4. MATERIALS AND METHODS

4.1 Diversity and traditional knowledge of Unani medicinal plants from Northwest Gujarat

The survey conducted in Northwest Gujarat from 2020 to 2021 aimed to investigate the diversity and traditional knowledge of Unani medicinal plant species. Study area includes 3 districts of Northwest Gujarat: Sabarkantha, Banaskantha and Aravalli (Fig. 5). In this survey, the numbers of medicinal plants were explored and emphasis given on Unani plants which are very useful for treatment of various diseases. Plant names have been arranged alphabetically and for correct nomenclature have been done after following the recent nomenclatural changes. Plant names are enumerated with their Botanical name, Family, Folk/Local name, Unani name, Ayurveda name and Siddha name. In addition, we have given information on traditional knowledge of plants and part of the plants used in various diseases or major ailments.

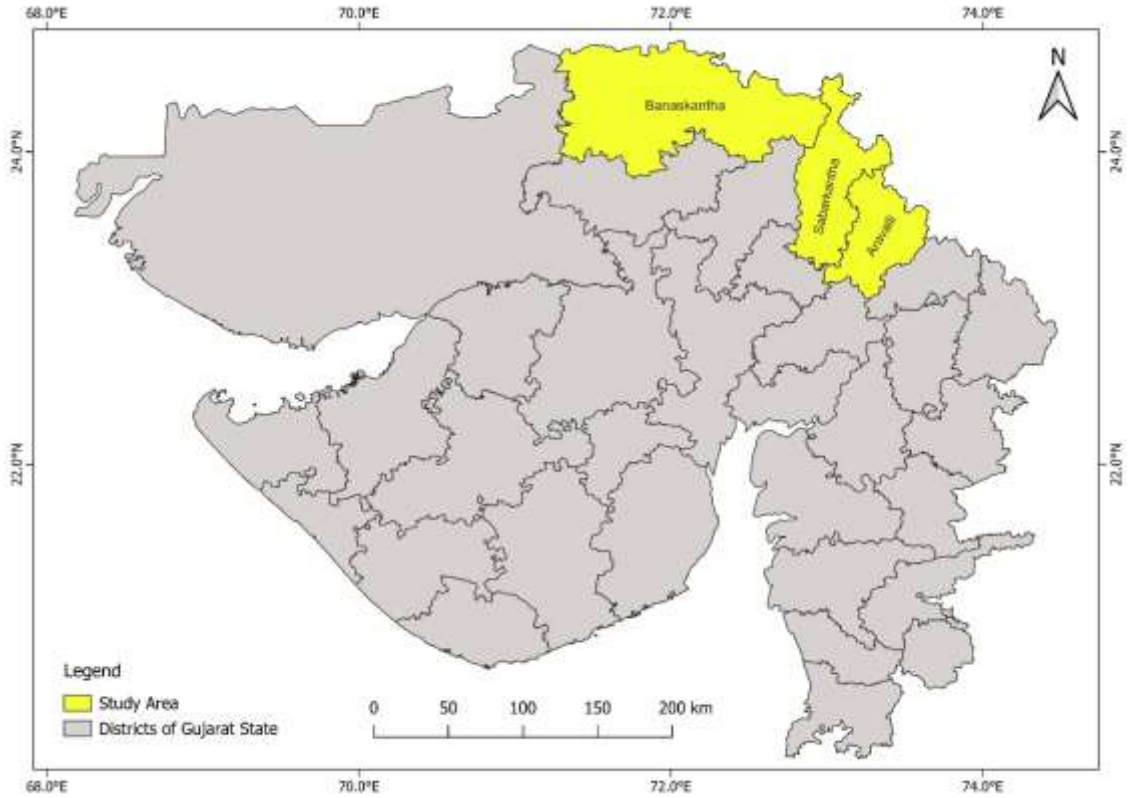

Fig. 5. Geographic Representation of Selected Districts in Northwest Gujarat

(https://onlinemaps.surveyofindia.gov.in/)

4.2 Batrisu Vasanu: A Polyherbal Formulation from Central Gujarat

The contemporary study was conducted in the central Gujarat region from 2020 to 2021. We have selected the commercial suppliers from 3 major districts Vadodara, Ahmadabad, and Panchmahal. From each district, randomly 3 shops of Saraiya (herbal suppliers) and Unani medicinal raw material suppliers were selected and visited. Each shop was enquired for the marketed product with local popular name Batrisu vasanu or Katlu and their raw material and ingredients for the same. Five products with on-pack information labels about ingredients were included in the study. We have also confirmed the same with Hakim (Unani practitioner) from the above places for their conformity with the ingredients used in this preparation (Hakim, 1991). Samples were then coded (PH01 to PH05) to maintain the confidentiality of the manufacturer for further research. Ingredients listed on the packets were noted and redundant names were removed. Names of the herb written in vernacular names were validated using standard local language and scientific literature. In addition to the above, we have also enquired from the hakims for the same purpose (Hakim, 1991). Further referencing was done with standard Unani books (Gupta, 2003). Ayurvedic Pharmacopoeia of India (API) – Part I (Joshi et al., 2017), The Unani Pharmacopoeia of India –Part I (Anonymous, 2007) & Bustanul Mufradat (Urdu edition) (Hakim, 1991) were used for confirming all plant details. The data were recorded in Microsoft Excel, which was then used for statistical analysis, and graphics were presented. Relative frequency citation (RFC) value was calculated by dividing the number of times a particular species was mentioned in plant samples i.e., PH01-PH05 (FC) by the total number of plant samples (N). RFC value was calculated for all plants by the following equation: RFC = FC/N X100

4.3 Selection of Unani Polyherbal Formulations

The selection of polyherbal Unani formulations for this study was carried out through a systematic two-step process to ensure a comprehensive representation of both traditional and innovative formulations.

