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Standardization and Quality Control Analysis of a Polyherbal Antidiabetic Formulation

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Abstract

Exploration of novel drugs from natural resources may not always result in the effective management of complex metabolic conditions like diabetes using the “one disease, one therapy, and one target” paradigm. As a result, the field of synergistic pharmacology, which includes polyherbal medicines, has become the preferred method for discovering new pharmaceuticals. This study aims to standardize a polyherbal antidiabetic formulation by combining *Costus pictus*, *Gymnema slyvestre*, and *Momordica charantia* via synergism to enhance the bioavailability of the active compounds, reduce the toxicity and promote therapeutic effects of the selected herbs for antidiabetic activity. The formulation was standardized by evaluating its physical characteristics, powder characteristics, physicochemical properties, phytochemical properties, chromatographic analysis, fluorescence analysis, and safety parameters concerning toxins, microbes, and residues. The loss on drying was 8%, which was within the prescribed limits, indicating that the product has a long storage life. Inorganic metallic salts, such as silica and phosphates, are negligible in the powder, as indicated by its ash value (7.51%), acid-insoluble ash (1.07%), water-soluble ash (2.97%), and sulphated ash (7.76%). The extractive value of water was the highest (12.66%), compared to alcohol (3.54%) and ether (3.34%). The polyherbal formulation was acidic with pH of 6.5 and 6.0 in 1% and 10% solution, respectively, which minimizes the possibility of microbial contamination. The phytochemical analysis confirmed the presence of alkaloids, triterpenoids, flavonoids, phenols, saponins, glycosides, tannins, and diterpenes. Based on the safety evaluation results, the formulation is safe to consume and free of heavy metal contamination, microbial load, pesticide residue, and aflatoxin. The established metrics are sufficient for assessing polyherbal formulation and can be used as reference standards for quality control and assurance. The lack of standardization studies has hampered the translation of new drug candidates into commercially viable therapies, so standardized alternatives are imperative for determining safety, efficacy, and reliability.

Keywords: Standardization; Herbal medicines; *Momordica charantia*; *Gymnema slyvestre*; *Costus pictus*; Polyherbal formulation.

Introduction

Diabetes mellitus, more often referred to as diabetes, is a severe and diverse category of endocrine dysfunction with numerous

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underlying effectuates that are distinguished by a consistent, unabating rise in blood glucose levels and irregularities in lipid, polypeptide, or sugar metabolism as a consequence of an array of glitches in insulin production, release, and activity (1–3). The prevalence of diabetes has dramatically increased around the globe over the past century due to modifications in human attributes and poor lifestyle choices (4, 5). The International Diabetes Federation's most recent 10th edition Diabetes Atlas documents a sustained rise in diabetes prevalence worldwide, reaffirming diabetes as a serious threat to human health and welfare on a global scale (6). According to the latest health statistics reported by the IDF, 53.7 crore adult population belonging to the peer range of 20-79 years are diagnosed with diabetes, and by 2030 and 2045, this figure is projected to incline to 64.3 and 78.3 crores, respectively (6). The disease is often linked with multiple complications and deleterious outcomes on multiple organs, such as the heart, kidney, eyes, liver, brain, and others (7). To reduce early mortality owing to diabetes, its subtypes, and its associated complications, novel therapeutic alternatives that are safe, effective, and sustainable with no side effects are urgently needed.

Existing therapy delivered through various routes exhibits low aqueous solubility, penetrability, stability, and fast metabolism, which leads to adverse consequences such as inadequate therapeutic activity and dose-limiting side effects. Exogenic insulin replacement therapy is the standard of care for all type 1 diabetic patient; however, for some patients, this strategy falls short of achieving ideal blood glucose levels (8). Significant progress has been made in treating type 2 diabetes mellitus, as there are medications available in the market that can be administered orally or in an injectable form. Ten classes of oral drugs are commercially accessible, including biguanides (Metformin), glucagon-like peptide 1 (GLP-1) receptor agonists (incretin mimetics), sulfonylureas (Glibenclamide), alpha-glucosidase inhibitors (Acarbose), meglitinides (Repaglinide), thiazolidinediones (TZDs) (Rosiglitazone), dipeptidyl peptidase IV (DPP-4) inhibitors (Gliptins), dopamine agonists, bile acid resins, sodium-glucose transport protein 2 (SGLT 2) inhibitors (Gliflozins) (9–11). The possible and severe adverse effects of taking these medications include weight gain, diarrhea, abdominal bloating and discomfort, gastrointestinal (stomach and bowel) problems, bone fractures, water retention, heart failure, headaches, nausea and vomiting, and hypersensitivity reactions (12–14). Until clinical trials are completed and the side effects of the above drugs are reduced, effective pharmacological management of diabetes and associated complications demands a highly individualized strategy that considers crucial aspects such as safety, cost-effectiveness, improved mechanisms of action, and natural alternatives with fewer or no unwanted side effects.

The Indian subcontinent is enriched with a vast range of indigenous medicinal and aromatic plant varieties, contributing to the region's cultural diversity, religious harmony, and economic values. In addition to Ayurveda and other traditional medicines, India has long used herbal remedies and made significant contributions to pharmaceutical research and development (15–19). Even though herbal medicine has been clinically practiced for ages, there is a lack of up-to-date, adequate scientific evidence, which has resulted in concerns about the quality, safety, herb-herb interactions, efficacy, adulterants, contaminants, and reliability of herbal drugs. As the name implies, herbal drug standardization is the detailed method of identifying and defining an array of standards that contain stable, unique, plant-specific, and unambiguous inherent phytochemical qualitative and quantitative characteristics of a plant that strongly indicate quality and safety, effectiveness, and reproducibility. A variety of factors have been reported to influence the quality of herbal drugs, including unknown knowledge about the active biocom-

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ponents present in plants, mixtures or blends of these active biocomponents to achieve a high therapeutic effect, a lack of standard reference marker compounds, natural variations in plant varieties, changing climate, location and time of raw material collection, transportation, processing and storage of raw materials, contamination with microbes, aflatoxins, pesticides, and other physical, chemical, and biological contaminants (20). There may be problems with the quality and safety of the raw materials used to prepare herbal formulations because they are close to sewage water, polluted areas, and application of pesticides, which can be absorbed by the plants or accumulate directly on the leaves. This makes it extremely important to standardize raw materials before formulating them to prevent adverse health effects. Many researchers working on herbal formulations begin by conducting in vitro and in vivo studies with their prepared formulation without first standardizing the raw materials and the formulation. As a result, many herbal formulations enter the market, but they do not have strong therapeutic effects and may cause side effects as these formulations are not standardized.

In this study, we standardized a polyherbal antidiabetic formulation by evaluating various parameters to improve the bioavailability of active compounds, reduce toxicity, and promote the therapeutic effects of the selected herbs for antidiabetic activity.

