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Original Article

Opponents Deployed Pharmacognostic, Physicochemical, and Chromatographic Techniques to Establish Dhatupaushtik Churna

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Abstract

Background: Dhatupaushtik Churna is a polyherbal powdered formulation that contains equal amounts of Gokhru (*Tribulus terrestris* L.; Family: Zygophyllaceae), Ashwagandha (*Withania somnifera* L.; Family: Solanaceae), and Safed musli (*Chlorophytum borivilianum* L.; Family: Asparagaceae). It is used to treat a myriad of ailments, including premature ejaculation, weakness, rejuvenation of the male reproductive system, and anti-aging. This study aimed to thoroughly investigate this Churna using the Ayurvedic Pharmacopoeia of India's (API's) criteria.

Methods: The fundamental aspiration of this investigation is to document Dhatupaushtik Churna's distinctive characteristics to legitimize its identification, quality, and purity. The innumerable specifications explored in this study included organoleptic characteristics, physicochemical parameters, physical characteristics of Churna, preliminary phytochemical, heavy metal, microbial analysis, metabolomic tracking using gas chromatography/mass spectrometry, and high-performance thin-layer chromatography assessment.

Results: It was revealed that the established parameters are informative tools for assisting regulatory authorities, scientific organizations, and manufacturers in developing high-efficacy standard formulations and can be utilized as reference standards in a pharmaceutical startup's quality control/quality assurance laboratory.

Conclusion: Based on the findings, every ingredient in Dhatupaushtik Churna might be identified. The characterization parameters documented in this dissertation might be employed as a standard benchmark for the quality control analysis of Dhatupaushtik Churna. To uphold batch-to-batch consistency, the criteria given in this study might be used to prepare a monograph on quality standards for Dhatupaushtik Churna.

Keywords: Chromatographic analysis, Dhatupaushtik Churna, Metabolomic tracking, Pharmacognostical, Physicochemical, Toxic contaminant

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Introduction

Traditional, complementary, alternative, or non-conventional treatments are primarily utilized by 70%–95% of the worldwide population in impoverished countries, according to the World Health Organization (WHO) (1). Furthermore, there has been a dramatic rise in the use of herbal medications by a global trend of individuals reverting to natural remedies. The public's increasing use of botanicals (drugs and other goods derived from plants) pushes efforts to evaluate the health claims made by these agents and set quality and manufacturing standards (2).

Standardization is the practice of prescribing a set of standards or inherent features, consistent parameters, and traditional qualitative and quantitative values that assure quality, efficacy, safety, and repeatability for herbal medicines. It is impossible to consider medicine scientifically genuine unless it is verified and characterized to ensure repeatability in product manufacture. Further, several serious and fatal side effects have recently been identified, including decisive toxic effects, allergic reactions, contaminant effects, and interactions with herbal drugs. Accordingly, standardization is a key step

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in establishing a consistent biological activity, chemical profile, or quality assurance program for herbal drug manufacturing (3).

The Indian medicinal practice, which includes Ayurveda, Siddha, and Unani, is one of the oldest comprehensive healthcare systems in the world, with well-documented cures. Ayurveda, a component of India's cultural legacy, is well-known for its originality and global recognition as a natural approach to treating ailments and improving health (4). Ironically, standardization and quality control in manufacturing Ayurvedic medications have remained ambiguous. Most Ayurvedic formulations have yet to be created regarding quality control standards and assessment procedures (5). As a result, proving the efficacy of herbal formulations is critical to justifying their usage in today's medical system. Standardization methods should consider sample authentication, organoleptic evaluation, pharmacognostic evaluation, volatile matter, quantitative evaluation (ash values and extractive values), phytochemical evaluation, xenobiotics test, microbial load testing, toxicity testing, and biological activity. Additionally, the phytochemical profile is of particular importance since it directly impacts the action of medicinal herbs. The fingerprint ensures the quality of the drug's phytochemical profile. At the same time, the quantity of the marker component is used as an additional criterion in determining the sample's quality. Chromatographic techniques are also utilized to assess the quality of herbal formulations. Methods for the standardization of plant material concerning marker compounds, such as high-performance thin-layer chromatography (HPTLC), high-performance liquid chromatography (HPLC), liquid chromatography-mass spectrometry, and gas chromatography-mass spectrometry (GC-MS), are significant (6,7).

Dhatupaushtik Churna is a powdered Ayurvedic polyherbal composition used to treat premature ejaculation, cure weakness, rejuvenate the male reproductive system, and eliminate aging, among other things (8). Gokhru (*Tribulus terrestris* L.; Family: Zygophyllaceae), Ashwagandha (*Withania somnifera* L.; Family: Solanaceae), and Safed musli (*Chlorophytum borivilianum* L.; Family: Asparagaceae) are three essential ingredients that are blended in equal proportions (Table 1). Considering that there is a scarcity of scientific data on Dhatupaushtik Churna, it was decided to characterize it to ensure its identification, quality, and purity. Thus, this research aims to thoroughly examine this Churna using the Ayurvedic Pharmacopoeia of India's (API's) criteria. The WHO, the European Agency for the Evaluation of Medicinal Products, and the United States Pharmacopoeia (USP) have all given interoperability criteria. Through quality control methods, the Churna was assessed for important physical, chemical, and analytical characteristics as part of the procedure to evaluate its safety and persistent efficacy (9-12).

Table 1. Composition of Dhatupaushtik Churna

Common Name	Scientific Name	Part	Quantity
Gokhru	*Tribulus terrestris* L.	Seed	1 Part
Ashwagandha	*Withania somnifera* L.	Root	1 Part
Safed musli	*Chlorophytum borivilianum* L.	Root	1 Part

Materials and Methods

Acquisition and Authentication of Plant Materials

Dhatupaushtik Churna's three ingredients were consumed from Ayurvedic medical stores in Kolkata, West Bengal, India. Dr. Suchandra Samanta Mondal, Assistant Professor, Department of Botany, Krishnanagar B. Ed. College, Krishnanagar, Nadia, West Bengal, India, validated its authenticity, and a voucher specimen was preserved in our research laboratory for prospective reference (Reference No. Cert/01-03/20).

Preparation of Dhatupaushtik Churna

The Dhatupaushtik Churna was made based on the Ayurvedic Formulary of India's standard procedure (13). According to the literature, all ingredients were shade dried and powdered individually and then sieved through a #80 sieve before being mixed consistently in equal proportions to produce uniformly blended Churna (Figure 1) (14).

Protocol for Standardization of Dhatupaushtik Churna

Determination of Foreign Matter

To identify the emergence of foreign matter, 100 g of coarsely powdered Dhatupaushtik Churna was distributed in a thin layer. Next, the samples were viewed with the unaided eye or a magnifying lens (6X or 10X) to painstakingly separate the foreign organic matter as precisely as possible and weigh it. The percentage of foreign organic matter had to be weighed and determined concerning the weight of the drug taken (15,16).

Organoleptic Parameters

The ability to acknowledge the acceptable crude drugs or adulterants relies immensely on organoleptic criteria. Colour, aroma, taste, appearance, and texture are all organoleptic virtues. These metrics are used to evaluate the



Figure 1. Dhatupaushtik Churna

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powdered samples, which is then followed by mainstream guidelines (17,18).