The initial selection phase involved identifying 15 polyherbal formulations based on the availability of Unani medicinal plants, with a particular focus on those sourced from the Northwest Gujarat region (Table 1). This regional focus allowed for the inclusion

of plants that are locally grown and commonly used in Unani practice, ensuring the authenticity and relevance of the formulations.

Following the initial identification, the selection was refined with guidance from experienced Hakims (Unani physicians), who recommended the inclusion of five polyherbal formulations for further investigation. Of these five formulations, three were traditional, classical formulations directly referenced in the Unani Pharmacopoeia. These formulations are grounded in established Unani principles, representing the conventional approach to polyherbal combinations and their therapeutic uses. The remaining two formulations, classified as novel, were introduced by Hakims, who modified traditional formulations based on their experiential knowledge and contemporary practices. These modifications reflect the evolving nature of Unani medicine, incorporating modern insights while retaining core traditional elements.

This balanced selection process ensures that both traditional and innovative aspects of Unani polyherbal formulations are represented, providing a holistic perspective for the study. The final selection of five formulations—three traditional (MN, SN, and SC) and two modified (F1 and F2)—will serve as the focus for the subsequent phases of the research.

Table 1. Unani Polyherbal formulations

Sr. No.	Unani polyherbal formulations	Therapeutic uses	References
1	Itrifal Kashneezi	Amraz Sar (Diseases of Head), Amraz Gosh (Diseases of Ear) due to Cold and Cough.	Morakabbat (Kabir, 2003)
2	Jawarish Tabasheer	Qai (Vomiting), Safravi Is-hal (Biliary Diorrhoea).	Morakabbat (Kabir, 2003)
3	Jawarish Zar-Ooni Sada	It is a unique drug as Muqavvi Gurda (Renal tonic). Muqavvi Jiger (Liver tonic), Muqavvi Meda (Gastric tonic), Muqavvi Dimagh (Brain tonic).	Morakabbat (Kabir, 2003)
4	Laooq Khayarshamber	It is beneficial in Khushoonat Halaq (Sore Throat), Sual (Cough), Zatul Janb (Pleurisy) and Zeequnnafas (Asthma). It is also used as Mullein (Laxative).	Morakabbat (Kabir, 2003)

Sr. No.	Unani polyherbal formulations	Therapeutic uses	References
5	Sufoof Mulayyin	Qabz, Waj-ul-Meda (Gastrointestinal system).	NFUM Pt.-VI (Anonymous, 2011).
6	Majoon-E-Dabeed-Ul-Ward	Istisqa (Dropsy), Zof-e-Kabid (Weakness of Liver), Waram-e-Kabid (Hepatitis), Waram-e-Rahem (Uteritis), Faqr-ud-Dam (Anemia).	UPI Pt.-2, VOL-1(Anonymous, 2009).
7	Sufoof-E-Mohazzil	Saman-e-Mufrit (Obesity).	UPI Pt.-2, VOL-3 (Anonymous, 2016).
8	Habb-E-Asgandh	Waja-ul- Mafasil (Arthralgia), Waja-ul-Warik (Coxalgia).	UPI Pt.-2, VOL-3 (Anonymous, 2016).
9	Sufoof Aslussoos	Riqqat Mani (Less Semen viscosity), Sur-ate Inzal (Premature Ejaculation), Jiryan (Spermatorrhoea).	Morakabbat (Kabir, 2003).
10	Majun Aarad Khurma	Riqqat Mani (Spermatorrhoea), Sur-ate Inzal (Premature ejaculation), Kasrat Ahtelam (Excessive nocturnal Emission).	Morakabbat (Kabir, 2003).
11	**Majoon-E-Najah (MN)**	Malikhuliya (Melancholia), Qulanj (Colic), Ikhtenaq-ur-Raham (Hysteria).	UPI Pt.-2, VOL-3 (Anonymous, 2016).
12	**Sufoof-E-Najah (SN)**	Malikhuliya (Melancholia), Qulanj (Colic), Ikhtenaq-ur-Raham (Hysteria).	-
13	**Sufoof-E-Chobchini (SC)**	Waj-ul-Mafasil (Joint pain), Niqras (Gout), Aatishak (Syphilis), Irq-un-Nisa (Sciatica), Fasad-ud-Dam (Putrefaction of Blood).	UPI Pt.-2, VOL-1 (Anonymous, 2009).
14	**Formulation 1 (F1)**	Nakisul Bah (Female infertility), Su-e-Mizaj (impotence), Ilaj-e-Marasim-e-Niswan (Female reproductive disorders), Muqawwi-e-Bah (Aphrodisiac) Qawi-e-Raham (Uterotonic), Haul-e-Tuklil-e-Tukl (Ovulation-inducing), Nabz-e-Androgin-o-Opigen (Phytoandrogen-oogenic), Munazzil-e-Fikr (Antistress) and Munazzil-e-Insulin (insulin-sensitizing).	-

Sr. No.	Unani polyherbal formulations	Therapeutic uses	References
15	**Formulation 2 (F2)**	Quwwat wa Mudāfat-e-Badan (Improves strength & immunity), Muqawwi-e-Bah (Aphrodisiac effect), Haul-e-Tuklil-e-Tukl (Improves female fertility), Dām-e-Su (Antidiabetic), Dāfid-ul-Wabā' (Antimicrobial), Hifzāniyat-e-Aa'sāb (Neuroprotective), Munazzil-e-Hisaab (Antioxidant).	-

4.4 Collection and Authentication of Unani Medicinal Plant Drugs

The majority of the ingredients were collected directly from their natural habitats, prioritizing commonly available species to maintain authenticity and quality. The remaining ingredients were sourced from reputable Unani commercial suppliers in Vadodara and Delhi. For proper identification and validation, herbarium specimens were meticulously prepared for each plant material. The identification of each plant species was verified using standard taxonomic references (Cooke, 1908; Shah, 1978) to ensure accuracy. These herbarium specimens were thoroughly examined and deposited at the BARO Herbarium, The Maharaja Sayajirao University of Baroda, with accession numbers BAROKS01–BAROKS15. This rigorous authentication process ensured the precise identification of plant species and upheld the quality standards of the raw materials utilized in the formulations.