Materials and Methods

Collection and processing of raw materials

Collected leaves were thoroughly washed using distilled water, and all damaged leaves were sorted out. The leaves were then dried under shade for 1 week. Once the leaves were dried entirely, they were ground using a mixer-grinder. The coarse powder obtained was passed through a sieve to acquire a moderately fine nature of powder, and it was stored in a closed airtight box and used for further analysis.

Preparation of the Polyherbal formulation

The prepared powders were combined in a ratio of 1:1:1. Formulations in powder form are recommended due to their minute-sized particles. It is proven that smaller particles absorb more quickly in the gastrointestinal tract, so smaller particles are more therapeutically effective (21).

Standardization of raw material

The raw materials were standardized as per the guidelines imposed by The Ayurvedic Pharmacopoeia of India and Guidelines prescribed by Drug Development of Ayurvedic Formulations, Central Council for Research in Ayurvedic Sciences, Ministry of AYUSH, Government of India.

Physical evaluation

Morphological and microscopical study of prepared plant powder

The external morphology and powder microscopy of all the raw materials were studied, photo-documented, and correlated with existing standard literature.

Particle size analysis

An optical microscope was used to visualize the particles, and to determine the size in micrometers, the Particle size analyser-Mastersizer 2000 instrument was used. In 100 ml of sterile distilled water, 0.1 grams of polyherbal powder was added. For microscopic examination, a few drops of prepared material were mounted on a clean slide, covered with a coverslip, and placed on the microscope stage. For determining the particle size, using the Particle size analyser-Mastersizer 2000 instrument, 100 ml of the prepared sample was allowed to pass through the instrument's inlet. A minimum of three observations were performed to determine the polyherbal powder's mean average particle size.

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Organoleptic characteristics

Evaluation of organoleptic characteristics, such as physical texture, color, odour, and taste of plant powders, were determined.

Powder characterization

Flow property measurements

It was assessed by analyzing parameters like Bulk density (ρb), Tapped density (ρt), Angle of repose, Hausner's ratio, and Carr's Compressibility index (CI) using the methods by (22). The experiments were done in triplicates.

Bulk density is denoted in g/ml and is calculated by, $\rho b = M/Vo$

Where, M- Mass of powder and Vo- Bulk volume of the powder

Tapped density is denoted in g/ml and is calculated by,

$$\rho t = M/Vt$$

Where, M - Mass of powder and Vt- Tapped volume of the powder

The (ρb) and (ρt) values are used to compute (CI) and Hausner's ratio

Carr's Compressibility index (CI)

Powder's compressibility refers to its ability to decrease volume under pressure. Density determines compressibility. Having compressibility means that the powder can reduce in volume under pressure.

$$\boldsymbol{CI = 100[(\rho t - \rho b/\rho b)]}$$

Where, $\boldsymbol{\rho b}$- Bulk density and $\boldsymbol{\rho t}$ -Tapped density

Hausner's ratio

It is expressed as the ratio of (ρt) to (ρb).

$$\boldsymbol{Hausner's\ ratio = \rho t/\rho b}$$

Where, $\boldsymbol{\rho b}$- Bulk density and $\boldsymbol{\rho t}$ -Tapped density

The angle of repose

Where, h- height, and d- diameter in cm

$$\boldsymbol{Angle\ of\ repose\ (\theta) = tan - [2h/d]}$$

Physico-chemical evaluation

A physicochemical evaluation of the raw material and the prepared polyherbal formulation was carried out to determine the purity and standards of raw materials that affect product quality and numerous other parameters. The analysis was carried out as per the General Guidelines for Drug Development of Ayurvedic Formulations (Guidelines series I).

Loss on Drying

$$Loss\ on\ drying\ (\%) = \frac{Final\ weight\ of\ the\ sample}{Initial\ weight\ of\ the\ sample} \times 100$$

Determination of Ash Values

It can be evaluated by testing the following mentioned four parameters:

Determination of Extractive Values

$$Total\ ash\ (\%) = \frac{Final\ weight\ of\ the\ residue}{Initial\ weight\ of\ the\ sample} \times 100$$

$$Acid\ insolube\ ash\ content\ (\%) = \frac{Final\ weight\ of\ the\ residue}{Initial\ weight\ of\ the\ sample} \times 100$$

$$Water\ soluble\ ash\ content\ (\%) = \frac{Final\ weight\ of\ the\ residue}{Initial\ weight\ of\ the\ sample} \times 100$$

$$Sulphated\ ash\ content\ (\%) = \frac{Final\ weight\ of\ the\ residue}{Initial\ weight\ of\ the\ sample} \times 100$$

It can be evaluated by testing the following mentioned three parameters:

Measurement of pH value

$$\%Water\ soluble\ extractive\ values = \frac{Weight\ of\ the\ residue\ obtained\ after\ drying}{Weight\ of\ the\ sample\ taken} \times 100$$

$$\%Alcohol\ soluble\ extractive\ values = \frac{Weight\ of\ the\ residue\ obtained\ after\ drying}{Weight\ of\ the\ sample\ taken} \times 100$$

$$\%Ether\ soluble\ extractive\ values = \frac{Weight\ of\ the\ residue\ obtained\ after\ drying}{Weight\ of\ the\ sample\ taken} \times 100$$

A 1% and 10% (w/v) polyherbal powder solution was made using distilled water, and the pH was measured using a digital pH metre.

Phytochemical evaluation

Preparation of extracts

The phytochemical analysis of the individual plants, i.e., *Costus pictus*, *Gymnema slyvestre*, and *Momordica charantia,* is well studied and reported in the literature. The pre-

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pared polyherbal powder (1:1:1) was evaluated for various secondary metabolites. Soak 1 gm of polyherbal powder in 10 ml of water, alcohol, and ether for 24 hours. Filter the extract using Whatman Grade 41 filter paper. The filtered extract was used for carrying out the phytochemical analysis for screening metabolites. The three solvents were selected as per the General Guidelines for Drug Development of Ayurvedic Formulations (Guidelines series I). The confirmation of triterpenoids was established through the execution of Salkowski's Test and Steroids test. The presence of flavonoids was determined using the Alkaline reagent test and Lead acetate test. Alkaloids were detected by conducting Hager's test and Wagner's test. Phenols and tannins were identified through the implementation of Ferric chloride and Lead Acetate test. Saponins were detected using the Froth test. Glycosides were identified using the Keller-Kilani test. The detection of Diterpenes was accomplished through the Copper acetate test.

Chromatographic evaluation

Thin Layer chromatography

The dried raw materials and polyherbal powder samples (15g) were milled and extracted for 6 hours in Soxhlet apparatus using (250 ml) methanol and ethanol, respectively. The temperature of Soxhlet apparatus was pre-set to the respective boiling points of the solvents (Methanol: 64-65 °C and Ethanol:75-78 °C). Utilizing a rotary evaporator and reduced pressure, each filtrate was concentrated until dry (Super Fit- Rotavap, model- PBU-6, India). Thin-layer chromatography (TLC) was performed using silica gel 60F254, 5x3 cm (Merck). The extract was spotted as a band on TLC plates using capillary glass tubes. The plates were sprayed with the appropriate derivatizing agents, given time to dry, and then heated on a hot plate for one minute. The resolution of extract components was evaluated by finding distinct spots on the chromatogram.

