
<tr>
<td>Cinnamomum tamala Nees &amp; Eberm.</td>
<td>Cinnamomum verum Presl</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Plant</th>
<th>Unofficial substituent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nardostachys jatamansi D. C. (rhizome)</td>
<td>Flickingeria macraei (Lindl.) Seidenf.</td>
</tr>
<tr>
<td>Acacia catechu Willd. (heartwood)</td>
<td>Myroxylon balsumum (L.) Harms.</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Plant</th>
<th>Adulterant</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cuminum cymminum L</td>
<td>Anethum graveolens L.</td>
</tr>
<tr>
<td>Embelia ribes Burm. f.</td>
<td>Embelia tsjeriam-cottam A. DC.</td>
</tr>
<tr>
<td>Alpinia officinarum Hance.</td>
<td>Alpinia calcarata Rosc.</td>
</tr>
</tbody>
</table>

Table-12 Official substitutents, unofficial substitutents and adulterants for some crude drugs used in DMA
3.4.1 *Nardostachys jatamansi* DC. and *Flickingeria macraei* (Lindley) Seidenf.

*Flickingeria macraei* (Lindley) Seidenf. is often used as an unofficial substitute for *Nardostachys jatamansi* DC. These two plants can be easily differentiated by TLC (TLC -33). Further identity of *Nardostachys jatamansi* DC. was established by GLC fingerprint (Fig -28) of its essential oil. *Flickingeria macraei* (Lindley) Seidenf. does not contain volatile oils.
3.4.2 *Hemidesmus indicus* R. Br. and *Cryptolepis bunchananii* Roem. & Schult.

The two plants are in the same family and their close relationship is indicated in the Sinhala name for the two plants, Kalu iramusu (*Cryptolepis bunchananii* Roem. & Schult.) and Sudu iramusu (*Hemidesmus indicus* R. Br.). The TLC's of the two plants (methanol extracts) are very similar. However, *Cryptolepis bunchananii* Roem.& Schult. displays an prominent orange spot of $R_f = 0.21$ not observed *Hemidesmus indicus* R. Br. (TLC-34). The official substituent, *Cryptolepis bunchananii* Roem. & Schult. is often used due to the shortage of *Hemidesmus indicus* R. Br.

3.4.3. Bees honey

Bees honey of different qualities are found in the market. It is often adulterated with glucose, fructose, sucrose and corn syrup. The quality of the honey is being determined by its diastase number, HMF content and invertase activity. The bees honey is a complex mixture. It contains fructose, glucose, sucrose, maltose, lactones, higher sugars, and free acids. Seventeen flavanoids namely, kaempferol, quercetin, 8-methoxy kaempferol, quercetin-3-methyl ether, isorhamentin, kaempferol-3-methylether, quercetin-3-3'-dimethyl ether, quercetin-3,7-dimethyl ether, galangin, luteolin, apigenin, genkwanin, chrysin, luteolin-7-methyl ether, pinocembrin, pinobanksin and hesperetin have been reported. The diethyl ether extract of six commercial samples of bees honey gave different coloured fluorescent zones under excitation at 366 nm (TLC-35, TLC-36 under white light) when chromatographed on silica using toluene/ethyl formate (5:5) as the solvent system and treatment thereafter with NP/PEG reagent on TLC. There is a wide variation of the flavonoids in the commercial samples. The flavonoid profile can be useful in determining the quality
and standardization of bees honey. Track no:3 in TLC-35 and 36 is of authentic bees honey collected from the Kekirawa. It is rich in flavonoids. The spots at $R_f = 0.51$ and 0.41 have been identified as kaempferol and quercetin respectively. These are found only in the bees honey from Ayurvedic Drug Corporation shown in tracks 1. The bees honey represented in tracks 2, 5, and 6 appears to be adulterated with another sugar source.

### 3.5 Chromatographic fingerprints for Dasamoolarista

The chromatographic “Fingerprinting” is widely accepted as the most informative method for monitoring the identity and purity of plant materials as well as finished products. The fingerprint (FP) is a powerful tool for assessing the consistency of product quality. It can be used for successful in-process control. If defects are found during processing there may be possibilities to take corrective action. Thin layer chromatography, High performance liquid chromatography and Gas chromatography are widely applied to establish fingerprints for medicinal plants and their preparations. The combination of fingerprints from the difference chromatographic methods give a comprehensive image of the plant extracts or their preparations. Among the different chromatographic techniques TLC- FP is widely used for the purpose of routine quality control of herbal products. The GLC-FP method is restricted to the volatile part of the drug or extract. The HPLC-FP is difficult to develop for poly herbal mixtures. It is not economical for routine quality control purposes. Complex poly herbal preparations contain different classes of compounds whose chromatographic behaviour is not uniform. Therefore it is necessary to fractionate and simplify the complexity of the material used for chromatography, in order to obtain meaningful FPs.
Fingerprints in which each peak represents a single compound, and its identity and source (s) are known represent an ideal situation which is rarely achieved. Due to the variable composition of biological materials, fingerprints of samples are not expected to match exactly the reference fingerprint. The range of variability to be expected can be assessed only by studying the variability in a number of reference samples. Attempts to develop a quantitative comparison of FP’s based on relative peak area of selected peaks was not successful due to the high variability in the data of reference samples. Comparison of peak areas of those to an internal standard may be more meaningful. The following fingerprints were developed for DMA:

1. Chloroform extract of DMA = section 2.14.1.6
   Chromatographic conditions = section 2.14.1.6
   Fluorimetric analysis was carried out at \( \lambda_{ex} = 340 \text{ nm} \) and \( \lambda_{em} = 400-500 \text{ nm} \).
   Fig-29 Fingerprint-1, umbelliferone (peak no:1) is the important marker compound in this FP.

2. Diethyl ether extract of DMA = section 2.14.1.1
   Chromatographic conditions = section 2.14.1.1
   Photometric analysis was carried out at \( \lambda = 435 \text{ nm} \)
   Fig-30 Fingerprint-2, kaempferol (peak no:05) and isoliquiritigenin (peak no: 06) are the important marker compounds in this FP.
3. Diethyl ether extract of DMA = section 2.14.1.1
Chromatographic conditions = section 2.14.1.1
Fluorimetric analysis was carried out at $\lambda_{ex} = 315$ nm and $\lambda_{em} = 400-600$ nm.

