RESEARCH ARTICLE

Development of Quality Control Parameters and Deciphering Phytochemical Profile of the *Messua Ferrea* Linn Stamens

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Abstract

Introduction: 'Nagakesara' aka 'Cobra's saffron' is recommended in many ayurvedic remedies, consisting of dried stamens of *Mesua ferrea* Linn (Fam. Calophyllaceae). Thus, we aimed to develop a comprehensive quality control toolkit for *M. ferrea* stamen to ensure its identity, purity, consistency and mitigate adulteration.

Materials and Methods: Collection, authentication, and pulverization of the stamens of *M. ferrea* was done for the determination of the physiochemical, qualitative and quantitative phytochemicals, HPLC and LC-MS fingerprint, as well as secondary-metabolites in the stamen extract in view of World Health Organization (WHO) and Indian Herbal Pharmacopoeia.

Results: Collected stamens of *M. ferrea* were authenticated by the Botanical Survey of India. Determined physicochemical attributes of the authenticated powdered stamens were found within the permissible limits. Yield of the hydroalcoholic extract of the stamens was found to 18.26 % w/w and was found enriched in secondary metabolites *viz.*, alkaloids, glycosides, coumarin, phenols, tannins, flavonoids, terpenoids, saponins, carbohydrates, phytosterols and amino acids. Quantified total phenolic, flavonoid and carbohydrate content were found to be 284.24 ± 8.18 µg equivalent of gallic acid/ mg, 242.52 ± 8.36 µg equivalent of quercetin/ mg and 92.34 ± 2.58 µg equivalent of glucose/ mg of extract, respectively, Further, HPTLC and LC-MS fingerprint profile of the extract were developed. Twenty phytochemicals were identified by the generated LC-MS data.

Discussion: Established physicochemical, qualitative and quantitative phytochemical, chromatographic fingerprints and identified phytochemical profile of the *M. ferrea* stamens would serve as definitive tools for quality control and assurance measures by the regulatory authorities.

Keywords: LC-MS, Fingerprint profile, Myricetin, Luteolin, Kaempferol, Gallic Acid, Coumaric acid, β-Sitosterol

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Introduction

Plants and herbs are the pivotal ingredients for the traditional healthcare systems *viz.,* African, Ayurveda, Brazilian, Chinese, Egyptian, Homeopathy, Japanese, Korean, Siddha, Unani medicine[1]. Indeed, the World Health Organization (WHO) is continuously inspiring the practice of natural healthcare resources, as herbal medications are preferred over synthetic pharmaceuticals because they are less costly, more readily available, have multi-components and multi-target activities, and possess better efficacy with less adverse effects possibly by holistic principles of synergism and/or antagonism of a range of available phytochemicals[2]. The WHO considers traditional and complementary medicines to include disciplines as wide-ranging as Ayurveda, yoga, homeopathy and complementary therapies[3]. Despite of time immortal dependency on the plant and/or -derived nutraceutical, pharmaceutical and medicinal products. In current evidence-based prospective, worldwide acceptance of such indigenous and traditional knowledge-based healthcare products and/or services is limited to a confined zone and population; might be due to material inconsistency-driven mutable impact on the recipients[2]. Besides, several attributes including, geographical, temporal, seasonal, climatic, harvesting, processing etc., are contributing in a great degree of variability, leading to poor global reliance[4]. Therefore, WHO and Ayurveda, Yoga, Unani, Siddha, and Homoeopathy (AYUSH), Ministry of Government of India are taking a lead, and advocated the development of robust quality control tools for plant and/or extract derived products to ensure the batch-to-batch content uniformity[5]. Which involves, physicochemical evaluations, qualitative (e.g., identification, phytochemicals and chromatographic profile) determinations, and quantitative (e.g., content

analyses) markers estimations[6]. Although, marker-based approaches are available on a case-to-case basis; but, for most plant materials, plant extracts, and herbal medicines, it is not possible to assure quality based only on the content or presence/absence of one compound[7]. Thus now-a-days pattern-oriented approaches have been extensively studied, introducing the use of multivariate data analysis on chromatographic/spectroscopic fingerprints[5,8]. Thus, development of the quality control tools for the content uniformity will enhance the societal confidence as well as trust of the end-user to these cost-effective, alternative therapeutic options primarily driven from indigenous and traditional knowledge system-based product and/or services.