Fluorescence Analysis

The powdered samples were exposed to ultraviolet (UV) light at 254 nm and 365 nm wavelengths. The standard methodology was followed for fluorescence analysis (19,20). The Dhatupaushtik Churna powder was treated with several standard reagents and then explored under visible and UV light in a UV cabinet. The colour was observed with unaided eyes.

Microscopic Overview of Dhatupaushtik Churna

The powdered sample (Gokhru, Ashwagandha, and Safed musli) was individually pretreated with various reagents before being put on glass slides with glycerol for histochemical identification of cell walls and other constituents for powder microscopy. Phloroglucinol with concentrated hydrochloric acid (1:1, v/v) was used for lignified cell walls. In addition, 60% chloral hydrate with 25% concentrated sulphuric acid, 2% iodine, and 5% alcoholic ferric chloride were utilized for calcium oxalate crystals, starch and aleurone grains, and tannins, respectively, and iodine solution with concentrated sulphuric acid was employed for cellulose. For the identification of distinct cell components, each of the glass slides was examined using an Olympus compound microscope with a camera (21-23).

Physicochemical Investigation

According to the pharmacopoeia, physicochemical evaluations include total ash, acid insoluble ash, alcohol soluble extractives, water-soluble extractives, loss on drying, and pH values. Powdered samples were employed in these physicochemical tests (24-26).

Exploration of Powder Physical Attributes

The powdered sample was obtained, and its physical characteristics were investigated, including bulk density, tap density, angle of repose, Hausner's ratio, and Carr's index (27).

Qualitative Phytochemical Investigation

Dhatupaushtik Churna and its ingredients were subjected to comparative qualitative phytochemical analyses on several extracts of varying polarities. Standard procedures were adopted to assess and classify the presence of diverse natural compounds and chemical classes, such as alkaloids, amino acids, anthraquinone glycoside, carbohydrates, flavonoids, phenols, phenolic compounds, proteins, resins, saponins, steroids, sterols, tannins, triterpenoids, and volatile oil (28,29).

Toxic Contaminants Assessment

a. Heavy Metal Perception

As part of routine protocols, arsenic (As), cadmium (Cd), lead (Pb), and mercury (Hg) were detected in the fine powder using atomic absorption spectroscopy (30). The heavy metal analysis was performed using the Perkin Elmer AAS-200 instrument. In essence, 0.5 g of powdered Dhatupaushtik formulation was thoroughly mixed with 5 mL concentrated hydrochloric acid and 5 mL nitric acid, respectively, and then agitated until the fumes were fully dissolved and a clear solution emerged. The digested material was then transferred to a 50 mL volumetric flask and filled with deionized distilled water to the required volume. Subsequently, the material was stored for further analysis. The instruments were calibrated using a blank solution set to zero. Next, the experimental and reference (As, Hg, Pb, and Cd purchased from Merck, Germany) samples were loaded, and the absorbance was monitored (31-33).

b. Microbial Limit Test

Plants cultivated in space and on Earth are subjected to different environmental conditions. According to emerging evidence indicating a close interaction between plants and their microbiota, plants have distinct and different microbial communities that are crucial for their survival (34). Microbiological exploration of the fine powder Dhatupaushtik formulation was performed to verify the total microbial load and the particular pathogen following USP protocols. Total aerobic microbial count and cumulative yeast and mould count were assessed for the total microbial burden. The test drug was examined for certain pathogens, such as *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Salmonella* species (35,36).

c. Pesticide Residue Examination

Pesticides, which are prevalently in herbs, are subject to restrictions specified by the WHO and the Food and Agriculture Organization. During the cultivation stages, these insecticides are blended in with the herbs. Pesticides, such as dichloro diphenyl trichloroethane, benzene hexachloride, toxaphene, and aldrin, induce substantial unfavorable effects in humans when they are mixed with crude drugs (37). The QuEChERS (quick, easy, cheap, effective, rugged, and safe) (38) solid phase extraction technique was used in coalescence with GC-tandem MS to quantify and evaluate particular pesticide residues, such as organochloride, organophosphorus, and pyrethroids toxic substances (39-41).

d. Aflatoxin Determination

Aflatoxin is the most prevalent mycotoxin carcinogenic to animals and humans. *Aspergillus flavus* and *Aspergillus parasiticus* are the most common sources of aflatoxins. This toxin may be present in the plant species. Identifying and quantifying aflatoxins in food and feed are major challenges to ensure food safety. The WHO-recommended aflatoxin test for herbal medications is designed to identify the presence of aflatoxins B_1, B_2, G_1, and G_2, which are harmful toxins prevalent in any plant material (42). As a

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result, finding practical, sensitive, and robust analytical techniques is crucial for detecting and quantifying aflatoxins present in low quantities in food and feed. Several chromatographic and sensor-based approaches are employed to detect aflatoxins (43). Consequently, in this study, HPLC methods were utilized to analyze the Dhatupaushtik formulation for aflatoxin testing (44,45). To this end, 40 µL of the samples were injected into a pre-heated HPLC column (Luna C_{18} column). For post-column electrochemical derivatization of the fluorescence detector, 1 L of water and methanol (60:40, v/v) with 119 mg of potassium bromide and 350 µL of 4M nitric acid were added to the mobile phase. With a total runtime of 20 minutes, the flow rate was fixed at 1 mL/min. The excitation and emission wavelengths for fluorescence detection were adjusted at 362 nm and 455 nm, respectively. Radiant Research Services Private Limited in India facilitated the calibration reference standards.

Chromatographically Anatomization

a. High-Performance Thin-Layer Chromatography Analysis

Marker compounds are chemical components of a pharmaceutical that can be employed to confirm its potency or identity. The active components or chemicals used to confirm the botanical identity of the starting material are commonly referred to as marker compounds. As a result, it is critical to produce reliable chromatographic fingerprints representing the herbal drug's pharmacologically active and chemically unique components (46).

HPTLC studies were conducted according to the standard methods (47,48). The HPTLC fingerprint approaches were implemented to identify potential polyphenolic components in the methanolic extract of Dhatupaushtik Churna (MEDC).

b. Stationary Phase and Sample Prepping

The MEDC was dissolved in methanol used for the sample. Silica gel 60 F 254 HPTLC plates (E. MERCK KGaA; 10.0 cm × 10.0 cm) were utilized as the stationary phase. Toluene, ethyl acetate, and formic acid (8.5:1.5:0.1) were employed for the mobile phase.

c. Sample Application

Different applied volumes of reference standards, such as 3,4,5-trihydroxybenzoic acid (gallic acid), 3,4-dihydroxy cinnamic acid (caffeic acid), and 4-hydroxy-3-methoxycinnamic acid (ferulic acid), were used to detect phytoconstituents present in Dhatupaushtik Churna. The reference standard, along with 6 µL and 12 µL of the extract, was applied as a band on the plates using a syringe (100 µL), with inert gas flow providing a delivery speed of 150 µL per second. The syringe was mounted on a CAMAG linomat V sample applicator attached to the CAMAG HPTLC system and was programmed through WIN CATS software. The length of the applied band was kept at 8 mm.

d. Development of Chromatogram

The plate for Dhatupaushtik Churna was developed in toluene: ethyl acetate: formic acid (8.5:1.5:0.1), and 10 mL of the mobile phase was utilized per chromatography run. The linear ascending development was performed in a (20 cm × 10 cm) twin trough glass chamber saturated with the mobile phase.

e. Detection of Spots and Photo-Documentation

The developed plate was dried by hot air with the help of a hair dryer at a temperature of 60 °C to evaporate the solvent from the plate. The plate was scanned using D2 lamp at 280 nm in CAMAG TLC Scanner 3. The R_f values and fingerprint data were recorded by WIN CATS software.