4.5 Preparation of Five Selected Unani Polyherbal Formulations

This study involves the preparation of five Unani polyherbal formulations, utilizing standardized protocols to maintain authenticity and ensure consistency across batches. Three of these formulations—Majoon-E-Najah (MN), Safoof-Najah (SN), and Safoof-E-Chobchini (SC) were prepared according to the methods outlined in the *National Formulary of Unani Medicine* (NFUM, Part I) and the *Unani Pharmacopoeia of India* (UPI), with MN and SN following Part II, Volume 3 and SC following Part II, Volume 1. Formulations F1 and F2 are novel formulations developed based on the recommendations of experienced Unani practitioners (Hakims), incorporating traditional wisdom with modern standards for herbal medicine preparation.

4.5.1 General Preparation Procedure for Unani Formulations

The following steps outline the standardized process used across all five formulations, ensuring uniformity and quality control. Variations specific to each formulation are detailed in the subsequent sections.

4.5.1.1 Ingredient Selection and Preparation

- Pharmacopoeial-grade ingredients were sourced to meet stringent quality standards for purity and potency.
- Ingredients were carefully cleaned to eliminate dust or impurities before further processing.

4.5.1.2 Drying

- Ingredients containing moisture were air-dried in a shaded, well-ventilated area to preserve active compounds. Exposure to direct sunlight was avoided to prevent degradation.

4.5.1.3 Powdering

- Each ingredient was individually pulverized using a mechanical grinder or mortar and pestle, ensuring a fine and uniform particle size necessary for even mixing and accurate dosing.

4.5.1.4 Sieving

- Powdered ingredients were passed through an 80-mesh sieve to achieve consistency. Any particles that did not pass were re-powdered and sieved again.

4.5.1.5 Mixing

- Sieved powders were transferred to a clean mixing container and thoroughly blended to create a homogenous mixture, ensuring consistency in color, texture, and distribution of active ingredients.

4.5.1.6 Packaging and Storage

- Final formulations were stored in airtight containers to prevent exposure to moisture, light, and air. Each container was labeled with the formulation name, preparation date, and storage instructions.

4.5.1.7 Quality Control and Consistency Checks

- Formulations were monitored for consistency in texture, color, and particle size to ensure they met standards outlined in the *Unani Pharmacopoeia of India* (UPI).

4.5.2 Specific Methodological Variations for Each Formulation

4.5.2.1 Majoon-E-Najah (MN) Polyherbal Formulation

Majoon-E-Najah is a traditional semi-solid Unani formulation composed of nine ingredients (Table 2). To achieve its characteristic consistency, a binding syrup known as *Qiwam* is prepared. This syrup plays a crucial role in binding the powdered ingredients together, ensuring the formulation's efficacy and stability (Afrin et al., 2017, 2019; Urooj et al., 2020; NFUM, Pt. I by Anonymous, 2006; UPI, Pt. II, Vol. 3 by Government of India, Ministry of AYUSH, 2016)

4.5.2.1.1 Preparation of Qiwam:

600 g of sugar was dissolved in 225 ml of water. The mixture was heated until it reached a concentration of 70° Brix, forming a thick syrup-like consistency.

4.5.2.1.2 Incorporation of Powdered Ingredients:

The powdered ingredients were gradually added to the Qiwam while continuously stirring to ensure a uniform and well-blended mixture.

4.5.2.1.3 Preservation:

To prevent microbial growth, 2-3 g of benzoic acid was added to the formulation.

4.5.2.1.4 Cooling and Storage:

The Majoon-E-Najah mixture was allowed to cool to room temperature before being stored in an airtight container.

Table 2. Ingredients of Majoon-E-Najah (MN)

Sr. No.	Unani Name	Botanical Name	Part used	Quantity (g)
1	Post-e-halela kabli	*Terminalia chebula* Retz.	Processed dried fruit	50
2	Post-e-Balela	*Terminalia bellerica* (Gaertn.) Roxb.	Fruit	50
3	Aamla	*Emblica officinalis* Gaertn.	Fruit	50
4	Halela Siyah	*Terminalia chebula* Retz.	Unprocessed dried fruit	50
5	Turbud	*Operculina turpethum* (L.) Silva Manso	Root	25
6	Bisfayej	*Polypodium vulgare* Linn.	Root	25
7	Aftimoon	*Cuscuta reflexa* Roxb.	Whole Plant	25
8	Ustukhuddus	*Lavandula stoechas* Mill.	Flowers	25
9	Qand safaid (Sugar)	*Saccharum officinarum* L.		600

4.5.2.2 Sufoof-e-Najah (SN)

Sufoof-e-Najah is a powdered version of Majoon-E-Najah, prepared without the use of Qiwam or any binding agent. It consists of 8 powdered ingredients only (Table 3), combined in equal parts for a dry powder formulation (NFUM, Pt. I by Anonymous, 2006, and UPI, Pt. II, Vol. 3 by Government of India, Ministry of AYUSH, 2016)

4.5.2.2.1 Method

The specified eight ingredients were powdered, sieved, and thoroughly mixed to create a homogeneous blend. The final formulation was stored in airtight containers as per the general preparation procedure.