**Fig-31** Fingerprint-3, gallic acid (peak no: 02), quercetin (peak no:04)
kaempferol (peak no:06), umbelliferone (peak no: 09), the isoshinanolone or the mixture of isoshinanolone and epiisoshinanolone (peak no:10) are the important marker compounds in this FP.

4. Chloroform extract of DMA = section 2.14.1.7
Chromatographic conditions = section 2.14.1.7
Fluorimetric analysis was carried out at $\lambda_{ex} = 384$ nm and $\lambda_{em} = 400-450$ nm.

**Fig-32** Fingerprint-4, isoshinanolone or the mixture of isoshinanolone and epiisoshinanolone (peak no: 1) are the important marker compounds in this FP. This FP also clearly shown that the variation of isoshinanolone/isoshinanolone and epiisoshinanolone in reference samples and commercial samples of DMA. The brand E shows a completely different pattern to the reference profile and does not show the peak for isoshinanolone or the mixture of isoshinanolone and epiisoshinanolone as well.

5. Chloroform extract of DMA = section 2.14.1.4
Chromatographic conditions = section 2.14.1.4
Photometric analysis was carried out at $\lambda = 283$ nm.

**Fig-33** Fingerprint-5, gallic acid (peak no:01) and 5-HMF (Peak no :02) are the important marker compounds in this FP.
6. Ethyl acetate extract of DMA = section 2.16.2
Chromatographic conditions = section 2.14.2
The HPLC analysis was carried out at $\lambda = 254$ nm.

Fig-34 Fingerprint-6, In this FP, individual peaks have not been assigned. It is however, a useful pattern to compare medium polarity compounds containing chromophores, in DMA. It can be seen that there is a good correspondence between the different brands. However, brand B appears to have a much lower concentration of compounds than the others.

7. Chloroform extract of DMA = section 2.14.3.2.3
Chromatographic conditions = section 2.14.3 see under GLC setting for DHC and DDHC)

Fig-35 Fingerprint-7, dihydrodehydrocostus lactone (peak no:07) and dehydrocostus lactone (peak no:08) are the important marker compounds in this FP. This FP shows the low polar volatile compound of DMA. Dihydrodehydrocostus lactone is not found in reference sample-3. Brand D shows a relatively larger amount of dihydrodehydrocostus lactone than dehydrocostus lactone. Commonly dehydrocostus lactone level is higher than dihydrodehydrocostus lactone level in the DMA. Wide variation in the intensity of peak-6 is observed in the reference samples and the commercial samples of DMA. The identity of this peak has not yet been established.
8. Chloroform extract of DMA = section 2.16.2

Chromatographic conditions = section 2.14.3

Fig–36 Fingerprint-8, Cinnamaldehyde (peak no: 01) eugenol (peak no: 02) and 5-HMF (peak no: 03) are the important marker compounds in this FP. This FP shows medium polar volatile compound profile of DMA. Cinnamaldehyde not found in brand D and E. Concentration of volatile compounds of brand-E seems to be the lowest. Although eugenol is found in reference samples as well as all commercial samples it varies 7.6-22.2 % (on the basis of percentage relative peak area). There seems to be a wide variation of 5-HMF content in reference samples and the commercial samples. A low content of 5-HMF is found in brand-C and D.
Fig-29 Fingerprint-1
Fig- 30 Finger print-2

Fig- 31 Finger print-3
Fig-32 Fingerprint-4
Fig-33 Finger print -5
Fig- 34 Finger print-6
Fig- 35  Finger print-7 (Cont. ......)
Fig- 35 Finger print-7
Fig- 36 Finger print-8 (Cont.....)
Fig-36 Finger print- 8
Conclusion

The present study has demonstrated that even complex mixtures such as Dasamoolarista (DMA) can be analysed chromatographically to obtain qualitative and quantitative data which can be used to devise tests for identity, potency and quality, necessary for standardization and quality assurance. The methods developed can be extended to study other drugs, as well as obtain further data on DMA. The study has also provided information for the first time on some of the chemical constituents of DMA.

The following compounds: Kaempferol, Quercetin, Isoliquiritigenin, Isoshinanolone, Epiisoshinanolone, Gallic acid, 5-Hydroxymethylfurfural, Alizarin, Purpurin, Umbelliferone, Dehydrocostus lactone, Dihydrodehydrocostus lactone, Cinnamaldehyde and Eugenol were identified in DMA in this study. Our procedure for identifying marker compounds starting from the chromatograms of the finished product rather than the plant ingredients, is expeditious, as marker compounds are not always the major secondary metabolites found in the plant ingredients. For example, in case of Plumbago indica L., the major constituent plumbagin is not incorporated into DMA during processing, and a mixture of isoshinonolone and epiisoshinanolone detected at 0.0044 mg/ml level by fluorescence serves as the marker compound. With Glycyrrhiza glabra L., the major compound, glycyrrhizin and its aglycone, glycyrrhetic acid is difficult to detect, whereas isoliquiritigenin can be observed easily with NP/PEG reagent. However, isoliquiritigenin is a representative compound for a group of plants consisting of Pterocarpus marsupium Roxb. Glycyrrhiza glabra L. and Myroxylon balsamum (L.) Harms. The major contributor of isoliquiritigenin to
the drug is *Pterocarpus marsupium* Roxb. Therefore a chromatographic system to
detect glycyrrhetic acid as a specific marker for *Glycyrrhiza glabra* L. needs to be
developed.

Quantitative measurement of selected marker compounds and representative
compounds, Dehydrocostus lactone, Umbelliferone, the mixture of Isoshinanolone
and epiisoshinanolone isoliquiritigenin, in market samples show that there is a wide
variation in the composition of DMA with respect to different brands and to different
batches of the same brand, indicating the need to establish raw material and process
control to obtain standardized products. The chromatographic systems described in
this thesis will be useful towards achieving this end. Draft specifications for a quality
preparation of DMA, based on the results of this work are given overleaf.
### 3.7 Draft Specifications for Dasamoolarista

