Research Article

Development and Minitab-Based Optimization of a Stress-Relief Herbal Extemporaneous Mixture

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ABSTRACT

The increasing demand for natural solutions to well-being has generated higher demand for ready-to-mix herbal blends intended for stress relief support. In the present work, the formulation and optimization of a new extemporaneous herbal blend containing *Withania somnifera* (Ashwagandha) as the key adaptogen were the focus with the aim of creating a stable, consumer-friendly, and convenient powder blend having desirable sensory and physical characteristics. Major natural compounds—Ashwagandha, Isapghula husk, milk powder, sugar, coffee, and microcrystalline cellulose (MCC)—were chosen for their therapeutic potential and compatibility. The identity and quality of Ashwagandha were tested by phytochemical screening, Thin Layer Chromatography (TLC), and High-Performance Thin Layer Chromatography (HPTLC) for the presence of active withanolides. Physicochemical and microbial quality analyses of raw materials were also carried out for ensuring the safety and uniformity of the formulation. MCC was found to be a better dispersing agent than benzyl alcohol for maximizing the mixability of Isapghula with enhanced dispersibility and mouthfeel. A step-wise formulation strategy was adopted, and sequential trials were done to optimize viscosity, reconstitution time, and organoleptic quality. Optimization was carried out using Design of Experiments (DOE) through Minitab software, which facilitated finding the best combination of ingredients and time to mix to achieve uniform results. The completed prototype displayed desirable properties, such as smoothness, good taste, appropriate viscosity, tolerable pH, and acceptable flow properties. Short-term stability studies validated the product's integrity under typical storage conditions, validating its potential for real-world application. In conclusion, this study illustrates an effective formulation strategy for a stress-relief herbal powder, presenting a natural, effective, and consumer-friendly solution in the arena of functional wellness products.

Keywords: Herbal formulation, *Withania somnifera*, Ashwagandha, Isapghula husk, Microcrystalline cellulose, Ready-to-mix powder, Extemporaneous mixture, Stress relief, Herbal adaptogen, Design of Experiments (DOE), Minitab optimization, Herbal wellness product.

INTRODUCTION

In the fast pace of the modern era, stress is now a ubiquitous problem affecting the physical and mental health of people. As more and more awareness developed regarding synthetic stress management measures having harmful side effects, there is an increasing demand for natural, safe, and effective remedies. Ready-to-mix (RTM) herbal products make for a convenient option because they are easy to prepare, convenient to carry, and there is growing consumer interest in plant-based wellness.[1]

This project focuses on the development of a new stress-relief herbal extemporaneous blend, with Ashwagandha—a well-known adaptogenic herb with anxiolytic and restorative action—as the core ingredient. Blending Ashwagandha with complementary ingredients like psyllium husk, milk powder, and microcrystalline cellulose (MCC) yields a synergistic blend that provides both function and convenience in a ready-to-mix form. The formulation strategy targets ease of use, quick dispersibility, and patient compliance.[2-5]

In addition to formulation, this work investigates key evaluation measures essential to product performance, including viscosity, reconstitution time, and powder flow properties. Optimization follows design of experiments (DoE) guidelines to provide quality and user consistency. These tests function to determine not only the effectiveness but also the manufacturability and shelf-readiness of the formulation.[6]

Extemporaneous ready-to-mix (RTM) herbal preparations provide individualized and on-demand preparation of herbal medicines with maximum efficacy and convenience. These preparations involve selection of high-quality herbal drugs, stabilizers, and dispersing agents to ensure solubility and uniformity. Infusion, decoction, and maceration are typical preparation techniques depending upon the herbal ingredients. Compliance with Standard Operating Procedures (SOPs), control measures for quality, and regulatory standards (e.g., USP <795>) provides protection, stability, and compliance. Documentation, ingredient testing, and good manufacturing practices (GMP) are important for ensuring consistency of the product and therapeutic efficacy. [7,8]

Ashwagandha: The Stress-Relief Adaptogen:

Ashwagandha (Withania somnifera), one of the most notable herbs in Ayurvedic medicine, is well known for its adaptogenic nature and ability to support the body in coping with stress and anxiety.

Role of Ashwagandha in Stress Relief:

Ashwagandha (Withania somnifera), the sacred herb in Ayurvedic medicine, has an important role in stress management owing to its adaptogenic nature. Adaptogens are such natural substances that enable the body to cope with stress through balancing physiological processes. Ashwagandha primarily functions through the regulation of the hypothalamic-pituitary-adrenal (HPA) axis, which is involved in the body's stress response. Ashwagandha reduces the levels of cortisol, a hormone secreted during stress or physical or emotional tension, thus inducing relaxation and calming sensations.[9] It has been shown through clinical trials that daily consumption of Ashwagandha root extract can reduce stress and anxiety levels, enhance quality of sleep, and overall well-being. Moreover, Ashwagandha has antioxidant qualities that shield the brain against oxidative stress, which aggravates anxiety and mental exhaustion.[10] Not only does its calming nature minimize stress, but also promotes cognitive function, mood stabilization, and levels of energy. Due to such advantages, Ashwagandha is commonly employed in herbal preparations intended to enhance mental acuity, emotional stability, and tolerance to day-to-day stressors.[11]

Phytochemical Composition and Mechanism of Action:

The Ashwagandha roots are rich in withanolides, steroidal lactones that are responsible for its medicinal action. These substances are thought to modulate brain stress pathways and hence alleviate stress and induce feelings of well-being.

Clinical Evidence Supporting Stress Relief:

There are various clinical trials that have explored Ashwagandha's effectiveness in managing stress:

Randomized Controlled Trials (RCTs): Individuals with chronic stress who took Ashwagandha extract had significant decreases in stress-assessment scores than those who took a placebo.

Cortisol Reduction: Supplementation with Ashwagandha has also been correlated with a considerable reduction in serum cortisol level, the hormone responsible for responding to stress. [2,11]

Safety and Dosage Considerations:

Ashwagandha is generally tolerated by most people when taken within suggested dosages. Side effects are minimal and can consist of gastrointestinal upset or sleepiness. Those who are pregnant, lactating, or have autoimmune diseases should seek the advice of healthcare providers prior to adding supplementation. [12,13]

Incorporating Ashwagandha into Herbal Extemporaneous Mixtures:

When formulating such mixtures, it is crucial to consider the compatibility of Ashwagandha with other herbs, the appropriate dosage, and the intended delivery method to ensure efficacy and safety. By integrating Ashwagandha into personalized herbal formulations, practitioners can offer tailored solutions to individuals seeking natural methods for stress management. However, it is essential to base such formulations on current scientific evidence and adhere to regulatory guidelines to ensure quality and effectiveness. [14,15]

The formulation of herbal extemporaneous mixtures has significant scientific, commercial, and therapeutic potential in the pharmaceutical, nutraceutical, and functional food industries.

Rationale:

The demand for Herbal formulation that offer health benefits and basic nutrition is growing rapidly. Ashwagandha, with its well-documented health benefits, represents an excellent ingredient for stress relieving products. A Herbal Extemporaneous Mixture formulation will provide consumers with a convenient, easy-to-use product that leverages the adaptogenic properties of Ashwagandha. The goal of a project is to formulate a novel stress relief herbal extemporaneous mixture, addressing formulation challenges and ensuring regulatory compliance as a herbal drug product.