Fluorescence analysis

Fluorescence analysis of raw materials and polyherbal powder

0.5 g of the raw materials and the prepared polyherbal powder was evaluated for physical change in color on exposure to UV light. Powders were evaluated for color change without any treatment, and with treatment of 5 ml alkaline solution, and acid solutions. To determine the fluorescence property, the samples were observed below ordinary light, blue light, and UV light (365 nm & 302 nm).

Safety evaluation of the polyherbal formulation

Quantitative analysis of heavy metals

The prepared polyherbal powder was also tested for contaminants like Lead (Pb), Mercury (Hg), Arsenic (As), and Cadmium (Cd) by Inductive coupled plasma-Optical emission spectroscopy. The analysis was done using Perkin Elmer Optima 5300 DV ICP-OES at Sophisticated Analytical Instrument Facility (SAIF), IIT Madras.

Microbial load analysis

The prepared polyherbal powder was evaluated to estimate the microbial load. The microbial load was calculated using the pour plate method. According to the Guidelines established by The Ayurvedic Pharmacopoeia of India, and The World Health Organization, the Total aerobic viable count test for the presence of Yeasts and molds, *Escherichia coli, Clostridia, Salmonellae, Shigella, Pseudomonas, Staphylococcus* was carried out.

Pre-treatment of polyherbal powder

1 gram of the polyherbal powder was mixed thoroughly by vortexing in 9 ml sterile saline solution for 10 minutes. Then, the mixture was centrifuged at 6000 rpm to remove debris, and the supernatant was used for microbial analysis. The dilutions were made by mixing 1 ml of supernatant with 9 ml of sterile saline solution; subsequently, dilutions up to 10^3 were

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made in the same manner.

1 ml of each dilution was transferred aseptically to separate sterile Petri plates, and 20-25 ml of differential media with respect to the detection of microbe was added. Agar plates were allowed to solidify before incubating for 48 hours at 35 °C. After incubation, colonies were detected and counted to determine the microbial load (CFU/gm).

The microbial load of the polyherbal formulation was determined by using Tryptic soy Agar, Potato Dextrose Agar, MacConkey Agar, *Salmonella* & *Shigella* agar, Mannitol Salt Agar, MacConkey Agar. The presence of specific pathogens in differential media was determined by observing the colony's distinguishing color, shapes and pattern.

Pesticide residual analysis

The polyherbal powder was examined for organochlorine, organophosphorus, organocarbamates, and pyrethroid pesticides.

Test for the presence of Aflatoxins

The World Health Organization, states that aflatoxins are a class of naturally occurring toxins synthesized by certain molds that infect numerous food crops and cause potential hazards to humans (World Health Organization, 2022). The powder was tested for Aflatoxins using reference standards (B1; B2; G1; G2).

GC-MS analysis

Sample preparation

To keep the volatile components in the mixture intact, the method selected for extraction was Cold maceration. 10% (w/v) polyherbal powder solution was made using 99% Ethanol and the solution was kept for 3 days on a shaker.

Compound Identification

National Institute of Standards and Technology Library (NIST 11.L) was used to interpret the GC-MS spectrum. The unidentified compounds mass spectra were compared with the identified compounds spectrum stored in the NIST Library and Rt values, structure, peak area and the name of the compound were determined.

Results and Discussion

Physical evaluation

Morphological and microscopical study of prepared plant powder

The morphology of all the crude plant materials and powder microscopy was studied, photo-documented, and compared with standard literature. *Costus pictus* is a herb, it has a spirally arranged phyllotaxy (spiromonostichous) with simple, large, and lanceolate leaves, which are ventrally dark green and dorsally light green. The leaf length was 14 to 25 cm and 5 to 8 cm broad. *Gymnema slyvestre* is a woody climber, and the leaves had oppositely arranged two ranked leaf arrangements; the leaves were acute, elliptical, hairy (pubescent), and green in colour. The leaf was 2 to 5.5 cm long and 2 to 3.5 cm broad. *Momordica charantia* is a climber with trailing tendrils; the leaves are alternately arranged, simple, 5-7 lobed, hairy (pubescent), with long petioles and green in colour. The length of the leaf was 5 to 12 cm and 5-9 cm broad. Powder microscopy analysis of the raw material displayed characteristic unicellular trichomes in *Costus pictus*. *Gymnema slyvestre*, in contrast, exhibited multicellular trichomes and distinctive calcium oxalate crystals in the form of a Rosette. In the case of *Momordica charantia*, non-glandular trichomes were found. The presence of non-glandular trichomes was observed in the case of *Momordica charantia.* The other microscopic structures, such as types of parenchymal cells, fibrovascular bundles, cork cells, epidermal cells, and others, were also observed and compared with the standard literature. The external morphology and powder microscopy results were consistent with the existing plant reports in the literature, indicating the purity of the raw materials used.

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Particle size analysis of the polyherbal powder

An optical microscope was used to visualize these particles of varied sizes, and a light microscopic image was created. The mean particle size of the polyherbal powder was 44.197 µm; moreover, the polyherbal powder contains particles ranging from 10.363 µm to 430.533 µm.

Organoleptic evaluation

Investigating the organoleptic parameters determines the level of quality and authenticity of the raw materials. It is done by sensory organs, including the sense of taste, texture, color, and smell of the material. The polyherbal formulation had a moderately fine texture, a greenish-brown colour, and a distinct odor with a mildly bitter taste.

Powder characteristics

Flow property assessment

A crucial factor to measure is the polyherbal powder's flow properties because it has an impact on the dose uniformness. The Bulk density and the Tapped density of the polyherbal formulation were 0.31 ± 0.01 and 0.43 ± 0.01, respectively. The frictional coefficient among the particles of the polyherbal powder is equal to the tangential angle of repose. The irregularity and roughness of the particles can be determined if the angle of repose is high. In this study, the angle of repose for the polyherbal powder was 26.58 ± 2.09%, which falls in the standard range of 25-30, indicating excellent flow property. Hausner's ratio was 1.37 ± 0.01%, which falls within the standard values of 1.26-1.46, indicating a passable flow rate. The Carr's compressibility index for the powder was 27.88 ± 0.02%, which falls in the standard range of 26-31, indicating poor flow property. The results from Carr's compressibility index (CI) and Hausner's ratio display the poor and passable flow properties, respectively, implying that certain excipients and fillers can be used to improve the flow properties.