Metabolomic Tracking With Gas Chromatography/Mass Spectrometry

One of the most widely used analytical platforms for metabolomics is GC-MS, which is employed to identify metabolites associated with chromatographic peaks as well as quantification to compare the abundance of a specific metabolite in different samples. It is also applied, in conjunction with pathway analysis, to understand the biochemical interrelationship between several metabolites that vary in a coordinated or differential manner (49,50). The GC-MS analysis of the MEDC was performed using Thermo GC-Trace Ultra (version 5.0, Thermo MS DSQ II). The column DB 35-MS Capillary Standard Non-Polar Column (30 m × 0.25 mm inner diameters and film thickness of 0.25 µm) was utilized to separate compounds. The column oven temperature was programmed from 70 °C (holding for 2 minutes) to 260 °C at a rate of 6 °C/min with a final hold time of 10 minutes. The injector temperature was maintained at 250 °C. The carrier gas was helium at a 1.0 mL/min flow rate. GC was performed in a splitless mode. For MS detection, the electron ionization mode with an ionization energy of 70 eV was used, with a mass range of 50–650 m/z. The mass spectrometer's ion source was operated at 220 °C, and the MS transfer line was maintained at a temperature of 280 °C. An injection volume of 1 µL of the sample was utilized, and the phytoconstituents in the MEDC were expressed as a percentage by the peak area. The identification and characterization of phytochemical compounds in the MEDC were based on GC retention time. The mass spectra were computer-matched with standards available in mass spectrum libraries, such as Wiley 9.

Statistical Analysis

Wherever feasible, the data compiled in the analyses were expressed as the mean ± standard error of the mean (SEM) using GraphPad InStat software.

Results

Pharmacognostical Study

Foreign Matter

Using unaided eyes and lenses, about 100 g coarsely

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powdered sample was spread out as a thin layer form, and the foreign matter was sorted out. Inconspicuous or discernible materials are appropriately identified rather than plant ingredients. The weight is estimated when the experiment is executed. The measured foreign matter level in the ingredients of Dhatupaushtik Churna was less than 0.9% (w/w), which is well below the maximum permissible limits as per API.

Organoleptic Parameters

The developed in-house Dhatupaushtik Churna was determined to be peach puff yellow in colour with a distinctive aroma and astringent test during the organoleptic examination. The organoleptic implications of the Churna, including its ingredients, are provided in Table 2.

Fluorescence Analysis

The fluorescence behavior of powdered Dhatupaushtik formulation under ordinary light and UV light (both long 365 nm and short 254 nm wavelengths) was observed during research exploration (distinctive colours displayed in Table 3).

Microscopic Study of Dhatupaushtik Churna

Different microscopical features were found after microscopic analysis of powder forms of Gokhru (*Tribulus terrestris*), ashwagandha (*Withania somnifera*), and safed musli (*Chlorophytum borivilianum*). Using these features, it is possible to assess microscopical characteristics and crude drugs and identify the appropriate crude drug from adulterant material. The powder of Gokhru consists of calcium oxalate prismatic crystals, sclereids fibre, with single long thick-walled trichomes, sclereids (a reduced version of sclerenchyma cells), and inner cells. Furthermore, the presence of ashwagandha is indicated by starch grains, pitted xylem vessels, tracheids, cork cells, and fibres. In contrast, starch, fibres, and sclereids fragments have been detected in the powder form of Safed musli (Figure 2).

Physicochemical Investigation

Dhatupaushtik Churna's physicochemical study revealed distinct features in different solvent treatments. It exhibited water soluble extractive value (8.4 ± 0.12)% w/w, whereas alcohol soluble extractive (2.56 ± 0.14)% w/w, total ash content (8.93 ± 0.12)% w/w, acid insoluble ash (8.01 ± 0.09)% w/w, a pH rate of 7.22 ± 0.21, and loss on drying (1.98 ± 0.06)% w/w at 105°C.

Physical Characteristics

The flowability of the in-house Dhatupaushtik formulation was also found to be extremely poor, with a bulk density of 0.4777 ± 0.01 g/mL, a tap density of 0.6858 ± 0.00 g/mL, and an angle of repose of 38.39° ± 0.88°. Conversely, the Hausner ratio and Carr's index were 1.4540 ± 0.00 (poor) and 45.541 ± 0.24% (extremely poor), respectively.

Qualitative Phytochemical Investigation

Dhatupaushtik Churna and its ingredients were phytochemically assessed in the solvents of varying polarities, the findings of which are reported in Table 4. Phytoconstituents, such as alkaloids, steroids, tannins, anthraquinone glycosides, flavonoids, saponins, and carbohydrates, were identified during the preliminary phytochemical screening of various extracts.

Table 2. Organoleptic Parameters of Raw Materials Present in Dhatupaushtik Churna

Sample Name	Colour	Odour	Taste	Appearance	Texture
Gokhru	Crayola lemon yellow	Odourless	Pungent	Powder	Fine
Ashwagandha	Whitish cream	Characteristics	Bitter	Powder	Fine
Safed musli	Whitish brown	Odourless	Pungent	Powder	Fine
Dhatupaushtik Churna	Peach puff Yellow	Characteristics	Astringent	Powder	Fine

Table 3. Fluorescence Scrutiny of Dhatupaushtik Churna

Powdered Drug	Visible/Day Light	254 nm	365 nm
Powder as such	Brown	Light green	Black
Powder + Concentrated HCl	Brown	Light green	Black
Powder + 1 M NaOH	Brown	Green	Blue
Powder + 1% $AgNO_3$	Brown	Very light green	Dark blue
Powder + Concentrated HNO_3	Brown	Greenish brown	Dark blue
Powder + Concentrated H_2SO_4	Dark brown	Dark green	Dark blue
Powder + Br_2 water	Reddish brown	Deep green fluorescence	Dark blue fluorescence
Powder + CH_3OH	White	Whitish brown	Dark green
Powder + CH_3COOH	White	Light green	Blue fluorescence
Powder + NH_3	White brown	Green fluorescence	Dark blue fluorescence
Powder + I_2	Dark brown	Green fluorescence	Dark blue fluorescence