The *Mesua ferrea* Linn, a flowering tree belonging to the Calophyllaceae family; commonly known as 'Ceylon ironwood', or 'cobra saffron', or 'Nagapushpa' or 'Nagkesher'[9]. It is a native plant to southern Nepal, Sri Lanka, Burma, New Guinea, Thailand, northeastern part of India, and extended towards eastern Himalayas Burma Andaman Islands, evergreen forests of South Konkan and north Cannara. *M. ferrea* is the state tree of Nagaland, and the national tree of Sri Lanka[10]. Its flower is considered as the state flower of Tripura. In Assam it is popular as 'Nahor'. In the Indian traditional knowledge system specially in Ayurveda, the stamens of the plant are indicated as 'Nagakeshara' for 'Vitarakta', 'Vastiroga', 'Raktapitta', and 'Sopharoga'. 'Nagakeshara' is used for the treatment of asthma, bleeding piles, dysentery, dyspepsia, fever, inflammation, rheumatism, and microbial infections[11,12]. Dried stamens of the *M. ferrea* are one of the ingredients in the several ayurvedic formulations *i.e.* Nagakesaradi Curna, Candanabalalaksadi Taila, Chyawanprash, Kumaryasava and Brahma-Rasayan[13]. Therefore, objective of the present study was to develop the quality control tool for *M. ferrea* stamens including physicochemical attributes, qualitative and quantitative parameters, and phytochemical profile of the hydroalcoholic extract of the dried stamens of the *M. ferrea* by liquid chromatography-mass spectrometry (LC-MS) technique as per WHO guidelines.

Materials And Methods

Collection and Identification of the Plant Specimen

The flowers of *M. ferrea* were collected from Dibrugarh University, Dibrugarh, Assam, India, in the month of March. Herbarium was prepared from the collected plant specimen, and was identified by Dr. N. Odyuo, Scientist- E & Ho. O., Botanical Survey of India (BSI), Shillong- 793 003, India as *Mesua ferrea* Linn (Calophyllaceae**)** through a reference no.: BSI/ERC/Tech/2022-2023/299 dated 10.10.2022. For future reference a voucher specimen (*M. ferrea*: DU/PSC/HRB/AS-01/2022, a specimen number 001) has been deposited in the Departmental herbarium. Moreover, the stamens were separated, washed and dried in the oven at 45 °C and pulverized into a coarse powder, stored in an airtight container for further use.

Physicochemical Attributes Determination

The physicochemical parameters were determined following the methods described in the WHO guidelines[14], Ayurvedic Pharmacopoeia of India[15], Indian Pharmacopoeia[16], and Indian Herbal Pharmacopoeia[17].

Foreign matter

The dried coarsely powdered plant materials (100 g) were evenly spread on a white sheet of paper and carefully monitored using a magnifying glass. Foreign matter like sand, clay and other particles etc. was separated from the plant parts, weighed and finally, the foreign matter was determined as the percentage weight/weight (w/w).

Loss on drying

A porcelain dish was taken and 1.5 g of the dried crude drug was placed on it. The porcelain dish was kept for 30 minutes at a temperature of 105 °C. The moisture lost due to drying is calculated from the loss in weight of the dried crude drug.

Total ash

About 2-4 g of the air-dried coarsely powdered plant material was taken in a tarred silica dish and incinerated at a temperature not exceeding 450 °C until free from carbon. The silica dish bearing the total ash was cooled and weighed. The percentage (w/w) of ash with reference to the air-dried drug was then calculated[17].

Acid-insoluble ash

A known amount of total ash obtained by incinerating powdered drug material at a temperature not exceeding 450°C was boiled with 25 ml of hydrochloric acid (70 g/L) for 5 min. The solution was filtered through an ash-less filter paper and later washed with hot water. The filter paper bearing insoluble matter was dried, ignited and later cooled in a desiccator and weighed. The percentage of acid-insoluble ash (w/w) with reference to the air-dried drug was calculated[17].

Water-soluble ash

The total ash was boiled with 25 ml of water for 5 min. The insoluble matter was collected on an ash less-filter paper washed with hot water and ignited for 15 min at a temperature not exceeding 450 °C. The weight of the insoluble matter was subtracted from the weight of the ash and the difference in weight represents the water-soluble ash. The percentage of ash (w/w) with reference to the air-dried drug was then calculated[17].