Table 3. Ingredients of Sufoof-E-Najah (SN)

Sr. No.	Unani Name	Botanical Name	Part used	Quantity (g)
1	Post-e-halela kabli	*Terminalia chebula* Retz.	Processed dried fruit	50
2	Post-e-Balela	*Terminalia bellerica* (Gaertn.) Roxb.	Fruit	50
3	Aamla	*Emblica officinalis* Gaertn.	Fruit	50
4	Halela Siyah	*Terminalia chebula* Retz.	Unprocessed dried fruit	50
5	Turbud	*Operculina turpethum* (L.) Silva Manso	Root	25
6	Bisfayej	*Polypodium vulgare* Linn.	Root	25
7	Aftimoon	*Cuscuta reflexa* Roxb.	Whole Plant	25
8	Ustukhuddus	*Lavandula stoechas* Mill.	Flowers	25

4.5.2.3 Sufoof-e-Chobchini (SC)

Sufoof-e-Chobchini is another powdered formulation, requiring no binding agents or Qiwam. It is composed of eight specified ingredients (Table 4), which were processed and combined as outlined in the general methodology (Government of India, Ministry of AYUSH, 2009).

4.5.2.3.1 Method

Ingredients were powdered, sieved, mixed, and stored following the general preparation procedure (Unani Pharmacopoeia of India, Part II, Volume 1 by Government of India, 2009).

Table 4. Ingredients of Sufoof-E-Chobchini (SC)

SR. NO.	Unani Name	Botanical Name	Part used	Quantity (g)
1	Chobchini	*Smilax china* Linn.	Root	50
2	Sana	*Cassia angustifolia* Vahl.	Leaf	50
3	Ushba Maghribi	*Smilax aristolochaefolia* Mill.	Root	50
4	Bisfayej	*Polypodium vulgare* Linn.	Root	50
5	Suranjan	*Colchicum luteum* Baker	Root	50
6	Aftimoon	*Cuscuta reflexa* Roxb.	Stem	50
7	Gul-e-Surkh	*Rosa damascena* Herrm.	Petal	50
8	Sandal Safaid	*Santalum album* L.	Wood	50

4.5.2.4 Formulation 1 (F1)

Formulation 1 is a novel powdered formulation containing four ingredients (Table 5). It was developed based on clinical experience and recommendations by an in-house Unani practitioner (Hakim).

4.5.2.4.1 Method

Each ingredient was individually powdered, sieved, and mixed to homogeneity, then stored in airtight containers according to the general preparation procedure.

Table 5. Ingredients of Formulation 1 (F1)

Sr. No.	Unani Name	Botanical Name
1	Baikh asgand	*Withania somnifera* (L.) Dunal
2	Baikh piyabansa	*Barleria prionitis* L.
3	Gule dhawa	*Woodfordia fruticosa* (L.) Kurz
4	Gule nilofar	*Nymphaea alba* L.

4.5.2.5 Formulation 2 (F2)

Formulation 2 is another novel powdered formulation comprising five ingredients selected by a Hakim based on traditional knowledge and clinical insights (Table 6).

4.5.2.5.1 Method

Following the general methodology, each ingredient was processed, combined, and stored to maintain quality and potency.

Table 6. Ingredients of Formulation 2 (F2)

Sr. No.	Unani Name	Botanical Name
1	Kocha	*Mucuna pruriens* (L.) DC.
2	Asgand	*Withania somnifera* (L.) Dunal
3	Gajar bij	*Daucus carota* L.
4	Satavar	*Asparagus racemosus* Willd.
5	Taalmakhana	*Hygrophila auriculata* (Schumach.) Heine

4.6 Extraction

The **Majoon-E-Najah** (MN) formulation was subjected to various extraction techniques to determine the optimal method for isolating its active constituents. Three different extraction methods were employed: Soxhlet extraction, sonication extraction, and reflux extraction, utilizing three solvents—water, hydroalcoholic, and ethanol. The selection of hot (reflux) or cold (sonication) extraction methods was based on the solubility characteristics of the respective solvents. For water extraction, both reflux and sonication methods were applied, while hydroalcoholic and ethanolic extractions were carried out using reflux and sonication methods (Belscak-Cvitanovic and Komes, 2017; Bommakanti, 2023; Ranjha et al., 2021; Sridhar et al., 2021). This resulted in six distinct extraction methods, each assigned a specific code for identification (Table 7).

Additionally, for other formulations such as Sufoof-e-Najah (**SN),** Sufoof-e-Chobchini **(SC), Formulation 1 (F1),** and **Formulation 2 (F2),** sonication was exclusively employed to yield three distinct extracts: aqueous (AQ), hydroalcoholic (HA), and ethanolic (ET) (Table 7). The powdered samples of **Majoon-E-Najah (MN)** Unani formulation were extracted using water, hydroalcoholic, and ethanol solvents. Specifically, the water extract (RF-AQ) and hydroalcoholic extract (RF-HA) were obtained via reflux extraction, while the ethanolic extract (SX-ET) was obtained using Soxhlet extraction for 6 to 8 hours. The resulting extracts were then subjected to rotary evaporation to remove excess solvents, and the solvent-free residues were dried and powdered for further analysis.

For the other four formulations (**SN, SC, F1, F2**), sonication was performed on powdered samples of each formulation, resulting in the preparation of three extracts: **SN (AQ), SN (HA)**, and **SN-(ET**). Each extract was dissolved in the respective solvent to achieve a concentration of 1 mg/mL, vortexed for 5 minutes, and sonicated for 20 minutes. The extracts were then filtered using Whatman filter paper No. 1**,** and the resulting filtrates were used for subsequent analyses (Ranjha et al., 2021).