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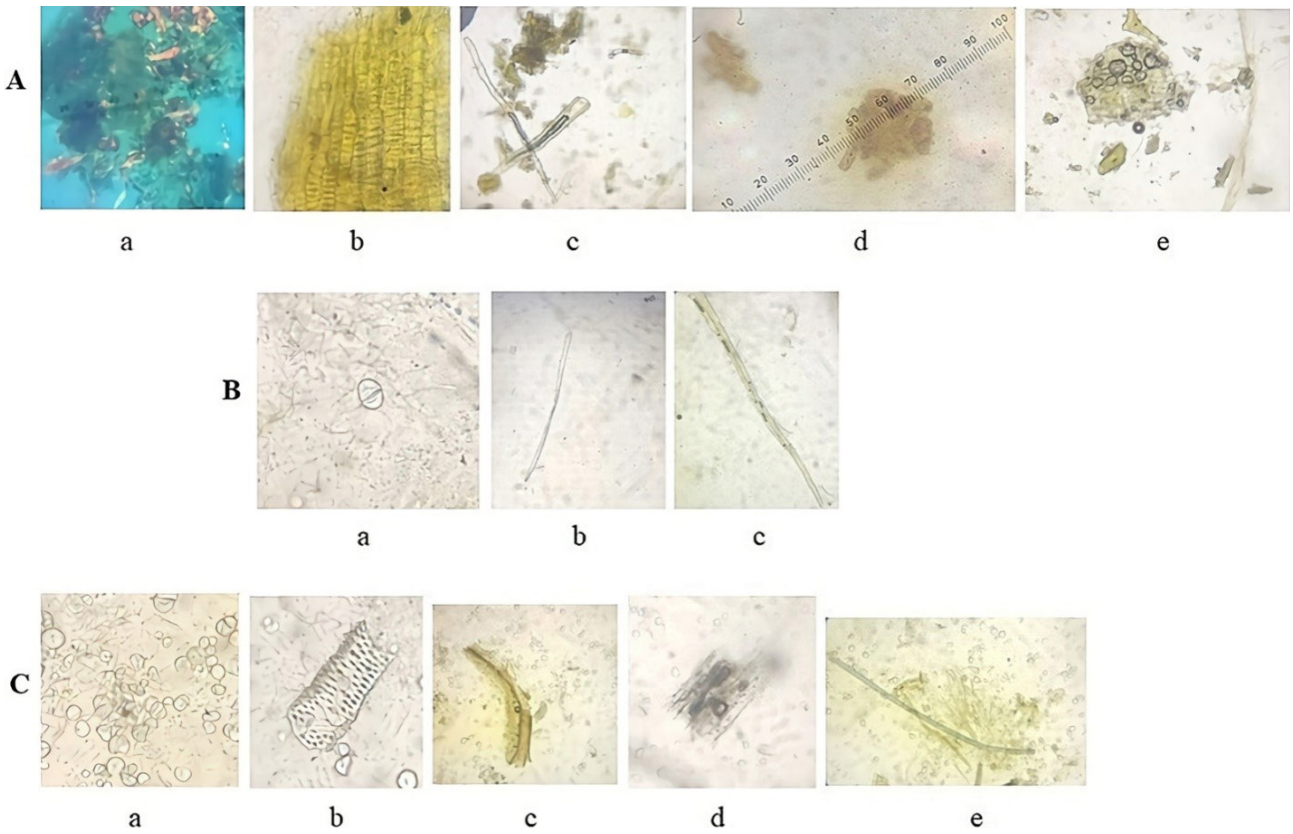


Figure 2. Microscopic Characters of Gokhru, Safed musli, and Ashwagandha. **(A)** Gokhru: (a) Calcium oxalate prismatic crystals, (b) Fiber sclereids, (c) Single long thick-walled trichome, (d) Sclereids in the powder, and (e) inner cells. **(B)** Safed musli: **(a)** Starch grains, **(b)** Fiber, and **(c)** Sclereids fragments. (C) Ashwagandha: (a) Starch grains, (b) Pitted xylem vessel, (c) Tracheids, (d) Cork cell, and (e) Fibres

Table 4. Results of Phytochemical Tests on Crude Materials Found in Dhatupaushtik Churna

Material	Extracts	Phytoconstituents Present
Gokhru	n-Hexane extract	A, F, and St
	Chloroform extract	A, St, and Ag
	Ethyl acetate extract	A, Sa, Ag, T, and St
	Methanol extract	A, Sa, T, F, C, and Ag
	Water extract	A, Sa, and T
Ashwagandha	n-Hexane extract	Ag, T, F, and St
	Chloroform extract	A, Sa, T, F, and St
	Ethyl acetate extract	A, Sa, T, Ag, F, C, and St
	Methanol extract	A, Sa, Ag, T, F, and St
	Water extract	Sa, F, and St
Safed musli	n-Hexane extract	A and St
	Chloroform extract	A, St, and T
	Ethyl acetate extract	A, Sa, and F
	Methanol extract	A, Sa, F, C, and St
	Water extract	Sa, T, C, and St
Dhatupaushtik Churna	n-Hexane extract	A, Sa, Ag, T, and St
	Chloroform extract	A, Sa, T, Ag, and St
	Ethyl acetate extract	A, Sa, Ag, T, C, and St
	Methanol extract	A, Sa, Ag, T, F, C, and St
	Water extract	A, Sa, T, C, and St

Note. A: Alkaloids; Ag: Anthraquinone glycoside; C: Carbohydrates; F: Flavonoids; Sa: Saponins; St: Steroid; T: Tannins.

Determination of Toxic Contaminants

a. Heavy Metal Determination

The heavy metal analysis of the fine powder using Dhatupaushtik Churna revealed that As was the most found toxic metal, but it was trace amounts less than 0.01 ppm, followed by Hg, Cd, and Pb, which were found to be 0.01 ppm, 0.01 ppm, and 0.02 ppm, respectively (Table 5). The heavy metal level of the Dhatupaushtik Churna was under the noxious limits established by the API (not more than 3 ppm for As, 10 ppm for Pb, 0.3 ppm for Cd, and 1 ppm for Hg).

b. Microbial Limit Test

The Dhatupaushtik Churna's microbiological profile was determined to be acceptable since it was devoid of pathogens such as *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Salmonella* species. The total aerobic microbial count and total yeast and mould count (TYMC) were both significantly lower than 10 colony-forming units per g per/mL when determining the total microbial load. The overall microbial load was within the acceptable limits stipulated by the USP (Table 6).

c. Pesticide Residual Study

We failed to find various pesticides, such as dieldrin, endosulfan, hexachlorocyclohexane (α, β, γ, and δ), dichlorodiphenyltrichloroethane, methyl parathion, monocrotophos, phorate, alachlor, chlorpyriphos, ethion,

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Table 5. Dhatupaushtik Churna's Heavy Metal Appraisal

Heavy Metal	Standard Limit (ppm)	Observed Value (ppm)
Arsenic	3	<0.01
Mercury	1	<0.01
Lead	10	<0.02
Cadmium	0.3	<0.01

Table 6. Microbial Limit Test Results of Dhatupaushtik Churna

Particulars	Limits	Results
Total aerobic microbial count CFU/gm/mL	<100000	340
Total yeast and mould count CFU/gm/mL	<1000	<10
Pathogen detection		
Escherichia coli	-	-
Staphylococcus aureus	-	-
Salmonella sp.	-	-
Pseudomonas aeruginosa	-	-

Note. (+) Present; (-) Absent.

atrazine, and butachlor, in our assessment of pesticide residues in Dhatupaushtik Churna. As a result, the formulation was free of harmful pesticide residues, such as organochlorine, organophosphorus, and pyrethroids (with a detection limit of 0.01 mg/kg).