Alcohol-soluble extractable matter

About 4 g of the coarsely powdered air-dried plant material was macerated with 100 ml of ethanol in a glass stopper conical flask for 24 h. The mixture was shaken frequently during the first 6 h and allowed to stand for 18 h. After 24 h the mixture was filtered and the final volume was make-up to the mark. 25 ml of the filtrate was taken in a flat bottom previously weighed dish, evaporated to dryness, dried at 105

°C and finally weighed. The percentage (w/w) of ethanol-soluble extractive with reference to the air-dried drug was calculated[17].

Water-soluble extractable matter

For the determination of the water-soluble extractable matter, the same method as above was followed using water as a solvent for extraction. The percentage (w/w) of water-soluble extractive with reference to the air-dried drug was calculated[17].

Extraction Procedure of the M. ferrea L. Stamens

The hydro-alcoholic extract of dried powdered *M. ferrea* L. stamens (HAMS) was prepared by the cold maceration method. In brief, the dry powdered stamen (500 g) was mixed with 5 L of 70% ethanol (ethanol:water :: 70:30) for 72 h with periodic shaking at 4 h intervals. The extract was filtered using Whatman No. 1 filter paper and then concentrated and evaporated in a rotary evaporator (Buchi, Rotavapor R-3) at 55 °C at 210 mbar, a brown paste was obtained. Furthermore, the extract was deep frozen to solidify, the residual aqueous solvent was then removed using a vacuum lyophilizer (SACNVAC CoolSafe, LABOGENE Scandinavian by design, Denmark). The percentage yield was found to be 18.26% w/w. The extract was stored at 2-4 °C and protected from direct sunlight until used for various investigations.

Qualitative Phytochemical Screening

Qualitative chemical test is a preliminary test designed to detect the presence of phytoconstituents[18,19]. This test is non-numerical; it is not descriptive but rather exploratory.

Test for alkaloids

(a) Mayer's test: Required volume of the extract i.e., HAMS and Mayer's the reagent was mixed. The formation of cream colour precipitate ensures the presence of alkaloids.

Preparation of Mayer's reagent: Accurately 1.358 g of mercuric chloride was dissolved in 60 ml of distilled water (Solution A). To another container, 5 g of potassium iodide was dissolved in 10 ml of distilled water (Solution B). Solution A and B were mixed and made up to 100 ml with distilled water to form Mayer's reagent.

(b) Dragendroff's test: Required volume of the extract (HAMS) and Dragendroff's reagent was mixed. The formation of orange to reddish colour precipitate ensures the presence of alkaloids.

Preparation of Dragendroff's reagent: Accurately 0.85 g of basic bismuth nitrate was dissolved in a mixture of 10 ml of acetic acid and 40 ml of distilled water (Solution A). To another container, 8 g of potassium iodide was dissolved in 20 ml of distilled water (Solution B). 5 ml each of solution A and solution B were mixed with 20 ml of acetic acid and the volume was made up to 100 ml with distilled to obtain Dragendroff's reagent.

(c)Wagner's test: Required volume of the extract (HAMS) and Wagner's reagent were mixed. The formation of reddish-brown colour precipitate ensures the presence of alkaloids.

Preparation of Wagner's reagent: 1.27 g of iodine, 2 g of potassium iodide was dissolved in 5 ml of water and the volume was made up to 100 ml with water.

Test for glycosides

Legal's test: To the HAMS extract, 1 ml of pyridine and a few drops of sodium nitroprusside were added and then it was made alkaline with NaOH solution. The appearance of a pink colour showed the presence of glycosides.

Test for coumarins

To the HAMS extract, 10% dilute NaOH was added and the mixture was monitored under UV light (366 nm). The appearance of the blue colour fluorescence confirmed the presence of coumarins.

Test for phenols

To the HAMS dissolved in methanol, few drops of freshly prepared solution of 5% ferric chloride were added. Formation of blue colour indicated the presence of phenol.

Test for tannins

To the HAMS extract dissolved in methanol, 0.5 ml of 10 % lead acetate was added. The appearance of white precipitate indicated the presence of tannins.

Test for flavonoids

To the HAMS extract dissolved in ethanol, the required amount of magnesium turnings and a few drops of concentrated hydrochloric acid were mixed. Formation of the pink colour solution indicates the presence of flavonoids.