Table 7. Extraction Methods for Selected Polyherbal formulations Using Different Solvents and Techniques

Sr. No.	Formulation	Solvent	Extraction method	Code given
1	Majoon-E-Najah (MN)	Aqueous	Reflux	RF (AQ)
		Aqueous	Sonication	SN (AQ)
		Hydro alcoholic	Reflux	RF (HA)
		Hydro alcoholic	Sonication	SN (HA)
		Ethanolic	Soxhlet	SX (ET)
		Ethanolic	Sonication	SN (ET)
2	Sufoof-E-Najah (SN)	Aqueous	Sonication	SN (AQ)
		Hydro alcoholic	Sonication	SN (HA)
		Ethanolic	Sonication	SN (ET)
3	Sufoof-E-Chobchini (SC)	Aqueous	Sonication	SC (AQ)
		Hydro alcoholic	Sonication	SC (HA)
		Ethanolic	Sonication	SC (ET)
4	Formulation 1 (F1)	Aqueous	Sonication	F1 (AQ)
		Hydro alcoholic	Sonication	F1 (HA)
		Ethanolic	Sonication	F1 (ET)
5	Formulation 2 (F2)	Aqueous	Sonication	F2 (AQ)
		Hydro alcoholic	Sonication	F2 (HA)
		Ethanolic	Sonication	F2 (ET)

4.7 Determination of Extract Percentage Yield

The percentage yield of each extract was calculated to assess the efficiency of the extraction methods. The formula used for calculating the yield is:

Percentage yield (%) = a/b × 100

where a is the dry weight of the extract, and b is the weight of the soaked sample material (Sembiring et.al., 2018).

4.8 Organoleptic Characteristics

Organoleptic properties, including color, taste, appearance, texture, and odor, were evaluated following standard methods (Aziz et.al., 2019; Maurya et.al.,2020; Wallis, 2004).

4.9 Evaluation of Invitro Antibacterial and Antifungal Activity

4.9.1 Chemicals, Reagents, Solvents, and Standards

All chemicals, reagents, and culture media used in this study were sourced from reliable suppliers to ensure the accuracy and reproducibility of antimicrobial evaluations. Nutrient agar, Luria broth, and Sabouraud dextrose agar (SDA) used for culturing microorganisms were procured from HiMedia Laboratories Pvt. Ltd., Mumbai, India. Ciprofloxacin, gentamicin, and amphotericin B, used as positive controls in the study, were also obtained from HiMedia Laboratories. All other chemicals employed were of analytical grade to maintain the integrity of the experimental outcomes.

4.9.2 Microorganisms

The bacterial strains *Escherichia coli* and *Bacillus megaterium* were obtained from the Department of Microbiology, Faculty of Science, The Maharaja Sayajirao University of Baroda. The fungal strains *Candida albicans* (MTCC 854) and *Aspergillus niger* (MTCC 281) were procured from Aakaar Biotechnologies Pvt. Ltd., Lucknow, India. The antimicrobial activity of the polyherbal formulations was assessed using the agar well diffusion method.

4.9.3 Culture and Maintenance of Microorganisms

Pure bacterial cultures were maintained on Nutrient agar (NA), and fungal cultures were maintained on Sabouraud dextrose agar (SDA). Both bacterial and fungal cultures were sub-cultured periodically to ensure viability and were stored at 4°C for preservation before their application in antimicrobial assays.

4.9.4 *In vitro* Antibacterial Activity by Agar Well Diffusion Assay

4.9.4.1 Test Microbial Strains

The antibacterial activity of five selected Unani polyherbal formulations was evaluated using the agar well diffusion method (Bhinge et al., 2017; Dev et al., 2019), with minor modifications. The MN formulation was prepared in six different extracts, while the other four formulations were prepared in three extract types: Aqueous (AQ), Hydro-alcoholic (HA), and Ethanolic (ET). The activity of these extracts was tested against *Escherichia coli* and *Bacillus megaterium*.

4.9.4.2 Preparation of Culture Media and Inoculation

Luria Broth (LB) was used for culturing bacterial strains, and Nutrient Agar (NA) was prepared as the solid medium for agar plate preparation. All media preparation steps were performed under aseptic conditions.

4.9.4.3 Preparation of Nutrient Agar and Broth

Nutrient Agar (NA) was prepared using a composition of 13 g of N-broth per 1000ml and 2.5 g of agar per 100 ml, based on the manufacturer's instructions. The required quantities of N-broth powder and agar were calculated to achieve the total volume needed for the experiment, considering that each petri plate required 22 ml of agar. The medium was prepared by dissolving the calculated quantities in double-distilled water, sterilized by autoclaving at 121°C and 15 psi for 20 minutes, and cooled to 45–50°C before pouring into sterile petri dishes. After solidification, 0.3 ml of standardized bacterial inoculum (adjusted to 1×10^7 CFU/ml) was evenly spread on the plates using a sterile glass spreader. Plates were rested at room temperature for 30 minutes to ensure adherence.

4.9.4.4 Preparation of Luria Broth (LB)

Luria Broth was prepared using 20 g of LB powder per 1000 ml, as per the manufacturer's instructions. The required amount of LB powder was calculated for 6 ml of broth per test tube. The medium was sterilized by autoclaving at 121°C and 15 psi for 20 minutes. Bacterial strains were inoculated into 6 ml of LB and incubated at 37°C for 18–24 hours to achieve active growth. Cultures were standardized to a turbidity equivalent to a 0.5 McFarland standard (approximately 1×10^7 CFU/ml) for subsequent use.

4.9.4.5 Agar Well Diffusion Assay

After inoculating the agar plates, wells of 6 mm diameter were bored using a sterile cork borer. Each well was filled with 50 µl of test extracts at varying concentrations. Ciprofloxacin (5 µg/well) and gentamicin (5 µg/well) were used as positive controls, and the solvents for extract preparation were used as negative controls. Plates were incubated at 37°C in a Bio-Oxygen Demand (BOD) incubator for 24 hours. The inhibition zones were measured in millimeters using a calibrated digital Vernier caliper. Each experiment was performed in triplicate, and the mean inhibition zone diameters and standard deviations (SD) were calculated. The antibacterial efficacy of the extracts was expressed as the size of the inhibition zones, enabling comparative analysis of the formulations and controls.