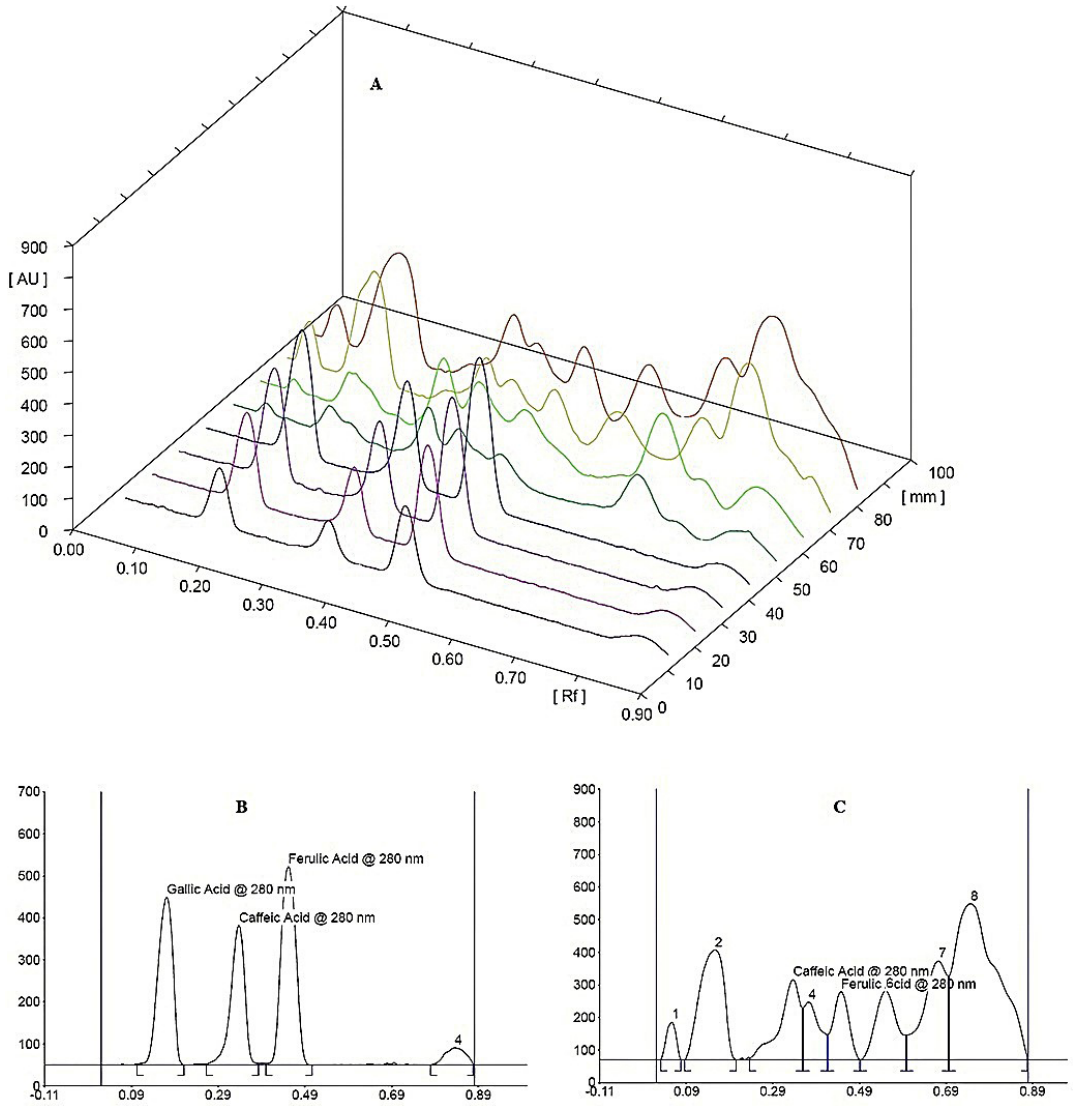
d. Aflatoxin Determination

Aflatoxin levels in the formulation were not identified, and the presence of many undesirable aflatoxins (B_1, B_2, G_1, and G_2) in Dhatupaushtik Churna was not intercepted, which is below the detection limit mapped by the API.

Chromatographic Analysis

a. High-Performance Thin-Layer Chromatography Analysis

The methanol extract from Dhatupaushtik Churna revealed myriad well-distinguished peaks under a wavelength of 280 nm in HPTLC fingerprinting, which was compared to the reference standards (gallic, caffeic, and ferulic acids). Based on the results (Figure 3), the R_f value of the test extract was identical to that of standard



Figures 3. (A) 3D Visualization of a Dhatupaushtik Churna Methanol Extract HPTLC Chromatogram for Polyphenolic Identification, **(B)** Reference Standard Peak Densitogram (Gallic, Caffeic, and Ferulic Acids), **(C)** The Peak Densitogram of Dhatupaushtik Churna Methanol Extract Clearly Demonstrating the Presence of Caffeic Acid (IUPAC) and Ferulic Acid (IUPAC). *Note*. HPTLC: High-performance thin-layer chromatography

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caffeic and ferulic acids. This is the first time that caffeic and ferulic acids have been found in the MEDC.

b. Gas Chromatography-Mass Spectrometry Analysis

GC-MS analysis was used to investigate the metabolomic composition of Dhatupaushtik Churna's crude methanolic extract, which represented a total of 38 peaks and led to the identification of 38 phytoconstituents in the extract. Table 7 lists the names, compound retention time, molecular formula, molecular weight, and percentage area of the obtained phytochemical compounds and their chemical structures (Figures 4 and 5). Alcohol, ketone, aldehyde, aromatic amine, heterocyclic amine, fatty acid, fatty acid esters, carbohydrate, and phytosterol derivative chemicals were the most abundant constituents in the methanolic extract.

Discussion

Standardization is a prerequisite in the Ayurvedic system of medicine. Traditional healthcare systems are functional,