Test for triterpenoids

To the HAMS extract dissolved in ethanol, the required amount of tin and few drops of thionyl chloride were mixed. Formation of pink colour indicated the presence of triterpenoids.

Test for saponins

Foam test: Extract (HAMS) were diluted with 1 ml of distilled water and was shaken vigorously. Formation of persistent foam/froth up to 10 min or more indicated the presence of saponin.

Test for carbohydrates

Molisch's test: The extracts (HAMS) were made to react with 0.5 ml of α–naphthol solution. The mixtures were vortexed and two drops of concentrated sulphuric acid were added from the side of the test tube. Formation of the purple ring at the junction of two liquids showed the presence of carbohydrates.

Test for phytosterols/steroids

Libermann Burchard test: Known volume of the extract (HAMS) was dissolved in 3 ml of acetic anhydride. To this solution, two drops of concentrated sulphuric acid were added slowly along the side of the test tube. The appearance of a bluish-green colour showed the presence of phytosterols/steroids.

Test for amino acids

To the known volume of extract (HAMS), 2 ml of ninhydrin solution was added and the solution was heated. Formation of violet colour indicated the presence of amino acids.

Quantitative Phytochemical Estimation

Quantitative estimation numerically depicts the quantity of various phytochemicals present in the crude drug by using mathematical and statistical methods. Following up with the findings from preliminary phytochemical screening, an analytical method was developed to determine the content of phenol, flavonoid and carbohydrates using the hydro-alcoholic extract of *M. ferrea* L. stamens (HAMS) using UV-Vis spectrophotometric method[19,20].

Estimation of total phenolic content in the HAMS

The determination of total phenolic content was done following the Folin-Ciocalteu method[2]. Briefly, aliquots of standard gallic acid (20-200 µg/ml) and HAMS (100 µg/ml) were initially made up to 0.5 ml volume with distilled water. To this solution, 0.25 ml of Folin-Ciocalteu reagent (2 N) and 1.25 ml sodium carbonate (20 %) were mixed vigorously and kept at room temperature for 40 min. The absorbances were recorded at 740 nm and methanol was used for carrying out the blank. The result was expressed as µg of gallic acid equivalent weight (GAE)/mg of the extract.

Estimation of total flavonoid content in the HAMS

The determination of the total flavonoid content was carried out based on the aluminium chloride method using quercetin as the standard reference[9]. The method was based on the formation of a flavonoid–aluminium complex having the absorption maxima at 415 nm. 100 µl of HAMS in methanol (10 mg/ml) was mixed with 100 µl of 20 % aluminium trichloride (20 %) in methanol. A drop of acetic acid was added and the mixture was diluted with methanol up to 5 ml. After 40 min the absorbance was measured spectrophotometrically at 415 nm. A blank sample was prepared using 100 µl of methanol in place of the extract. The absorbance of the standard quercetin solution (0.5 mg/ml) in methanol was also measured under the same conditions and the total flavonoid content (µg quercetin equivalent/mg extract) was calculated.

Estimation of total carbohydrate content in the HAMS

The total carbohydrate content was determined as per the colorimetric method described by Gerwig[22]. In brief, anthrone reagent was prepared by dissolving 200 mg of anthrone in 100 ml of water. The reagent was allowed to stand for 30-40 min with occasional shaking until it was clear. The reagent was prepared freshly and should be used within 12 h from the time of preparation. The anthrone reagent (5 ml) was pipetted out into a thick-walled pyrex tube (150 × 25 mm) and chilled in ice-cold water. The solution under test (HAMS, 1 ml) was layered on the acid medium, cooled for further 5 min and finally mixed thoroughly while cooling. The tubes were then loosely fitted with corks, heated in boiling water-bath and later cooled for 5 min and the absorbance were measured at 600 nm. The total carbohydrate content (µg equivalent of glucose/mg HAMS) was quantified.

Characterization of HAMS by High Performance Liquid Chromatography (HPLC)-Fingerprinting

The HPLC-fingerprint is a comprehensive method for the assessment of the quality and reliability of food and plant extracts. The fingerprint profile of the extract can be used as a reference to compare and identify the components of plant extract samples[23]. The 0.1 mg of HAMS is dissolved in 1 ml of HPLC grade methanol by sonication for 10 min. Dissolved samples pass through the filtering HPLC syringe, to avoid undissolved material if any. The injection volume of 10 µl was employed and the detection wavelength was set at 254.4 nm. Graded solvent system consists of methanol: water with 50:50, 80:20, 90:10 and 50:50 was eluted for 0 min, 5 min, 10 min and 5 min, respectively.