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<tr>
<th>Specification</th>
<th>Major compound(s)/Classes of Compound(s)/Fraction(s)</th>
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<tr>
<td>a) TLC</td>
<td>Isoshinanolone/mixture of isoshinanolone and episoshinanolone</td>
<td>Matches with reference</td>
<td>32-33,77-79,123,175,181</td>
</tr>
<tr>
<td>i. TLC-1/2/3/25</td>
<td>Isoshinanolone/mixture of isoshinanolone and episoshinanolone</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ii. TLC-4/5</td>
<td>Umbelliferone</td>
<td></td>
<td>32-33,79-81,122-123,175</td>
</tr>
<tr>
<td>iii. TLC-6</td>
<td>Dehydrocostus lactone and dihydrolecostus lactone</td>
<td></td>
<td>32-33,81-82,176</td>
</tr>
<tr>
<td>iv. TLC-7/8/9/10/11/13/14/15</td>
<td>Quercetin,kaempferol and other flavonoids</td>
<td></td>
<td>32-33,82-85,122-123,176-178</td>
</tr>
<tr>
<td>v. TLC-12</td>
<td>Alizarin and purpurin</td>
<td></td>
<td>32-33,84,177</td>
</tr>
<tr>
<td>vi. TLC-14</td>
<td>Isoliquiritigenin</td>
<td></td>
<td>32-33,85-86,122,178</td>
</tr>
<tr>
<td>vii. TLC-16</td>
<td>Eugenol</td>
<td></td>
<td>32-33,85,179</td>
</tr>
<tr>
<td>viii. TLC-18/19/20/23</td>
<td>Gallic acid</td>
<td></td>
<td>32-33,87,123,179-180</td>
</tr>
<tr>
<td>xi. TLC-21/22</td>
<td>5-Hydroxymethylfurfural</td>
<td></td>
<td>32-33,88,123,180</td>
</tr>
<tr>
<td>b) GLC</td>
<td>Dehydrocostus lactone and dihydrolecostus lactone</td>
<td></td>
<td>72,124,131-132</td>
</tr>
<tr>
<td>i. Figure :35</td>
<td>Dehydrocostus lactone and dihydrolecostus lactone</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ii. Figure : 36</td>
<td>Eugenol,Cinnamaldehyde and 5-hydroxy methyl furfural</td>
<td></td>
<td>75,86-87,125,133-134</td>
</tr>
<tr>
<td>c) HPLC</td>
<td>Ethyl acetate soluble fraction</td>
<td></td>
<td>.75,124,130</td>
</tr>
<tr>
<td>i. Figure: 34</td>
<td>Ethyl acetate soluble fraction</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>B. Quantitative</strong></td>
<td>Limits (µg/ml)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>i. Gallic acid content</td>
<td>990-1705</td>
<td>49-52,61-63,89-92</td>
<td></td>
</tr>
<tr>
<td>ii. 5-HMF content</td>
<td>Not more than 284</td>
<td>53-54,61,64-65,92-94</td>
<td></td>
</tr>
<tr>
<td>iii. Isoliquiritigenin content</td>
<td>3.2-4.6</td>
<td>46-48,110-112,</td>
<td></td>
</tr>
<tr>
<td>iv. Umbelliferone content</td>
<td>0.67-1.68</td>
<td>46,57-58,108-109</td>
<td></td>
</tr>
<tr>
<td>v. Total costus lactone content</td>
<td>3.51-6.44</td>
<td>46,55-56,67-72,101-107</td>
<td></td>
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<tr>
<td>vi. Total isoshinanolone and episoshinanolone content</td>
<td>5.3-44.6</td>
<td>46,59-60,94-100</td>
<td></td>
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</tbody>
</table>
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Antioxidative and superoxide scavenging activities of retrochalcones in


## Thin layer chromatograms

### Key for photographs of thinlayer (TLC) chromatograms

<table>
<thead>
<tr>
<th>TLC-1 Tracks</th>
<th>1 = chloroform extract of DMA</th>
<th>2 = chloroform extract of <em>Plumbago indica</em> L. root</th>
<th>3 = mixture of isoshinanolone and epiisohinanolone ($R_f = 0.68$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Solvent system</td>
<td>Toluene/diethyl ether (1:1, saturated with 10 % acetic acid)</td>
<td>Detection</td>
<td>Anisaldehyde sulfuric acid reagent</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>TLC-2 Tracks</th>
<th>1 = chloroform extract of DMA</th>
<th>2 = chloroform extract of <em>Plumbago indica</em> L. root</th>
<th>3 = mixture of isoshinanolone and epiisohinanolone ($R_f = 0.63$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Solvent system</td>
<td>Toluene/diethyl ether (1:1, saturated with 10 % acetic acid)</td>
<td>Detection</td>
<td>Without chemical treatment</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>TLC-3 Tracks</th>
<th>1 = chloroform extract of DMA</th>
<th>2 = chloroform extract of <em>Plumbago indica</em> L. root</th>
<th>3 = mixture of isoshinanolone and epiisohinanolone ($R_f = 0.63$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Solvent system</td>
<td>Toluene/diethyl ether (1:1, saturated with 10 % acetic acid)</td>
<td>Detection</td>
<td>Without chemical treatment</td>
</tr>
</tbody>
</table>
### TLC-4

**Tracks**

1 = chloroform extract of DMA  
2 = chloroform extract of *Aegel marmelos* Correa. root  
3 = umbelliferone ($R_f = 0.59$)

**Solvent system**  
Toluene/ethyl methyl ketone (9:1)

**Detection**  
Without chemical treatment

### TLC-5

**Tracks**

1 = chloroform extract of DMA  
2 = chloroform extract of *Aegel marmelos* Correa. root  
3 = umbelliferone ($R_f = 0.58$)

**Solvent system**  
Toluene/ethyl methyl ketone (9:1)

**Detection**  
Without chemical treatment

### TLC-6

**Tracks**

1 = chloroform extract of DMA  
2 = chloroform extract of *Saussera lappa* C. B. Clarke. rhizome  
3 = mixture of dehydrocostus lactone and dihydrodehydrocostus lactone ($R_f = 0.84$)