4.9.5 In vitro Antifungal Activity by Agar Well Diffusion Assay

The *In vitro* antifungal activity of five selected Unani polyherbal formulations was evaluated using the agar well diffusion method, following the protocols of Magaldi et al. (2004) and Bauer et al. (1966), with modifications. Three types of extracts—Aqueous (AQ), Hydroalcoholic (HA), and Ethanolic (ET)—were prepared for each formulation and tested against *Candida albicans* and *Aspergillus niger*.

4.9.5.1 Preparation of Culture Media and Inoculation

Sabouraud Dextrose Agar (SDA) was prepared by dissolving SDA powder in double-distilled water and sterilizing it at 121°C and 15 psi for 20 minutes. The sterilized SDA medium was poured into sterile petri dishes (20 ml per dish) and allowed to solidify at room temperature under aseptic conditions. Fungal cultures of *Candida albicans* and

Aspergillus niger were prepared by adjusting them to a 0.5 McFarland standard, which corresponds to approximately 1.5×10^8 CFU/ml. Each petri dish was inoculated with 100 µl of the fungal suspension, which was spread evenly across the surface of the agar plate using a sterile spreader.

4.9.5.2 Agar Well Diffusion Assay

After inoculation, 6 mm diameter wells were bored into the agar surface of each plate. Test extracts were applied at concentrations ranging from 62.5 to 1000 µg/ml for *Candida albicans* and from 312.5 to 5000 µg/ml for *Aspergillus niger*. Each well was filled with 10 µl of test formulation. Amphotericin B (25 µg/well) was used as a positive control, while the respective solvents for each formulation served as negative controls. The plates inoculated with *Candida albicans* were incubated at 37°C for 24 hours, while plates with *Aspergillus niger* were incubated under the same conditions for 48 hours. After incubation, the diameters of the inhibition zones were measured in millimeters using a digital Vernier caliper. All measurements were conducted in triplicate, and the results were expressed as the mean ± standard deviation (SD) of the inhibition zone diameters to assess the antifungal efficacy of the formulations.

4.10 *In vitro* Antioxidant Activity by DPPH Assay

4.10.1 Chemicals and Reagents for DPPH Assay

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) reagent, solvents, and ascorbic acid (used as the standard) for the assay were procured from HiMedia Laboratories Pvt. Ltd., Mumbai, India. All chemicals were of analytical grade, ensuring the accuracy and reproducibility of the antioxidant evaluation.

4.10.2 DPPH Assay Procedure

The antioxidant potential of five distinct Unani polyherbal formulations was evaluated using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay. Three different extracts—Aqueous (AQ), Hydroalcoholic (HA), and Ethanolic (ET)—were prepared for each formulation, and ascorbic acid (AS) was used as a standard antioxidant for comparison.

For the assay, extracts were prepared at specific concentration ranges based on formulation type. The MN, SC, and F2 formulations were tested within a range of 20

to 120 µg/mL; the SN formulation was tested from 5 to 50 µg/mL; and the F1 formulation was tested at 10 to 60 µg/mL. Each concentration was mixed with a DPPH solution, and the mixture was incubated in the dark for 30 minutes to allow for the reaction. After incubation, absorbance was measured at 517 nm using a microplate reader, with decreased absorbance values indicating higher antioxidant activity.

Percentage inhibition of the DPPH radical was calculated as follows:

% DPPH radical scavenging activity = {(OD control – OD sample)/OD control} × 100

where OD control is the absorbance of the control (DPPH solution without extract) and OD sample is the absorbance of the formulation extract or ascorbic acid. A bar graph of percentage inhibition was plotted against concentration for each extract and ascorbic acid to visualize comparative antioxidant potential.

Additionally, a non-linear regression analysis was conducted, with one combined graph displaying three curves for the extracts along with the ascorbic acid curve for direct comparison. The IC50 values were determined from these regression curves. All tests were conducted in triplicate, with results presented as mean ± SD (Blois, 1958).

4.11 Total Phenolic Content (TPC) and Total Flavonoid Content (TFC)

4.11.1 Chemicals and Reagents for TPC and TFC

The chemicals and reagents used for the Total Phenolic Content (TPC) and Total Flavonoid Content (TFC) assays were sourced from HiMedia Laboratories Pvt. Ltd., Mumbai, India. All reagents were of analytical grade to ensure the accuracy and reproducibility of the assays. For TPC determination, Folin-Ciocalteu reagent, sodium carbonate, and gallic acid (as the standard) were used. For TFC determination, aluminum chloride, sodium acetate, and quercetin (as the standard) were employed. The use of high-quality chemicals ensured the reliability and precision of the experimental results.

4.11.2 Determination of Total Phenolic Contents (TPC)

The total phenolic content of five selected Unani polyherbal extracts (Aqueous (AQ), Hydroalcoholic (HA), and Ethanolic (ET)) was determined using the Folin-Ciocalteu reagent method, with gallic acid as the standard, following the procedure by Singleton

et al. (1999). For each measurement, 100 µL of extract was mixed with 100 µL of Folin-Ciocalteu reagent in a microplate well. The mixture was allowed to react for 5 minutes, after which 500 µL of a 20% sodium carbonate solution was added. The resulting mixture was incubated in the dark for 2 hours at room temperature.

After incubation, the absorbance was measured at 760 nm using a Versamax Microplate Reader. A gallic acid calibration curve, prepared within the concentration range of 10-70 µg/mL, was used to quantify the phenolic content, which was expressed as milligrams of gallic acid equivalents (GAE) per gram of extract. Each test was performed in triplicate, and the results are presented as mean ± SD

4.11.3 Determination of Total Flavonoid Contents (TFC)

Total flavonoid content in the selected five Unani polyherbal extracts (Aqueous (AQ), Hydroalcoholic (HA), and Ethanolic (ET)) were determined using the aluminum chloride colorimetric method, with quercetin as the standard (Meda et al., 2005). For each extract, 100 µL was mixed with 2% aluminum chloride solution and incubated for 5 minutes. Subsequently, 100 µL of 1 M sodium acetate solution was added, and the mixture was incubated in the dark for 40 minutes at room temperature.