Phytochemical Profiling of the HAMS by LC-MS Technique

The Agilent 1290 Infinity II LC system i.e. UPLC-ESI-QTOF-MS coupled to a 6546 Quadrupole time-of-flight (QTOF) (Agilent Technologies, Santa Clara, USA) using a Dual-AJ Selectrospray Ion Source (Jet Stream Technology) was used to analyse the phytochemical profile of the HAMS. In brief, chromatographic separation was carried out on a reversed-phase C18 column (Poroshell 120, 2.1 x 100 mm, 2.7 µm pore size) at 40 °C. Water + 0.1% formic acid (Phase A) and acetonitrile (Phase B) as mobile phases with a flow rate of 0.3 mL/min. A gradient from 5% B to 100% B in 20 minutes, and the gradient came back to the initial conditions in 5 minutes with a re-equilibration time of another 5 minutes was used. The injection volume was 5 µL. The optimal conditions of the electrospray ionization (ESI) were as follows: gas temperature 320 °C, drying gas 8 L/min, nebulizer 45 psi, sheath gas temperature 350 °C, and sheath gas flow 11 L/min. Spectra were acquired in the m/z range 100–1200 in positive mode, fragmentor voltage was 175 V, and an acquisition rate of 1.5 spectra/s. The obtained MS data, molecular peaks and fragmentations pattern were identified *via* untargeted identification of mass spectra using the library of reported phytochemicals of the *M. ferrea*[24]. The selected library of the phytochemical was prepared by robust datamining from the PubChem, ChemSpider, METLIN, and MassBank database[8].

Results And Discussion

The demand for herbal medicine is ever-increasing, but the absence of evidence-based research and the lack of standardization is the main obstacle in the globalization of herbal products[4,7]. Therefore, the evaluation of any crude drug based on pharmacognostical aspects is considered to be the primary steps in establishing the quality control profile of any crude drug[14].

Physicochemical Characterization

Various physicochemical constants were evaluated, as they are important parameter, which helps in detecting adulteration or improper handling of the drug. The ash values are quantitative standards that represent the presence of various impurities like carbonate, oxalate and silicate which may be naturally occurring or deliberately added to the crude drug as a form of an adulterant[25]. Likewise, extractive values are also useful to assess the amount of active chemical constituents present in the plant/plant parts using different solvents. Physicochemical parameters were evaluated as per the guidelines of the official guidelines and compiled in Table 1. The values of estimated physicochemical parameters were found to be within the limits as prescribed by the Ayurvedic Pharmacopoeia of India[15] and Indian Herbal Pharmacopoeia[17].

Extraction of the Pulverized Dried Stamens of the *M. ferrea*

The hydroalcoholic extract of the coarsely powdered dried stamens of the *Mesua ferrea* (HAMS) was made by the cold maceration method. The yield of HAMS was found to be 18.26% w/w.

Qualitative Phytochemical Characterization of HAMS

For the maintenance of the standard of herbal medicine, WHO stresses the importance of the qualitative and quantitative methods for characterizing herbal medicine[3]. The phytochemical screening is helpful in the prediction of the chemical nature of active constituents present in the tested drugs since phytochemicals are proven to be responsible for the various pharmacological activities of the drugs[26]. This technique of phytochemical screening is helpful in identifying the class of phytochemicals and detecting the presence of adulterants[25]. The HAMS was found to contain a wide range of secondary metabolites including alkaloids, glycosides, coumarin, phenols, tannins, flavonoids, terpenoids, saponins, carbohydrates, phytosterols and amino acids.

Quantitative Phytochemical Characterization of HAMS

For quantitatively estimating the amount of a substance, the preparation of a standard curve is first necessary. This is followed by measuring the optical densities of serially diluted standard solutions of the pure compound[13,18,20].