**Solvent system**  
n-Hexane/diethylamine (99:1)

**Detection**  
Anisaldehyde sulfuric acid reagent

White light
| TLC-7  | Tracks  | 1 = ethyl acetate extract of DMA  
|       |        | 2 = ethyl acetate extract of *Woodfordia fruticosa* (L.) Kurz. flower  
|       | Solvent system | Chloroform/ethyl formate/formic acid (5:4:1)  
|       | Detection | Natural products-polyethyleneglycol reagent  
|       |         | UV-366 nm  

| TLC-8  | Tracks  | 1 = ethyl acetate extract of DMA  
|       |        | 2 = ethyl acetate extract of *Woodfordia fruticosa* (L.) Kurz. flower  
|       |        | 3 = ethyl acetate extract of resin  
|       |        | 4 = ethyl acetate extract of bees honey  
|       |        | 5 = quercetin ($R_f = 0.47$)  
|       |        | 6 = kaempferol ($R_f = 0.61$)  
|       | Solvent system | Toluene/ethyl formate (5:5)  
|       | Detection | Natural products-polyethyleneglycol reagent  
|       |         | UV-366 nm  

| TLC-9  | Tracks  | 1 = ethyl acetate extract of DMA  
|       |        | 2 = ethyl acetate extract of Musthakarista  
|       | Solvent system | Toluene/ethyl formate/formic acid (5:4:1)  
|       | Detection | Natural products-polyethyleneglycol reagent  
|       |         | UV-366 nm  

**TLC-10**

**Tracks**
1 = ethyl acetate extract of DMA
2 = ethyl acetate extract of *Pterocarpus marsupium* Roxb. heart wood

**Solvent system** Toluene/ethyl formate/formic acid (5:4:1)
**Detection** 5 % aqueous ferric chloride reagent White light

**TLC-11**

**Tracks**
1 = ethyl acetate extract of DMA
2 = ethyl acetate extract of *Pterocarpus marsupium* Roxb. heart wood

**Solvent system** Toluene/ethyl formate/formic acid (5:4:1)
**Detection** Natural products-polyethyleneglycol reagent UV-366 nm

**TLC-12**

**Tracks**
1 = chloroform extract of DMA
2 = chloroform extract of *Rubia cordifolia* Linn.
3 = alizarin ($R_f = 0.73$) and purpurin ($R_f = 0.56$)

**Solvent system** Toluene/ethyl methyl ketone/ formic acid (8:1.9:0.1)
**Detection** 25 % ammonia vapour White light
TLC-13

**Tracks**

1 = diethyl ether extract of DMA

2 = diethyl ether extract of *Glycyrrhiza glabra* Linn. stem

3 = diethyl ether extract of *Woodfordia fruticosa* (L.) Kurz. flower

**Solvent system**  Toluene/ethyl formate (5:5)

**Detection**  Natural products-polyethyleneglycol reagent  UV-366 nm

---

TLC-14

**Tracks**

1 = diethyl ether extract of DMA

2 = diethyl ether extract of *Glycyrrhiza glabra* Linn. stem

3 = diethyl ether extract of *Pterocarpus marsupium* Roxb. heartwood

4 = diethyl ether extract of *Myroxylon balsamum* (L.) Harms. heart wood

5 = quercetin ($R_f = 0.44$), kaempferol ($R_f = 0.58$), isoliquiritigenin ($R_f = 0.67$)

**Solvent system**  Toluene/ethyl formate (5:5)

**Detection**  Natural products-polyethyleneglycol reagent  White light
TLC-15

Tracks
1 = diethyl ether extract of DMA
2 = diethyl ether extract of *Glycyrrhiza glabra* Linn. stem
3 = diethyl ether extract of *Pterocarpus marsupium* Roxb. heartwood
4 = diethyl ether extract of *Myroxylon balsamum* (L.) Harms. heartwood
5 = quercetin ($R_f = 0.44$), kaempferol ($R_f = 0.58$)

Solvent system: Toluene/ethyl formate (5:5)
Detection: Natural products-polyethylene glycol reagent, UV 366 nm

TLC-16

Tracks
1 = chloroform extract of DMA
2 = chloroform extract of *Eugenia caryophyllata* Thumb. flower bud
3 = chloroform extract of *Cinnamomum verum* J. Presl. leaf
4 = chloroform extract of *Cinnamomum verum* J. Presl. bark
5 = eugenol ($R_f = 0.47$)

Solvent system: n-Hexane/diethylamine (8:2)
Detection: Anisaldehyde sulfuric acid reagent, White light
**TLC-17**

**Tracks**
1 = ethyl acetate extract of DMA

2 = gallic acid ($R_f = 0.48$)

**Solvent system** Chloroform/ethyl formate/formic acid (5:4:1)

**Detection** 5% aqueous ferric chloride reagent  
White light

---

**TLC-18**

**Tracks**
1 = ethyl acetate extract of DMA

2 = gallic acid ($R_f = 0.51$)

**Solvent system** Chloroform/ethyl formate/formic acid (5:4:1)

**Detection** Without chemical treatment  
UV 254 nm

---

**TLC-19**

**Tracks**
1 = ethyl acetate extract of DMA

2 = gallic acid ($R_f = 0.51$)

**Solvent system** Chloroform/ethyl formate/formic acid (5:4:1)

**Detection** Natural products-polyethyleneglycol reagent  
UV 366 nm
### TLC-20

**Tracks**
1 = ethyl acetate extract of DMA  
2 = gallic acid \( (R_f = 0.51) \)

**Solvent system** Chloroform/ethyl formate/formic acid (5:4:1)  
**Detection** Natural products-polyethylene glycol reagent  

**UV 254 nm**

### TLC-21

**Tracks**
1 = chloroform extract of DMA  
2 = 5-HMF \( (R_f = 0.43) \)

**Solvent system** Toluene/ethyl acetate (7:3)  
**Detection** Anisaldehyde sulfuric acid reagent  

**White light**

### TLC-22

**Tracks**
1 = chloroform extract of DMA  
2 = 5-HMF \( (R_f = 0.44) \)

**Solvent system** Toluene/ethyl acetate (7:3)  
**Detection** Without chemical treatment  

**UV 254 nm**
TLC-23

Tracks
1 = ethyl acetate extract of Dasamoolarista
2 = ethyl acetate extract of Musthakarista
3 = ethyl acetate extract of Draksharista
4 = ethyl acetate extract of Saraswatharista
5 = ethyl acetate extract of Nimbarista
6 = ethyl acetate extract of Asokarista
7 = ethyl acetate extract of Kadirarista
8 = ethyl acetate extract of Danthyarista
9 = ethyl acetate extract of Amurtharista
10 = ethyl acetate extract of Arjunarista
11 = ethyl acetate extract of Aswagandharista
12 = ethyl acetate extract of Balarista
13 = ethyl acetate extract of Abeyarista

Solvent system  Chloroform/ethyl formate/formic acid (5:4:1)
Detection    Without chemical treatment    UV 254 nm
TLC-24