Table 7. Dhatupaushtik Churna Methanolic Extract GC-MS Interpretation

Compound	Nature	Retention Time (min)	Molecular Formula	Molecular Weight (g/mol)	% Area
Furfural	Aldehyde	3.113	$C_5H_4O_2$	96	2.38
2-Furanmethanol	Alcohol	3.291	$C_5H_6O_2$	98	1.10
Butanedioic acid, 2,3-bis(acetyloxy)-, [R-(R*,R*)]-	Polyunsaturated acid	3.533	$C_8H_{10}O_8$	234	0.29
Propanal, 2,3-dihydroxy-	Aldehyde	3.690	$C_3H_6O_3$	90	1.24
6,7-Dioxabicyclo[3.2.2]non-8-ene	Bicyclic	4.018	$C_7H_{10}O_2$	126.15	0.45
2-Furancarboxaldehyde, 5-methyl-	Aldehyde	4.621	$C_6H_6O_2$	110	1.15
2,4-Dihydroxy-2,5-dimethyl-3(2H)-furan-3-one	Alcohol	4.808	$C_6H_8O_4$	144	0.29
2®,3(S)-1,2,3,4-Butanetetrol	Alcohol	5.746	$C_4H_{10}O_4$	122.12	0.61
6-Methyl-2-pyrazinylmethanol	Alcohol	6.160	$C_6H_8N_2O$	124.14	2.10
Furyl hydroxymethyl ketone	Ketone	6.289	$C_6H_6O_3$	126	0.27
4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl-	Alcohol	7.176	$C_6H_8O_4$	144	4.75
Benzenecarboxylic acid	Aromatic acid	7.654	$C_7H_6O_2$	122	15.62
2-Furancarboxaldehyde, 5-(hydroxymethyl)	Aldehyde	8.386	$C_6H_6O_3$	126	25.39
Pentanoic acid, 3-hydroxy-4-methyl-, methyl ester	Ester	8.636	$C_7H_{14}O_3$	146.18	1.07
d-Glycero-d-ido-heptose	Carbohydrate	9.130	$C_7H_{14}O_7$	210	0.33
2-Methoxy-4-vinylphenol	Aromatic Alcohol	9.528	$C_9H_{10}O_2$	150	0.39
Propanamide, 3-(1-piperazinyl)-	Amide	9.678	$C_7H_{15}N_3O$	157	1.91
3-Cyclopentylpropionic acid, 2-tetrahydrofurylmethyl ester	Ester	10.534	$C_{13}H_{22}O_3$	226	0.71
Ecgonine	Alkaloid	11.324	$C_9H_{15}NO_3$	185	0.48
2,5-Methylene-d,l-rhamnitol	Heterocyclic alcohol	11.803	$C_7H_{14}O_5$	178	1.90
D-Allose	Carbohydrate	12.174	$C_6H_{12}O_6$	180	1.10
Benzoic acid, 4-hydroxy-3-methoxy-	Aromatic acid	12.730	$C_8H_8O_4$	168	0.53
Azelaic Acid	Fatty acid	13.460	$C_9H_{16}O_4$	188	0.83
Hydrazinecarboxamide, 2-(2-methylcyclohexylidene)	Aromatic amine	14.178	$C_8H_{15}N_3O$	169.22	6.63
Pyrimidine-2,4(1H,3H)-dione, 6-hydroxy-5-methyliminomethyl-	Pyrimidines	14.464	$C_5H_7N_3O_2$	169	8.79
4-O-Methylmannose	Carbohydrate	14.790	$C_7H_{14}O_6$	194.18	2.14
2-Furancarboxylic acid, tert-butyldimethylsilyl ester	Furan derivative	14.990	$C_{11}H_{18}O_3Si$	226	0.64
Hexadecanoic acid, methyl ester	Fatty acid derivative	16.522	$C_{17}H_{34}O_2$	270.5	0.86
Pentadecanoic acid	Fatty acid	16.886	$C_{15}H_{30}O_2$	242.40	5.45
9,12-Octadecadienoic acid, methyl ester	Fatty acid derivative	18.189	$C_{19}H_{34}O_2$	294.5	1.55
9,12-Octadecadienoic acid (Z,Z)-	Fatty acid derivative	18.560	$C_{18}H_{32}O_2$	280.4	5.69
Octadecanoic acid	Fatty acid	18.759	$C_{18}H_{36}O_2$	284.5	1.21
Furan, tetrahydro-2-isopentyl-5-propyl-	Furan derivative	20.343	$C_{12}H_{24}O$	184.32	0.24
1,3-Dioxane, 2-phenyl-	Aromatic-Heterocyclic	20.604	$C_{10}H_{12}O_2$	164.20	0.42
Bicyclo[3.1.0]hexane-2-undecanoic acid, methyl ester	Fatty acid derivative	21.237	$C_{18}H_{32}O_2$	280.4	0.52
Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester	Fatty acid derivative	21.729	$C_{19}H_{38}O_4$	330.5	0.23
β-Stigmasterol	Steroid derivative	28.658	$C_{29}H_{48}O$	412.7	0.34
β.-Sitosterol	Phytosterols	29.471	$C_{29}H_{50}O$	414.7	0.41

Note. GC-MS: Gas chromatography-mass spectrometry.

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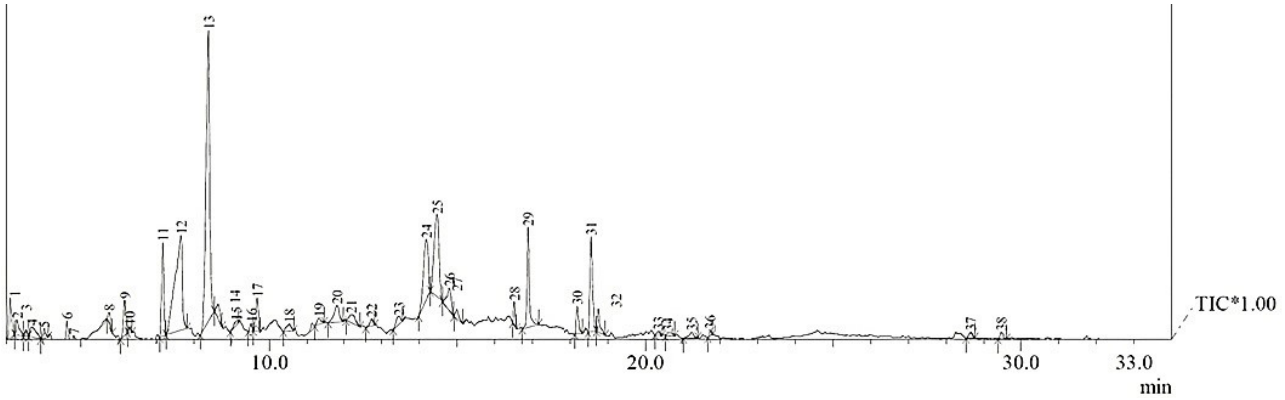


Figure 4. GC-MS Chromatogram of Dhatupaushtik Churna Methanol Extract. *Note*. GC-MS: Gas chromatography-mass spectrometry