This Herbal Extemporaneous Mixture is convenient and customizable, allowing to add other beneficial ingredients like fruits, nuts, or seeds to enhance the nutritional profile and taste according to your preferences.

MATERIALS AND METHODS

Ingredient Studies:

List of Ingredients for Herbal Extemporaneous Mixture:

All the ingredients were procured from the local market. The Herbal Extemporaneous Mixture was formulated using the following natural ingredients which are tabulated in Table 1.

Extraction and Quantification of Free Withanolides from Ashwagandha Dry Extract

A methodical extraction protocol was applied to isolate free withanolides from *Withania somnifera* (Ashwagandha) dry extract using a solvent-based approach. An accurately weighed 5 g sample was initially mixed with equal parts methanol and water (25 mL each) in a separating funnel. This mixture underwent successive defatting with 50 mL hexane, repeated four times to remove non-polar constituents. Post-defatting, residual aqueous methanolic solution was further treated with 25 mL ether, repeated five times.

All ether fractions were pooled and subjected to dual water washes to eliminate aqueous residues. The purified ether

extract was vacuum-dried to remove solvent, yielding a residue containing free withanolides.

The dried residue was cooled in a desiccator and weighed. The percentage of free withanolides was then calculated using:

Free Withanolides (%) = (Residue Weight / Initial Extract Weight) × 100

This method provides a reliable approach for quantitative determination of free withanolides in herbal extract preparations.[14]

Table 1: List Ingredients used in the formulation of Herbal Extemporaneous Mixture

Sr. No.	Ingredients	Biological Source	Use/Function
1.	Ashwagandha	Roots of *Withania somnifera*	Adaptogen, reduces stress and improves relaxation.
2.	Isapghula (Psyllium Husk)	Seeds of *Plantago ovata*	Natural fiber, Viscosity modifier.
3.	Milk Powder	Dairy (Cow/Buffalo milk)	Enhances Taste and Texture
4.	Sugar	*Saccharum officinarum* (Sugarcane)	Sweetening agent, improves palatability
5.	Microcrystalline Cellulose (MCC)	Derived from Plant fibres	Enhances powder flow and solubility, Prevents caking,
6.	Coffee	Beans of *Coffea arabica*	Flavourings and taste enhancement
7.	Sodium Benzoate	Benzoic Acid derivative	Preservative, prevents microbial growth

Identification of Withanolides in Ashwagandha Extract Using TLC and Phytochemical Screening

To confirm the presence of withanolides in *Withania somnifera* extract, both chromatographic and chemical tests were conducted.

a. Thin Layer Chromatography:

A TLC analysis was performed using manually coated silica gel plates. A small volume (2–5 µL) of the previously extracted sample was spotted onto the baseline. The mobile phase comprised chloroform and methanol in a 9:1 ratio. The plate was developed in a beaker acting as a chamber and allowed to run until the solvent front reached ~8 cm. After air-drying, visualization was achieved using vanillin-sulfuric acid spray, revealing coloured spots under visible light. Under UV (254/366 nm), multiple fluorescent spots were noted. The Rf values ranged between 0.2 and 0.6, indicating the presence of various withanolides.

b. Phytochemical Tests:

The Liebermann-Burchard reaction was used for steroidal lactone detection. Upon addition of acetic anhydride and concentrated sulfuric acid to the extract, a bluish-green colour developed, suggesting a positive reaction. Further, the Salkowski test produced a reddish-brown ring at the interface of chloroform and sulfuric acid layers, confirming the presence of withanolides. These tests collectively validated the presence of withanolides in the Ashwagandha extract through both qualitative and chromatographic evidence. [16,17]

Physicochemical Analysis of Crude Drug Ashwagandha:

Besides the identification of active constituents, physicochemical examination of crude drug Ashwagandha is also critical, as it guarantees the quality, purity, and identity of the raw material.

The parameters including the content of moisture and ash values give invaluable information regarding the stability, safety, and effectiveness of the herb. The examination is also useful in identifying adulteration and guaranteeing adherence to regulatory standards, which is necessary to ensure consistency in formulation and therapeutic effectiveness.

Total Ash (Limit: ≤15%): To determine total ash, 1 gram of the sample is accurately weighed and placed in a silica crucible. The sample is ignited at a temperature range of 500–600°C until it becomes white, indicating the removal of all organic matter. The crucible is then cooled in a desiccator and weighed. This process is repeated until a constant weight is achieved to ensure complete ashing.

Acid Insoluble Ash (Limit: ≤4%): The total ash is treated with 25 mL of hydrochloric acid and boiled for 5 minutes. The mixture is filtered using ashless Whatman filter paper, and the residue is thoroughly washed with hot water. This residue is then ignited in a crucible, cooled, and weighed. The remaining ash indicates the acid-insoluble portion, typically representing siliceous matter like sand.

Water Insoluble Ash (Limit: ≤6%): The total ash is boiled with 25 mL of distilled water for 5 minutes. After boiling, it is filtered and the residue is washed with hot water. This residue is then ignited at below 450°C, cooled, and weighed. The result represents the portion of ash that is not soluble in water, indicating potential impurities like soil or silicates.

Sulfated Ash (Limit: ≤10%): For this test, 2 grams of the sample are weighed into a silica crucible. 3 mL of concentrated sulfuric acid (H_2SO_4) is added to char the organic matter. The mixture is then incinerated gradually until the sample is free of carbon and a white residue remains. The crucible is cooled and weighed to determine the Sulfated ash content.

Moisture Content (Limit: ≤5%): The sample is weighed in a pre-weighed tarred China dish and dried in a hot air oven at 100–105°C. After drying, the dish is cooled in a desiccator and reweighed. This drying and weighing process is repeated until a constant weight is obtained. The loss in weight represents the moisture content of the sample. [18]

Evaluating the Dispersion-Enhancing Agents for Improved Water Dispersibility of Isapghula Husk:

Isapghula (Psyllium Husk), obtained from Plantago ovata, is appreciated during formulation development for its gelation property and swelling behaviour, which helps in stabilizing herbal preparations. Though it increases viscosity and contributes towards reconstitution in Herbal Extemporaneous Mixtures (HEMs), its poor water dispersibility restricts its performance.

To enhance dispersibility, two dispersing agents, benzyl alcohol and microcrystalline cellulose (MCC), were tested. During the first trial, 1 g of Isapghula (100 µm) was mixed with 1 mL of benzyl alcohol and was added into 200 mL water under stirring. Partial agglomeration was seen, reflecting poor dispersion.