After incubation, the absorbance was measured at 415 nm using a Versamax Microplate Reader. Total flavonoid content was quantified using a quercetin standard curve (10-70 µg/mL), and the results were expressed as milligrams of quercetin equivalents (QE) per gram of extract. Methanol was used as the blank. The procedure was performed in triplicate, and results are presented as mean ± SD.

4.12 Pearson Correlation Analysis

Pearson correlation analysis was performed to explore the relationships between various pharmacological activities and phytochemical content across five Unani polyherbal formulations, with three extracts: Aqueous (AQ), Hydroalcoholic (HA), and Ethanolic (ET). The specific pharmacological activities examined were antibacterial, antifungal, and antioxidant activities, while the phytochemical content included Total Phenolic Content (TPC) and Total Flavonoid Content (TFC) for each extract.

The parameters used in this analysis included the Minimum Inhibitory Concentration (MIC) values for antibacterial and antifungal activities, the IC50 values for antioxidant

activity, and the concentrations (mg/g) for TPC and TFC. Correlation coefficients (r) and p-values (set at 0.05) were calculated for each formulation to assess the strength and significance of the relationships between the pharmacological activities and phytochemical content. The analysis was performed using PAST software (version 4.03.exe). The results are presented in both a tabular format and a correlogram, providing a comprehensive view of the correlations for each formulation. Statistical significance was considered at $p < 0.05$.

4.13 Phytochemical Profiling of Selected Formulations through Thin-Layer Chromatography (TLC) and High-Performance Thin-Layer Chromatography (HPTLC)

4.13.1 Sample Preparation for TLC and HPTLC

For both TLC and HPTLC analysis, Formulation 1 (F1) and Formulation 2 (F2) powders were extracted using ethanol, hydroalcoholic, and aqueous solvents to prepare three different extracts. Additionally, the individual ingredients were separately extracted in methanol. Each extraction was carried out using 1 gram of sample in 10 mL of the respective solvent. The mixtures were vortexed for 5 minutes, followed by sonication for 20 minutes. The extracts were then filtered through Whatman filter paper No. 1. The resulting filtrates were used to develop chromatographic fingerprints in both TLC and HPTLC.

4.13.2 TLC Pre-screening and Solvent System Optimization

Initial Thin-Layer Chromatography (TLC) was performed on the three extracts **(Aqueous (AQ), Hydroalcoholic (HA)**, and **Ethanolic (ET))** as well as their individual ingredients, all analyzed on a single plate for both **Formulation 1 (F1)** and **Formulation 2 (F2)**. The purpose of this preliminary TLC was to screen and optimize solvent systems for the separation of phytoconstituents in these formulations and their individual ingredients. Several solvent systems were tested to identify the most effective one for the separation and resolution of compounds. The optimized solvent systems selected for further analysis were as follows:

- **Formulation 1 (F1)**: Toluene: Ethyl acetate: Formic acid: Methanol (9:9:2.4:0.6 v/v/v/v)

- **Formulation 2 (F2)**: Toluene: Ethyl acetate: Formic acid (10:8:2 v/v/v)

These solvent systems provided clear and distinct separations, suitable for the subsequent High-Performance Thin-Layer Chromatography (HPTLC) analysis.

4.13.3 HPTLC Analysis

Following the TLC optimization, HPTLC was performed on the **AQ, HA,** and **ET** extracts of **Formulation 1 (F1)** and **Formulation 2 (F2),** along with their individual ingredients. The goal was to create comprehensive chromatographic fingerprints of these formulations, ensuring their authenticity and quality. The HPTLC analysis was carried out on silica gel 60 F254 plates (Merck, Germany) using the optimized solvent systems mentioned above. The plates were generated in a twin trough chamber (CAMAG, Muttenz, Switzerland), lined on one of the inner walls with Whatman filter paper no. 1**.** The chamber was tilted 45 degrees to ensure even distribution of the mobile phase in both troughs, and was saturated with the mobile phase for 20 minutes. The plates were then developed ascendingly in the pre-saturated chamber up to a height of 85 mm from the base of the plate. After development, the plates were air-dried and scanned at 254 nm**,** 366 nm**,** and 540 nm using the CAMAG TLC Scanner 4**.** Visualization was achieved with a 10% methanolic sulfuric acid spray reagent, followed by derivatization using the Automated CAMAG Derivatizer and heating at 105°C on the CAMAG Plate Heater to develop color and enhance spot visibility. For the analysis, a CAMAG HPTLC system with a Linomat V Automatic Sample Spotter (CAMAG, Muttenz, Switzerland) and a CAMAG TLC Scanner IV with winCATS planar chromatography manager software was used (Wagner and Bladt, 2004; Stahl, 2007).

4.13.4 Rf Value Analysis and Schematic Representation

The Rf (Retention Factor) values of the phytoconstituents separated during HPTLC were recorded for all three extracts **(AQ, HA, ET)** and their individual ingredients. These Rf values were compared to identify common compounds present across the extracts and ingredients. A schematic representation was created for **Formulation 1 (F1),** highlighting the common Rf values (marked in red) that were present in all extracts and ingredients, confirming the authenticity and consistency of the formulation. This process was similarly applied to **Formulation 2 (F2).**

4.13.5 Densitogram Analysis

Densitograms for each HPTLC track were generated, offering a detailed representation of the chromatographic peaks, which correspond to the relative abundance of each compound. The Rf values**,** peak heights**,** and abundance of the separated compounds were recorded for both formulations. These Densitograms were analyzed, and the data was compiled into a table, allowing for a thorough comparison of the extracts and their individual ingredients. This comparison ensured the authenticity and quality of **Formulation 1 (F1)** and **Formulation 2 (F2).**

4.14 HR-LCMS-QTOF Analysis of Unani Polyherbal Formulations

The High-Resolution Liquid Chromatography-Mass Spectrometry with Quadrupole Time-of-Flight (HR-LCMS-QTOF) analysis was performed to profile the metabolites in two Unani polyherbal formulations, Formulation 1 (F1) and Formulation 2 (F2). The ethanolic extracts were selected for analysis based on their superior phytochemical composition, as indicated by Thin Layer Chromatography (TLC) and High-Performance Thin Layer Chromatography (HPTLC) fingerprinting. The ethanolic extracts showed a greater number of separation bands and better separation compared to the aqueous and hydroalcoholic extracts, suggesting a richer and more distinct phytochemical profile.