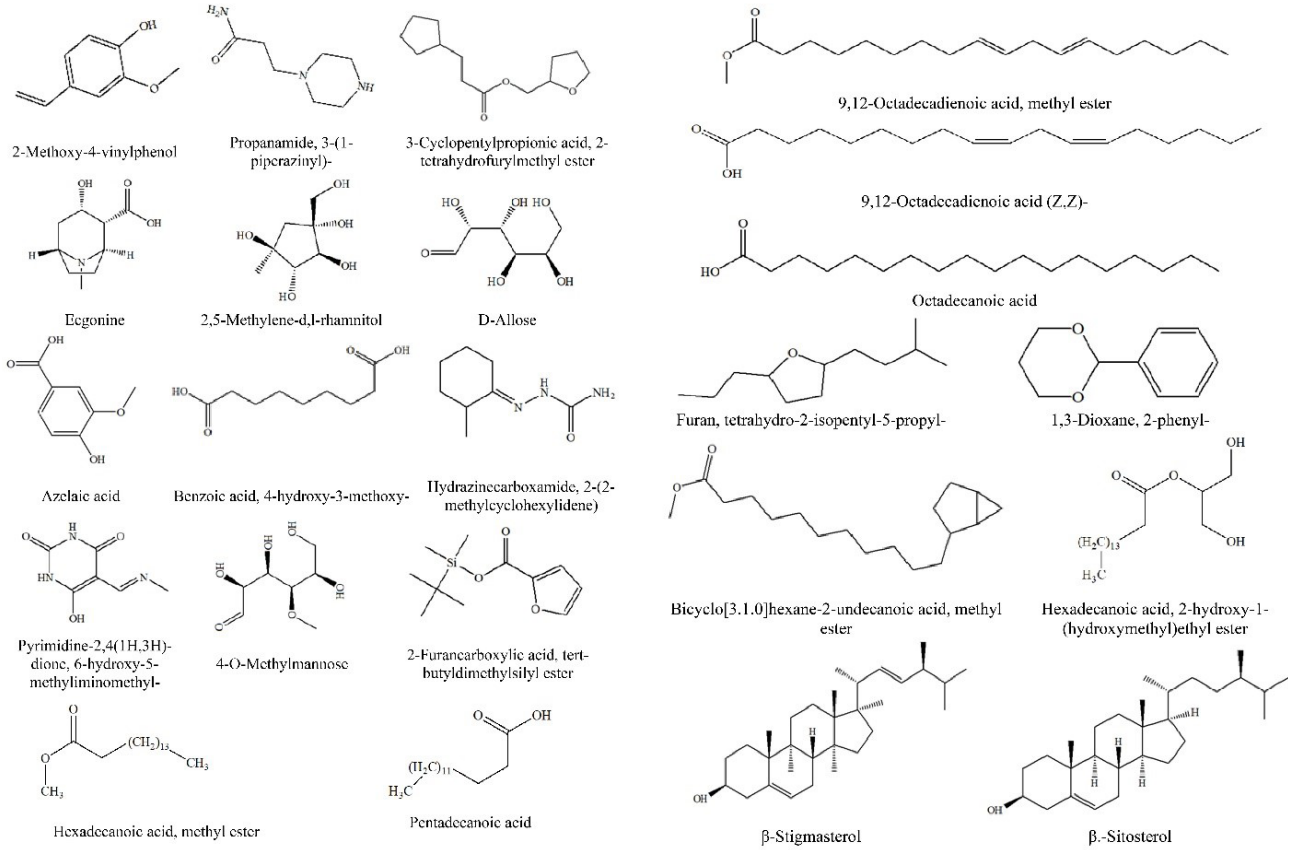


Figure 5. GC-MS Analysis of Dhatupaushtik Churna Methanol Extract Identifying the Chemical Compositions of Metabolites. Note. GC-MS: Gas chromatography-mass spectrometry

but a lack of quality assurance plagues them, allowing us to determine the formulation's quality. The Central Council of Research has set the basic rules for verifying the quality of these formulations in Ayurveda and Siddha. A procedure or method for evaluating herbal formulations must be developed to ensure consistency across batches during production (51-53).

Standardization's importance should not be underestimated. It is a crucial phase in the formulation process since it impacts the final product's quality. A technique for standardizing every product on the market must be established to avoid batch-to-batch variance. Specimen authenticity, organoleptic evaluation, pharmacognostic evaluation, volatile matter, quantitative evaluation (ash values and extractive values), phytochemical evaluation, xenobiotic evaluation, microbial test automation, toxicity screening, and biological activity should be taken into consideration when using standardization methods (54). As a result, our most contemporary research focuses on Dhatupaushtik Churna, a unique powdered Ayurvedic polyherbal formulation used to treat premature ejaculation, heal weakness, rejuvenate the male reproductive system, and erase aging, among other things. The standardization of this polyherbal Churna is in accordance with the API guidelines. The WHO, the European Agency for the Evaluation of Medicinal Products, and the USP have all presented standardization criteria. Through quality

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control methods, the Churna was evaluated for important physical, chemical, and analytical parameters as part of the procedure to ensure its safety and consistent efficacy.

Foreign matter, such as other parts of the same plant or other plants, moulds, or insects, including excreta and apparent impurities such as sand and stones, hazardous and toxic foreign matter, and chemical residues, should be avoided in herbal medicines (55). In our investigation, the tested foreign matter content of all the ingredients of Dhatupaushtik Churna was less than 0.9% (w/w), which is well below the maximum permissible limits as per API. The sensory aspects of food or other substances, such as taste, sight, smell, and touch, are organoleptic properties. The diversity in these attributes is a good measure of quality. Fluorescence is a phenomenon demonstrated by various substances that glow when exposed to light. During the organoleptic evaluation, the prepared in-house Dhatupaushtik Churna was peach puff yellow, with a unique aroma and astringent test. The fluorescence behaviors of powdered Churna samples were examined, displaying distinctive colours after being exposed to ordinary light and UV light (both long 365 nm and short 254 nm wavelengths) and treated with various reagent solutions. Fluorescence is a phenomenon presented by a variety of chemicals that clearly fluoresce in light. Further, fluorescence is an important criterion in pharmacognostical evaluation because it is a phenomenon displayed by a variety of chemicals that clearly fluoresce in light. Several plant components sparkle in the visible spectrum of daylight. Moreover, UV light produces fluorescence in many more natural products, or they may often be converted into fluorescent derivatives by applying different reagents (56). Similarly, using various staining reagents, powder microscopy is utilized to explore the microscopic features of medicinal plants. These investigations provide a useful diagnostic tool for both standardization and adulterant detection. This approach is also effective for determining the presence of components in a polyherbal powder (57). The presence of all Dhatupaushtik Churna's herbal ingredients, such as Gokhru (which is composed of calcium oxalate prismatic crystals), sclereids fibers (which have a single, long thick-walled trichome), sclereids (a reduced version of sclerenchyma cells), and inner cells, was confirmed in our powder microscopic interpretation. Likewise, the presence of ashwagandha has been indicated by starch grains, pitted xylem channels, tracheids, cork cells, and fibres, whereas starch, fibres, and sclereids fragments have been detected in the powder form of Safed musli. These microscopic investigations might be beneficial for entrepreneurial purposes and verify Dhatupaushtik Churna's compliance. Furthermore, established preliminary and physicochemical specifications provide crucial data for subsequent research and facilitate the identification of formulations in industrial output (58). Both water and volatile materials are detected using the percentage of moisture content test. The proportion of materials left after ignition is usually measured as total ash. The quantity of silica present, especially sand and siliceous materials, is measured using acid-insoluble ash. Extractive values are important for determining the kind and quantity of chemical components contained in a drug (59). Dhatupaushtik Churna was evaluated for extractive values, total ash content, acid insoluble ash, pH, and loss on drying due to the relevance of these physicochemical properties. Similarly, powder flow is fundamental for mixing, packing, and transportation in pharmaceutical manufacturing. The angle of repose, bulk density, tapped density, Carr's compressibility index, and Hausner's ratio are the characteristics used to assess powder flow (60). Fine particles with a lower bulk/tapped density are less free-flowing than heftier, denser particles. The angle of repose is another fast test for determining relative flowability and monitoring powder flow. The angles of repose below 30°, 30°-45°, 45°-55°, and beyond 55° indicate flow-able powders, considerable cohesiveness, real cohesiveness, and extremely high cohesiveness and restricted flowability, respectively (61). A Hausner's ratio of less than 1.25 suggests a free-flowing powder, whereas values greater than 1.25 represent a powder with poor flowability (62). Finally, the flow qualities improve with a decline in Carr's index (63). Contrarily, Dhatupaushtik Churna powder has a low tap and bulk density, making it difficult to flow. This assertion was backed by the high angle of repose, Hausner's ratio, and Carr's index values.

Consequently, the use of homemade and commercial herbal remedies poses a major risk to the health of elderly people who take these drugs due to the lack of microbiological quality criteria. Microbial testing revealed the presence of *E. coli*, *P. aeruginosa*, *S. aureus*, and *Salmonella* species, all of which imply fecal contamination and poor hygiene in the manufacturing and storage of herbal formulations. Herbal drugs have a high level of bacterial contamination, which might be owing to the poor quality of the water used during product manufacturing (64,65). Microbial contamination in herbal medicines can reduce or inactivate their therapeutic effect, exposing patients using these therapies to risk. To maintain good quality, safety, and efficacy of natural products, manufacturers should ensure the lowest possible amounts of microorganisms in the raw material, finished dosage forms, and packaging components (66). The microbiological load of Dhatupaushtik Churna was determined to be within API limits, with pathogens such as *E. coli*, *P. aeruginosa*, *S. aureus*, and *Salmonella* species completely undetectable. This implies that it was microbiologically safe for human consumption.