Physico-chemical evaluation

Determining the Physico-chemical properties of a potential drug is of utmost importance primarily to know the intrinsic risk of that compound, including its ability to impede standard biological activities, as well as the physiological risks and impact on the environment, whether causing any particular physical or toxicological hazards which depends on the system it interacts with and its inherent physico-chemical characteristics. It is also determined by the compound's molecular structure, composition, size, structure, and morphology. This data is crucial and getting it is relatively simple, quick, and doable at a preliminary level analysis (24). Out of the various possible applications of pharmacognosy, the pharmaceutical industry's prominent concern is evaluating crude drugs for their Physico-chemical parameters.

Loss on drying

The moisture content in the product delivers insights into the overall product shelf-life, stability and storage duration. This information enables us to understand the amount of powder's volatile and H2O content. The loss on drying values were 9.03 ± 0.384% for *Costus pictus*, 6.69± 0.591% for *Gymnema slyvestre*, 5.72 ± 0.557% for *Momordica charantia* and 8 ± 1.632 % for the prepared polyherbal powder. The moisture content of the herbal medicines at the end of the drying process must be between 10 and 14% (25). The prepared polyherbal powder has a moisture content within the prescribed limits and thus can be kept in storage for a longer time without deteriorating.

Determination of ash values

Inorganic metallic salts, such as silica, and phosphates, are negligible in the polyherbal powder, as indicated by its results of total ash (7.51%), acid-insoluble ash (1.07%), water-soluble as (2.97%), and sulphated ash (7.76%).

Total ash content

Based on the amount of leftover residual ash, af-

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ter incineration, the total amount of ash in crude herbal drug is calculated. The total amount of ash content of raw materials and polyherbal powder were 8.26 ± 0.056 % for *Costus pictus*, 9.60 ± 0.044 % for *Gymnema slyvestre*, 5.25 ± 0.028 % for *Momordica charantia* and 7.51± 0.018% for the prepared polyherbal powder. This is a crucial factor to consider when evaluating the raw materials before using them for formulation.

Acid Insoluble ash

This information is crucial because it establishes the powder's physiochemical characteristics and helps estimate the quantity and type of minerals in the raw powder. The ash values were 1.92 ± 0.016 % for *Costus pictus*, 0.07± 0.036 % for *Gymnema slyvestre*, 1.84 ± 0.061% for *Momordica charantia* and 1.07 ± 0.023% for the prepared polyherbal powder.

Water soluble ash

The ash values were 2.39 ± 0.024 % for *Costus pictus*, 2.20± 0.016% for *Gymnema slyvestre*, 2.88 ± 0.004% for *Momordica charantia* and 2.97 ± 0.012% for the prepared polyherbal powder. The potential to prevent the development of microorganisms in the raw powder increases with the amount of minerals in the plant powder, which is water soluble.

Sulphated ash

The test helps us to determine the content of inorganic impurities in raw materials and polyherbal powder. The ash values were 8.47 ± 0.016% for *Costus pictus*, 5.4± 0.08% for *Gymnema slyvestre*, 9.14 ± 0.016% for *Momordica charantia* and 7.76 ± 0.008 % for the prepared polyherbal powder. Sulphuric acid is used in the test to oxidize and decompose organic matter, producing only sulphate salts of cations; also, compared to other ash tests, the sulphated ash test is anticipated to produce reproducible results, due to the stability of inorganic sulphate salts at high temperatures (26).

Determination of extractive values

The extractive values assist us in determining which solvent (water, alcohol, ether) exhibits the highest phytoconstituent yield. The water, alcohol and ether soluble extractives of raw materials and the polyherbal powder were determined. This step offers an approach for estimating the amount of biologically active compounds in raw powders and the polyherbal powder when extracted with different solvents. The extractive value enables us to understand that maximum yield is obtained in which solvent (water, alcohol, or ether) is at a preliminary level before moving forward with a wide range of solvents based on polarity for extraction.

The extractive values for water-soluble content were 8.65 ± 0.004% for *Costus pictus*, 12.26± 0.008% for *Gymnema slyvestre*, 13.28± 0.008% for *Momordica charantia* and 12.66± 0.012% for the prepared polyherbal powder. The results indicate that maximum extraction (higher extractive values) was observed with water as a solvent for raw materials and the polyherbal formulation. A lower value suggests the raw materials are exhausted or adulterated during drying, storing, or processing (27).

The alcohol-soluble extractive value content of raw materials and the polyherbal powder were 3.64 ± 0.016% for *Costus pictus*, 4.82± 0.012% for *Gymnema slyvestre*, 3.64 ± 0.016% for *Momordica charantia* and 3.54 ± 0.016% for the prepared polyherbal powder.

The ether-soluble extractive value content of raw materials and the polyherbal powder were 3.53 ± 0.012% for *Costus pictus*, 3.66± 0.016 % for *Gymnema slyvestre*, 3.56 ± 0.012% for *Momordica charantia* and 3.34 ± 0.008% for the prepared polyherbal powder. The drug's ether-soluble extractive value implies the presence of lipids, fats, and steroids (27).

In the current study, the extractive values are more in water, followed by alcohol and ether. Water attracts microbial contamination if used for extraction for longer than 24 hours.

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Therefore, different ratios of alcohol or hydro alcohols are used to achieve maximum extraction while minimizing the risk of contamination. So, for extraction, alcohol is preferred over water. Ethers are known to be toxic, and since they are also highly volatile, extraction requires a large volume of solvent. Ethers are, therefore, not particularly preferred in this situation for extraction.

Determination of pH

The pH of the prepared polyherbal solution (1% solution) had a pH of 6.5 ± 0.1 and the 10% solution had a pH of 6.0 ± 0.1. The pH values indicate that the polyherbal powder is lightly acidic, which enables fewer chances of microbial contamination.

Preliminary phytochemical analysis of polyherbal powder

Table 1 lists the test procedure, observations and inferences that were obtained. The prepared polyherbal powder's phytochemical analysis revealed the existence of triterpenoids, flavonoids, saponins, alkaloids, phenols and tannins, glycosides, and diterpenes. Alcohol extracts had the highest screening for these phytochemicals, followed by water and ether extracts.