Table 1: List of determined physicochemical attributes of the coarsely powdered dried stamens of the *Mesua ferrea*

S. No.	*Parameters*	*Results (% w/w) Mean ± SD (n=3)*
1.	Foreign matter	0.10 ±0.02
2.	Loss on drying	5.38 ±0.24
3.	Total ash	2.48 ± 0.28
4.	Acid insoluble ash	0.34 ± 0.06
5.	Water soluble ash	1.98 ± 0.48
6.	Alcohol soluble extractive	15.64 ± 0.64
7.	Water soluble extractive	19.82 ± 0.86

Total phenolic content

Among natural products, gallic acid is the most significant polyphenol. The Folin-Ciocalteu technique was utilized to measure the total phenolics content equivalent of gallic acid[21]. The HAMS extract was found to contain 284.24 ± 8.18 µg equivalent of gallic acid/ mg of HAMS dry extract. The Standard calibration curve for total phenolic content is shown in Figure 1.

Total flavonoid content

The total flavonoid content was calculated as µg of quercetin equivalent to per mg of dry weight[19]. The HAMS contained 242.52 ± 8.36 µg equivalent of quercetin/ mg of dry extract. The standard calibration curve of quercetin for total flavonoid content is shown in Figure 2.

Total carbohydrate content

The total carbohydrate content in HAMS was found to be 92.34 ± 2.58 µg equivalent of glucose/ mg of HAMS dry weight. The standard calibration curve of glucose for total carbohydrate content is shown in Figure 3.

Characterization of HAMS by HPLC Fingerprinting

Development of HPLC-fingerprints is a pivotal tool in the quality control of complex plant extract and formulations[27]. Chemical fingerprints attained by chromatographic techniques are strongly recommended for the purpose

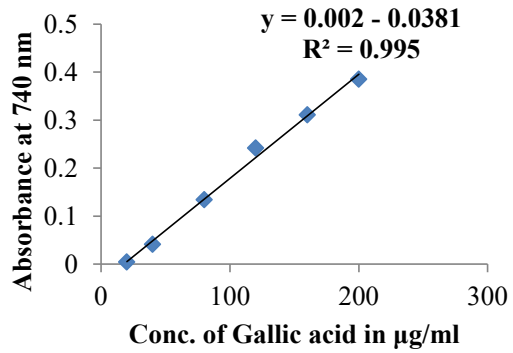

Figure 1: Standard calibration curve of gallic acid for total phenolic contents.

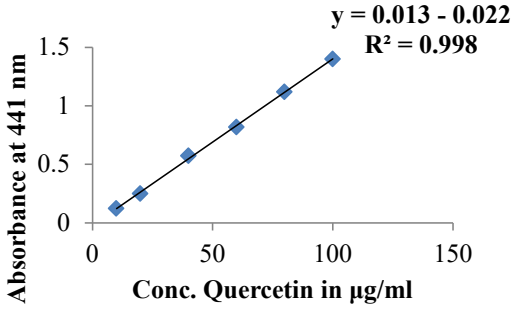

Figure 2: Standard calibration curve of quercetin for total flavonoids contents.

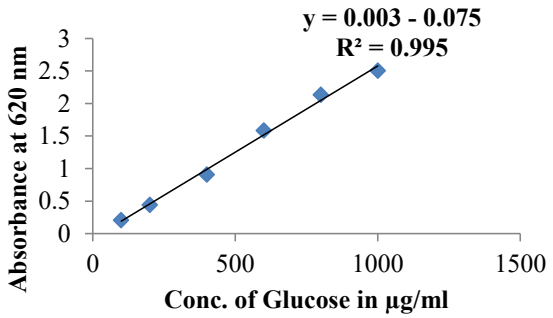

Figure 3: Standard calibration curve of glucose for total carbohydrate contents

of quality control of plant extracts because they might represent appropriately the chemical integrities of the extract, thus used for authentication and identification of the herbal extracts and derived-products[28]. The developed HPLC-fingerprinting profile suggests presence of thirty (30) separately resolved peaks as per aforesaid instrumental conditions. The HPLC-fingerprint profile of the HAMS is depicted in the Figure 4 and the distinctive retention time ($R_{t)}$ values along with relative percentage of corresponding area under the curve were compiled in the Table 2. The percentage area under the curve signifies the relative abundancy of the corresponding phytochemicals in the HAMS in above-mentioned experimental conditions.