In the second configuration, 145 mg of MCC was mixed with 1 g of Isapghula and also dispersed homogeneously in water. This mixture displayed homogeneous dispersion without clumping, which showed better performance. Therefore, according to these observations, MCC is a more appropriate dispersion enhancer for aqueous herbal formulations using Isapghula.[19,20]

Formulation, Development & Optimization:

Formulation Methodology:

1. **Sieving of Ingredients:**
   - All procured ingredients were initially passed through Sieve No. 20 to ensure uniform particle size distribution, enhancing the homogeneity of the final formulation.
2. **Trituration of Specific Ingredients:**
   - Isapghula, being fibrous and coarse in nature, was subjected to intensive trituration using a grinder to achieve a fine consistency.
   - Additional trituration was carried out using a mortar and pestle to further refine the texture, ensuring uniformity and ease of mixing.
3. **Exemption of Certain Ingredients from Sieving and Trituration:**
   - Sodium Benzoate, Microcrystalline Cellulose (MCC), and Coffee were exempted from the sieving and trituration steps.
   - Coffee is hygroscopic in nature and tends to absorb moisture from the environment; hence, an appropriate quantity of MCC was incorporated to counteract this property.
   - The remaining exempted ingredients were already available in a micronized form and did not require further size reduction.
4. **Preparation of Mixtures:**
   - Mixing played a crucial role in ensuring uniform dispersion of ingredients.
   - Two separate mixtures were prepared in different crucibles, with special consideration of the mixing time for uniform blending:
     - Mixture-1: Isapghula was blended thoroughly with MCC, ensuring adequate mixing time for homogeneity.
     - Mixture-2: Coffee was combined with MCC, maintaining an optimal mixing time to prevent excessive exposure to environmental moisture.
5. **Final Mixing and Order of Ingredient Addition:**
   - After the preparation of Mixture-1 and Mixture-2, all ingredients were combined in a systematic order within a mortar and pestle to ensure homogeneity.
   - The ingredients were added sequentially in the following order with continuous and uniform mixing after each addition:
     - Sugar
     - Milk Powder
     - Ashwagandha
     - Mixture-1 (Isapghula + MCC)
     - Mixture-2 (Coffee + MCC)
     - Sodium Benzoate
   - Each ingredient was thoroughly incorporated before the addition of the next to ensure a consistent and evenly distributed blend.
6. **Final Processing and Storage Considerations:**
   - The prepared herbal extemporaneous mixture was stored in an airtight container to prevent moisture absorption and degradation.

Formulation Trials were taken based on trial-and-error basis:

Four Batches was formulated at the laboratory on trial-and-error basis out of which No. 4 batch were found to be ideal for the further proceeding for the optimization.

Evaluation Parameters for Herbal Extemporaneous Mixture:

Organoleptic Properties of formulation:

Organoleptic Evaluation on Herbal Extemporaneous Mixture were carried out like appearance, taste, aroma and other properties by vision and touch sensations.

Table 2: Formulation Trials

Sr. No.	Ingredient	Trial 1	Trial 2	Trial 3	Trial 4 [Prototype]
1.	Milk Powder	5 g	10 g	15 g	15 g
2.	Sugar	5 g	10 g	10 g	10 g
3.	Ashwagandha	500 mg	500 mg	500 mg	500 mg
4.	Isapghula	3 g	2 g	1.5 g	12 mg
5.	MCC [Mixture 1]	140 mg	140 mg	140 mg	140 mg
6.	MCC [Mixture 2]	200 mg	200 mg	200 mg	200 mg
7.	Coffee	1.5 g	1.5 g	1 g	1 g
8.	Sodium Benzoate	31 mg	49 mg	57 mg	57 mg
Remarks		Low milk powder and sugar affected taste and texture. High Isapghula increased viscosity, reducing dispersibility.	Sugar was adequate; milk powder still low. Isapghula slightly reduced but still caused high viscosity.	Milk powder and sugar improved. Isapghula better adjusted. Coffee reduced, leading to better taste and consistency.	Balanced formulation. Good sweetness, texture, and dispersibility. Ready for final optimization through further testing.

Powder Flow Properties:

a. Angle of Repose:

Good flow properties are critical for the development of any pharmaceutical powder formulation. Interparticle forces or forces between particles as well as flow characteristics evaluated by the angle of repose. The angle of repose is defined as the maximum angle possible between the surface of the pile of samples and the horizontal plane.

The fixed funnel and free-standing cone methods employ a funnel that is secured with its tip at a given height, H, which was kept 5 cm, above graph paper that is placed on a flat horizontal surface. With r, being the radius of the base of the conical pile.

Formula for calculating angle of repose: t a n θ = h / r ,

The flow property of a powder can be assessed by measuring its angle of repose. An angle between 25° to 30° indicates excellent flow**,** while 31° to 35° reflects good flow properties**.** Angles in the range of 36° to 40° suggest fair flow**,** typically not requiring any external aid. If the angle lies between 41° to 45°**,** the flow is considered passable, though the material may experience slight obstruction. A value between 46° to 55° denotes poor flow**,** often requiring agitation or vibration to improve movement. Angles of 56° to 65° are classified as very poor**,** and any value above 66° is regarded as extremely poor, indicating severe flow issues.

b. Procedure for Determination of Bulk Density, Tapped Density, and Hausner's Ratio & Carr's Index:

A pre-weighed quantity of 10 g of Herbal Extemporaneous Mixture (HEM) was carefully poured into a 100 mL graduated cylinder without applying any external pressure. The initial volume occupied by the powder was recorded as the bulk volume (V_o)**.**

Next, the cylinder was tapped 50 times in a controlled manner until the powder-bed volume reached a minimum, ensuring uniform settling. The final volume after tapping was recorded as the tapped volume (Vt)**.**

The bulk density**,** tapped density**,** and Hausner's ratio were calculated using the following formulas:

Bulk Density (g/mL) = Mass of Sample (10 g) / Bulk Volume (V_o) in mL

Tapped Density (g/mL) = Mass of Sample (10 g) / Tapped Volume (Vt) in mL

Hausner's Ratio = Tapped Density / Bulk Density

The calculated values were analysed to evaluate **the** flow properties of the HEM formulation. A Hausner's ratio close to 1.0 indicates excellent flowability, while a higher value (>1.25) suggests poor flow properties, which may require further optimization.

The Carr's Index (%) was then determined using the equation:

Carr's Index (%) = (Tapped Density - Bulk Density) / Tapped Density × 100

The calculated Carr's Index was analysed to evaluate the flow properties of the HEM formulation. A Carr's Index below 10% indicates excellent flowability, while a value greater than 25% suggests poor flow properties, requiring further formulation optimization.

Reconstitution Time:

To measure the reconstitution time of the prototype formulation, 200 mL of water is taken in a 500 ml glass beaker at room temperature. A stopwatch is kept ready and reset to 00:00 before starting the test. The measured quantity of the prototype formulation is then added into the water in one go, and the stopwatch is immediately started once the powder touches the water surface. A glass stirrer or spoon is used to stir the solution in a consistent circular motion to facilitate dispersion. The stopwatch is stopped when the entire formulation has completely dissolved/dispersed without visible lumps or sedimentation. The time recorded is noted as

Reconstitution Time. Observations such as the appearance of the final solution (whether clear, cloudy, smooth, or with undissolved particles) are also noted.