Table 1: Preliminary Phytochemical Screening of Polyherbal Powder

Preliminary Phytochemical Screening			**The solvent used for extraction**		
Phytochemicals	Test	Observation	Water	Alcohol	Ether
Triterpenoids	Salkowski test	Yellow colour observed	++	+	-
	Steroids test	Formation of green colouration	-	++	+
Flavonoids	Alkaline Reagent test	Yellow colour formed that turns colourless, after adding dilute acid	++	+	-
	Lead acetate test	Formation of a yellow colour precipitate	++	++	
Alkaloids	Hager's test	Formation of yellow coloured precipitate	+	++	-
	Wagner's test	Formation of a brown-reddish precipitate	++	++	+
Phenols and Tannins	Ferric Chloride test	Formation of bluish-green or black colouration	++	+++	+
	Lead acetate test	The appearance of bulky white precipitate	++	++	-
Saponins	Froth test	The foam produced persists for ten minutes	+++	++	-
Glycosides	Keller-kilani test	Brown ring formation at the interface	+	++	+++
Diterpenes	Copper acetate test	Green colour observed	+	++	+++

+++ indicates Excellent, **++** indicates Very Good, **+** indicates Good, **-** indicates Absent

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Chromatographic evaluation

Thin-layer chromatography

Preliminary separation of flavonoids, saponins, steroids, and phenols in the individual plant extracts of *Costus pictus*, *Gymnema slyvestre*, and *Momordica charantia,* and polyherbal mixture in ethanol and Methanol by TLC analysis have been studied and reported to show significant antidiabetic activity (28–31). Literature suggests that the 3 selected plants have a good separation rate for methanolic extract. However, Ayurveda does not permit using methanol as a solvent for extraction; instead, it recommends using water or alcohol (ethanol) or hydro alcohol. So, the TLC analysis was performed using methanol and ethanol to check which solvent yields better separation. TLC investigation was performed to examine the qualitative composition of reported active compounds such as flavonoids, lignans, saponins, cardiac glycosides, anthracene derivatives, triterpenes, valepotriates, essential oils, coumarin drugs, bitter drugs, and pungent tasting principles.

The results of thin-layer chromatography with their respective solvent systems, derivatizing agents are displayed in Table 2. The plates are displayed in Fig. 1-5. Thin-layer chromatography was performed as a preliminary analysis to separate and confirm the existence of different secondary metabolites. The results indicate that the separation is better in ethanolic solvents than in methanol.

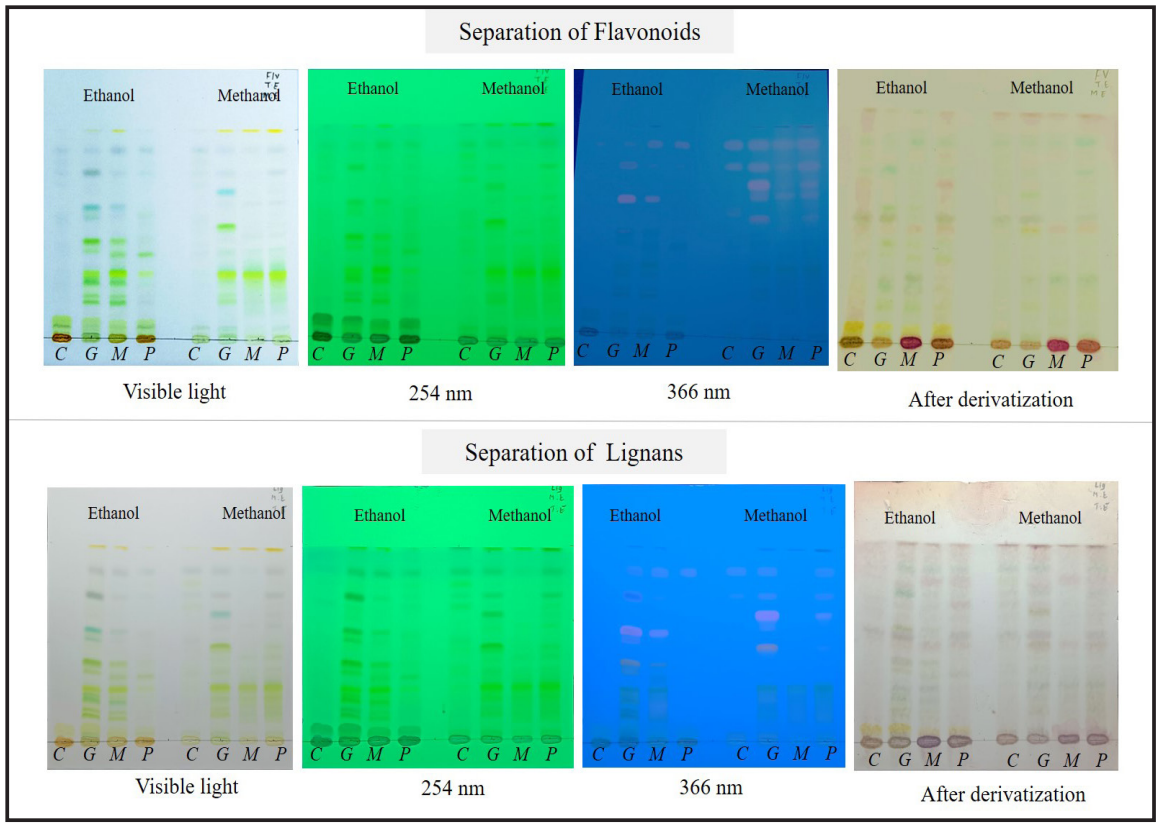

Fig. 1: Chromatographic fingerprint of Flavonoids and Lignans (*C- Costus pictus, G-Gymnema slyvestre, M- Momordica charantia, P*-Polyherbal formulation)

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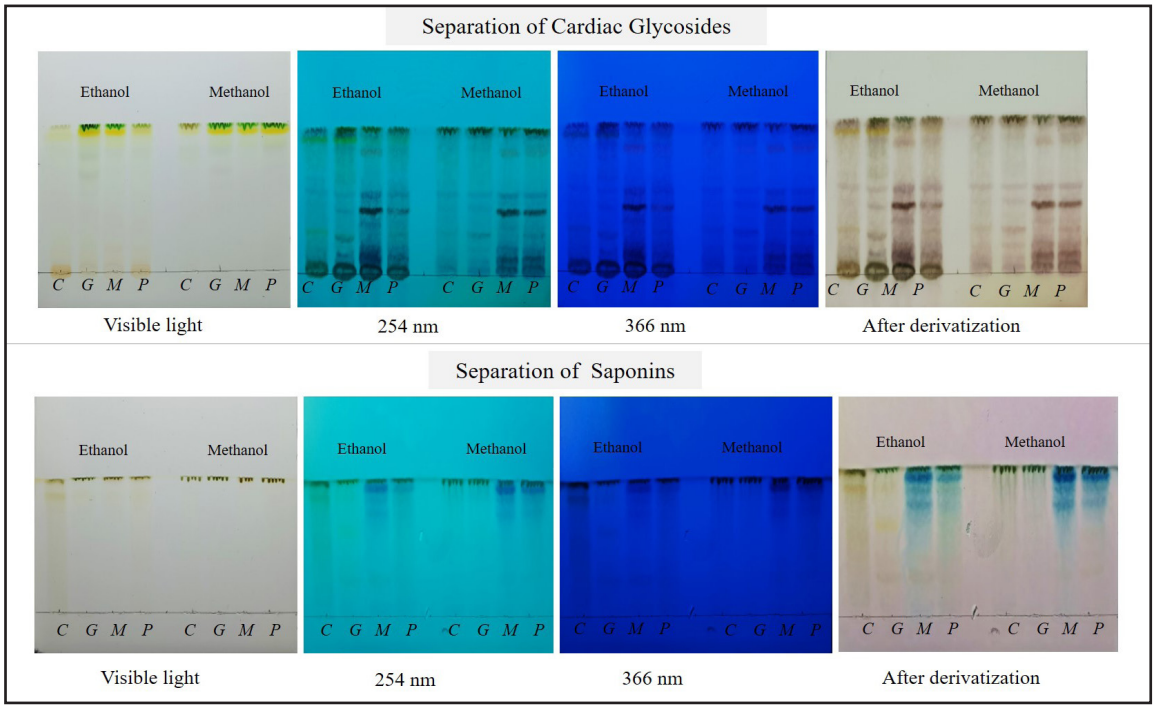