As researchers have well documented, mycotoxins are secondary metabolites produced by a range of fungus and mould species. They infect various foods and regional crops during pre- and post-harvesting periods when circumstances are favorable (e.g., high temperatures and moisture). Aflatoxin is the most frequent mycotoxin that causes cancer in animals and humans (67). Aflatoxins are

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mostly generated by *Aspergillus flavus* and *Aspergillus parasiticus* and are classified as Aflatoxins B_1, B_2, G_1, and G_2. As a result, numerous aspects of mycotoxin contamination in herbal drugs, as well as current tactics used to avoid or reduce contamination, are being investigated because the WHO advises that aflatoxin levels be kept as low as feasible (68). In contrast, no various unwanted Aflatoxins were detected in the Dhatupaushtik Churna, below the detection limit specified in the Indian Herbal Pharmacopoeia (69,70). Likewise, pesticides commonly found in herbs are monitored by the WHO and the Food and Agricultural Organization (71). During the growing process, these insecticides are blended with herbs. Pesticides (e.g., Dichloro diphenyl trichloroethane, benzene hexachloride, toxaphene, and aldrin) create massive deleterious effects in humans when combined with crude drugs (72). However, our Dhatupaushtik Churna formulation was free of hazardous pesticide residues, including organochlorine, organophosphorus, and pyrethroids.

Over the last decade, there has been a huge surge in interest in using medicinal plants to cure ailments worldwide. The ingestion of tainted medicinal plants and herbal items is thought to cause heavy metal poisoning in humans and animals. In medicine, heavy metals are commonly misunderstood, yet they refer to all hazardous metals. Both natural and artificial sources emit them into the environment. The availability and concentration of heavy metals in soil determine the presence of heavy metals in plant tissues. They can also naturally tumble from the atmosphere to plant surfaces (73). Due to their extended biological half-life, heavy metals are persistent in nature. As, Cd, Pb, and Hg are the most harmful heavy metals. They are inactive redox metals that cause toxicity by attaching to sulfhydryl protein groups and lowering glutathione levels. Cultivating and harvesting medicinal plants close to industrial locations that use these metals and their compounds, as well as regions where these metals have been improperly disposed of, are strictly banned to protect the purity and safety of herbal remedies. This is because plants from these areas are prone to high amounts of heavy metals, posing a risk of human contamination if ingested (74,75). Interestingly, heavy metal contamination screening of plant extracts, herbal formulation products, and medicinal plants, in general, is of critical medical value and must be given top priority in phytotherapy. In our investigation, the restrictions of all the specified heavy metals in the Churna were found within an acceptable range (76).

Phytochemicals are phenotypically active, naturally occurring chemical compounds found in plants that offer additional health benefits for humans than macronutrients and micronutrients (77). The chemical composition of crude material extracted from the plant is affected by factors such as geographic location, harvest time, partly utilized, and isolation technique (78). Dhatupaushtik Churna and its ingredients in different polarity solvents are phytochemically examined in this approach. Alkaloids, steroids, tannins, anthraquinone glycosides, flavonoids, saponins, and other phytoconstituents have all been proven beneficial in treating sexual and other neurological complications previously (79-82). According to the phytochemical assessment, the presence of the above constituents in Dhatupaushtik Churna may be responsible for its effectiveness in treating impotence, erectile dysfunction, and premature ejaculation. Accordingly, preliminary phytochemical analysis provides support in detecting the chemical component class contained in the extract, which can lead to quantitative estimation and identification of pharmacologically active phytocompounds. Similarly, the most widely used approaches for the standardization of herbal products are chromatography and spectroscopy. Nonetheless, the herbal system is difficult to assess due to its tricky chemical composition. The use of chemometrics in medicinal plants is, therefore, logical and essential. In medicinal plants, comprehensive methods and hyphenated techniques associated with chemometrics are increasingly utilized for extracting useful information and providing various data processing methods, with chemometrics resolution methods and principal component analysis being the most commonly applied techniques (83). In this study, the MEDC was analyzed using HPTLC, and the results demonstrated many distinctive peaks and retardation factor (R_f) values that might help in the quality monitoring of Churna. The presence of caffeic and ferulic acids in Dhatupaushtik Churna has not been documented in the existing literature. To the best of our knowledge, the current research is the first to identify and quantify the extracts of the above-mentioned compounds. Each plant or herbal medicine has a unique phytochemical profile of secondary metabolites that can help identify and standardize the product (84). Hence, it can be predicted that the presence of caffeic and ferulic acids in Dhatupaushtik Churna might be effective in the treatment of impotence and erectile dysfunction because caffeic acid and sodium ferulate play interventional roles in reversing erectile dysfunction through the metabolic regulation of free radicals, oxidative insult antagonism, and nitric oxide production enhancement (85,86). Likewise, matrix-matched calibration standards are utilized to account for the matrix effect in GC, and this is one of the simplest and cheapest procedures. GC-MS is inappropriate for thermo-sensitive compounds. Non-volatile components must be derivatized before analysis. The performance of the GC-tandem MS technology is influenced by the purity of the extract under examination. It is put into the system since the biochemical spectrum of the herbs is vast, and the nature of the herb is complex (87,88). GC-MS analysis has become more common in evaluating medicinal plants and herbal formulations in recent years since it has shown to be particularly effective in analyzing volatile essential oils, fatty acids, lipids, non-polar components, and alkaloids (89). Our GC-MS exploration revealed that the

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MEDC encapsulated alcohol, ketone, aldehyde, aromatic amine, heterocyclic amine, fatty acid, fatty acid esters, carbohydrate, alkaloid, and phytosterol derivatives. Fatty acids are known to serve as energy sources and storage for lipophilic substances and may affect semen quality (90). Research studies may assume that Dhatupaushtik Churna is composed of extinctive fatty acids (e.g., azelaic acid, pentadecanoic acid, etc.), fatty acid esters, aromatic acids (e.g., vanillic acid), and the like. Both lipid attributes have been connected to cellular and spermatic biology (91), as well as decreased neuronal apoptosis and neuroinflammation, relieved synaptic and cognitive deficits, and avoided premature aging (92).

Dhatupaushtik Churna was vindicated through pharmacognostic, physicochemical, pharmacological, microbiological, toxicological, and chromatographic parameters. The product's analytical criteria were defined in terms of quality-based raw ingredients. This research might be used as a benchmark for quality control analysis of various Churna formulations and a set of standard operating procedures.

Conclusion

This investigation utilized pharmacognostic, physicochemical, pharmacological, microbiological, toxicological, and chromatographic parameters to evaluate the Dhatupaushtik Churna. Authentic raw materials were used to develop the product's analytical specifications. All the constituents in the Dhatupaushtik Churna formulation were present, as were their specific retention properties, according to the chromatographic data. To ensure batch-to-batch uniformity, the parameters provided in this study might be employed to draw a monograph on Dhatupaushtik Churna quality standards. This document may also efficiently evaluate formulations using HPTLC, GC, and MS.

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Competing Interests

The authors declare that they have no conflict of interests.

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