Viscosity:

The viscosity of the prototype formulation was measured using a Brookfield Viscometer with Spindle No. 63 at 30 RPM in a beaker. The formulation was prepared by dissolving the measured quantity in 200 mL of water, ensuring uniform dispersion before testing. A suitable beaker was used, allowing the spindle to be fully immersed without touching the bottom or sides. The viscometer was set to 30 RPM, and the viscosity reading in centipoise (cP) was recorded once a stable value appeared. The final viscosity value was recorded, and any observations regarding flow behaviour, consistency, and dispersion characteristics were noted.

pH:

The pH of the Herbal Extemporaneous Mixture (HEM) was measured to ensure stability and compatibility. The formulation was prepared by dissolving the required quantity in 200 mL of water and stirring it gently to achieve uniform dispersion.

A clean beaker was used to hold the prepared solution. The pH meter electrode was rinsed with distilled water, then directly immersed into the solution, ensuring it was fully submerged without touching the bottom or sides of the beaker. The pH reading was allowed to stabilize, and the value was recorded.

This measurement was repeated three times and mean was taken.

The recorded pH was compared with the desired range (6.0 to 7.5, ideally around 6.7). If necessary, minor adjustments could be made to optimize the formulation for stability, solubility, and taste balance.

Interaction studies using FTIR:

Procedure for ATR-FTIR Analysis of HEM Ingredients with Ashwagandha:

Attenuated total reflectance (ATR) is the most widely used sampling methodology for Fourier transform infrared (FTIR) spectroscopy.

a. Preparation of Reference Standard (Ashwagandha):

- A pure sample of Ashwagandha (500 mg) was taken as a reference standard. Which was placed in aluminium foil-packed Petri plates and stored in a hot air oven at 70°C for 8 days.
- The sample was placed onto the ATR sample holder without adding KBr or any other diluents.
- The ATR-FTIR spectrum was recorded in the range 4000 cm^{-1} to 400 cm^{-1} with a resolution of 4 cm^{-1} using an ATR-equipped FTIR spectrometer.
- This spectrum served as a baseline reference for comparison with other ingredient mixtures.

b. Preparation for Ingredient Mixtures:

- Each HEM ingredient (Milk Powder, Sugar, Isapghula, MCC, Coffee, and Sodium Benzoate) was separately mixed with Ashwagandha in a 1:1 ratio (500 mg: 500 mg) and triturated thoroughly to ensure uniform mixing.
- The prepared mixtures were placed in aluminium foil-packed Petri plates and stored in a hot air oven at 70°C for 8 days to promote potential chemical interactions.

c. ATR-FTIR Spectroscopy Measurement for Each Mixture:

- After 8 days, a small, required quantity of each oven-treated mixture was directly placed onto the ATR sample holder without any additional preparation.
- The ATR-FTIR spectrum was recorded for each Ashwagandha-Ingredient combination in the range 4000 cm^{-1} to 400 cm^{-1} with a resolution of 4 cm^{-1}.
- The following binary mixtures were tested:
    1. Ashwagandha + Milk Powder
    2. Ashwagandha + Sugar
    3. Ashwagandha + Isapghula
    4. Ashwagandha + MCC
    5. Ashwagandha + Coffee
    6. Ashwagandha + Sodium Benzoate

d. Data Interpretation:

- The obtained ATR-FTIR spectra were analysed for functional group peaks of Ashwagandha and each ingredient.
- Any peak shifts, broadening, disappearance, or appearance of new peaks were noted, indicating possible chemical interactions or compatibility issues.
- The results were evaluated to determine ingredient compatibility, providing crucial data before proceeding with final formulation optimization.

Microbiological TAMC:

Procedure for Total Aerobic Microbial Count (TAMC):

The Total Aerobic Microbial Count (TAMC) of the herbal formulation was determined using serial dilution and plate count methods as per pharmacopeial guidelines, including USP <61>, WHO, and AYUSH standards. To ensure reliable microbial enumeration, four different dilutions (10^{-1}, 10^{-2}, 10^{-3}, and 10^{-4}) were prepared and analysed.

To begin, 1 g of the sample was accurately weighed and transferred into 9 mL of sterile Buffered Peptone Water (BPW) or sterile saline, creating a 1:10 dilution (10^{-1}

dilution**).** The suspension was vortexed to ensure proper mixing. Further serial dilutions were performed by transferring 1 ml from the 10^{-1} dilution into 9 ml of sterile diluent, yielding a 10^{-2} dilution (1:100 dilution). This process was repeated sequentially to obtain 10^{-3} (1:1000 dilution) and 10^{-4} (1:10,000 dilution). Each dilution was thoroughly mixed before proceeding to microbial plating.

For microbial enumeration, Plate Count Agar (PCA) or Nutrient Agar (NA) was used as the growth medium. The spread plate method was employed, where 0.1 ml from each dilution (10^{-1}, 10^{-2}, 10^{-3}, and 10^{-4}) was pipetted onto the agar surface and evenly spread using a sterile glass spreader. In an alternative pour plate method, 1 ml of each diluted sample was transferred to a sterile Petri dish, followed by the addition of molten agar (cooled to ~45°C), which was then gently mixed and allowed to solidify.

The inoculated plates were incubated at 30–35°C for 24–48 hours under aerobic conditions. After incubation, colonies were manually counted using a colony counter, and the Total Aerobic Microbial Count (CFU/g) was calculated using the formula:

CFU per gram (CFU/g) = (Number of Colonies × Dilution Factor) / Volume Plated (ml)

Plates with 30–300 colonies were considered valid for enumeration, and the final TAMC was determined based on the most appropriate dilution. The results were compared with regulatory limits to assess microbial acceptability, ensuring that the sample met the microbial safety standards **(**≤100,000 CFU/g for unprocessed herbal products and ≤10,000 CFU/g for processed herbal products**)**. If microbial contamination exceeded these limits, corrective actions such as sterilization, raw material quality improvement, and preservative addition were considered.

HPTLC for Identification and Authentication:

The analysis of the herbal extemporaneous mixture containing Ashwagandha was carried out using High-Performance Thin Layer Chromatography (HPTLC) for the purpose of qualitative identification of phytoconstituents, with a specific focus on detecting Withaferin A as a marker compound. For this purpose, approximately 3 grams of the formulation sample was accurately weighed and extracted using 25 mL of methanol. The mixture was heated on a water bath for about 10–15 minutes to ensure proper extraction of active constituents, then cooled and filtered to obtain a clear test solution.

The chromatographic analysis was performed on precoated silica gel 60 F_{254} TLC plates. A standard solution of Withaferin A was prepared by dissolving 10 mg in 10 mL of methanol, which served as the reference standard for comparison. Using a micropipette, 10 µL of both the standard and the test solution were spotted on the TLC plate as 10 mm bands. The plate was then developed in a twin-trough chamber using a mobile phase consisting of Chloroform : Methanol in the ratio of 9.0 : 1.0. The development was carried out up to a distance of 8 cm from the point of application.

After development, the plate was air-dried and visualized under UV light at 254 nm and 366 nm to detect any naturally fluorescent or UV-absorbing compounds. Subsequently, the plate was derivatized by spraying with anisaldehyde sulphuric acid reagent and was heated at 110°C for 5 minutes to develop visible coloured bands. The chromatograms were evaluated visually and digitally using the HPTLC system to compare the retention factors (Rf values) and band patterns of the test sample with those of the standard Withaferin A.