Fig. 2: Chromatographic fingerprint of Cardiac glycosides and Saponins (*C- Costus pictus, G-Gymnema slyvestre, M- Momordica charantia, P*-Polyherbal formulation)

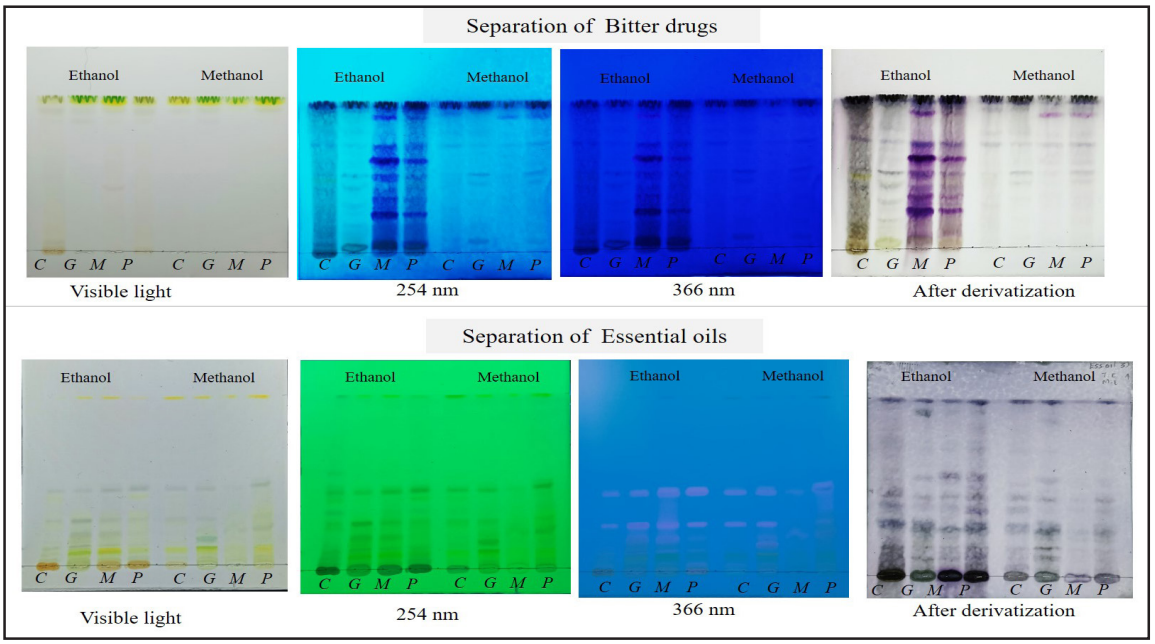

Fig. 3: Chromatographic fingerprint of Bitter drugs and Essential oils (*C- Costus pictus, G-Gymnema slyvestre, M- Momordica charantia, P*-Polyherbal formulation)

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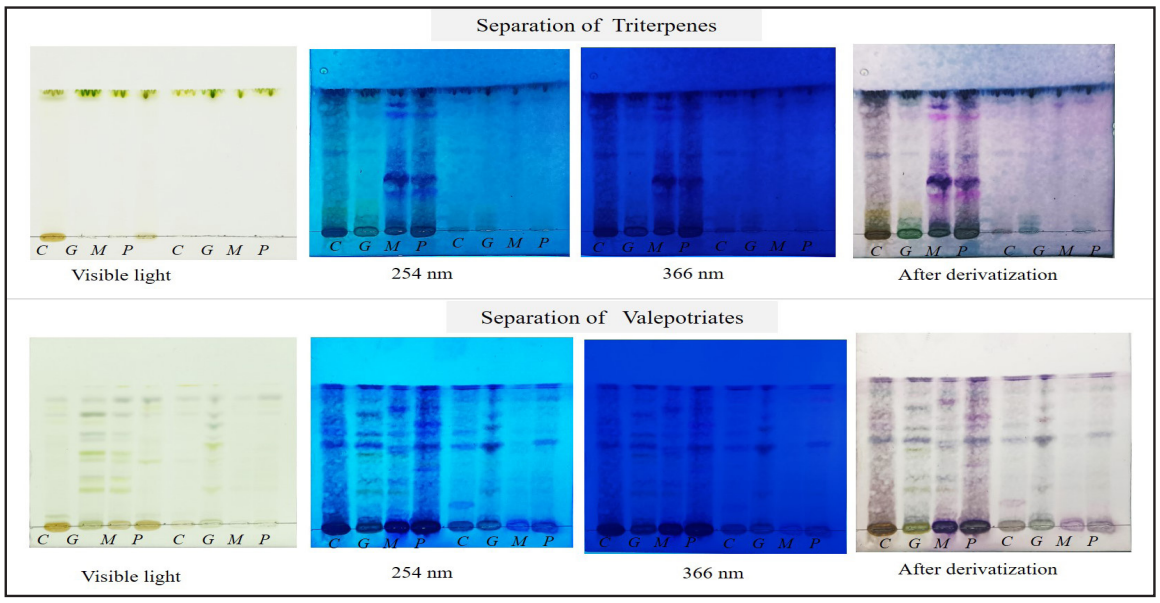

Fig. 4: Chromatographic fingerprint of Triterpenes and Valepotriates (*C- Costus pictus, G-Gymnema slyvestre, M- Momordica charantia, P*-Polyherbal formulation)

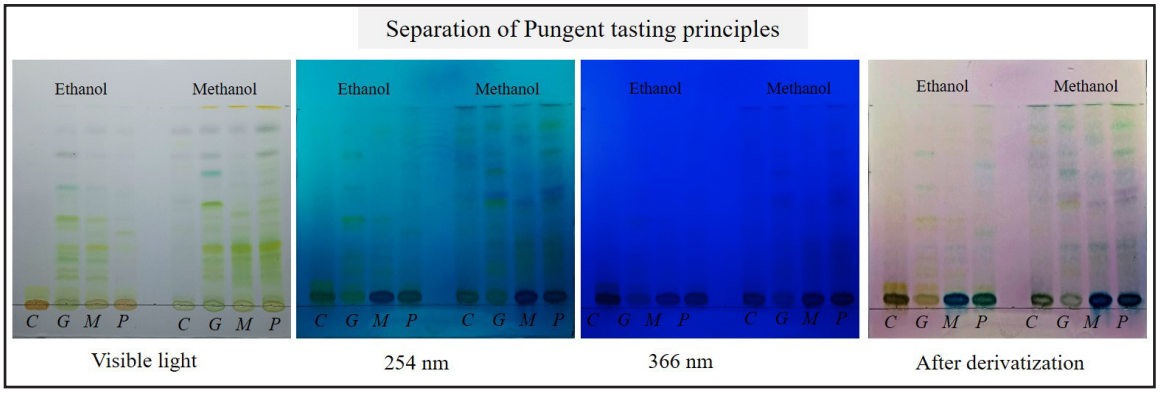

Fig. 5: Chromatographic fingerprint of Pungent tasting principles (*C- Costus pictus, G-Gymnema slyvestre, M- Momordica charantia, P*-Polyherbal formulation)