Tracks
1 = chloroform extract of *Plumbago indica* L. root (Before shodhna)
2 = chloroform extract of neutralized calcium hydroxide solution
3 = chloroform extract of *Plumbago indica* L. root (After shodhna)
4 = plumbagin ($R_f = 0.89$), mixture of isoshinanolone and epiisoshinanolone ($R_f = 0.62$)

Solvent system: Toluene/diethyl ether (1:1, saturated with 10 % acetic acid)
Detection: Anisaldehyde sulfuric acid reagent  White light

TLC-25

Tracks
1 = chloroform extract of reference DMA
2 = chloroform extract of commercial sample DMA (1)
3 = chloroform extract of commercial sample DMA (2)
4 = chloroform extract of commercial sample DMA (3)
5 = mixture of isoshinanolone and epiisoshinanolone ($R_f = 0.59$)

Solvent system: Toluene/diethyl ether (1:1, saturated with 10 % acetic acid)
Detection: Without chemical treatment  UV 366 nm
TLC-26

Tracks
1 = methanol extract of *Myroxylon balsamum* (L) Harms. heartwood

2 = methanol extract of *Acacia catechu* Willd. heartwood

Solvent system: Toluene/ethyl acetate (9.8:0.2)
Detection: Anisaldehyde sulfuric acid reagent White light

TLC-27

Tracks
1 = methanol extract of *Myroxylon balsamum* (L) Harms. heartwood

2 = methanol extract of *Acacia catechu* Willd. heartwood

Solvent system: Toluene/ethyl acetate (9.8:0.2)
Detection: Phosphomolybidic acid reagent White light

TLC-28

Tracks
1 = methanol extract of *Myroxylon balsamum* (L) Harms. heartwood

2 = methanol extract of *Acacia catechu* Willd. heartwood

Solvent system: Chloroform/toluene/ethyl formate/formic acid (5:3:2:1)
Detection: Anisaldehyde sulfuric acid reagent White light
<table>
<thead>
<tr>
<th>Track</th>
<th>Description</th>
</tr>
</thead>
</table>
| TLC-29 | 1 = methanol extract of *Myroxylon balsamum* (L) Harms heartwood  
2 = methanol extract of *Acacia catechu* Willd. heartwood  
3 = quercetin ($R_f = 0.39$), kaempferol ($R_f = 0.47$), isoliquritigenin ($R_f = 0.55$) |
| Solvent system | Toluene/ethyl formate (5:5) |
| Detection | Natural products-poly ethyleneglycol reagent  
White light |

<table>
<thead>
<tr>
<th>Track</th>
<th>Description</th>
</tr>
</thead>
</table>
| TLC-30 | 1 = methanol extract of *Myroxylon balsamum* (L) Harms. heartwood  
2 = methanol extract of *Acacia catechu* Willd. heartwood  
3 = quercetin ($R_f = 0.39$), kaempferol ($R_f = 0.47$) |
| Solvent system | Toluene/ethyl formate (5:5) |
| Detection | Natural products-poly ethyleneglycol reagent  
UV 366 nm |

<table>
<thead>
<tr>
<th>Track</th>
<th>Description</th>
</tr>
</thead>
</table>
| TLC-31 | 1 = chloroform extract of *Saussuera lappa* C. B. Clarke rhizome  
2 = chloroform extract of *Inula racemosa* Hook. rhizome |
| Solvent system | n-Hexane/diethyl ether (7:3) |
| Detection | 5 % ethanoilic sulfuric acid reagent  
White light |
TLC-32

Tracks
1 = chloroform extract of *Saussuera lappa* C. B. Clarke. rhizome
2 = chloroform extract of *Inula racemosa* Hook. rhizome

Solvent system n-Hexane/diethyl ether (7:3)
Detection: Anisaldehyde sulfuric acid reagent White light

TLC-33

Tracks
1 = chloroform extract of *Nardostachys jatamansi* D.C. rhizome
2 = *Nardostachys jatamansi* D.C. oil
3 = chloroform extract of *Flickingeria macraei* (Lindley) Seidenf.

Solvent system n-Hexane/diethyl ether (9:1)
Detection Anisaldehyde sulfuric acid reagent White light

TLC-34

Tracks
1 = methanol extract of *Cryptolepis bunchanaiii* Roem & Schult
2 = methanol extract of *Hemidesmus indicus* R. Br.

Solvent system n-Hexane/diethyl ether (9:1)
Detection Anisaldehyde sulfuric acid reagent White light
TLC-35

Tracks
1 = diethyl ether extract of bees honey from Ayurvedic drug corporation
2 = diethyl ether extract of commercial bees honey (imported from China)
3 = diethyl ether extract of commercial bees honey
4 = diethyl ether extract of commercial bees honey
5 = diethyl ether extract of commercial bees honey
6 = diethyl ether extract of commercial bees honey
7 = quercetin \( (R_f = 0.41) \), kaempferol \( (R_f = 0.51) \), isoliquiritigenin \( (R_f = 0.66) \)

Solvent system: Toluene/ethyl formate (5:5)
Detection: Natural products-polyethyleneglycol reagent, White light

TLC-36

Tracks
1 = diethyl ether extract of bees honey from Ayurvedic drug corporation
2 = diethyl ether extract of commercial bees honey (imported from China)
3 = diethyl ether extract of commercial bees honey
4 = diethyl ether extract of commercial bees honey
5 = diethyl ether extract of commercial bees honey
6 = diethyl ether extract of commercial bees honey
7 = quercetin \( (R_f = 0.41) \), kaempferol \( (R_f = 0.51) \),

Solvent system: Toluene/ethyl formate (5:5)
Detection: Natural products-polyethyleneglycol reagent, UV 366 nm
Photographs of thin layer chromatograms (TLC)
Appendix--ii