Stability:

a. Stability Testing Studies for 1 Hour After Mixing the Formulation with Water:

The stability testing of the Herbal Extemporaneous Mixture (HEM) was conducted for 1 hour after mixing the formulation with water to evaluate its short-term physical stability. A pre-weighed quantity of HEM was accurately measured and dispersed in 200 mL of water at room temperature (25°C ± 2°C) with continuous stirring to ensure uniform mixing. Observations were recorded at different time intervals, including immediately after mixing (0 minutes), 15 minutes, 30 minutes, 45 minutes, and 60 minutes. The evaluation focused on identifying any physical changes that might impact the formulation's quality and performance.

Parameters Tested:

1. Visual Appearance – Checked for any colour change, phase separation, or precipitation.
2. Sedimentation – Observed if undissolved particles settled at the bottom over time.
3. Viscosity – Measured at different time points to track consistency changes.
4. pH Measurement – Recorded at 0 minutes and 60 minutes to identify any fluctuations.
5. Reconstitution Time – Noted how quickly the formulation dispersed or dissolved completely.
6. Odour and Taste (if applicable) – Evaluated to detect any undesirable changes during the study period.

The recorded observations were analysed to determine the formulation's short-term stability. Any significant deviations, such as excessive sedimentation, viscosity shifts, or pH changes, were considered for further optimization before proceeding with extended stability studies.

b. Evaluation Testing for 3-Month Stability Study of Powdered Formulation

The stability evaluation of the powdered formulation was conducted over a 3-month period to assess its physical, chemical, and microbiological properties under standard storage conditions. The study aimed to determine any

potential changes in the formulation that could affect its quality, safety, and efficacy over time.

1. Visual Appearance and Colour

2. Moisture Content

3. Odour and Taste

4. Microbial Growth (Bacterial Contamination Test)

5. Reconstitution Time

Quality by Design:

Quality by Design (QbD) is a contemporary, scientific, and risk management strategy that assures the quality of pharmaceutical products through enhanced understanding of the formulation and manufacturing process. It encourages a systematic approach to development using statistical methods like Design of Experiments (DoE) to determine and control the variables affecting the performance of the final product. This approach is not only proactive but also increases consistency, reproducibility, and compliance. QbD begins with the Quality Target Product Profile (QTPP), which outlines the desired properties of the final product in terms of safety, efficacy, and convenience for the patient. For the Herbal Extemporaneous Mixture (HEM), the QTPP was formulated to facilitate rapid reconstitution in 200 mL of water, smooth texture with acceptable viscosity, stress-relieving action by the addition of Ashwagandha, palatable taste with good dispersibility, and stable pH and appearance during its shelf life. From the QTPP, Critical Quality Attributes (CQAs) were determined. These are particular physical, chemical, biological, or microbiological characteristics that need to be regulated in order to maintain the quality of the final product. The CQAs of this formulation comprised particle size, bulk density, flowability, viscosity, sedimentation, reconstitution time, pH, active content (e.g., withanolides of Ashwagandha), microbial safety, and general product stability.

In order to control these CQAs, Critical Process Parameters (CPPs) were addressed. These are the process parameters which have significant impact on product quality and must be monitored and regulated. In this project, critical processing steps like sieving (to ensure uniform particle size), mixing (to ensure homogeneity), blending (to avoid ingredient segregation), packing (to provide protection against moisture and contamination), and storage (to preserve stability with controlled temperature and humidity) were properly optimized. Likewise, the formulation was guided by Critical Material Attributes (CMAs), which are the raw material's physical, chemical, or microbiological attributes. Each ingredient was chosen for its particular functional benefit—Ashwagandha for its adaptogenic (stress-relief) activity, Isapghula husk to provide viscosity, Microcrystalline Cellulose (MCC) to improve powder flow and uniformity, Milk Powder and Sugar to enhance taste and palatability, Coffee for added flavour, and Sodium Benzoate as a preservative to provide microbial safety.

An extensive risk analysis was carried out according to ICH Q9 guidelines. The Ishikawa (Fishbone) Diagram was used as a tool to illustrate possible sources of variability—anything from the raw materials to equipment and staff. Failure Modes and Effects Analysis (FMEA) was utilized to sort out risks in terms of the severity, occurrence likelihood, and detectability, arriving at a Risk Priority Number (RPN). Through this analysis, mitigation measures were established, such as standardization of raw materials, mixing parameter optimization, and maintenance of proper storage conditions, packaging integrity, and trained staff. To guarantee consistent quality and process control, a clear control strategy was put into practice in accordance with ICH Q8–Q10. This involved monitoring material attributes, critical processing operations such as sieving and mixing, and environmental conditions including humidity and temperature. The final product was evaluated for uniformity, reconstitution performance, and microbial safety. This approach ensured that product quality was sustained throughout its lifespan.

Lastly, formulation optimization was done employing Minitab software, with guidance from experts. A Design of Experiments (DoE) strategy was employed to investigate systematically the impact of independent factors like Isapghula, MCC, and Coffee concentration, as well as mixing time. Using Response Surface Methodology (RSM), a design space was developed that delineated optimal conditions for desired CQAs such as viscosity, powder flow, and reconstitution time. The ultimate formulation satisfied all the quality targets established ahead of time, indicating the successful implementation of QbD concepts in the development of this herbal extemporaneous preparation.

The optimization strategy encompassed the application of Minitab Design of Experiments (DOE) by implementing a Plackett-Burman factorial design. This was followed by analysis of data, response optimization, and construction of contour plots to establish the optimal ranges for the variables of selected formulation.

- Isapghula concentration.
- MCC + Coffee concentration.
- MCC + Isapghula concentration.
- Mixing Time 1 (Coffee and MCC).
- Mixing Time 2 (Isapghula and MCC).

Optimal Ranges: Identified through contour plots ensuring desired values for dependent factors:

- Viscosity
- Angle of repose
- Reconstitution time

Table 3: Independent Factors

Independent Factors	-1	0	1
Isapghula	0.7 gm	1.2 gm	1.7 gm
Conc. of MCC + Coffee	150 mg	200 mg	250 mg
Mixing Time 1	8 min.	10 min.	12 min.
Conc. of MCC + Isapghula	140 mg	145 mg	150 mg
Mixing Time 2	10 min.	15 min.	20 min.

Table 4: Factorial Design: Independent and Dependent Factors

Std. Order	Run Order	Pt Type	Blocks	Isapghula	Conc. of MCC + Coffee	Mixing Time 1	Conc. of MCC + Isapghula	Mixing Time 2	Viscosity	Angle of Repose	Reconstitution Time
1	1	1	1	1.7	150	12	140	10	304	42.93	77.5
2	2	1	1	1.7	250	8	150	10	312	40.91	91
3	3	1	1	0.7	250	12	140	20	224	42.34	72
4	4	1	1	1.7	150	12	150	10	282	38.21	74
5	5	1	1	1.7	250	8	150	20	282	42.27	59
6	6	1	1	1.7	250	12	140	20	280	37.4	86
7	7	1	1	0.7	250	12	150	10	204	34.99	77
8	8	1	1	0.7	150	12	150	20	210	33.42	62
9	9	1	1	0.7	150	8	150	20	230	39.92	58.5
10	10	1	1	1.7	150	8	140	20	326	31.57	53
11	11	1	1	0.7	250	8	140	10	224	35.59	61
12	12	1	1	0.7	150	8	140	10	216	28.92	66.5
13	13	0	1	1.2	200	10	145	15	260	35.48	70

Table 3 outlines the independent variables along with their respective ranges used for optimizing the formulation. Table 4 presents the 13 batches generated using a factorial design, incorporating five independent variables and three dependent variables evaluated through practical experimentation.