Table 2: The results of TLC with the solvent systems and the derivatizing agents used for respective phytoconstituents

Phyto-constituents	Derivatizing agent	Observations and Inference	Results
Flavonoids	Sulphuric acid reagent	Blue, green, and red fluorescence under UV-366 nm	Present
Lignans	Sulphuric acid reagent	Fluorescence at 366 nm	Present
Cardiac glycosides	Sulphuric acid reagent	Yellow, brown, and blue zones under visible light	Present
Saponins	Vanillin sulphuric acid	Blue, blue-violet, and yellow-brown zones in visible light	Present

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Pungent tasting principles	Vanillin sulphuric acid	Lemon yellow and blue to violet bands in visible light	Present
Anthracene derivatives	Potassium hydroxide reagent	Yellow or red-brown fluorescence at 366nm	Absent
Coumarin	Potassium hydroxide reagent	Blue, blue-green, and yellow fluorescence under 366 nm	Absent
Bitter drugs	Anisaldehyde sulphuric acid	Red-violet, brown, blue-green, blue, and grey bands under visible light	Present
Essential oils	Anisaldehyde sulphuric acid	Blue, red, green and brown coloration in visible light.	Present
Triterpenes	Anisaldehyde sulphuric acid	Blue-violet bands under 366 nm	Present
Valepotriates	Anisaldehyde sulphuric acid	Violet and blue zone in visible light	Present

Fluorescence analysis

After being treated with alkali and acid, the polyherbal powder's colours and distinctive fluorescent properties could be used as a standard for identifying and authenticating its raw form. It can also be used to check samples for adulteration. After being treated with acid or alkali, some adulterants emit fluorescence. In the present case, no fluorescence was emitted in the polyherbal formulation, indicating the purity of the raw materials used.

Safety evaluation of the polyherbal formulation

The results of safety parameters are displayed in Table 3.

Table 3: The results of the Safety evaluation of the polyherbal formulation

Safety evaluation of the polyherbal formulation					
Heavy Metal Analysis					
Sample code	**Element symbol and Wavelength (nm)**	**Weight of sample in gms / Volume in ml**	**Dilution Factor**	**Concentration in ppm µg/ml (or) mg/litre**	**Maximum Limit as per WHO**
Polyherbal formulation	As 188.979	0.0506g/50ml	1	BDL	5 ppm
	Cd 228.802	,,	,,	BDL	0.3 ppm
	Hg 253.652	,,	,,	BDL	0.5 ppm
	Pb 220.353	,,	,,	BDL	10 ppm
Microbial Contamination Test					
Test parameter		**Result**		**Specification limits as per AYU specification**	
Total Bacterial Count		TFTC (Too few to count)		NMT 105CFU/g	
Total Fungal Count		Absent		NMT 103CFU/g	
Test for Specific pathogens					
Test parameter		**Result**			
E. coli		Absent			
Salmonella		Absent			
Pseudomonas		Absent			
Staphylococcus		Absent			
Shigella		Absent			

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Pesticide Residual Analysis				
Sl. No.	**Parameter**	**Unit**	**LOQ**	**Result**
1.	Carbofuran	mg/kg	0.005	BQL
2.	Chlorpyrifos	mg/kg	0.005	0.224
3.	BHC (alpha isomer)	mg/kg	0.005	BQL
4.	BHC (beta isomer)	mg/kg	0.005	BQL
5.	Cypermethrin	mg/kg	0.005	BQL
6.	Dichlorvos	mg/kg	0.005	BQL
7.	Lindane (BHC gamma isomer)	mg/kg	0.005	BQL
8.	Malathion	mg/kg	0.005	BQL
9.	DDT (sum of p,p´-DDT, o,p´- DDT, p-p´-DDE and p,p´-TDE (DDD) expressed as DDT)	mg/kg	0.005	0.108
10.	Endosulfan (sum of alpha- and beta-isomers and endosulfan- sulphate expressed as endosulfan)	mg/kg	0.005	BQL

Aflatoxin Residual Analysis		
Aflatoxin Type	**Result**	**Prescribed Limits by AYUSH**
B1	Not Detected	0.5 ppm
B2	Not Detected	0.1 ppm
G1	Not Detected	0.5 ppm
G2	Not Detected	0.1 ppm

BDL-Beyond detectable limits; NMT-Not more than

Heavy metals quantitative analysis

Heavy metal is a class of reactive metallic elements with proportionally denser mass, which even in small amounts (parts per million) are hazardous, due to their high density. Due to the collection of the plant material from polluted areas, contaminated sites, waste-water supplies, etc., heavy metals may be present in the herbal formulation in trace amounts. Because heavy metals are denser, they are difficult for our bodies to digest and can build up in soft tissues (32). As a result, various health risks to different organs, including the liver, heart, lungs, central nervous system, brain, and kidney, arise, leading to a variety of respiratory complications, cancers, and cardiovascular, renal, and nervous system disorders (33). The concentration of the mentioned heavy metals was beyond detectable limits, this proves that the polyherbal formulation is safe for consumption and free of the aforementioned heavy metal contamination.

Microbial load analysis

The mentioned microbes are not present, indicating no microbial contamination in the polyherbal powder. The results indicate that the polyherbal powder meets the standards given by The World Health Organization, indicating that the polyherbal formulation is safe for consumption.

Pesticide residual analysis

Pesticides of various classes are applied to the plant as a chemical control to eliminate various pests. Based on their chemical composition, pesticides have four main groups: Organochlorines, carbamate and pyrethrin, organophosphorus, and pyrethroids (34). When pesticides are applied to herbal plants during agricultural practices, they can accumulate

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directly on the leaves or be absorbed by the plants, contaminating them. Because pesticides in herbal raw materials can cause a variety of health problems, their safety must be assessed. The results of pesticide residue analysis were within prescribed limits.

Aflatoxin analysis

Fungi produce certain toxins known as mycotoxins. Our environment contains a number of fungi like *Aspergillus* species, including *Aspergillus parasiticus* and *Aspergillus flavus,* that produce aflatoxins, a type of mycotoxin (a toxin produced by the fungi) (35). The major aflatoxins which adversely affect human life are Aflatoxins-(B1;B2;G1;G2) (36). The aflatoxin assay results show that the polyherbal formulation is free of Aflatoxin.