$^1H$ nuclear magnetic resonance spectra ($^1H$ NMR)

and

$^{13}C$ nuclear magnetic resonance spectra ($^{13}C$ NMR)

of

Marker, representative compounds

and

compounds other than markers and representatives

of

Dasamoolarista
Fig. 42 13C NMR spectrum of Isoliquiritigenin
Fig. 43 1H NMR spectrum of plumbagin
Fig. 44. 13C NMR spectrum of plumbagin.
Fig -45 1 H NMR spectrum of mixture of isoshinanolone and epiisoshinanolone
Fig. 47 1 H NMR spectrum of isoshinanolone
Fig. 49 1H NMR spectrum of 5-HMF
Fig. 50 13C NMR spectrum of 5-HMF
Fig - 52 13C NMR spectrum of Umbelliferone
Fig. 53: 1H NMR spectrum of Gallic acid
Fig - 55 1H NMR spectrum of dehydrocostuslactone
Fig. 58  13 C NMR spectrum of Dihydrodehydrocostus lactone
Fig - 59 DEPT $45^\circ$ spectrum of Dihydrodehydrocostus lactone
Fig. 60 DEPT 90° spectrum of Dihydrodehydrocostus lactone
Fig. 62  HETERO COSY spectrum of Dihydrodehydrocostus lactone
Fig. 63 HOMOCOSY spectrum of Dihydrodehydrocostus lactone
Fig. 64 1H NMR spectrum of the mixture of Dihydrocostus lactone and Dihydrodihydrocostus lactone
Fig. 65 13C NMR of the mixture of Dehydrocostus lactone and Dihydrodehydrocostus lactone
Fig - 67  13 C NMR spectrum of Alizarin
Appendix—iii

List of plants used in the preparation of Arista

<table>
<thead>
<tr>
<th>1. ABEYARISTA</th>
<th>2. AMURTHARISTA</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Primary plant materials</strong></td>
<td><strong>Primary plant materials</strong></td>
</tr>
<tr>
<td><em>Embelia ribes</em> Burm. f.</td>
<td><em>Aerva lanata</em> (L.) Juss. ex Schult.</td>
</tr>
<tr>
<td>Water</td>
<td><em>Stereospermum suaveolens</em> (Roxb.) DC.</td>
</tr>
<tr>
<td>Sugar</td>
<td><em>Oroxylum indicum</em> (L.) Vent.</td>
</tr>
<tr>
<td><strong>Secondary plant materials</strong></td>
<td><strong>Secondary plant materials</strong></td>
</tr>
<tr>
<td><em>Premna integrifolia</em> Linn.</td>
<td><em>Alysircarpus vaginalis</em> (L.) DC.</td>
</tr>
<tr>
<td><em>Tribulus terrestris</em> L.</td>
<td><em>Gmelina arborea</em> Roxb.</td>
</tr>
<tr>
<td><em>Coriandrum sativum</em> L.</td>
<td><em>Tribulus terrestris</em> L.</td>
</tr>
<tr>
<td><em>Cucumis melo</em> var. <em>agrestis</em> Naud.</td>
<td><em>Solanum melongena</em> L.</td>
</tr>
<tr>
<td><em>Piper chaba</em> L.</td>
<td>Water</td>
</tr>
<tr>
<td><em>Anethum graveolens</em> L.</td>
<td>Sugar</td>
</tr>
<tr>
<td><em>Zingiber officinale</em> Roscoe.</td>
<td><em>Secondary plant materials</em></td>
</tr>
<tr>
<td><em>Baliospermum montanum</em> (Willd.) Muell.-Arg.</td>
<td><em>Carum copticum</em> Benth &amp; Hook. f.</td>
</tr>
<tr>
<td><em>Salmalia malabarica</em> (DC.) Schott &amp; Endl.</td>
<td><em>Hedyotis corymbosa</em> Linn</td>
</tr>
<tr>
<td><em>Ipomea turpethum</em> Br.</td>
<td><em>Alstonia scholaris</em> (L.) R. Br.</td>
</tr>
<tr>
<td><em>Madhuca longifolia</em> (L) J. F. Macbr.</td>
<td><em>Cyprus rotundus</em> L.</td>
</tr>
<tr>
<td><em>3. NIMBARISTA</em></td>
<td><em>Messua ferrea</em> L.</td>
</tr>
<tr>
<td><em>Azadirachta indica</em> A. Juss.</td>
<td><em>Picrorhiza kurrooa</em> Royle</td>
</tr>
<tr>
<td><em>Terminalia chebula</em> Retz.</td>
<td><em>Holarrhena antidysenterica</em> (Roxb.) Wall.</td>
</tr>
<tr>
<td><em>Terminalia bellerica</em> (Gaertn.) Roxb.</td>
<td><em>Aconitum heterophyllum</em> L.</td>
</tr>
<tr>
<td><em>Phyllanthus emblica</em> L.</td>
<td><em>Piper longum</em> L.</td>
</tr>
<tr>
<td>Water</td>
<td><em>Piper nigrum</em> L.</td>
</tr>
<tr>
<td>Sugar</td>
<td><em>Zingiber officinale</em> Roscoe.</td>
</tr>
<tr>
<td><strong>Secondary plant materials</strong></td>
<td></td>
</tr>
<tr>
<td><em>Terminalia chebula</em> Retz.</td>
<td></td>
</tr>
<tr>
<td><em>Terminalia bellerica</em> (Gaertn.) Roxb.</td>
<td></td>
</tr>
<tr>
<td><em>Phyllanthus emblica</em> L.</td>
<td></td>
</tr>
<tr>
<td><em>Azadirachta indica</em> A. Juss.</td>
<td></td>
</tr>
</tbody>
</table>
### 4. ASVAGANDHARISTA

<table>
<thead>
<tr>
<th>Primary plant materials</th>
<th>Secondary plant materials</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Curculigo orchiodes</em> Gaertn.</td>
<td>Water</td>
</tr>
<tr>
<td><em>Rubia cordifolia</em> L.</td>
<td></td>
</tr>
<tr>
<td><em>Terminalia chebula</em> Retz.</td>
<td>Sugar</td>
</tr>
<tr>
<td><em>Coscinium fenestratum</em> (Gaertn.) Colebr.</td>
<td></td>
</tr>
</tbody>
</table>

### 5. ASOKARISTA

<table>
<thead>
<tr>
<th>Primary plant materials</th>
<th>Secondary plant materials</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Terminalia chebula</em> Retz.</td>
<td><em>Coscinium fenestratum</em> (Gaertn.) Colebr.</td>
</tr>
<tr>
<td><em>Glycyrrhiza glabra</em> L.</td>
<td><em>Terminalia chebula</em> Retz.</td>
</tr>
<tr>
<td><em>Curcuma longa</em> L.</td>
<td><em>Terminalia chebula</em> Retz.</td>
</tr>
<tr>
<td><em>Santalum album</em> L.</td>
<td><em>Terminalia chebula</em> Retz.</td>
</tr>
<tr>
<td><em>Pterocarpus santalinus</em> L.</td>
<td><em>Terminalia chebula</em> Retz.</td>
</tr>
<tr>
<td><em>Acorus calamus</em> L.</td>
<td><em>Terminalia chebula</em> Retz.</td>
</tr>
<tr>
<td><em>Plumbago indica</em> L.</td>
<td><em>Terminalia chebula</em> Retz.</td>
</tr>
<tr>
<td><em>Water</em></td>
<td><em>Terminalia chebula</em> Retz.</td>
</tr>
<tr>
<td><em>Sugar</em></td>
<td><em>Terminalia chebula</em> Retz.</td>
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### 6. BALARISTA