RESULTS

Extraction of Withanolides from Ashwagandha:

The isolation and quantitation of free withanolides from an ashwagandha dry extract was effectively accomplished with a sequential solvent extraction method. It entailed preliminary extraction with methanol and water, followed by defatting using hexane and selective extraction using ether. The resultant purification and concentration were followed by weighing the final residue to calculate the content of free withanolides. According to procedure, a yield of ca. 120 mg of free withanolides was achieved from 5 g of dry extract, reflecting an estimated 2.4% content of withanolides. This procedure presents an efficient and reproducible means of quantifying bioactive compounds with consistency in herbal preparations and quality control in nutraceutical and pharmaceutical uses.

Result of Thin Layer Chromatography:

The Thin Layer Chromatography (TLC) determination of the withanolides extracted from Ashwagandha effectively established the occurrence of withanolides, with an Rf value of 0.32. This proves a clear resolution of the compound under the chosen Chloroform: Methanol (9:1 v/v) mobile phase on self-prepared silica-coated glass plates. The development of indicative blue, violet, or reddish spots upon spraying with Vanillin-Sulfuric Acid Reagent further verified the occurrence of withanolides. The outcome indicates that the TLC and extraction process worked well in separating and detecting the withanolides composition in Ashwagandha.

Phytochemical Screening for Withanolides:

The positive results observed in both the Liebermann-Burchard and Salkowski tests confirmed the presence of withanolides in the plant extract. These findings suggest that the extract contains steroidal lactones, which are characteristic of withanolides.

Physicochemical analysis of Crude Drug Ashwagandha:

The sample was evaluated for various ash and moisture parameters as per standard limits. The total ash content was found to be 0.20 g**,** which corresponds to 12.8% of the total sample weight, remaining within the acceptable limit of not more than 15%**.** The acid insoluble ash measured 0.23 g**,** equivalent to 3.2%**,** which complies with the limit of not more than 4%**.** The water insoluble ash content was 0.12 g**,** translating to 5.1%**,** staying within the prescribed limit of not more than 6%**.** The Sulfated ash was found to be 0.18 g or 8.3%**,** which is under the allowable limit of 10%**.** Lastly, the moisture content was determined to be 0.20 g, equivalent to 4.5%**,** which is within the limit of not more than 5%**.**

Isapghula's Dispersion Enhancing Agents:

Out of the two agents under test, microcrystalline cellulose greatly enhanced the dispersibility of Isapghula Husk in water. It facilitated equal mixing without the formation of clumps, showing improved wetting and hydration properties. On the other hand, the utilization of benzyl alcohol yielded partial clump formation, and hence it was less effective for obtaining a smooth dispersion. As such, microcrystalline cellulose was found to be the better agent for improving the dispersibility of Isapghula Husk in the formula.

Optimization results:

Analysis of Factorial Design:

Pareto charts were used to understand which formulation variables had the most effect on product quality. These charts helped to visualize which factors were statistically significant by showing bars that cross a reference line (set at p = 0.05). Any factor crossing this line was considered to have a strong influence on the response. This made it easier to focus on the most important variables, saving time and effort. Overall, the Pareto analysis supported better decision-making in optimizing the formulation and improving product quality.

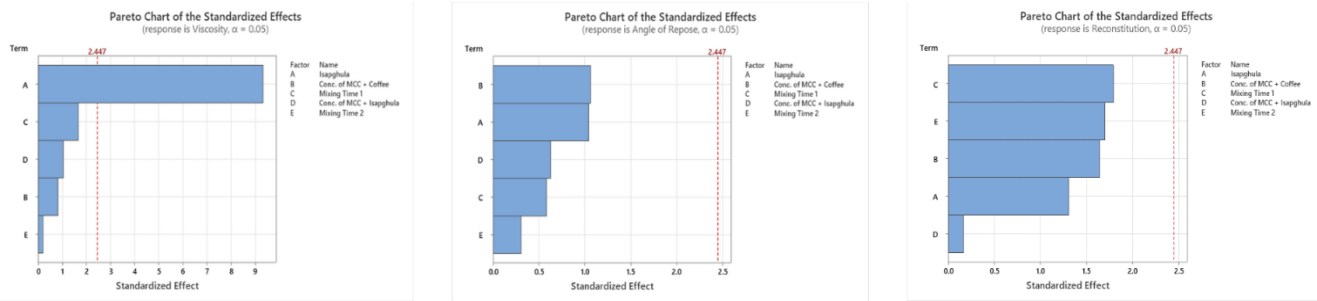

Figure 1: Pareto Charts

Figure 1 illustrates the Pareto charts generated through factorial design analysis, followed by ANOVA to identify the significant factors. The analysis revealed that viscosity was the most significantly affected parameter.

In the study, Isapghula (A) clearly stood out as the main factor affecting viscosity, showing a strong positive influence. This suggests that higher Isapghula levels increase thickness, impacting product texture. Other variables like MCC + Coffee and mixing times showed minimal or no significant effect on viscosity.

For angle of repose, no factor was statistically dominant, but MCC + Coffee (B) and Isapghula (A) showed a mild effect on powder flow, hinting they may affect pourability. In terms of reconstitution, mixing times (C and E) and MCC + Coffee (B) had a moderate impact, though not statistically significant. Longer or optimized mixing may still improve how well the product dissolves.

Summary: Isapghula strongly affects viscosity, while MCC + Coffee and mixing times influence flow and reconstitution. Careful control of these elements is essential for a well-balanced formulation.

Response Optimization:

In this study, response optimization was performed using *Minitab* software to identify the optimal combination of formulation and process parameters for achieving a high-performance ready-to-mix herbal product. The goal was to optimize three critical quality attributes (dependent variables): Reconstitution Time, Angle of Repose, and Viscosity. These parameters were selected as they directly affect the ease of use, flowability, and consistency of the product — all of which are vital for consumer acceptability and potential commercial scalability.

Using Minitab's Response Optimizer, the formulation was optimized by setting the objective for each response: *Reconstitution Time* was set to be minimized, while *Angle of Repose* and *Viscosity* were set to fall within specific target ranges that reflect ideal flow and consistency. Equal weight and importance were assigned to each response, indicating that all three outcomes were equally critical to the final formulation's success.

Upon execution, Minitab calculated and predicted an optimized solution that met all the predefined criteria. The software suggested the following optimal conditions: 1.2 g of Isapghula, 200 mg of MCC + Coffee, with a mixing time of 10 minutes, and 145 mg of MCC + Isapghula with a second mixing time of 15 minutes. Under these conditions, the software predicted a Reconstitution Time of 70 seconds, an Angle of Repose of 35.48°, and a Viscosity of 260 cps, each fitting well within the desired targets. The overall Composite Desirability score was 0.713767, which indicates a reasonably good fit across all responses.