GC-MS Reports

The active compounds in the formulation were identified using GC-MS technique. The chromatogram reveals multiple peaks of compounds, as displayed in Fig. 6. The compounds associated with the peaks were found by examining and comparing the mass spectra and peaks of existing compounds from NIST 11 library.

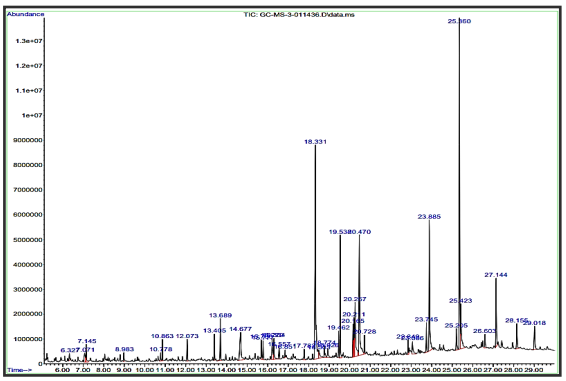

Fig. 6: GC–MS chromatogram of the polyherbal formulation

According to GC-MS analysis, from the available chromatogram, it is known that there are 39 compounds contained in the ethanolic extract of the Polyherbal formulation. The compound's Retention time, Peak area (%) and Similarity (%) are listed in Table 4. The compounds were detected at 6.327 minutes to 29.018 minutes.

Table 4: Compounds identified by GC-MS analysis

Peak	Retention Time	Area (%)	Compound	Similarity (%)
1	6.327	0.37	4-Hexenoic acid	60.28%
2	7.071	0.85	Glycerin	11.01%
3	7.145	0.95	Propane, 1,1,3-triethoxy	93.46%
4	8.983	0.40	Cyclohexane, octyl	77.90%
5	10.778	0.38	1-Octadecanesulphonyl chloride	64.37%
6	10.863	0.78	6-Tetradecene, (Z)	77.83%
7	12.073	1.20	Caprolactam	74.40%
8	13.405	0.98	2-Tetradecene, (E)-	84.94%
9	13.689	1.76	Phenol, 2,4-bis(1,1-dimethylethyl)-	30.59%
10	14.677	3.55	Butanal, 2-methyl	42.73%
11	15.700	0.84	1-Nonadecene	99.44%
12	15.777	0.83	Diethyl Phthalate	20.39%
13	16.220	1.54	Oxalic acid, cyclobutyl heptadecyl ester	74.54%
14	16.304	1.26	2-Cyclohexen-1-one, 4-(3-hydroxy-1-butenyl)-3,5,5-trimethyl	28.76%
15	16.557	0.66	Benzophenone	72.79%
16	16.851	0.29	11-Dodecen-2-one	74.15%

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17	17.782	0.57	5-Eicosene, (E)-	99.74%
18	18.331	16.13	n-Hexadecanoic acid	82.03%
19	18.503	0.92	-Ascorbic acid 2,6-dihexadecanoate	94.50%
20	18.774	0.73	9-Borabicyclo[3.3.1]nonane, 9-(2-propen-1-yloxy)-	68.80%
21	18.926	0.75	7,9-Di-tert-butyl-1-oxaspiro(4,5)deca-6,9-diene-2,8-dione	69.73%
22	19.462	1.06	Dibutyl phthalate	8.63%
23	19.538	5.29	Phytol	30.56%
24	20.165	1.65	cis-Vaccenic acid	72.66%
25	20.211	2.30	Octadecanoic acid	87.55%
26	20.267	4.51	9,12-Octadecadienoic acid (Z,Z)	90.54%
27	20.470	9.17	9,12,15-Octadecatrienoic acid, (Z,Z,Z)-	71.13%
28	20.728	0.82	5-Ethyl-1-nonene	85.16%
29	22.849	0.68	Dichloroacetic acid, heptadecyl ester	86.66%
30	23.066	1.05	1H-Indene, 1-hexadecyl-2,3-dihydro	38.86%
31	23.745	1.34	Tetracosane	73.39%
32	23.885	7.63	Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester	95.57%
33	25.205	1.30	Tetracosane	71.84%
34	25.360	15.11	Squalene	51.19%
35	25.423	3.76	Octadecanoic acid, 2,3-dihydroxypropyl ester	98.55%
36	26.603	0.88	Octadecane	70.51%
37	27.144	4.07	2H-1-Benzopyran-6-ol, 3,4-dihydro-2,8-dimethyl-2-(4,8,12-trimethyltridecyl)-, [2R-[2R*(4R*,8R*)]]-	83.69%
38	28.155	1.78	gamma-Tocopherol	55.07%
39	29.018	1.85	Vitamin E	58.48%

Vitamin E was reported at the end of the Retention time (29.018 minutes). The most prevalent compounds are n-Hexadecanoic acid; Phytol; Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester; 9,12,15-Octadecatrienoic acid, (Z,Z,Z)-; Squalene; 2H-1-Benzopyran-6-ol, 3,4-dihydro-2,8-dimethyl-2-(4,8,12-trimethyltridecyl)-, [2R-[2R*(4R*,8R*)]]-; Cis-vaccenic acid; Gamma-tocopherol.

Conclusion

Every day, new herbal products enter the market, each claiming to have different therapeutic effects for different ailments. However, the majority of these products are not standardized or evaluated for safety parameters, which may result in various side effects and health issues over time. Increasing public interest in herbs and herbal products necessitates that researchers provide standardized recommendations for their safe and effective usage, quality, and adverse effects. Though several herbal extracts or their bioactive compounds are available in the market for managing diabetes, their efficacy remains clinically unsatisfactory. For instance, *Costus pictus* (Insulin plant), *Gymnema slyvestre* (Gurmar plant), and *Momordica charantia* (Bitter Melon plant) have been reported for their antidiabetic activity. However, there are certain shortcomings with each plant that prevents its dependability. As a result, it is prophesied that the synergistic effect of these plants will aid in effective diabetes treatment. Many plants have been individually explored for their poten-

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tial antidiabetic activities; however, combining medicinal plants will help to achieve maximum synergistic antidiabetic potential. Therefore, this research aims to develop and standardize a polyherbal formulation to attain the synergistic effect of three antidiabetic plants. The present study establishes quality control limits for the polyherbal formulation and is an effort toward the phytochemical profiling of selected plants. The chromatographic fingerprints developed can serve as a quality control method to identify and authenticate the raw powders. This study will aid in the integration of the traditional system with the modern system of medicine, thus making science out of ethnomedicine. Once the polyherbal powder is evaluated for its antidiabetic potential using various in vitro, in vivo, and toxicity assays, it can be commercialized as a product in the market. It is anticipated that this formulation will be a safe alternative therapy with less or no adverse effects.

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