4.14.1 Sample Preparation

For the HR-LCMS-QTOF analysis, **Formulation 1 (F1) and Formulation 2 (F2)** powders were extracted in ethanol (1 mL) to obtain a concentration of 1 mg/mL**.** The mixtures were vortexed for 5 minutes, sonicated for 20 minutes, and then filtered using Whatman filter paper no. 1**.** The filtrate was then subjected to HR-LCMS-QTOF analysis to profile the metabolites of both formulations.

The ethanolic extracts of both formulations were prepared using sonication for efficient extraction, followed by filtration to remove any solid particulates. These extracts were chosen for HR-LCMS-QTOF analysis due to their superior band profiles and high separation quality observed during HPTLC fingerprinting, which indicated the presence of more diverse metabolites compared to the other extracts.

4.14.2 Chromatographic and Mass Spectrometric Conditions

- The HR-LCMS-QTOF analysis was carried out at the Sophisticated Analytical Instrument Facility (SAIF), IIT Bombay, following their established protocols for metabolite profiling of two Unani polyherbal formulations (F1, F2), with the following instrumental setup:
- **Instrument:** Agilent 6550 Q-TOF Mass Spectrometer
- **Ion Source:** Dual AJS Electrospray Ionization (ESI)
- **MS Mode:** Positive and Negative Ion Modes (ESI+ and ESI−)
- **Sampling Method:** High-Throughput Sampler (HiP Sampler) G4226A

4.14.3 Chromatographic Conditions

- **Column:** Agilent 1290 Infinity LC system
- **Column Type:** Reverse phase column
- **Mobile Phase A:** Water with 0.1% Formic Acid (FA)
- **Mobile Phase B:** Methanol/Acetonitrile mixture
- **Flow Rate:** 0.300 mL/min
- **Gradient Program:** The mobile phase gradient started with 95% water (A) and 5% methanol (B) at 1 minute, gradually increasing the percentage of methanol to 100% by 25 minutes. This was followed by a post-run phase with 95% water for re-equilibration.
    - **Time Segments:**
        1. 0–1 min: 95% A, 5% B
        2. 1–25 min: 0% A, 100% B
        3. 25–30 min: 0% A, 100% B
        4. 30–31 min: 95% A, 5% B
        5. 31–35 min: 95% A, 5% B
    - **Column Temperature:** 40°C

4.14.4 Mass Spectrometry Settings

- **Scan Mode:** Full scan MS and MS/MS
- **Mass Range:** 50–1200 m/z
- **Absorption Threshold:** 200 counts
- **MS/MS Threshold:** 5 counts
- **Ion Mode:** Both Positive and Negative ESI modes
- **Resolution:** High resolution to facilitate the precise identification of low-abundance metabolites
- **Data Acquisition:** Data was collected using Mass Hunter software, with settings optimized for the detection of metabolites across a wide mass range.

4.14.5 Data Processing and Compound Identification

The obtained MS data was processed using Mass Hunter Qualitative Analysis software to identify metabolites based on their molecular formulas, retention times, and mass-to-charge ratios (m/z). The metabolites were classified into groups, including alkaloids, phenols, fatty acyls, flavonoids, and others. Further identification was validated by matching the acquired data against several public and commercial databases such as: KEGG, DrugBank, HMDB (Human Metabolome Database), ChemSpider, PubChem.

4.14.6 Metabolite Profiling and Data Analysis

For both formulations, the identified metabolites were categorized by their chemical classes. The metabolites detected in the positive ESI mode included alkaloids, fatty acyls, amino acids, and their derivatives, while in the negative ESI mode, metabolites such as benzene derivatives, tannins, and terpenes were identified. A comparative analysis of the metabolites between Formulation 1 and Formulation 2 was conducted to highlight the similarities and differences in their chemical compositions.

4.14.7 Presentation of Results

The metabolite data for each formulation were compiled in tables, providing information on the molecular formula, retention time, observed mass, m/z ratio, peak height, and area. These data were further supported by database IDs such as KEGG ID,

Drug Bank ID, HMDB ID, Chemspider ID, and PubChem CID, ensuring reliable compound identification and annotation.

Charts illustrating the distribution and abundance of major metabolite groups were generated to compare the relative abundance of compounds between both formulations. The analysis indicated the presence of diverse and abundant metabolites in both formulations, with some metabolites being more prevalent in one formulation than the other.

4.15 Data Analysis

Statistical analyses were performed using Graph Pad Prism version 9.5.1. Results are expressed as mean ± SD, with each experiment conducted in triplicate. Antibacterial, antifungal, antioxidant activities, as well as total phenolic and flavonoid contents, were analyzed using two-way ANOVA followed by Tukey's multiple comparison test to determine significant differences between groups. Correlation analyses, expressed as Pearson correlation coefficients, were carried out using PAST software (version 4.03.exe) to evaluate the relationships between the different assays. The IC50 values for antioxidant activity were calculated from regression equations based on percentage inhibition versus concentration curves. Statistical significance was considered at $p < 0.05$, $p < 0.01$, and $p < 0.001$, varying across assays. For clarity, statistical significance between groups is indicated using alphabetic lettering (a, b, c, d,) in the study results.