Phytochemical profile of the HAMS by LC-MS techniques

Continued research and discovery necessitate the use of high-throughput analytical methods to screen and identify

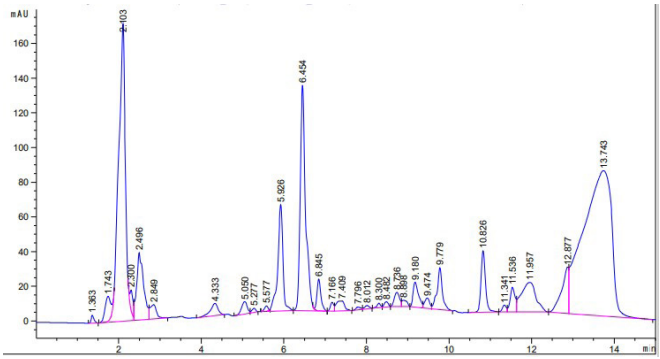

Figure 4: Chromatogram of the high-performance liquid chromatography (HPLC)-fingerprint profile of the hydroalcoholic extract of the Mesua ferrea stamens (HAMS)

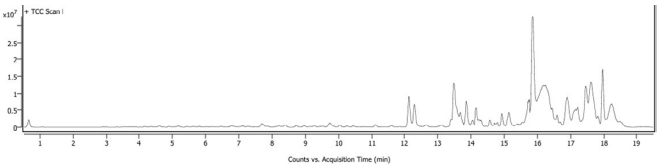

Figure 5: Chromatogram of the LC-MS-fingerprint profile of the hydroalcoholic extract of the *Mesua ferrea* stamens (HAMS)

Table 2: The HPLC-fingerprint profile of the hydroalcoholic extract of the Mesua ferrea stamens (HAMS) with distinctive retention time (R_t, min) values and their relative area (%) at 254.4 nm.

Peak	*Retention time (R_t min)*	*Area (%)*
1	1.363	0.2029
2	1.743	1.2860
3	2.103	18.8880
4	2.300	0.5827
5	2.496	4.2537
6	2.849	0.8325
7	4.333	0.8759
8	5.050	0.6423
9	5.277	0.1462
10	5.577	0.1697
11	5.926	5.0718
12	6.454	11.2447
13	6.845	1.1307
14	7.166	0.2694
15	7.409	0.7477
16	7.796	0.1262
17	8.012	0.1410
18	8.300	0.1443
19	8.482	0.1886
20	8.736	0.6740
21	8.898	0.3030
22	9.180	1.2281
23	9.474	0.4568
24	9.779	1.9988
25	10.826	2.5030
26	11.341	0.2376
27	11.536	1.1361
28	11.957	3.9535
29	12.877	3.0113
30	13.743	37.5533

bioactive components and potential therapeutic molecules from plants[28]. Utilizing a pre-generated plant extract library, we subjected botanicals to LC-MS/MS-based molecular networking to determine their chemical composition of already known metabolites[8]. The obtained LC-MS chromatogram is a typical fingerprinting profile (shown in Figure 5) for the HAMS in a given set of experimental conditions. Phytochemical profiling of the HAMS was done by the untargeted identification of the phytochemical using MS data, MS/MS fragmentation patterns and molecular formula proposed by the MassHunter by comparing with the literature data[24] and some databases, such as PubChem, ChemSpider,

Table 3: List of identified phytochemicals in the hydroalcoholic extract of the *Mesua ferrea* stamens (HAMS) by untargeted LC-MS technique.