This Minitab-driven optimization confirms that the selected levels of formulation ingredients and mixing times can lead to a balanced product with quick reconstitution, acceptable powder flow, and ideal viscosity, making it suitable for consumer use and potential scale-up in commercial production.

Contour Plot of Angle of Repose, Reconstitution, Viscosity vs Isapghula vs Viscosity:

The contour plots illustrate the relationship between Isapghula concentration and viscosity on key response

parameters. The plot of Angle of Repose shows that as viscosity increases with moderate levels of Isapghula, the angle of repose remains within the desired range (34–38°), indicating good powder flow properties essential for packaging and handling. The Reconstitution plot reveals that higher levels of viscosity and Isapghula improve reconstitution values, achieving above 90%, which reflects better dispersibility and consumer convenience during preparation. The Viscosity plot confirms a consistent increase with higher Isapghula, indicating controllable thickening behaviour. Overall, the plots confirm that optimal formulation with moderate to high Isapghula and viscosity ensures enhanced flowability, quick reconstitution, and stable mouthfeel—making the product more effective and are more consumer user-friendly.

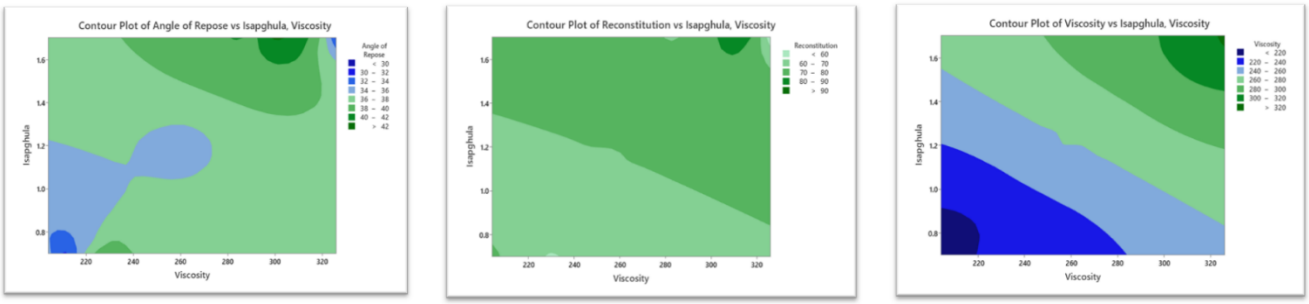

Figure 2: Contour Plots

Figure 2 illustrates the contour plot, which aids in the generation of the design space, a crucial element for formulation scale-up

Table 5: Final Optimized Batch

Sr. No.	Ingredients	Quantity
1.	Milk Powder	15 g
2.	Sugar	10 g
3.	Ashwagandha	500 mg
4.	Isapghula	1.2 g
5.	MCC (Mixture 1)	145 mg
6.	Coffee	1 g
7.	MCC (Mixture 2)	200 mg
8.	Sodium Benzoate	57 mg

Table 5 presents the final optimization results, showcasing the optimized formulation batch that meets the desired Quality Target Product Profile (QTPP)

Formulation Evaluations:

Organoleptic Evaluation:

The appearance of the mixture was observed to be a beige-coloured powdered form with black coffee dots, while the reconstituted liquid exhibited a beige-coloured smooth liquid consistency. The taste was reported to be sweet, accompanied by a pleasant milky smell. The texture of the powdered mixture was described as smooth with no grittiness, and upon reconstitution, the liquid texture was noted to be smooth and easily flowable.

Flow Properties:

The evaluated flow properties of the powdered formulation indicate favourable characteristics. The angle of repose was found to be 34.70°**,** which suggests good flowability of the powder. The bulk density and tapped density were determined to be 0.93 g/ml and 0.96 g/ml**,** respectively. Based on these values, the Hausner's ratio was calculated as 1.04**,** and the Carr's Index was 3.70%**.** Both of these values fall within the range that indicates excellent to good flow properties**,** confirming that the powder blend has minimal compressibility and is likely to perform well in processing and handling operations.

Reconstitution Time:

The reconstitution time of the Optimized formulation was recorded as 1 minute, 9 seconds, and 51 milliseconds (01:09.51). The formulation completely dispersed in 200 mL of water at room temperature without visible lumps or sedimentation, Solution appeared smooth, indicating good dispersibility. and recorded time met the expected Quality Target Product Profile (QTPP), confirming the formulation's suitability for reconstitution.

Viscosity:

The viscosity of the Optimized formulation was determined using a Brookfield Viscometer with Spindle No**.** 63 at 30 RPM. The mean viscosity value from three readings (256 cP, 267 cP, and 278 cP) was found to be 267 cP, and the formulation exhibited smooth flow behaviour with uniform dispersion, meeting the expected consistency requirements.

pH:

The pH of the Herbal Extemporaneous Mixture (HEM) was recorded as 6.6, which falls within the desired range of 6.0 to 7.5 and is close to the ideal value of 6.7.

- The measured pH confirms the stability and compatibility of the formulation.
- No further adjustments are required as the pH is well-balanced for solubility and taste.
- The formulation meets the expected Quality Target Product Profile (QTPP) for pH.

Excipient Compatibility Study of Ashwagandha using FTIR:

Based on the FTIR spectral analysis, the interaction of Ashwagandha with various excipients (Milk Powder, MCC, Sugar, Coffee, and Isapghula) was evaluated. The following key conclusions were drawn in figure 3.

It was found that Ashwagandha is compatible with all tested excipients, as no significant chemical degradation or strong interaction was observed. Coffee exhibits the strongest interaction, likely due to π-π stacking and hydrogen bonding, which may slightly alter the pharmacological effect by influencing bioavailability. Isapghula showed moderate interaction (O-H shifts), which may affect solubility and mucilage formation but does not pose a stability concern. Overall, Ashwagandha can be formulated with these excipients, but minor formulation adjustments may be needed based on the desired pharmacokinetic profile. *Refer Table No.6*