<table>
<thead>
<tr>
<th>Secondary plant materials</th>
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<tbody>
<tr>
<td><em>Sida cordifolia</em> L.</td>
<td><em>Withania somnifera</em> Dunal.</td>
</tr>
<tr>
<td><em>Woodfordia fruticosa</em> (L.) Kurz</td>
<td><em>Zingiber officinale</em> Roscoe.</td>
</tr>
<tr>
<td><em>Myristica borsfieldia</em> L.</td>
<td><em>Water</em></td>
</tr>
<tr>
<td><em>Mesua ferrea</em> L.</td>
<td><em>Sugar</em></td>
</tr>
<tr>
<td><em>Piper longum</em> L.</td>
<td><em>Secondary plant materials</em></td>
</tr>
<tr>
<td><em>Piper nigrum</em> L.</td>
<td></td>
</tr>
<tr>
<td><em>Pogostemon heyneanus</em> Benth.</td>
<td></td>
</tr>
<tr>
<td><em>Elettaria cardamomum</em> Maton.</td>
<td></td>
</tr>
<tr>
<td><em>Cinnamomum verum</em> Presl.</td>
<td></td>
</tr>
<tr>
<td><em>Alpinia galanga</em> Willd.</td>
<td></td>
</tr>
<tr>
<td><em>Elettaria cardamomum</em> Maton.</td>
<td></td>
</tr>
<tr>
<td><em>Barleria prionitis</em> L.</td>
<td></td>
</tr>
<tr>
<td><em>Vetiveria zizanioides</em> (L.) Nash.</td>
<td></td>
</tr>
<tr>
<td><em>Tribulus terrestris</em> L.</td>
<td></td>
</tr>
<tr>
<td><em>Cinnamomum verum</em> Presl.</td>
<td></td>
</tr>
<tr>
<td>7. DANTHYARISTA</td>
<td>8. MUSTHAKARISTA</td>
</tr>
<tr>
<td>------------------</td>
<td>------------------</td>
</tr>
<tr>
<td><strong>Primary plants materials</strong></td>
<td><strong>Primary plants materials</strong></td>
</tr>
<tr>
<td>Solanum melongena L.</td>
<td>Cyperus rotundus L.</td>
</tr>
<tr>
<td>Stereospermum suaveolens DC.</td>
<td>Water</td>
</tr>
<tr>
<td>Oroxyllum indicum L.</td>
<td>Sugar</td>
</tr>
<tr>
<td>Premna integrifolia Linn.</td>
<td>Secondary plant materials</td>
</tr>
<tr>
<td>Gmelina arborea Roxb.</td>
<td>Woodfordia fruticosa (L.) Kurz</td>
</tr>
<tr>
<td>Alyssicarpus vaginalis L. DC.</td>
<td>Carum copticum Benth &amp; Hook. F.</td>
</tr>
<tr>
<td>Aegle marmelos Correa</td>
<td>Zingiber officinale Roscoe.</td>
</tr>
<tr>
<td>Aerva lanata Juss.</td>
<td>Piper nigrum L.</td>
</tr>
<tr>
<td>Tribulus terrestris L.</td>
<td>Eugenia caryophyllata Thumb.</td>
</tr>
<tr>
<td>Baliospermun montanum (Willd.) Muell.-Arg</td>
<td>Trigonella foenum-graecum L.</td>
</tr>
<tr>
<td>Plumbago indica L.</td>
<td>Plumbago indica L.</td>
</tr>
<tr>
<td>Sugar</td>
<td>Camum cuminum L.</td>
</tr>
<tr>
<td>Water</td>
<td>Secondary plant materials</td>
</tr>
<tr>
<td><strong>Secondary plant materials</strong></td>
<td><strong>Secondary plant materials</strong></td>
</tr>
<tr>
<td>Terminalia belerica (Gaertn.) Roxb.</td>
<td>Asparagus racemosus Willd.</td>
</tr>
<tr>
<td>Phyllanthus emblica L.</td>
<td>Ipomea digitata</td>
</tr>
<tr>
<td>10. ARJUNARISTA</td>
<td>9. SARASWATHARISTA</td>
</tr>
<tr>
<td><strong>Primary plants materials</strong></td>
<td><strong>Primary plants materials</strong></td>
</tr>
<tr>
<td>Terminali arjuna W &amp; A.</td>
<td>Zingiber officinale Roscoe.</td>
</tr>
<tr>
<td>Vitis vinifera L</td>
<td>Water</td>
</tr>
<tr>
<td>Madhuca longifolia (L.) J. F. Macbr.</td>
<td>Bees honey</td>
</tr>
<tr>
<td>Sugar</td>
<td>Sugar</td>
</tr>
<tr>
<td><strong>Secondary plant materials</strong></td>
<td><strong>Secondary plant materials</strong></td>
</tr>
<tr>
<td>Woodfordia fruticosa (L.) Kurz</td>
<td>Woodfordia fruticosa. (L.) Kurz.</td>
</tr>
<tr>
<td>Piper cubeb L.</td>
<td>Piper cubeb L.</td>
</tr>
<tr>
<td>Ipomea turpethum R. Br.</td>
<td>Ipomea turpethum R. Br.</td>
</tr>
<tr>
<td>Withania sominifera (L.) Dunal.</td>
<td>Withania sominifera (L.) Dunal.</td>
</tr>
<tr>
<td>Eugenia caryophyllata Thumb.</td>
<td>Eugenia caryophyllata Thumb.</td>
</tr>
<tr>
<td>Acorus calamus L.</td>
<td>Acorus calamus L.</td>
</tr>
<tr>
<td>Saussurea lappa C. B. Clarke</td>
<td>Saussurea lappa C. B. Clarke</td>
</tr>
<tr>
<td>Cinnamomum verum Pres</td>
<td>Cinnamomum verum Pres</td>
</tr>
<tr>
<td>Elettaria cardamomum Maton.</td>
<td>Elettaria cardamomum Maton.</td>
</tr>
<tr>
<td>Tinospora cordifolia (Willd) Miers</td>
<td>Tinospora cordifolia (Willd) Miers</td>
</tr>
<tr>
<td>ex.Hook.f. &amp; Thoms.</td>
<td></td>
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<tr>
<td>11. KADIRARISTA</td>
<td>12. DRAKSHARISTA</td>
</tr>
<tr>
<td>-----------------</td>
<td>-----------------</td>
</tr>
<tr>
<td><strong>Primary plants materials</strong></td>
<td><strong>Primary plants materials</strong></td>
</tr>
<tr>
<td><em>Acacia catechu</em> Willd.</td>
<td><em>Vitis vinefera</em> L.</td>
</tr>
<tr>
<td><em>Cedrus deodara</em> Roxb.</td>
<td><em>Water</em></td>
</tr>
<tr>
<td><em>Psoralia corylifolia</em> L.</td>
<td><em>Sugar</em></td>
</tr>
<tr>
<td><em>Coscinum fenestratum</em> (Gaertn.) Colebr.</td>
<td></td>
</tr>
<tr>
<td><em>Terminalia chebula</em> Retz.</td>
<td><strong>Secondary plant materials</strong></td>
</tr>
<tr>
<td><em>Terminalia bellerica</em> (Gaertn.) Roxb.</td>
<td><em>Cinnamomum verum</em> Presl.</td>
</tr>
<tr>
<td><em>Phyllanthus emblica</em> L.</td>
<td><em>Eletaria cardamomum</em> Maton.</td>
</tr>
<tr>
<td><em>Water</em></td>
<td><em>Cinnamomum verum</em> Presl.</td>
</tr>
<tr>
<td><em>Bees honey</em></td>
<td></td>
</tr>
<tr>
<td><em>Sugar</em></td>
<td></td>
</tr>
<tr>
<td><strong>Secondary plants materials</strong></td>
<td><em>Mesua ferrea</em> L.</td>
</tr>
<tr>
<td><em>Woodfordia fruticosa</em> (L.) Kurz</td>
<td></td>
</tr>
<tr>
<td><em>Mesua ferrea</em> L.</td>
<td><em>Piper longum</em> L.</td>
</tr>
<tr>
<td><em>Myristica fragrans</em> Houtt.</td>
<td></td>
</tr>
<tr>
<td><em>Eugenia caryophyllata</em> Thumb.</td>
<td></td>
</tr>
<tr>
<td><em>Eletaria cardamomum</em> Maton</td>
<td></td>
</tr>
<tr>
<td><em>Cinnamomum verum</em> Presl</td>
<td></td>
</tr>
<tr>
<td><em>Pogostemon heyneanus</em> Benth.</td>
<td></td>
</tr>
<tr>
<td><em>Piper longum</em> L.</td>
<td></td>
</tr>
<tr>
<td><em>Hibiscus abelmoschus</em> L.</td>
<td></td>
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</table>
### 13. **DASAMOOLARISTA**