S. No.	*RT*	*m/z*	*Molecular Weight (g/mol)*	*Chemical Name*	*Molecular Formula*	*Compound identifier (CID)*
1	2.908	[M+H]+: 303.1978 [M+Na]+: 324.1765	302.19	Ellagic acid	$C_{14}H_6O_8$	5281855
2	4.354	[M+H]+: 311.1682, 312.1641 [M+Na]+: 333.1501, 334.1539	310.3	6-Deoxyjacareubin	$C_{18}H_{14}O_5$	5281629
3	5.106	[M+H]+: 415.25 [M+Na]+: 437.23, 434.23	414.7	β-Sitosterol	$C_{29}H_{50}O$	222284
4	6.463	[M+H]+: 427.2150, 429.2069 [M+Na]+: 449.1970, 450.2003	426.7	α-Amyrin	$C_{30}H_{50}O$	73170
5	6.989	[M+H]+: 165.1396 [M+ NH4]+: 180.1583	164.16	p-Coumaric acid	$C_9H_8O_3$	637542
6	8.738	[M+H]+: 449.1049, 450.1096 [M+Na]+: 471.0873, 472.0904	448.4	Quercetin 3-O-rhamnoside	$C_{21}H_{20}O_{11}$	5280459
7	9.324	[M+H]+: 433.4385, 434.415	432.4	Kaempferol 3-O-rhamnoside	$C_{21}H_{20}O_{10}$	5316673
8	10.642	[M+H]+: 287.05, 288.06	286.24	Luteolin	$C_{15}H_{10}O_6$	5280445
9	11.584	[M+H]+: 449.1995, 450.2029 [M+NH4]+: 466.2261, 467.2292 [M+Na]+: 471.1812, 472.1853	448.4	Luteolin 8-C-glucoside (Astragaline)	$C_{21}H_{20}O_{11}$	5281675
10	11.799	[M+H]+: 287.2534, 288.2561	286.24	Kaempferol	$C_{15}H_{10}O_6$	5280863
11	11.858	[M+H]+: 543.1258, 544.1290	542.5	Rhusflavanone	$C_{30}H_{22}O_{10}$	466314
12	13.322	[M+H]+: 171.1371, 172.1408 [M+NH4]+: 188.1632, 189.1661 [M+Na]+: 193.1189	170.12	Gallic acid	$C_7H_6O_5$	370
13	14.691	[M+H]+: 303.30, 304.30 [M+Na]+: 324.28	302.23	Quercetin	$C_{15}H_{10}O_7$	5280343
14	15.995	[M+H]+: 611.518, 612.52 [M+Na]+: 633.50, 634.50	610.5	Rutin	$C_{27}H_{30}O_{16}$	5280805
15	16.763	[M+H]+: 543.5064, 544.5092	542.496	Mesuaferrone A	$C_{30}H_{22}O_{10}$	273591759
16	17.210	[M+H]+: 319.2823 [M+NH4]+: 336.3089, 337.3121 [M+Na]+: 341.2643, 342.2674	318.23	Myricetin	$C_{15}H_{10}O_8$	5281672
17	17.352	[M+H]+: 275.2564, 276.2600 [M+NH4]+: 292.2829, 293.2862 [M+Na]+: 297.2385, 298.242	274.22	Tovopyrifolin-C	$C_{14}H_{10}O_6$	5480342
18	17.421	[M+H]+: 541. 5635, 542.5656	540.5	Mesuaferrone B	$C_{30}H_{20}O_{10}$	90472563
19	17.940	[M+H]+: 427.3867, 428.3900 [M+Na]+: 449.3666, 450.3659	426.7	Lupeol	$C_{30}H_{50}O$	259846
20	18.328	[M+H]+: 395.4179, 396.4212	394.4	Macluraxanthone	$C_{23}H_{22}O_6$	5281646

METLIN, and MassBank to annotate the phytochemicals analysed from the extracts, and a maximum error of 8 ppm was accepted[29]. Through LC-MS-based untargeted analysis twenty phytochemicals (see the Table 3) were identified *viz*., Ellagic acid, 6-Deoxyjacareubin, β-Sitosterol, α-Amyrin, p-Coumaric acid, Quercetin 3-O-rhamnoside, Kaempferol 3-O-rhamnoside, Luteolin, Luteolin 8-C-glucoside (Astragaline), Kaempferol, Rhusflavanone, Gallic acid, Quercetin, Rutin, Mesuaferrone A, Myricetin, Tovopyrifolin-C, Mesuaferrone B, Lupeol, Macluraxanthone from the phytochemical profile of the HAMS.

Conclusion

Authentication and chemical characterization of the *M. ferrea* stamens aka 'Nagakeshara' has been established for the effective quality control of this herbal drug. Which includes, quantification of physicochemical features of the powdered drug, characterization of qualitative and quantitative phytochemical parameters as well as fingerprint profile *via* HPLC and LC-MS of the hydroalcoholic extract of the *M. ferrea* stamens (HAMS). Further, twenty phytochemicals of HAMS have been identified using selective untargeted

LC-MS technique. Developed quality control tools could be significant in validating the content uniformity of the *M. ferrea* stamens in a range of pharmaceutical preparations and products. In addition, the study supports the marker compound-based quantification of the HAMS using identified phytochemicals *via* HPLC, HPTLC and of LC-MS/MS to develop the robust monograph of the *M. ferrea* stamens.

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Conflict of Interest

The authors declare that they have no known conflicts of interest and/or competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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