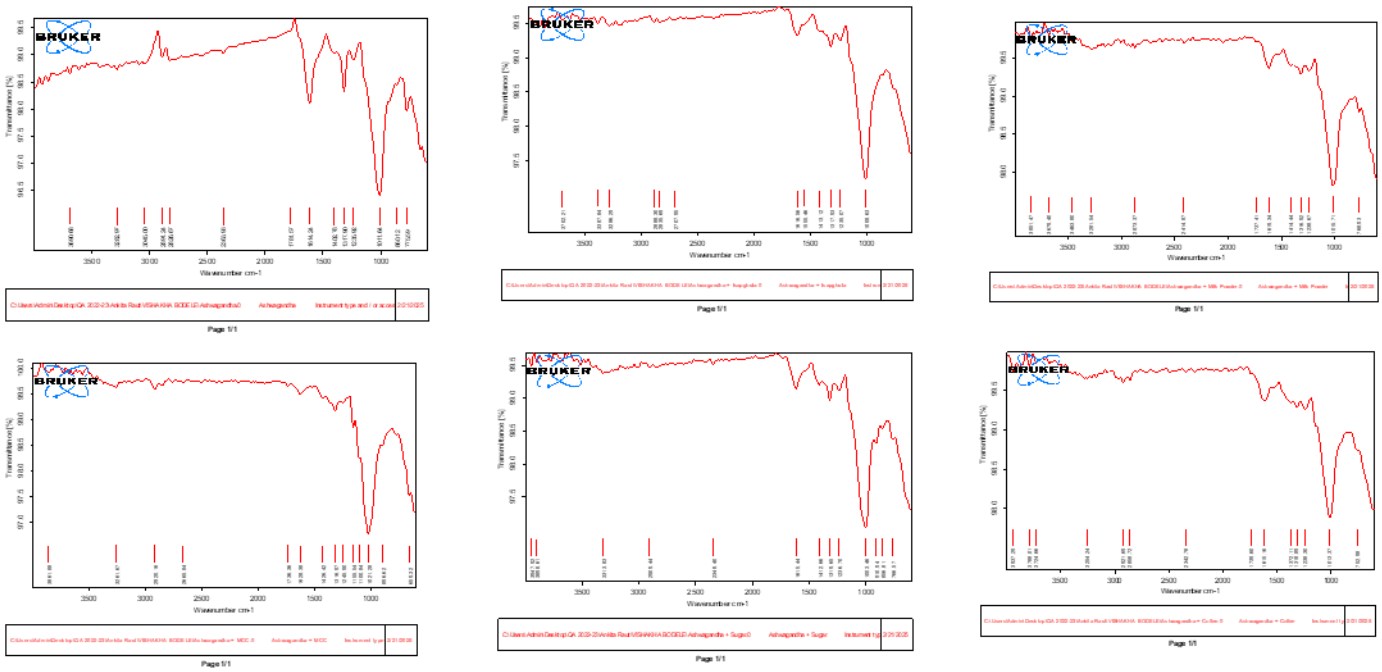

Figure 3: Graphs generated through FTIR

Table 6: FTIR Observations

Functional Group	Ashwagandha (cm^{-1})	+ Milk Powder (cm^{-1})	+ MCC (cm^{-1})	+ Sugar (cm^{-1})	+ Coffee (cm^{-1})	+ Isapghula (cm^{-1})	Interaction Type
O-H Stretching (3200-3600 cm^{-1})	3375	3362 (-13)	3355 (-20)	3350 (-25)	3345 (-30)	3353 (-22)	Hydrogen Bonding
C=O Stretching (1600-1750 cm^{-1})	1650	1645 (-5)	1648 (-2)	1644 (-6)	1642 (-8)	1646 (-4)	Slight interaction
C-N & C-O Stretching (1200-1350 cm^{-1})	1255	1252 (-3)	1250 (-5)	1249 (-6)	1248 (-7)	1251 (-4)	Minor interaction
Aromatic C=C (1450-1600 cm^{-1})	1505	1502 (-3)	1501 (-4)	1500 (-5)	1498 (-7)	1503 (-2)	π-π Stacking (only with Coffee)
New Peaks Formation	None	None	None	None	None	None	No chemical incompatibility

Microbiological TAMC Results:

The Total Aerobic Microbial Count (TAMC) of the herbal formulation was found to be 78,000 CFU/g based on the valid 10^{-2} dilution plate. This value is within the acceptable limit of ≤100,000 CFU/g as per USP/WHO guidelines, indicating that the formulation meets microbiological safety standards.

Authentication of the Presence of Ashwagandha in Final Formulation using HPTLC:

The HPTLC analysis confirmed the presence of Withaferin A and related phytoconstituents in the herbal extemporaneous mixture. Bands observed at Rf values ~0.56, 0.58, and 0.81 closely matched the standard, validating the identity of Ashwagandha in the formulation and indicating phytochemical consistency and authenticity.

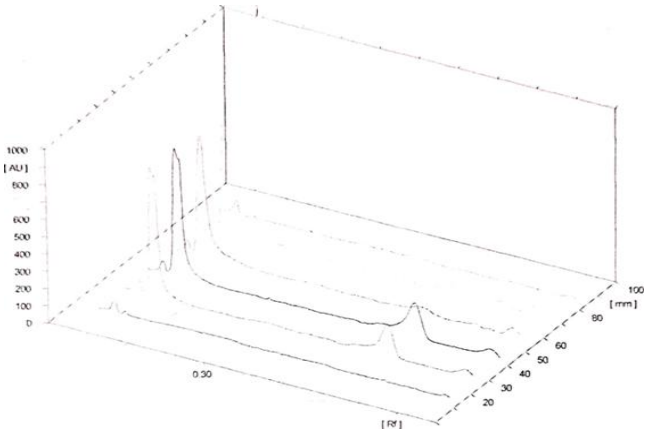

Figure 4: HPTLC Tracks 3D View

Stability Studies Results:

a. Short Term Stability Studies:

This short-term stability study was performed to evaluate the behaviour of the ready-to-mix herbal formulation over a one-hour period under ambient conditions. The primary goal was to assess the product's physical and organoleptic stability immediately after mixing and at time intervals of 15, 30, 45, and 60 minutes.

The short-term stability study indicates that the ready-to-mix herbal formulation is physically, chemically, and organoleptically stable for up to 60 minutes after mixing. Key observations include:

- Consistent appearance and viscosity throughout.
- No pH change, indicating no chemical degradation.
- No odour or taste changes, ensuring sensory stability.
- Minimal sedimentation, which is acceptable and does not affect usability.
- Quick reconstitution time, making it user-friendly.

The formulation remains stable, effective, and consumer-acceptable during the typical use period after mixing, supporting its readiness for further development and market potential.

b. Long Term Stability Studies:

A stability study was performed on a ready-to-mix herbal product for the long term to assess its physical, chemical, sensory, and microbiological stability for a period of three months under ambient storage conditions. This evaluation guarantees that the preparation retains its desired quality and safety level throughout its shelf life.

The 3-month stability study indicates that the formulation remains stable and safe during the storage period. Key findings include:

- No changes in appearance, odour, or taste, ensuring sensory consistency.
- Moisture content remained within acceptable limits, preventing spoilage.
- No microbial growth detected, confirming microbiological safety.
- Reconstitution time remained consistent, indicating ease of use.
- Minimal sedimentation observed after 45–60 minutes, which is acceptable for herbal powders.

The formulation is physically, chemically, and microbiologically stable over a 3-month period, demonstrating its suitability for long-term storage and supporting its potential for commercial use**.**

DISCUSSION

The optimized herbal product demonstrated good dispersibility, optimum viscosity, and quick reconstitution, validating the efficacy of the chosen ingredients and process. MCC dramatically enhanced Isapghula's dispersion, whereas mixing time and proportion impacted flow as well as preparation ease. Stability and compatibility studies further validated the integrity and usability of the formulation in actual practice.

CONCLUSION

This research efficiently developed a ready-mix herbal extemporaneous preparation for stress relief intended to be reconstituted in 200 mL of water. The optimized combination of Ashwagandha, Isapghula, MCC, and other supportive materials reached the required quality characteristics of rapid reconstitution, smoothness, and good flowability. The end product provides an easy, natural, and efficient method of managing daily stress.

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