<table>
<thead>
<tr>
<th><strong>Primary plants materials</strong></th>
<th><strong>Secondary plants materials</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Pterocarpus marsupium</em> Roxb.</td>
<td><em>Hibiscus abelmoschus</em> L.</td>
</tr>
<tr>
<td><em>Alysicarpus vaginalis</em> L.</td>
<td><em>Ipsos caprifolius</em> L.</td>
</tr>
<tr>
<td><em>Inula racemosa</em> Hook.</td>
<td><em>Vetiveria zizanioides</em> (L.) Nash</td>
</tr>
<tr>
<td><em>Cedrus deodara</em> Roxb.</td>
<td><em>Santalum album</em> L.</td>
</tr>
<tr>
<td><em>Aerva lanata</em> Juss.</td>
<td><em>Cinnamomum verum</em> Presl. (bark)</td>
</tr>
<tr>
<td><em>Solanum melongena</em> L.</td>
<td><em>Myristica fragrans</em> Houtt.</td>
</tr>
<tr>
<td><em>Areca catechu</em> L.</td>
<td><em>Eugenia caryophyllata</em> Thumb.</td>
</tr>
<tr>
<td><em>Curcuma longa</em> L.</td>
<td><em>Mesua ferrea</em> L.</td>
</tr>
<tr>
<td><em>Holarrhena antialyseric</em> (Roxb.) Wall.</td>
<td><em>Anethum graveolens</em> L.</td>
</tr>
<tr>
<td><em>Sida cordifolila</em> L.</td>
<td><em>Elettaria cardamomum</em> Maton.</td>
</tr>
<tr>
<td><em>Holarrhena antialyseric</em> (Roxb.) Wall.</td>
<td><em>Piper longum</em> L.</td>
</tr>
<tr>
<td><em>Mesua ferrea</em> L.</td>
<td><em>Plumbago indica</em> L.</td>
</tr>
<tr>
<td><em>Feronia limonia</em> (L) Swingle.</td>
<td><em>Cinnamomum verum</em> Presl. (leaf)</td>
</tr>
<tr>
<td><em>Glycyrrhiza glabra</em> L.</td>
<td><em>Asparagus racemos</em> Willd</td>
</tr>
<tr>
<td><em>Terminalia bellirica</em> (Gaertn.) Roxb.</td>
<td><em>Woodfordia fruticosa</em> (L.) Kurz</td>
</tr>
<tr>
<td><em>Rubia cordifolia</em> L.</td>
<td><em>Vitis vinifera</em> L.</td>
</tr>
<tr>
<td><em>Nigella sativa</em> L.</td>
<td><em>Phyllanthus emblica</em> L.</td>
</tr>
<tr>
<td><em>Bees honey</em></td>
<td><em>Tragia involucrata</em> L.</td>
</tr>
</tbody>
</table>
Appendix--iv

Structural formulae of marker, representative compounds and compounds other than markers and representatives.

(i) Kaempferol

(ii) Quercetin

(iii) Isoliquiritigenin

(iv) Plumbagin

(v) Isoshinanolone (cis-isomers)

(vi) Epiisoshinanolone (trans-isomers)

(vii) 5-Hydroxymethyl furfural

(viii) Umbelliferone
Dehydrocostus lactone

Gallic acid

Dihydrodehydrocostus lactone

Alizarin

Purpurin